The Cloning and Characterization of MADD-4, A Novel Guidance Cue in *Caenorhabditis elegans*

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Molecular Genetics

University of Toronto

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Abstract

Directed cell migration is fundamental to the development of all multicellular organisms including humans. To investigate the molecular underpinnings of directed cell migration, our lab primarily exploits plasma membrane extensions called muscle arms, from the body wall muscles in the tiny nematode *Caenorhabditis elegans*. Through genetic screens for genes required for muscle arm extension, we found that the UNC-40/DCC netrin guidance receptor directs muscle arm extension to the midlines. Surprisingly, neither the UNC-6/netrin cue (the canonical ligand for UNC-40) nor other well-characterized guidance cues such as the slits, ephrins and semaphorins were found to be the primary cue for muscle arm extension. This suggested that muscle arms were likely responding to a novel guidance cue.

In this thesis, I describe the cloning and characterization of MADD-4, a novel secreted cue that diffuses and attracts muscle arms and sensory axons along the dorsoventral axis in *C. elegans*. MADD-4 is a member of the non-enzymatic ADAMTSL family of proteins and is well
conserved among animals. Very little is known about the biological role of any of MADD-4’s orthologs.

Together with Kevin Chan, I found that MADD-4’s guidance function is dependent on an EVA-1-UNC-40 co-receptor complex. We found that MADD-4 interacts with both EVA-1 and UNC-40. Similarly, we found that EVA-1 and UNC-40 likely physically interact and this interaction is critical for the MADD-4 response. Furthermore, we found that the binding of EVA-1 to UNC-40 increases UNC-40’s sensitivity to MADD-4. This enhanced sensitivity becomes especially meaningful within a field of other ligands capable of binding UNC-40 like UNC-6. In the absence of UNC-6, UNC-40’s responsiveness to MADD-4 becomes less dependent on EVA-1. Hence, by regulating UNC-40’s sensitivity to MADD-4, EVA-1 may increase the precision by which UNC-40-directed processes can reach MADD-4-expressing target cells.

Collectively, the work discussed in this thesis recounts the first description of a novel guidance cue and its mechanism of action. Furthermore, since the biological role of any ADAMTSL family member outside of MADD-4 is largely unknown, it is very likely that my work on MADD-4 will broaden our understanding of the biological role of the ADAMTSL family of proteins.
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Dedication:

To My Loving Parents
Table of Contents
Abstract ............................................................................................................................................................................ ii
Acknowledgements ........................................................................................................................................................ iv
Dedication ......................................................................................................................................................................... vi
List of Figures ................................................................................................................................................................... ix
List of Tables ...................................................................................................................................................................... xi
List of Abbreviations ...................................................................................................................................................... xii

Chapter One: General Introduction ................................................................................................................................. 1
1.1 Directed Cell Migration .................................................................................................................................................. 2
1.2 Directed Membrane Extension- An Overview ................................................................................................................ 4
1.3 Challenges Faced by the Guidance Field .................................................................................................................... 6
1.4 The Netrin Family of Guidance Cues and their Receptors: A Brief Overview ....................................................... 8
   1.4.1 Discovery of Netrins ........................................................................................................................................ 10
   1.4.2 Netrin Structure ............................................................................................................................................. 14
   1.4.3 Molecular Regulators of Netrin Signaling .................................................................................................. 15
1.5 The Slit Family of Guidance Cues and Their Receptors .............................................................................................. 21
   1.5.1 Intracellular Modulators of Slit-Robo Signaling .......................................................................................... 24
1.6. Extracellular Matrix (ECM) Regulators of Directed Migration During Development ..................................... 25
   1.6.1 Heparan Sulfate Proteoglycans (HSPGs) ................................................................................................. 26
   1.6.2 The TSR Superfamily ................................................................................................................................... 28
1.7 Caenorhabditis elegans is an Ideal Model System for Genetic Analyses ............................................................. 32
   1.7.1 Muscle Arm Extension in C. elegans as a Model for Directed Membrane Extension ............................. 33
1.8. Overview of Doctoral Research Project .................................................................................................................. 37

Chapter Two: The Discovery and Characterization of ADAMTSL/MADD-4, A Novel Secreted 
Cue Required for Midline-Oriented Guidance in Caenorhabditis elegans ................................................................. 39
2.1 Abstract ........................................................................................................................................................................ 40
2.2 Introduction ............................................................................................................................................................... 41
2.3 Results ......................................................................................................................................................................... 45
   2.3.1 A Forward Genetic Screen for Madd Mutants Reveals MADD-4, an ADAMSL Ortholog ......................... 45
   2.3.2 MADD-4B is Necessary for Muscle Arm Extension .................................................................................. 53
   2.3.3 MADD-4 Likely Functions from the Midline Motor Neurons to Guide Muscle Arm Extension ... 54
   2.3.4 MADD-4 localization to the Dorsal Midline is Dependent on the UNC-104 Anterograde Kinesin .......... 59
   2.3.5 Ectopic Expression of MADD-4 Redirects Muscle Arm Extension in an UNC-40-Dependent Manner .................................................. 62
   2.3.6 A Domain Analysis of MADD-4 Indicates that TSR4 and the IG Domain are both Necessary and Sufficient for Activity ..................................................................................................................... 65
   2.3.7 MADD-4 is a Secreted Cue that Diffuses .................................................................................................. 68
   2.3.8 MADD-4 Cooperates with UNC-6 to Guide Ventral Muscle Arm Extension ........................................ 68
   2.3.9 MADD-4 Guides Axons along the Dorsal-Ventral Axis ............................................................................ 70
   2.3.10 Determining the influence of Heparan Sulfate Proteoglycan Core Proteins on MADD-4’s Ability Direct Migrations Along the Dorsoventral Axis ......................................................................................... 74
   2.3.11 An ADAMTSL3 Mutation implicated in Colorectal Cancer Disrupts MADD-4 Activity ...................... 77
2.4. Discussion ................................................................................................................................................................. 81
   2.4.1 MADD-4 Attracts Cell-Extensions in an UNC-40 Dependent Fashion ..................................................... 81
Chapter Three: MADD Attracts Midline-Oriented Migrations through an EVA-1-UNC-40 co-Receptor Complex in *Caenorhabditis elegans* ................................................. 93

3.1 Abstract ................................................................................................................. 94
3.2 Introduction ............................................................................................................. 95
3.3 Results ...................................................................................................................... 98
  3.3.1 EVA-1 Functions in a MADD-4 Pathway ......................................................... 98
  3.3.2 EVA-1 is a MADD-4 Receptor ....................................................................... 104
  3.3.3 EVA-1 Functions within an UNC-40 Complex ............................................. 114
  3.3.4 UNC-6 Interferes with UNC-40’s Interaction with MADD-4 ................. 118
  3.3.5 EVA-1 Sensitizes UNC-40 to the MADD-4 Cue ........................................ 119
  3.3.6 EVA-1 and UNC-40 Direct Axon Extension Towards MADD-4 .......... 121

3.4. Discussion ............................................................................................................. 126

3.5 Materials and Methods ....................................................................................... 131
  3.5.1 Nematode Strains, Counts, and Microscopy ............................................... 131
  3.5.2 Statistical Analyses ......................................................................................... 132
  3.5.3 A Forward Genetic Screen for Mutants that Suppress Muscle Arm Redirection towards CAN-expressed MADD-4 .................................................. 132
  3.5.4 Molecular Biology and Transgenics ................................................................. 134
  3.5.5 Co-immunoprecipitation and Western Analysis ........................................... 135
  3.5.6 HEK293T Cell Surface Binding Experiments .............................................. 137
  3.5.7 MADD-4-EVA-1 Interaction Analysis in vivo ............................................. 138
  3.5.8 Characterizing Sub-Cellular Localization of EVA-1 and UNC-40 ............. 138

Chapter 4: Discussion .................................................................................................. 139

4.1 MADD-4 is a Novel Guidance Cue Required for Midline-Oriented Guidance .... 140
4.2 Human Orthologs of MADD-4 and UNC-40 may have a Common Function .... 141
4.3 An EVA-1-UNC-40 Co-Receptor Complex Mediates Attraction Towards the MADD-4 Guidance Cue ................................................................................... 143
4.4 The EVA-1 Cytoplasmic Region is Critical for Muscle Arm Extension Towards MADD-4 .............................................................. 145
4.5 Exploring the role of ECM Components in Regulating MADD-4 Mediated Guidance .............................................................. 147
4.6 Extracellular Sugar Modifications May Regulate Muscle Arm Extension Towards MADD-4 .............................................................. 150
4.7 Concluding Remarks ............................................................................................. 151

References .................................................................................................................. 153
List of Figures

Figure 1.1 Regulation of Rho GTPases During Directed Membrane Extension: An Overview .......................................................... 7

Figure 1.2 Netrin Mediated Axon Migration to the Ventral Midline is Evolutionarily conserved ............................................................... 9

Figure 1.3 Canonical Receptors of Netrin Signaling .......................................................... 11

Figure 1.4 Embryonic Spinal Axon Outgrowth Assay ....................................................... 13

Figure 1.5 Molecular Regulators of Netrin Signaling ........................................................ 19

Figure 1.6 Slit-Robo Signaling ....................................................................................... 22

Figure 1.7 Muscle Arm Extension in Caenorhabditis elegans ........................................ 35

Figure 2.1 A Schematic of Selected Details of the C. elegans Neuromuscular Anatomy .. 43

Figure 2.2 Muscle Arm Extension Defects of madd-4 Mutants ........................................ 46

Figure 2.3 The Cloning of madd-4 ................................................................................. 49

Figure 2.4 A Multiple Sequence Alignment of MADD-4 and its Drosophila and human orthologs .................................................................. 51

Figure 2.5 Constructs Designed to Investigate madd-4’s Expression Pattern .............. 55

Figure 2.6 MADD-4’s Post-Embryonic Expression Pattern ........................................... 56

Figure 2.7 The Expression Pattern Driven by madd-4b Promoter Elements ............... 58

Figure 2.8 The Embryonic Expression Pattern Driven by madd-4b Promoter Elements. 60

Figure 2.9 Expression from a madd-4 Fosmid Reporter ................................................ 61

Figure 2.10 The UNC-104 Kinesin is Required for MADD-4 Localization to the Dorsal Nerve Cord .................................................................. 63

Figure 2.11 Ectopic Expression of MADD-4 from the CAN Neurons Attracts Muscle Arms to the Lateral Line ........................................... 66

Figure 2.12 Dorsally-Expressed MADD-4 Diffuses to the Ventral Midline .................... 69

Figure 2.13 Ectopic Expression of MADD-4 from the Dorsal Muscles Redirects the AVM and PVM Mehanosensory Axons Dorsally .................................................. 71

Figure 2.14 Determining the Domains Required to Mediate MADD-4’s ability to Direct Sensory Axon Guidance ................................................. 75
Figure 2.15 Determining the Ability of Selected HSPG Core Protein Mutants to Modulate MADD-4’s Guidance Function ................................................................. 78

Figure 3.1 EVA-1 Functions with UNC-40 to Mediate Muscle Arm Attraction Towards MADD-4........................................................................................................................................... 99

Figure 3.2 EVA-1 Functions in a MADD-4 Pathway to Counteract UNC-6 Interference . 103

Figure 3.3 EVA-1 Mediates MADD-4 Activity.......................................................................................................................... 105

Figure 3.4 Eva-1 Functions Cell-Autonomously in Muscles and Interacts with MADD-4107

Figure 3.5 Analyses of EVA-1 Domain Function..................................................................................................................... 110

Figure 3.6 MADD-4 Induces EVA-1 Endocytosis ................................................................................................................... 113

Figure 3.7 Characterizing the Sub-cellular Localization and Interaction Specificity of EVA-1 and UNC-40 ................................................................. 115

Figure 3.8 Analyses of the EVA-1 –UNC-40 Interaction........................................................................................................ 116

Figure 3.9 MADD-4’s Ability to Attract AVM Axons is Dependent on EVA-1 and UNC-40 ........................................................................................................... 122
List of Tables

Table 3.1. A Screen for Suppressors of MADD-4 Redirected Muscle Arms............101
List Of Abbreviations

Abl: Abelson
ADF: Actin Depolymerization Factor
ADAMTS: A Disintegrin and Metalloproteinase with Thrombospondin Repeats
ADAMTSL: A Disintegrin and Metalloproteinase with Thrombospondin Repeats Like
AVM: Anterior Ventral Microtubule

BWM: Body Wall Muscle

CAN: Canal Associated Neuron
CAM: Cell Adhesion Molecule
CFP: Cyan Fluorescent Protein
CNS: Central Nervous System
CoIP: Co-Immunoprecipitation

DCC: Deleted in Colorectal Cancer
DTC: Distal Tip Cell

ECM: Extracellular Matrix
Egl: Egg-laying
EMS: Ethyl Methane Sulfonate
EVA: Enhancer of unc-40 Ventral Axon Guidance defect

FGF: Fibroblast Growth Factor
FNIII: Fibronectin type-III

GABA: Gamma Aminobutyric Acid
GAG: Glycosaminoglycan
GAP: GTPase Activating Protein
GEF: Guanine nucleotide Exchange Factor
GFP: Green Fluorescent Protein
GDI: Guanine nucleotide Dissociation Inhibitor
GPI: Glycophosphatidylinositol

HEK293T: Human Embryonic Kidney cells expressing the simian virus 40 T antigen
him: high incidence of males
HS: Heparan Sulfate
HSN: Hermaphrodite Specific Neuron
HSPG: Heparan Sulfate Proteoglycan

IG: Immunoglobulin
IP: Immunoprecipitation

Ion: long
L-AchR: Levamisole-sensitive Acetylcholine Receptor
Madd: Muscle arm development defective
MAPK: Mitogen Activated Protein Kinase
mig: migration abnormal

PI3K: Phosphatidylinositol 3-Kinase
PLAC: Protease and Lacunin
PTK: Protein Tyrosine Kinase
PVM: Posterior Ventral Microtubule

RFP: Red Fluorescent Protein
Robo: Roundabout

SFK: Src-Family of Kinases
Sos: Son of Sevenless

TM: Trans Membrane
TSP: Thrombospondin
TSR: Thrombospondin Type I Repeat

UNC: Uncoordinated

VEGF: Vascular Endothelial Growth Factor
VL11: Ventral Left Muscle 11
VL19: Ventral Left Muscle 19

WASP: Wiskott-Aldrich Syndrome Protein
WAVE: WAS-family Verprolin homologous protein
WT: Wild-Type

YFP: Yellow Fluorescent Protein
Chapter One: General Introduction
1.1 Directed Cell Migration

Directed cell migration is a fundamental process that governs numerous aspects of normal animal development including tissue morphogenesis, wound healing and immune responses (Lara & Schneider 2013). During directed cell migration, migrating cells and plasma membrane extensions, such as axons of neurons, become polarized and are subsequently directed along a specific path towards a target site. The migration of cells and membrane extensions is tightly regulated by the action of attractive and repulsive chemotrophic guidance cues (Tessier-lavigne et al. 1996). For example, the axons of the commissural spinal neurons are attracted towards the ventral floor plate of the developing vertebrate spinal cord by the netrins. Repulsive cues on the other hand, facilitate directed cell migration by providing a repulsive spatial barrier such that migrating cells and cell extensions are steered away from the source of the repulsive cue (Song & Poo 2001). Importantly, it is the combined action of both attractive and repulsive cues that enable migrating cells and membrane extensions to successfully reach their target sites (Song & Poo 2001). In most multi-cellular organisms including humans there are four well-established families of guidance cues namely the netrins, slits, semaphorins and the ephrins. Netrins primarily function as attractive guidance molecules while the slits, semaphorins, and ephrins act as repulsive cues (Kolodkin et al. 2011).

Errors in genes that act within guidance pathways are known to result in a variety of developmental abnormalities and diseases. Importantly, in humans the disruption of directed cell migration results in a host of severe congenital diseases including Opitz Syndrome, Miller-Dieker Syndrome, X-linked Kallman’s Syndrome and Down Syndrome (Alexander et al. 2010; Nigro et al. 1997; Hardelin et al. 1999; Yamakawa et al. 1998). Furthermore, in metastatic
cancers, tumor cells often rely on the protein machinery normally used by migrating cells to invade and colonize distant tissues where they initiate growth of new tumors (Muller et al. 2001; Ellis & Hicklin 2008; Yang & Weinberg 2008; Lara & Schneiderw 2013). Hence, a thorough understanding of the underlying genetic mechanisms governing directed cell migration is central to our ability to develop more powerful therapeutic measures against migration-related diseases.

Given the fundamental role of directed cell migration in animal development, it is unsurprising that many key molecular components of the guidance machinery are evolutionarily conserved among a wide range of vertebrate and invertebrate animal species. This has provided a fertile ground for research initiatives aimed at interrogating the molecular underpinnings of directed cell migration by employing relatively simple organisms like the fruit fly *Drosophila melanogaster* and the free-living tiny nematode *Caenorhabditis elegans* as animal models to investigate the genetic regulation of directed cell migration.

The use of animal models has been central to the discovery of several key genetic regulators of directed cell migration that have conserved roles in more complex animals including humans. For example, the slit guidance cue and its receptor robo, which play a central role in the normal development of the nervous system in most complex animals, including humans, were first discovered from genetic screens carried out in *Drosophila* (Dickson & Gilestro 2006). Similarly, the netrin guidance cue along with its receptors DCC and UNC-5 were first identified and understood in *C. elegans* as key components required for the proper wiring of the nervous system. Later studies revealed that the orthologs of netrin and its receptors DCC and UNC-5 also played a central role in the wiring of the mammalian nervous system through similar molecular mechanisms (Moore et al. 2007). Hence, the use of animal
model systems has been invaluable towards furthering our understanding of directed cell migration.

1.2 Directed Membrane Extension- An Overview

Directed membrane extension is a highly sophisticated process that involves a series of sequential changes occurring within the migrating cell resulting in the directed extension of a plasma membrane in response to extracellular guidance cues. The first step towards the directed extension of a membrane involves sensing and interpreting attractive and repulsive guidance molecules in the environment.

The binding of an attractive or repulsive guidance cue to specific cell-surface receptors on the migrating cell is thought to result in the activation of distinct signaling cascades that converge upon the modulation of cytoskeletal elements within the cell (Bouquet & Nothias 2007). The initial step in the signaling cascade often involves phosphorylation of the guidance receptor (Tong et al. 2001; Palmer et al. 2002; Round & Stein. 2007), which then leads to the activation of the RHO GTPases either directly or indirectly. Members of the RHO GTPase family, which include RHO, Rac and Cdc42, primarily function as modulators of the actin cytoskeletal dynamics (Bouquet & Nothias 2007).

Regulation of the Rho-GTPases

The RHO GTPases function as molecular switches by alternating between a GTP bound active state and a GDP bound inactive state (Hall. 1998). The activity of these GTPases is regulated by three factors: Guanine nucleotide exchange factors (GEFs) function as activators of Rho GTPases by binding to a GTPase substrate and promoting the release of GDP in exchange for GTP. GTPase activating proteins (GAPs) on the other hand, favor the inactive
state of the GTPases by catalyzing the hydrolysis of GTP into GDP. Finally, Guanine nucleotide
dissociation inhibitors (GDIs) also function to maintain GTPases in an inactive state by
preventing the dissociation of GDP (Moon & Zheng 2003; Schmidt et al. 2002). In addition, Rho
GTPase activity is also regulated by cyclic second messenger nucleotides within the cell. For
example, the cAMP-dependent protein kinase A (PKA) has been shown to modulate Rho
GTPase activity by inhibiting Rho GEF function (Manganello et al. 2003) while activating the
Rho-GDIs (Ellerbroek et al. 2003). RHO GTPase activation leads to important changes in the
cytoskeletal dynamics. Their effects mainly converge on two key outcomes: actin
polymerization/depolymerization (Bouquet & Nothias 2007). Upon activation, Cdc42 and RAC
stimulate the activity of the Arp2/3 (Actin related protein) complex, a key regulator of the actin
cytoskeleton (Dickson. 2001; Ridley. 2001). The Arp2/3 complex promotes the formation of
elaborate networks of actin by stimulating de novo polymerization of actin monomers and by
binding existing actin filaments and stimulating the formation of new actin branches (Bouquet
& Nothias 2007). Nucleation promoting factors Wiskott–Aldrich syndrome protein (WASP),
and WAS-family verprolin-homologous protein (WAVE) function to bring actin monomers
together with the Arp2/3 complex (Bouquet & Nothias 2007).

Depolymerization of actin is induced by the actin depolymerization factor (ADF) cofillin.
The activity of cofilin/ADF is stimulated by the slingshot (SSH) phosphatase and inhibited by
the LIM kinase (Bouquet & Nothias 2007). RAC and Cdc42 promote actin polymerization by
stimulating LIM kinase activity (Edwards et al. 1999) while Rho and its downstream effector
ROCK promote actin depolymerization by stimulating the activity of SSH (Hsieh et al. 2006).

Upon binding to their receptors, repulsive guidance cues in general induce localized
membrane collapse at the leading edge by promoting actin depolymerization (Bouquet &
Nothias 2007). Work done by several groups suggests that chemorepellent-induced membrane collapse is achieved by inducing the activity of Rho while simultaneously inhibiting the activity of Cdc42 and RAC (Bouquet & Nothias 2007). For example, the binding of semaphorins to the plexin receptors has been shown to activate RhoA while simultaneously inactivating Rac through sequestration (Hu et al. 2001). Hence, upon encountering repulsive guidance cues, membrane extensions like axonal growth cones collapse and turn away from the source of the inhibitory factors (Liu & Strittmatter 2001; Aizawa et al. 2001; Sasaki et al. 2002; Jurney et al. 2002). On the other hand, attractive guidance cues promote the activity of Cdc42 and RAC while inhibiting Rho function. This in turn stimulates actin polymerization at the leading edge, resulting in the directed extension of a plasma membrane towards the source of cue (Bouquet & Nothias 2007) [Figure 1.1]. However, it is the combined action of both attractive and repulsive cues that ensures the successful navigation of migrating membrane extensions towards their target sites.

1.3 Challenges Faced by the Guidance Field

Despite the discovery and characterization of several key molecules involved in the transduction of extracellular spatial information towards the directed migration of a plasma membrane, many gaps still remain in our understanding of directed cell migration and directed membrane extensions. For instance, there are directed migration events where neither the guidance cues nor the receptors involved are known (Honigberg & Kenyon 2000; Yu et al. 2002; Bülow et al. 2008; James et al. 2013). Furthermore, many guidance receptors have been found to be capable of binding multiple guidance cues within the same spatial environment and similarly many guidance cues have been shown to bind multiple receptors.
Figure 1.1. Regulation of the Rho GTPases During Directed Membrane Extension: An Overview. The binding of attractive (orange circles) or repulsive (purple rectangles) guidance cues to specific cell-surface receptors gives rise to distinct cellular responses resulting from the modulation of Rho GTPase activity. Attractive cues promote membrane extension by activating Cdc42 and RAC, which in turn induce the activity of components of the WASP and WAVE families, which promote the assembly of the actin cytoskeleton by activating the Arp2/3 complex. Repulsive cues promote membrane collapse at the leading edge by stimulating the activity of Rho and downstream effectors like Rock, which promote actin depolymerization by inducing the activity of cofilin. Schematics adapted from Bouquet & Nothias 2007.
(Ahmed et al. 2011; Haddick et al. 2014). Hence, how migrating cells are able to interpret and integrate spatial information from several distinct guidance cues such that they are able to extend a single stereotypical trajectory towards their target sites is not well understood. Addressing this question has been one of the central focuses of my doctoral research. In the following sections, I will review the current state of knowledge on a select few families of guidance molecules, their receptors and downstream signaling pathways. I have focused on those guidance molecules that are most relevant to the body of my thesis. This will serve as a foundation towards understanding the mechanism of action of the novel guidance cue MADD-4, which will be described in detail in Chapters two and three.

1.4 The Netrin Family of Guidance Cues and their Receptors: A Brief Overview

The netrins comprise a family of guidance proteins that direct the migration of cells and membrane extensions during development. In mammals, four secreted forms of netrin (netrin-1, 2, 3 & 4) and two membrane-anchored forms (netrin-G1 &G2) have been identified (Rajasekharan & Kennedy 2009). Netrins are evolutionarily conserved and netrin orthologs share highly conserved roles at the midline of developing vertebrate and some invertebrate species to ensure the proper development of the central nervous system (CNS), (Rajasekharan & Kennedy 2009). In vertebrates, netrin-1 is secreted by the floor plate cells at the ventral midline and forms a ventral to dorsal gradient that is sensed by migrating axonal growth cones (Kennedy et al. 1994; Serafini et al. 1996). Similarly, in invertebrates like Drosophila and C. elegans, netrin is expressed from cells located at the ventral midline and attracts axonal growth cones towards the ventral midline (Rajasekharan & Kennedy 2009; Moore et al. 2007) [Figure 1.2].
Figure 1.2. Netrin-Mediated Axon Migration to the Ventral Midline is Evolutionarily Conserved. (A). In vertebrates, netrin is secreted by the floor plate cells at the ventral midline and forms a ventral-dorsal gradient and attracts several classes of migrating axons towards the ventral midline. (B). In the fruit fly Drosophila, netrins A and B are expressed by the midline glial cells and attracts the axons of commissural neurons towards the ventral midline. (C). Early neural development in the nematode C. elegans, is marked by the migration of neuronal axons towards a row of ventral epidermoblast cells that express UNC-6/netrin. The netrin gradient is represented in blue in A, B and C. Schematics adapted from Moore et al 2007.
Interestingly, the netrin gradient has been shown to have a dual role in that it functions as an attractant for some classes of axons and as a repellent for others, based on the expression of specific cell-surface receptors (Chan et al. 1996; Colamarino & Tessier-lavigne 1995). A wide variety of receptors including integrins, the Down Syndrome Cell Adhesion Molecule (DSCAM) and the Adenosine A2b receptor are implicated in mediating the axon guidance functions of netrins (Ly et al. 2008; McKenna et al. 2008). However, two families of receptors, namely DCC (Deleted in Colorectal Cancer) and UNC-5 (which includes UNC-5A, B, C & D) are widely considered to be canonical receptors for secreted netrins (Song & Poo 2001). Axons expressing DCC are attracted towards netrin while those expressing any of the UNC-5 receptors or UNC-5 together with DCC are repelled from the source of netrin (Song & Poo 2001) [Figure 1.3]. Outside of their role in the nervous system, netrins have also been found to effect tissue morphogenesis by directing cell migration and modulating interactions between migrating cells and the extracellular matrix (ECM).

1.4.1 Discovery of Netrins

Towards the end of the 19th century, the Spanish neuroscientist Santiago Ramón Cajal proposed that the migration of axonal growth cones were directed by secreted chemotropic factors. Using fixed sections, he noticed that dorsal commissural spinal axons within the embryonic spinal cord projected towards the floor plate cells found at the ventral midline. This finding led Cajal to hypothesize that the ventral floor plate was the source of a secreted chemoattractant that diffused and formed a gradient in the surrounding environment. He further postulated that this chemoattractant gradient was required to attract commissural axonal growth cones towards the ventral midline (Moore et al. 2007). His insight would later
Figure 1.3. Canonical Receptors of Netrin Signaling. The DCC family of transmembrane receptors mediates the netrin-induced chemoattractive response while the UNC-5 family mediates the netrin-induced chemorepulsion. FNIII domains IV and V of DCC and both IG domains of UNC-5 are implicated in netrin binding.
serve as a keystone in the discovery of the netrins and other secreted guidance cues towards the close of the 20th century.

Compelling evidence for the chemotropic model of axon guidance started to emerge during the late 1980’s and the early 1990’s. Dorsal explants from an embryonic rat spinal cord, when cultured at a distance from ventral floor plate explants, resulted in axon outgrowth directed towards the floor plate explants regardless of its position (Placzek et al. 1990; Tessier-Lavigne. 1988) [Figure 1.4]. This strongly suggested that the ventral floor plate is indeed a source of secreted chemotrophic factors that attracts axon extensions.

In parallel, genetic screens carried out in the nematode C. elegans by Sydney Brenner revealed several key genes involved in the regulation of circumferential axon guidance (Brenner 1974). One of the genes to come out of his screen, unc-6, was found to encode a secreted protein with a high degree of homology to the vertebrate laminins (Ishii et al. 1992). UNC-6 is expressed by the motor neurons along the ventral nerve cord and unc-6 loss-of-function mutants display an uncoordinated movement phenotype due to extensive motor axon guidance defects along the dorsoventral axis (Wadsworth et al. 1996; Hedgecock et al. 1990). Commissural axons of the DA, DB, VD and DD class of motor neurons that normally migrate towards the dorsal midline, away from the source of UNC-6 are disrupted in unc-6 loss-of-function mutants. In addition, the axon trajectories of the AVM mechanosensory neuron and the hermaphrodite specific neuron (HSN) that typically extend towards the source of UNC-6 at the ventral midline are also disrupted in unc-6 loss-of-function mutants (Hedgecock et al. 1990). These observations suggested that UNC-6 is a central regulator of axon guidance along the dorsoventral axis where it functions to attract certain classes of axons while repelling others.
Figure 1.4. Embryonic Spinal Axon Outgrowth Assay. A dorsal explant of an embryonic rat spinal cord, containing the cell bodies of commissural neurons, was fixed within a collagen matrix. Under control conditions as shown on the left, commissural axon extensions were found to remain within the explant. However, when an explant containing the cells of the ventral floor plate was added to the collagen matrix (As shown on the right), commissural axon extensions were found to emerge out of the dorsal explant and grow into the collagen matrix in the direction of the ventral floor plate explant. This suggested that the cells of the ventral floor plate were the source of a secreted chemoattractant (shown in blue) that attracted the commissural spinal axons through the collagen matrix towards the floor plate explant. Schematics adapted from Moore et al 2007.
In 1994 Marc Tessier-Lavigne’s group purified two secreted proteins from chick embryonic brain extracts that elicited commissural axon outgrowth in vitro, mimicking the properties of the rat ventral floor plate explants. Subsequently, the two secreted proteins were found to be homologs of UNC-6. In recognition of their ability to guide axonal growth cones, they were named netrin-1 and netrin-2 based on the Sanskrit word ‘netr’ which means, “to guide” (Kennedy et al. 1994; Serafini et al. 1994). Further studies conducted using the chick model revealed that netrin-1 is expressed at the ventral midline of the embryonic chick spinal neuroepithelium and attracts extending commissural spinal axons towards the ventral midline. Similar studies conducted using rodent models showed that netrin-1 activity is also critical for the proper migration of commissural spinal axons towards the ventral midline within the embryonic mouse spinal cord (Serafini et al. 1996; Kennedy et al. 2006). Hence, a century later, the chemotropic model of axon guidance originally proposed by Cajal was finally validated by the discovery of the netrins.

1.4.2 Netrin Structure

All netrins are comprised of roughly 600 amino acid residues and share an overall similar domain organization. Three characteristic domains found on all netrins include the N-terminal domains V and VI that are homologous to the corresponding N-terminal domains found in laminins and a C-terminal domain, C, that shares homology with members of the complement protein family and tissue inhibitors of metalloproteinases (TIMPS) (Rajasekharan & Kennedy 2009). The laminins are large trimetric secreted proteins that are composed of a α, β, and γ chain (Miner & Yurchenco 2004). The domains V and VI of netrins 1-3 are highly similar to the γ chain of the laminins while those of netrin-4 and netrins G1 & G2 are similar to the
laminins β chain (Yurchenco & Wadsworth 2004). Orthologs of netrins 1, 3, 4, G1 & G2 are widely expressed in mammals while the expression of netrin-2 orthologs have so far been detected only in zebrafish and chick (Park et al. 2005; Serafini et al. 1994). Apart from vertebrates, orthologs of netrins 1-3 are also found in evolutionarily divergent animals including *C. elegans, D. melanogaster* and the planarian *Schmidtea mediterranea* (Ishii et al. 1992; Mitchell et al. 1996; Harris et al. 1996; Cebrià & Newmark 2005).

The domain V of netrins is comprised of three-tandem epidermal growth factor (EGF) repeats; V-1, V-2 and V-3 that are composed of roughly 150 amino acids. In *C. elegans*, the V-3 domain primarily mediates the UNC-6/netrin attractive response while the V-2 domain mediates repulsion (Lim & Wadsworth 2002). Domain VI has been shown to play a central role in both the attractive and repulsive response of UNC-6. The C-terminal domain C of UNC-6 is rich in basic amino acids and is thought to interact with the negatively charged sugar residues associated with ECM components such as the heparan sulfate proteoglycans (HSPGs) (Lim & Wadsworth 2002; Rajasekharan & Kennedy 2009). Removal of UNC-6’s C-terminal domain C while having little impact on axon guidance, results in increased axon branching. It is speculated that the C-terminal domain C of UNC-6 may function to repress signaling events that promote axon branching. Further investigation is required to elucidate the role of the C-terminal domain C of netrins (Lim & Wadsworth 2002; Rajasekharan & Kennedy 2009).

**1.4.3 Molecular Regulators of Netrin Signaling**

Netrins are known to interact with a diverse array of cell-surface receptors to elicit distinct chemotropic responses. The binding of netrin-1 to its receptors has been shown to modulate cytoskeletal assembly primarily through regulating the actin cytoskeletal dynamics.
Attraction to secreted netrins is mediated through the DCC family of transmembrane proteins while the UNC-5 family mediates repulsion (Rajasekharan & Kennedy 2009) [refer Figure 1.3]. In vertebrates, members of the DCC family include DCC and neogenin (Rajasekharan & Kennedy 2009). DCC orthologs in the invertebrate models C. elegans and Drosophila are known as UNC-40 and Frazzled respectively (Chan et al. 1996; Kolodziej et al. 1996). DCC, neogenin and their orthologs are single pass transmembrane proteins that are comprised of four extracellular Immunoglobulin (IG) domains followed by six fibronectin type-III (FNIII) repeats [see Figure 1.3], where the fourth and fifth FNIII repeat of DCC have strongly been implicated in mediating binding to netrin-1 (Cho 1994; Vielmetter 2009; Geisbrecht et al. 2003; Kruger et al. 2004; Finci et al. 2014). Immediately following the transmembrane domain is a cytoplasmic region comprising three short conserved motifs named P1, P2 and P3 [see Figure 1.3]. The cytoplasmic motifs of DCC contain several conserved phosphorylation sites and are thought to play a critical role in the recruitment of downstream signaling components (Rajasekharan & Kennedy 2009). However, the functional role of each of the three cytoplasmic motifs of DCC is not fully understood and awaits further investigation.

In vitro studies conducted using embryonic rat spinal explants have shown that blocking DCC activity via an anti-DCC antibody inhibits axon outgrowth in response to netrin-1. Furthermore, DCC inactivation in mice disrupts the migration of spinal commissural motor axons towards the source of netrin-1 at the ventral midline. Finally, DCC has also been shown to physically interact with netrin-1 (kd= 10^{-8}) (Keino-masu et al. 1996). A recent study has elucidated the crystal structure of human netrin-1 bound to the DCC receptor (Finci et al. 2014). The crystal structure reveals that netrin-1 can simultaneously associate with two DCC molecules through a DCC- specific bindings site and a generic binding site located within the V
domain of netrin-1 (Finci et al. 2014). Consistent with the findings from vertebrate model systems, genetic removal of UNC-40 in *C. elegans* disrupts the migration of axon trajectories towards sources of UNC-6 (Chan et al. 1996). In *C. elegans*, UNC-40 is expressed by many neurons including the AVM and HSN whose axons migrate towards sources of UNC-6 at the ventral midline. Genetic removal of UNC-40 disrupts the ventral migration of the AVM and HSN axons (Chan et al. 1996; Alexander et al. 2009; Gitai et al. 2003). UNC-40’s ability to mediate the UNC-6 response in the AVM neuron is dependent on the activity of UNC-115 (A LIM domain containing protein), UNC-34/Enabled and CED-10/RAC (Gitai et al. 2003). Together, these proteins function to modulate the actin cytoskeletal dynamics at the leading edge of the AVM axonal growth cone in response to UNC-6.

Additional studies from vertebrate models have highlighted a key role for the Src-family of kinases (SFKs) and protein tyrosine kinase 2 (PTK2 also called as focal adhesion kinase, FAK) in mediating netrin attraction. Both PTK2 and Src modulate netrin signaling by phosphorylating tyrosine residues within the cytoplasmic domain of DCC. In addition, Src when activated by netrin-1 phosphorylates PTK2, giving rise to a binding site for Grb2, an adaptor protein. The Grb2-PTK2 interaction is thought to result in the activation of the mitogen activated protein kinase (MAPK) cascade, which has been implicated in regulating axon outgrowth and turning responses towards netrins. Furthermore, PTK2 has also been shown to bind and activate phosphatidylinositol 3-kinase (PI3K), another key modulator of netrin signaling (Round & Stein 2007).

In *C. elegans*, the PI3K homolog AGE-1 was shown to function downstream of UNC-40 in mediating netrin-induced axon outgrowth from the HSN neuron. The HSNs comprise a pair of bilaterally symmetrical neurons that regulate the egg laying behavior of the worm. The
HSNs express UNC-40 and extend axons that are directed towards sources of UNC-6 at the ventral midline (Chang et al. 2006). In addition to AGE-1, UNC-40’s ability to mediate the UNC-6 response in the HSNs is dependent on the Rho GEF UNC-73/Trio, UNC-34/Enabled and MIG-10 (the C. elegans homolog of lamellipodin). AGE-1 is a stimulator of MIG-10 activity, which in turn modulates the function of Rho GTPases resulting in the reorganization of the actin cytoskeletal dynamics and the extension of axon outgrowth towards UNC-6 (Adler et al. 2006; Bouquet & Nothias 2007). Finally, studies conducted in vertebrate models have also shown that PTK2 can bind and phosphorylate N-WASP, a modulator of the actin cytoskeletal dynamics at the leading edge of several axonal growth cones (Wu et al. 2004; Round & Stein 2007).

Together, these studies illustrate a possible signaling mechanism by which netrin-induced axon attraction is mediated by the DCC receptor [Figure 1.5].

The UNC-5 family of receptors was first recognized as mediators of the UNC-6 repulsive response based on studies conducted in C. elegans. UNC-5 is the sole member of the UNC-5 receptor family in C. elegans and loss-of-function mutations in unc-5 were found to disrupt the migration of axons that normally project away from sources of UNC-6 at the ventral midline (Leung-hagesteijn et al. 1992). Furthermore, misexpression of UNC-5 in neurons that normally extend an axon ventrally, towards the source of UNC-6, caused their axon trajectories to be redirected dorsally away from the source of UNC-6 (Hamelin et al. 1993). As in C. elegans, a single member represents the UNC-5 receptor family in D. melanogaster (Keleman & Dickson 2001). In mammals, four members: UNC-5A, B, C and D, represent the UNC-5 receptor family. UNC-5 is comprised of two extracellular IG domains that are implicated in netrin binding followed by two extracellular thrombospondin type I repeats (TSRs) (Rajasekharan & Kennedy 2009; Geisbrecht et al. 2003; Hong et al. 1999). The UNC-5 intracellular region is composed of
Figure 1.5. Molecular Regulators of Netrin Signaling. Key signaling components that function downstream of the DCC and UNC-5 receptor families is shown. The binding of netrin to the DCC receptor activates a signaling cascade involving several classes of proteins (color coded according to their family and function) that ultimately converge upon the reorganization of the actin cytoskeletal dynamics resulting in the directed extension of a plasma membrane towards the source of netrin. Signaling events downstream of the UNC-5 receptor remain poorly understood. The Shp2 tyrosine phosphatase has been implicated in mediating the netrin-induced chemorepulsion through UNC-5, but further investigation is required to elucidate its role in the UNC-5 signaling cascade. Question marks (??) denote gaps in our current understanding of some of the molecular events associated with the netrin-induced attractive and repulsive responses. Schematics adapted from Rajasekharan & Kennedy 2009.
a ZU5 domain, a DCC-binding domain and a death domain (refer Figure 1.4). The ZU5 domain shares sequence homology with the Zona Occludens-1 scaffolding protein found at the tight junctions, but its function remains to be elucidated (Rajasekharan & Kennedy 2009).

Studies conducted in both vertebrate and invertebrate models suggest that UNC-5 on its own is capable of mediating short-range repulsion from sources of netrin (Rajasekharan & Kennedy 2009). UNC-5 has also been shown to physically interact with netrin-1 through its IG domains (Hong et al. 1999). Furthermore, a recent study has demonstrated that UNC-5 can simultaneously interact with the generic receptor-binding site on the netrin-1 V domain and to DCC via its cytoplasmic DCC-binding domain (Finci et al. 2014). The resulting netrin-1/DCC/UNC-5 ternary complex is thought to initiate a signaling cascade that results in the long-range netrin repulsion response (Finci et al. 2014). One explanation for the cooperation between the UNC-5 and DCC receptors in mediating the long-range netrin repulsion response may be that the binding of UNC-5 to DCC sensitizes the complex to lower concentrations of netrin as the axonal growth cones migrate away from the source of netrin. Several molecules have been implicated in UNC-5-mediated netrin repulsion including the Src1 tyrosine kinase, the Shp2 tyrosine phosphatase and the F-actin capping protein Mena (Round & Stein 2007). Studies using mice have shown that Shp2 can physically interact UNC-5c (also known as RCM) in response to netrins. However, the functional relevance of the Shp2-UNC-5c interaction is currently unclear and awaits further investigation (Round & Stein 2007). In addition, Shp2 has also been shown to modulate the activities of PI3K and RhoA in response to netrins. Further investigation is required to disentangle the roles of the different intracellular proteins implicated in regulating the UNC-5-mediated repulsive response to secreted netrins. Figure
1.5 provides a graphical overview of some of the molecular regulators implicated in the UNC-5 mediated netrin repulsion pathway.

1.5 The Slit Family of Guidance Cues and Their Receptors

The *slit* gene was first discovered from genetic screens conducted in *Drosophila* aimed at uncovering regulators of embryonic cuticle patterning and from subsequent screens for regulators of commissural axon guidance. In *slit* loss-of-function mutants, commissural axonal tracts fail to exit the midline upon entering it and instead collapse onto the midline. This suggested a role for *slit* as a repulsive guidance factor required to prevent axonal tracts from stalling at the midline. Later studies identified the midline glial cells as the site of *slit* expression in flies. Slit is widely conserved between both invertebrate and vertebrate species including humans. Invertebrates such as *C. elegans* and *Drosophila* contain just single *slit* protein, while mammals have at least three *slit* proteins Slit-1, Slit-2 and Slit-3 (Dickson & Gilestro 2006).

The *slit* proteins are large secreted glycoproteins (~195KD) that share a common domain organization comprising an N-terminal signal peptide followed by four leucine rich repeats (LRRs), seven to nine EGF-like repeats, a laminin G-like domain and a C-terminal cysteine rich domain. Most *slit* proteins are proteolytically cleaved within the N-terminal EGF repeats, but the functional relevance of this cleavage remains to be elucidated (Dickson & Gilestro 2006; Brose et al. 1999) [Figure 1.6]. In mice, the genetic removal of the three *slit* proteins results in axon guidance defects similar to but less severe than those observed in *Drosophila slit* mutants. In *C. elegans*, SLT-1 (the worm ortholog of *slit*) is expressed mainly from the dorsal body-wall muscles and functions to repel the axon of the AVM...
Figure 1.6. Slit-Robo Signaling. The binding of slit to the robo receptor results in the recruitment of slit-robo GAP1(SrGAP1) and Vilse/cross GAP to the CC3 and CC2 intracellular domains of robo respectively. SrGAP1 inhibits the activity of RhoA and Cdc42 while Cross/Vilse GAP inhibits Cdc42 function. In addition, the slit-robo interaction also results in the recruitment of an adaptor protein DOCK to the CC2 and CC3 robo domains and in turn serves as a docking site for the son of Sevenless (Sos) GEF protein, which activates RAC1 GTPase. Together, these signaling events converge upon the modulation of the actin cytoskeletal dynamics. In addition, the Abelson (Abl) tyrosine kinase binds the CC3 robo domain and antagonizes robo signaling via phosphorylation of the CC1 domain. Schematics adapted from Ypsilanti et al 2010.
mechanosensory neuron towards the source of UNC-6/netrin at the ventral midline (Hao et al. 2001). Hence the studies from vertebrate and invertebrate animal models indicate a conserved midline repulsion role for the slit proteins.

The mechanism of action of slit largely remained obscure until further genetic and biochemical studies identified it as a ligand for the roundabout (robo) family of cell-surface receptors. Domain function analyses in vertebrate and invertebrate models like Drosophila have shown that the N-terminal LRRs of slit are both necessary and sufficient for its repulsive role in axon guidance (Chen et al. 2001; Ba-charvet et al. 2001). Furthermore, detailed biochemical studies in Drosophila have identified the second N-terminal LRR of slit as a binding site for the robo receptors (Howitt et al. 2004).

The first robo gene to be discovered, robo1 was identified from genetic screens conducted in Drosophila for regulators of midline crossing in the CNS. Subsequent studies revealed the existence of two additional robo genes, robo2 and robo3 that also have roles in midline crossing (Dickson & Gilestro 2006). In C. elegans, the sax-3 gene encodes the sole member of the robo receptor family (Zallen et al. 1998) while mammals have at least four robo genes (Dickson & Gilestro 2006). In sax-3 loss-of-function mutants, axons from several classes of neurons were found to cross the ventral midline multiple times, similar to the robo mutants in Drosophila. This suggested that the robo receptors were mediators of inhibitory guidance information at the ventral midline whereby midline- oriented axonal growth cones are prevented from re-crossing the midline upon their initial entry (Zallen et al. 1998; Zallen et al. 1999).

The robo receptors are members of the IG superfamily of cell adhesion molecules (CAMS) and are typically comprised of five N-terminal IG domains followed by three FNIII
domains, a single-pass transmembrane domain and a C-terminal cytoplasmic region that is devoid of any obvious sign of catalytic function (Kidd et al. 1998). The mammalian robo4 is the only exception in that it is comprised of two IG and two FNIII domains (Dickson & Gilestro 2006). *In vitro* biochemical assays suggest that the first two IG domains of robo likely mediate its interaction with slit (Liu et al. 2004). Although the cytoplasmic region of the robo receptors is poorly conserved, four conserved motifs named CC0 through CC3 have been identified (Dickson & Gilestro 2006; Bashaw et al. 2000), (See Figure 1.6 for the domain organization of robo).

**1.5.1 Intracellular Modulators of Slit-Robo Signaling**

The cytoplasmic CC motifs of robo are implicated in binding various intracellular signaling proteins. For instance, in the presence of slit, the CC1 and CC2 motifs of robo have been shown to interact with the Ena/VASP proteins that are known modulators of the actin cytoskeleton (Bashaw et al. 2000). In the presence of slit, the Rho GTPase activating protein (GAPs) Vilse/cross GAP has been shown to bind the CC2 robo motif and inactivate the Rac1 and Cdc42 GTPases (Hailan Hu et al. 2005; Lundström et al. 2004; Wong et al. 2001). Rac-1 function is also modulated by the GEF Sos (Son of Sevenless) which is recruited to the CC2-3 robo motif via the adaptor protein Dock (Dreadlocks). The SrGAP1 (slit-robo GAP1) protein has been shown to inhibit RhoA and Cdc42 activity by binding the CC3 robo motif. The Abl (Abelson) tyrosine kinase has been shown to phosphorylate the CC1 robo motif, resulting in the down regulation of robo signaling (Ypsilanti et al. 2010) (See Figure 1.6). These intracellular effectors orchestrate the slit-robo mediated repulsive axon guidance response through regulation of cell polarity and cytoskeletal dynamics.
The robo receptors are not the only receptors known to bind slit and vice versa. In *C. elegans*, SLT-1/slit has been shown to interact with EVA-1 (Enhancer of Ventral Axon guidance defects), a conserved transmembrane protein with extracellular galactose binding lectin domains. EVA-1 was shown to act as a co-receptor for SAX-3/robo in mediating the SLT-1-induced repulsion of the AVM axon towards the ventral midline. Through *in vitro* assays, EVA-1 was found to be capable of binding both SAX-3 and SLT-1. Genetic analysis suggests that EVA-1’s role within the slit pathway is bifunctional, in that it promotes SLT-1 signaling by enhancing the sensitivity of SAX-3 to SLT-1 while simultaneously dampening the UNC-6/netrin signaling through the UNC-40 receptor (Fujisawa et al. 2007). EVA-1’s ability to modulate the activity of the netrin pathway was found to be dependent on the cytoplasmic region of SAX-3 (Fujisawa et al. 2007). The ability of EVA-1 to sensitize guidance receptors to guidance cues will be the main subject of discussion in Chapter 3.

1.6. Extracellular Matrix (ECM) Regulators of Directed Migration During Development

The organization of neuronal connections into functional networks relies on the precise navigation of axonal growth cones through the ECM towards their target sites. The ECM is comprised of a heterogeneous mixture of diverse protein families including collagens, heparan sulfate proteoglycans (HSPGs), laminins, fibronectins, elastins, fibrillins and thrombospondins (Myers et al. 2011; Adams & Tucker 2000). The ECM proteins that regulate axonal growth cone migrations are generated from diverse cell types including neurons and the supporting glial cells and are secreted into the surrounding interstitial space where they serve as a source of biochemical and structural support (Myers et al. 2011). Below, I will focus on two classes of
ECM proteins: the HSPGs and the thrombospondin type I repeat (TSR) superfamily and will discuss their role in regulating directed migration events during development.

1.6.1 Heparan Sulfate Proteoglycans (HSPGs)

The proteoglycan superfamily is comprised of molecules that are characterized by the presence of one or more highly sulfated glycosaminoglycan (GAG) side chains that are covalently attached to a central protein core. HSPGs have been shown to modulate the activity of diverse signaling pathways mainly by regulating the distribution of secreted ligands and mediating ligand-receptor interactions (Kramer & Yost 2003; Myers et al. 2011). The ability of HSPGs to modulate axon guidance was first recognized from in vitro studies using cultured cockroach embryos, where it was observed that both the addition of exogenous heparan sulfate (HS) and its enzymatic removal disrupted the migration of pioneer axons (Kramer & Yost 2003). This led to the speculation that HS functioned as a modulator of axon guidance likely by influencing the activity of secreted guidance cues in the cellular environment. Later, investigations into the slit family of guidance molecules offered further evidence for the involvement of HSPGs in regulating directed migration events during development (Wang et al. 1999).

Biochemical studies using rodent models showed that mammalian Slit2 interacts with the GPI-anchored HSPG glypican-1 and the interaction becomes greatly diminished upon the enzymatic removal of HS side chains attached to glypican-1 (Ronca et al. 2001; Liang et al. 1999). Additional studies indicated that the interaction between slit proteins and HS may be biologically relevant. The removal of HS side chains following a heparinase treatment diminished the affinity of Slit2 for the robo receptor and abolished the Slit2 mediated-
repulsion of cultured olfactory axons, demonstrating a key role for HSPGs in mediating the Slit2 repulsive response (Huaiyu Hu 2001). Similarly, a recent study in *C. elegans* showed that highly specific rerouting of the DB7 motor axon along the dorsoventral axis could be achieved by modifying HS side chains attached to specific HSPG core proteins. The rerouting of the DB7 motor axon was found to be dependent on the HSPG core protein LON-2/glypican together with the SLT-1/slit guidance cue and the EVA-1 receptor (Bülow et al. 2008).

In addition, parallel studies conducted in *Drosophila* have also identified a role for the transmembrane HSPG core protein syndecan in the slit-mediated repulsive axon guidance at the midline (Steigemann et al. 2004; Johnson et al. 2004). Removal of syndecan activity in the neurons was found to induce midline-oriented axon guidance defects similar to those observed in *slit* loss-of-function mutants. Furthermore, in syndecan mutant flies, the presence of slit was detected on the surface of the midline-glial cells that typically secrete slit but not on the surface of migrating axons. This suggested that loss of syndecan activity disrupts the extracellular distribution of slit. Finally, syndecan expression from neurons but not the midline glial cells was able to rescue the mutant phenotype of *Drosophila* syndecan mutants (Steigemann et al. 2004; Johnson et al. 2004).

Taken together, the results from studies in both vertebrate and invertebrate model systems have identified a central role for HSPGs as regulators of axon guidance by their ability to modulate the Slit response. It is presently unclear as to whether HSPGs can also modulate the cellular response to other signaling pathways involved in axon guidance like the netrin pathway.
1.6.2 The TSR Superfamily

The thrombospondins (TSPs) comprise a family of proteins that are abundant in the ECM. Like most ECM proteins, thrombospondin family members are modular and are characterized by a series of repeated domains, the most prominent of which are the thrombospondin type I repeats or TSRs (Adams & Tucker 2000). Over the years, studies conducted by several groups have identified a diverse array of protein families that share TSRs. These include members of semaphorin 5, F-Spondin, UNC-5 gene families and many others including the ADAMTS and ADAMTSL family members (Adams & Tucker 2000; Tucker 2004; Burstn-cohen et al. 1999). Most of these proteins are expressed by neuronal tissues and display in vitro and in vivo properties that suggest a key role for these protein families in regulating axonal outgrowth and growth cone migrations. Furthermore, previous studies have identified both cell and ECM-binding motifs within the TSRs of thrombospondin-1 (TSP-1). Hence, it has been proposed that the presence of the TSRs may be integral to the biological role of these diverse protein families (Adams & Tucker 2000; Tucker 2004).

In the following sections I will review the literature on the biology of the TSR module and focus on two prominent TSR containing protein families namely the F-spondin and the ADAMTS proteases and their role in guidance.

The TSR Domain

The TSR was first identified among members of the TSP family (Adams & Tucker 2000). The TSP family comprises five multidomain proteins TSP-1, TSP-2, TSP-3, TSP-4 & TSP-5 that are characterized by the presence of several EGF-type repeats also known as the thrombospondin type II repeats and a set of E/F hand-type calcium binding repeats or thrombospondin type III repeats. TSP-1 and TSP-2 are unique in that in addition to the EGF-type repeats commonly
found on TSP family members, they contain an additional set of three domains, which came to be known as the TSRs (Adams & Tucker 2000). Studies from invertebrate models like C. elegans have shown that the TSR is an ancient domain that arose before the evolutionary divergence of nematodes and chordates (Hutter et al. 2000). The human genome encodes 41 TSR-containing proteins while the Drosophila and C. elegans genomes encode 14 and 27 TSR-containing proteins respectively. On the other hand, TSR-containing proteins are entirely absent from the genomes of the plant model Arabidopsis thaliana, the yeast Saccharomyces sp and several diverse prokaryotic organisms (Tucker 2004).

The TSR domain typically spans sixty amino acids in length and is related to a protein module present in the thrombospondin related anonymous protein or TRAP, the coat protein of the malarial parasite Plasmodium sporozoites and the members of the complement family of proteins C6, 7, 8 and 9. X-ray crystallography studies conducted on human TSP-1 reveal that each TSR is composed of three antiparallel strands A, B and C where A has a less defined and an irregular structure while B and C are β-sheets. Furthermore, computational studies reveal that the typical features of the TSRs found in human TSP-1, that allow for it’s compacted folding is highly conserved within the animal kingdom (Adams & Tucker 2000; Tucker 2004).

1.6.2.1 The TSR superfamily and Directed Migrations

Since the original discovery of TSRs in TSP-1, numerous other proteins that have TSR domains have been identified. Among these, the F-spondins and the ADAMTS proteases are two prominent TSR containing families of ECM proteins that are implicated in regulating migration events during development.

F-spondins
The F-spondins are TSR-containing ECM proteins that are predominantly expressed by the floor plate cells of the developing vertebrate neural tube. *In vitro* studies demonstrated that F-spondin can promote attachment and initiate neurite outgrowth from cultured rodent spinal explants. The ability of F-spondin to promote attachment was found to be dependent on HSPGs. F-spondins are well conserved mainly among vertebrates and have been detected at the floor plate in developing mice, zebrafish and chick embryos (Adams & Tucker 2000; Higashijima et al. 1997; Burstyn-cohen et al. 1999). A previous study using the chick model showed that inhibiting the TSRs of F-spondin using an antibody lead to the abnormal turning of several commissural axonal tracts as they reached the ventral floor plate. The injection of either the purified F-spondin or just the F-spondin TSRs into the lumen of the developing chick embryo resulted in the disruption of commissural axon trajectories (Burstyn-cohen et al. 1999).

Whether F-spondin plays an instructive role in directing commissural axonal tracts towards the floor plate or whether it mainly acts as an ECM attachment factor remains unclear and warrants further investigation. Nevertheless, these studies indicate a potential role for F-spondin as a guidance molecule that provides spatial information to migrating growth cones during development.

**The ADAMTS Proteases**

The ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Repeats) family of ECM proteins comprises a group of secreted metalloproteinases that were originally identified in mice by Kuno and colleagues in 1997 (Kouji Kuno et al. 1997). Subsequent studies have shown that ADAMTS family members are well conserved among the animal kingdom and influence a wide range of developmental processes including cell migration. ADAMTS proteases are characterized by the presence of an N-terminal signal peptide, a proteolytic pro-
domain followed by a region that shares 25-45% sequence identity to the snake venom disintegrins, one or more TSRs, a cysteine rich domain (CRD) and a C-terminal spacer domain.

In some ADAMTS proteins, a protease and Lacunin (PLAC) domain follows the C-terminal spacer domain. Since the pro-domain of all ADAMTS family members contain a furin-cleavage site, it is generally thought that ADAMTS proteases are synthesized as inactive zymogen forms that are cleaved intracellularly prior to secretion, resulting in the mature active form (Jones & Riley 2005).

Structure function analysis on the murine ADAMTS-1 has revealed that the TSRs play a central role in mediating ADAMTS-1’s ability to interact with HSPGs in the ECM (Kuno & Matsushima 1998). In addition, ADAMTS-1 has also been shown to possess anti-angiogenic properties by way of suppressing the activity of two prominent angiogenic factors, namely the fibroblast growth factor 2 (FGF-2) and the vascular endothelial growth factor (VEGF). *In vitro* studies have shown that ADAMTS-1 physically interacts with VEGF, but fails to cleave it, suggesting that ADAMTS-1 inhibits VEGF function by likely sequestering it from its cell-surface receptor (Jones & Riley 2005).

More recently, members of the ADAMTS family have also been found to have a role in regulating cell migration. Studies in *C. elegans* have offered novel insights into the role of ADAMTS proteases in regulating directed migration events during development. Loss-of-function mutations in GON-1, the *C. elegans* homolog of ADAMTS-20, were found to disrupt the stereotypical migration of the pathfinding gonadal distal tip cells (DTCs) along the dorsoventral axis, resulting in gonad defects (Somerville et al. 2003; Blelloch et al. 1999).

Interestingly, studies in mice have also identified a similar role for ADAMTS-20 in regulating directed migration events during development. In mice lacking functional ADAMTS-20, the
stereotypical migration of melanoblasts during embryogenesis is severely disrupted, resulting in a ‘belted’ coat color defect (Rao et al. 2003). Additional studies in *C. elegans* have shown that another ADAMTS family member, MIG-17, also plays a key role in regulating the migration of the gonadal DTCs along the dorsoventral axis.

MIG-17 is secreted from the worm muscles and is localized to the basement membrane (BM) of the somatic gonad, where it regulates the directional migration of the DTCs. MIG-17 was also found to recruit two other ECM components, fibulin-1 (FBL-1C) and nidogen (NID-1) to the BM of the gonad. Furthermore, the recruitment of FBL-1C and NID-1 to the gonadal BM was found to be critical for MIG-17’s ability to regulate the migration of the gonad arms along the dorsoventral axis (Kubota et al. 2008; Ihara & Nishiwaki 2008). How FBL-1C and NID-1 influence the activity of MIG-17 remains unexplained and awaits further investigation. Nevertheless, collectively, these studies highlight an important role for the ADAMTS family in the regulation of cell migration events during development.

### 1.7 Caenorhabditis elegans is an Ideal Model System for the Genetic Analyses of Directed Migrations

The tiny free-living soil nematode *C. elegans* possesses several traits that make it a powerful model system for genetic analyses. First, the transparent nature of the animal allows for the visualization of cells and cell-extensions *in vivo* (Brenner 1974). Second, its stereotypical development allows for the efficient isolation of mutant forms that are distinct from the wild type. Third, its diploidy allows us to maintain mutations that induce sickness/death in a heterozygous condition. Fourth, there is widespread conservation between the proteome of *C. elegans* and more complex animals including humans. Finally, *C.
**C. elegans** is easy to maintain under laboratory conditions and highly amenable to genetic manipulations (Sulston & Horvitz 1977; Sulston et al. 1983; Brenner 1974). These traits make **C. elegans** an ideal animal model for a variety of genetic analysis including forward genetic screens, which forms the foundation for most of the fundamental discoveries made using **C. elegans**.

**C. elegans** has also repeatedly proven to be a powerful model system for the study of directed membrane extensions during development. Due to its transparent nature, the extension of pioneer axon trajectories emerging from individual neurons can be visualized in high resolution in live animals. For example, the extension of the axon of the AVM mechanosensory neuron has proven to be an ideal model to investigate the interplay between multiple spatial cues to ensure the formation of a single stereotypical trajectory (Fujisawa et al. 2007). Similarly, studies conducted on the migration of commissural motor axons along the dorsoventral axis have helped illuminate the central role of the netrin-signaling pathway in regulating directed migration events during development. As described above, the key components of the netrin pathway such as UNC-6, UNC-40 and UNC-5 were first discovered and understood in **C. elegans** and were later shown to play analogous roles in higher animals (Hedgecock et al. 1990; Hedgecock et al. 1987; Rajasekharan & Kennedy 2009). The UNC-6, UNC-40 and UNC-5 story nicely illustrates the power of the nematode model system and offers a striking example of genetic conservation between worms and higher animals.

### 1.7.1 Muscle Arm Extension in **C. elegans** as a Model for Directed Membrane Extension

Our group specializes in the use of actin-based plasma membrane extensions from the body wall muscles (BWMs) of **C. elegans**, called muscle arms, as a model system to interrogate
the underlying molecular machinery governing directed membrane extensions during development. The 95-monomonucleated BWMs of *C. elegans* are organized into four distinct quadrants with two quadrants flanking the left and right sides of the dorsal and ventral nerve cords, which define the dorsal and ventral midlines of the animal. Each quadrant is further subdivided into two rows of BWMs: a proximal row that is closest to the nerve cords and a distal row that is further from the nerve cords (Dixon & Roy 2005) [Figure 1.7A & B]. Typical of nematodes, the motor axons in *C. elegans* do not arborize onto the muscles to establish neuromuscular junctions; instead they extend presynaptic specializations along the dorsal and ventral midlines. Therefore, BWMs project muscle arm extensions towards the midline motor axons to establish neuromuscular junctions. The components of the post-synaptic machinery are harbored within the muscle arm termini (Dixon & Roy 2005).

During the early phase of embryonic development, myoblast cells, which are the progenitors of BWMs, are born juxtaposed to the motor axons. As development proceeds, the myoblasts begin to move away from the motor axons and in this process are thought to typically leave behind a single trailing muscle arm that is attached to the motor axon. Next, during the larval developmental stage, each BWM actively projects a stereotypical number of muscle arms that are directed towards the midline motor axons located within the dorsal and ventral nerve cords respectively (Dixon & Roy 2005) [Figure 1.7C].

The nerve cords comprise a bundle of axons that run along the dorsal and ventral midlines. The dorsal nerve cord is formed by the axons of the motor neurons, which control the body movements. The motor neuron cell bodies reside exclusively at the ventral nerve cord and extend axons, called commissures, along the circumference of the worm towards the dorsal midline.
Figure 1.7. Muscle Arm Extension in *Caenorhabditis elegans*. (A). Shows a schematic of an adult stage *C. elegans*. The distal body wall muscles are colored in dark yellow and neurons in red. The boxed region is shown in greater detail in B. The anterior is to the right and dorsal is up. (B). Shows a cross sectional view of the neuro-muscular anatomy of *C. elegans*. On average body wall muscles extend around four muscle arms towards the nearest motor axon targets located at the dorsal and ventral midlines respectively. (C). A schematic depicting a two-phase model of muscle arm extension. During embryonic development, myoblast cells are born juxtaposed to motor axons and as worm development proceeds, these myoblasts gradually move away from the motor axon and in this process leave behind a single trailing muscle arm that is attached to the motor axon. Later during larval development, body wall muscles actively extend muscle arms towards the midline motor axons in response to a muscle arm chemoattractant secreted by the midline motor axons at the dorsal and ventral midlines. Schematics adapted from Dixon & Roy 2005.
Two important earlier observations suggested that muscle arm extension to the nerve cords was likely guided by chemotropic cues. First, in *unc-5* and *unc-6* null mutants, the circumferential migration of the motor axons to the dorsal cord are disrupted, causing them to migrate along the lateral sides of the animal. However, BWMS continue to extend muscle arms to these laterally displaced motor axons, implying that the displaced axons are the source of an attractive guidance cue (Hedgecock et al. 1990). Second, in *unc-104* mutants, the kinesin motor protein required for the anterograde transport of vesicle within axons is disrupted, causing an abnormal accumulation of synaptic vesicles within the axonal cell bodies at the ventral nerve cord (Hall & Hedgecock 1991; Zhou et al. 2001). In these mutants, muscle arms migrate to these regions of vesicle accumulation (Hall & Hedgecock 1991), indicating that the vesicles contained a then unidentified muscle arm chemoattractant.

The observation that the dorsal and ventral nerve cords are the source of a muscle arm chemoattractant inspired us to launch a detailed investigation to identify the neuronally-expressed muscle arm guidance cue, the muscle-expressed guidance receptor and other downstream signaling components involved in the regulation of muscle arm extension. We anticipated that our findings would help define a novel guidance pathway that maybe conserved with higher animals.

To identify key genes required for muscle arm extension, we carried out forward genetic screens for mutant animals that extended fewer muscle arms towards the midlines, known as muscle arm development defective (Madd) mutants. We discovered that the UNC-40/DCC netrin receptor directs muscle arm extension through a downstream Rho-GEF, UNC-73/Trio. In *unc-40* mutants, the body wall muscles extend very few muscle arms towards the nerve cords at the dorsal and ventral midlines (Alexander et al. 2009). In addition, a novel
gene, *madd-2*, was discovered in our screens and found to function genetically upstream of *unc-40* and *unc-73* to direct muscle arm extensions to the midline (Alexander et al. 2010). MADD-2 is a member of the C1-subfamily of TRIM (*Tripartite motif*) proteins and is homologous to the human Opitz Syndrome protein MID-1 protein (Alexander et al. 2010). Interestingly, MID-1 is also required for midline-oriented development (Trockenbacher et al. 2001; Schweiger & Schneider 2003), indicating that C1-TRIM proteins may have a conserved biological role in regulating midline-oriented developmental events.

Using various genetic and biochemical analyses, some of which I conducted, we were able to better understand MADD-2’s role within the UNC-40 pathway. Our results demonstrated that MADD-2 likely physically interacts with both UNC-40 and UNC-73 *in vivo*. Furthermore, we found that MADD-2 likely facilitates the interaction between UNC-40 and UNC-73, suggesting that a key function of MADD-2 within the UNC-40 pathway may be to potentiate the interaction between UNC-40 and UNC-73 (Alexander et al. 2010).

Given that *unc-6* null mutants do not have obvious defects in muscle arm extension towards the ventral nerve cord (Alexander et al. 2009), we considered the exciting possibility that the UNC-40 netrin guidance receptor maybe responding to a non-netrin guidance cue to direct muscle arms towards the midlines. The work that I carried out towards my doctoral degree has identified this long sought after muscle arm guidance cue as MADD-4.

### 1.8. Overview of Doctoral Research Project

For my doctoral work, I have elucidated the biological role of MADD-4, a novel secreted guidance cue required for midline-oriented migrations in *C. elegans*. MADD-4 is secreted from the dorsal and ventral nerve cords and attracts muscle arms towards the dorsal and ventral
midlines. In addition, MADD-4 also has a role in attracting the pioneer axons of the AVM and PVM mechanosensory neurons towards the ventral midline. In chapter two, I describe the identification of MADD-4 as a novel guidance cue that attracts muscle arms and sensory axons of *C. elegans* along the dorsoventral axis. In chapter three, I describe MADD-4’s mechanism of action. In collaboration with Kevin Chan, another graduate student from our lab, I have determined that attraction towards MADD-4 is mediated by the netrin receptor UNC-40 together with a co-receptor EVA-1. My investigation of the role of EVA-1, together with that of Kevin Chan’s, has revealed that the EVA-1 receptor enhances the sensitivity of UNC-40 to MADD-4.

Taken together, these studies have offered novel insights into how migrating cells are able to interpret and integrate spatial information towards the directed extension of a plasma membrane towards a target site. Furthermore, given that no biological role has been ascribed to any of MADD-4’s orthologs, it is highly likely that my work on understanding the guidance activities of MADD-4 will lead to novel insights into this class of guidance proteins.
Chapter Two: The Discovery and Characterization of MADD-4, A Novel Secreted Cue Required for Midline-Oriented Guidance in Caenorhabditis elegans

This chapter is adapted from the following published research article:


The work discussed in this chapter was a collaborative effort between myself and other members of our lab. The experiments described in Figures 2.4, 2.6, 2.7, 2.8, 2.9, 2.12 & 2.15 were entirely performed by me. Louis Barbier (a former undergraduate thesis student in our lab) together with Serena D’ Souza (a graduate student in our lab) performed the forward genetic screen for muscle arm mutants and isolated 3 alleles of madd-4 (tr158, tr182 and tr185). Louis and Serena mapped madd-4 to a region on chromosome I. Eric Wong (a former undergraduate thesis student in our lab) performed few of the early experiments towards characterizing madd-4’s muscle arm phenotype. Dr. Peter Roy performed the experiments presented in figures 2.10, 2.11, 2.13 & 2.14. Dr. Guillermo Selman (a former research associate in our lab) generated most of the constructs used in this study. Rachel Puckrin (our former lab manager) provided technical support by generating several strains where the madd-4 null mutation was incorporated into different genetic backgrounds. Please refer to the figure legends for more details on the specific contribution made by different members of our lab towards the experiment in question.
2.1 Abstract

The netrins and slits are two families of widely conserved guidance cues that direct membrane extensions along the dorsoventral axis of animals. These cues typically emanate from the ventral or dorsal midlines and provide spatial information to migrating cells and membrane extensions by forming gradients along the dorsoventral axis. Some cell types, however, extend processes to both the dorsal and ventral midlines even in the absence of the netrins and slits, suggesting the existence of additional guidance molecules that are secreted from both midlines. In this chapter, I report the discovery and characterization of MADD-4, which is a heretofore poorly characterized protein that we have shown to be secreted by the dorsal and ventral nerve cords of the nematode C. elegans to attract sensory axons and muscle arms. Our work shows that MADD-4’s guidance activity is dependent on the UNC-40/DCC netrin receptor. The biological role of MADD-4 orthologs, including ADAMTSL1 and 3 in mammals, is unknown. Hence, elucidating the biological role of MADD-4 may offer key insights into the broader role of the ADAMTSL family of proteins.
2.2 Introduction

The midline of bilaterally symmetrical animals is where the left and right sides meet and is a key landmark around which development along the dorsoventral axis is organized. The cells found at the midline secrete several diffusible guidance molecules that establish gradients along the dorsoventral axis. Migrating cells and membrane extensions like axons that can sense these gradients travel either up or down the concentration gradient depending on whether they interpret the cue as attractive or repulsive. In this way, migrating cells and membrane extensions can position themselves along the dorsoventral axis in a stereotypical manner.

The slits and netrins are two well-established families of secreted guidance cues that have conserved roles in orchestrating midline-oriented migrations during development (Hao et al. 2001; Kidd et al. 1999; Rothberg et al. 1988). The slits typically function as chemorepellants while the netrins can act as either attractive or repulsive cues based on the receptors expressed on the surface of the migrating cell (Colamarino & Tessier-lavigne 1995; Hedgecock et al. 1990; Kennedy et al. 1994; Serafini et al. 1994). The coordinate expression of netrin and slit within the developing fruit fly ventral cord and the vertebrate spinal cord ensures the proper navigation of midline-oriented axons towards their target sites. Commissural axons expressing the netrin receptor, DCC in vertebrates and frazzled in flies, are attracted towards the source of netrin at the midline (Keino-Masu et al. 1996). By contrast, the expression of the slit receptor robo by axons in both flies and vertebrates causes their axons to be repelled from the source of slit at the midline (Kidd et al. 1999; Kidd et al. 1998). Upon traversing the midline, commissural axons up-regulate robo expression and consequently get repelled from
the midline. This interplay between netrin attraction and slit repulsion allows the migrating axons to successfully complete their contralateral trajectory (Kidd et al. 1998).

Unlike their coincident expression at the ventral midline of flies and vertebrates, the slit and netrin gradients are diametrically opposed along the dorsoventral axis of the nematode Caenorhabditis elegans. UNC-6/netrin likely reaches a maximum concentration at the ventral midline (Wadsworth et al. 1996) while SLT-1/slit likely reaches a maximum at the dorsal midline (Hao et al. 2001). Both guidance cues are global cues in that they provide guidance information along the entire dorsoventral axis. For example, commissural motor axons originate from the ventral midline and migrate down the UNC-6 gradient towards the dorsal midline (Hedgecock et al. 1990; Ishii et al. 1992). Similarly, the axons of the AVM and PVM mechanosensory neurons, which originate from their laterally positioned cell bodies, migrate down the SLT-1 gradient to reach the ventral midline (Hao et al. 2001; Zallen et al. 1998).

Some cells and membrane extensions that migrate along the dorsoventral axis use both UNC-6 and SLT-1 as guidance cues, others use either cue, and some apparently use neither (Alexander et al. 2009; Hao et al. 2001; Hedgecock et al. 1990; Ishii et al. 1992).

Our group specializes in employing muscle arm extension in C. elegans as a model system to investigate midline-oriented guidance (Dixon & Roy, 2005). As mentioned in the previous chapter, the body wall muscles used for locomotion are arranged in longitudinal rows that flank the dorsal and ventral nerve cords, which mark the dorsal and ventral midlines, respectively [Figure 2.1]. The body wall muscles extend actin-based membrane projections, called muscle arms, to the motor axons within the nearest nerve cord of young larvae (Dixon & Roy 2005; White et al. 1986). Once at the motor axon, the muscle arm terminus provides the post-synaptic machinery of the neuromuscular junction (White et al. 1986).
Figure 2.1. A schematic of selected details of *C. elegans* neuromuscular anatomy. The distal body muscles that express our YFP marker for muscle arms are indicated in yellow. Neurons are depicted in red. Relevant features are labeled. Worm schematic adapted from Dixon & Roy 2005.
The dorsal and ventral muscles extend arms in opposite directions. The regulation of muscle arm extension represents a symmetrical guidance event that occurs within a field of asymmetrically distributed guidance molecules along the dorsoventral axis. If positional information from either global guidance cues (i.e. netrin or slit) is to be used by extending muscle arms, the dorsal muscles must interpret this information differently than the ventral muscles. Alternatively, cells at the dorsal and ventral midlines may secrete another guidance cue to attract muscle arms. Previous work has shown that muscle arms can extend to motor axons that are misguided along the lateral body wall (Hedgcock et al. 1990), suggesting that the motor axons at the dorsal and ventral midlines likely secrete a heretofore unidentified cue to attract muscle arms.

Our group has previously demonstrated that the netrin receptor UNC-40 functions within the body muscles to direct muscle arm extension to the dorsal and ventral nerve cords (Alexander et al., 2009). In this chapter, I describe the discovery and characterization of a previously uncharacterized gene product called MADD-4 that is secreted from both the dorsal and ventral nerve cords to attract extending muscle arms and sensory axons along the dorsoventral axis. MADD-4 is needed for normal muscle arm extension to the dorsal midline, but functions redundantly with UNC-6 to direct muscle arms and axons to the ventral midline. Hence, MADD-4 provides symmetrical information along the dorsoventral axis so that muscle arms can extend to both the dorsal and ventral midlines.
2.3 Results

2.3.1 A Forward Genetic Screen for Madd Mutants Reveals MADD-4, an ADAMTS1 Ortholog

Using an ethyl methane sulfonate (EMS) based mutagenesis screen, our group previously identified several genes that function cell-autonomously to direct muscle arm extension (Alexander et al. 2009). However, this screen was not saturated for mutant genes, indicating the possibility that additional genes involved in directing muscle arm extension were yet to be discovered. In an effort to identify additional genetic regulators of muscle arm extension, Louis Barbier (a former undergraduate thesis student in our lab) together with Serena D’ Souza (a graduate student in our lab) performed another EMS based forward genetic screen for muscle arm mutants. Louis and Serena screened 20,000 randomly mutagenized genomes and isolated three alleles of a gene we call \textit{madd-4}(tr158, tr182, and tr185) that represented a novel class of muscle arm mutants. Madd is an acronym for the \textit{muscle arm development defective} phenotype (Alexander et al. 2009).

The \textit{madd-4} mutants are unique in that they display a strong dorsal Madd phenotype coupled with a weak ventral Madd phenotype [Figure 2.2]. Typically, this phenotype is observed among mutants with extensive errors in commissural motor axon guidance. However, \textit{madd-4} mutants display an intact dorsal cord [Figure 2.2B] and wild-type body movement, suggesting that their dorsal Madd phenotype is unlikely to be a secondary consequence of errors in motor axon guidance. A second unique feature of the \textit{madd-4} mutants is that they behave in a semi-dominant manner [Figure 2.2I], suggesting that these animals are sensitive to the dose of MADD-4. Using a combination of well-established
Figure 2.2. The Muscle Arm Extension Defects of madd-4 mutants. (A-H). Fluorescent micrographs of the four distal muscles of dorsal (A-D) or ventral (E-H) quadrants that express the muscle arm reporter (in white) most brightly from the trls30 integrated transgenic array. The nervous system is false coloured blue and the nerve cord is indicated with a yellow arrowhead. The genotype is indicated. In all micrographs, anterior is to the right. Dorsal right muscle 15 (Dr15) is indicated with a white arrow in B-E. Ventral left muscle 11 (Vl11) in F-I is indicated with a white arrow. The muscle arms of these two muscles are indicated with red arrowheads. The scale bar represents 40 μm. (I). The average number of muscle arms per Dr15 for the tr185 and ok2854 madd-4 alleles or large chromosomal deficiency (Df). Data for nDf23 (see Figure 2.3A) is similar to that of nDf29. (J). Transgenic rescue of the muscle arm extension defects of madd-4(tr185); [fosmid+RFP tag] indicates an extra-chromosomal array harbouring the madd-4 fosmid WMR0626cA02 in which madd-4 is tagged with a bicistronic mCherry tag; [madd-4p::MADD-4B::YFP] indicates an extra-chromosomal array harbouring the pPR680 construct that drives the expression of MADD-4B fused C-terminally with YFP from the madd-4A promoter. [neuro::MADD-4B::YFP] indicates a chromosomally integrated array (trls57) that expresses a MADD-4B-YFP fusion from the pan-neuronal promoter unc-119. (K). The average number of muscle arms for Vl11 is indicated for the genotypes shown. For I-K, the standard error of the mean is shown. Statistical significance (p < 0.001) is indicated with a filled asterisk whose color is matched with a dot above the data point to which the comparison was made. A lack of significant difference (p > 0.05) is indicated with an open asterisk whose color is matched with a dot above the data point to which the comparison was made. All fluorescent worm images were captured by me, except for 2.2C, G and H, which were captured by Eric Wong. I performed all the analysis shown in this figure except for the following: Analysis of the muscle arm extension defects in the madd-4(tr185); unc-6(ev400) and madd-4(tr185); trls57/+ genotypes were done by Eric Wong. Louis Barbier performed the complementation tests and the analysis of the madd-4 muscle arm phenotype in the background of the large chromosomal deficiencies shown in 2.2I. Dr. Guillermo Selman generated all the rescue constructs used in this study. Rachel Puckrin built the madd-4; slt-1 and madd-4; unc-129 doubles mentioned in 2.2K. Figure adapted from Seetharaman et al. 2011.
molecular mapping techniques, Louis mapped \textit{madd-4} to a 0.2 map unit interval on chromosome I (Wicks et al. 2001), [Figure 2.3A]. Approximately 40 genes reside within this 0.2 map unit interval. The \textit{F53B6.2} gene immediately became of interest to us because a previous study involving large-scale expression analyses in \textit{C. elegans} suggested that it is expressed in the midline motor neurons (Hunt-newbury et al. 2007), which are the targets of extending muscle arms and the predicted protein suggested that it was secreted, immediately raising the possibility that it could be a secreted guidance cue. However, apart from this, the \textit{F53B6.2} gene was otherwise uncharacterized. Several lines of evidence indicate that \textit{F53B6.2} is \textit{madd-4}.

First, each of the three \textit{madd-4} alleles isolated from our screen was found to contain a missense mutation within the \textit{F53B6.2} gene [Figure 2.3B and Figure 2.4]. Second, Louis determined that the \textit{madd-4(tr185)} allele failed to complement two independent deletion alleles of \textit{F53B6.2 (ok2854 and ok2862)} [Figure 2.2I]. Finally, I found that expressing the \textit{madd-4} cDNA under the control of the \textit{madd-4} promoter and enhancer elements rescues the muscle arm extension defects of \textit{madd-4} mutants [Figure 2.2J]. Henceforth, we refer to \textit{F53B6.2} as \textit{madd-4}.

The \textit{madd-4} locus is predicted to encode three secreted isoforms (Pinan-Lucarre et al. 2014; Reboul et al., 2001) [Figure 2.3B]. MADD-4A has 1045 amino acid residues and is comprised of a signal peptide followed by nine thrombospondin type I repeats (TSRs) that are interspersed by a single IG domain and a C-terminal PLAC domain of unknown function [Figure 2.3C and Figure 2.4]. MADD-4C is composed of 1043 residues and shares a nearly identical domain organization with MADD-4A, except for the absence of two amino acid residues (Pinan-Lucarre et al. 2014), [Figure 2.3C]. MADD-4B is a shorter isoform composed of 711 residues and it lacks the three N-terminal TSRs found in MADD-4A & C [Figure 2.3C].
A. Cloning madd-4

B. Known madd-4 Transcripts

C. Predicted MADD-4 Isoforms and Homologs

<table>
<thead>
<tr>
<th>MADD-4A/C (1045, 1043 aa)</th>
<th>MADD-4B (711 aa)</th>
<th>Drosophila CG31619 (1394 aa)</th>
<th>Human ADAMTS1/1/Punctin-1 (1762 aa)</th>
<th>Human ADAMTS3/Punctin-2 (1691 aa)</th>
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<td>T2</td>
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<td>52%</td>
<td>29%</td>
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Overall Blastp
Identity Score
26% 2xe-65
24% 3xe-51
23% 1xe-60
Figure 2.3. The Cloning of madd-4. (A). The cloning of madd-4. Molecular mapping techniques were used to map madd-4(tr185) between 3.01 and 3.80 centimorgans (cM) on the right arm of chromosome 1. Large chromosomal deficiencies (Df) were used in complementation tests to refine the madd-4 region. tr185 failed to complement nDf23 and nDf29, but not qDf9 (see Figure 2.2I). The region uncovered by nDf29 consists of ~200 kb. One of the genes in this 200 kb region, F53B6.2, harboured mutations in the background of each of our madd-4 alleles. The dotted red line defines the madd-4a promoter. The dotted yellow and green lines defines the madd-4b promoter. The dotted blue lines indicate the deleted sequence of the indicated allele. (B). The predicted transcripts of madd-4 are shown. (C). The predicted protein isoforms of MADD-4 and its orthologs in Drosophila and human. The green asterisk denotes the region where two amino acid residues are absent in the MADD-4C isoform. The percentage identity between the domains of MADD-4 and the corresponding domains of its orthologs is shown below the orthologs’ domains. The multiple IG domains of the ADAMTS-Ls are compared to the single IG domain of MADD-4. Similarly, TSR2 of MADD-4 is compared to the second and third TSR of the corresponding ortholog (indicated with a ‘2’ superscript) and TSR8 of MADD-4 is compared to the 9th and 10th TSR of the fly and ADAMTS-L3 orthologs (indicated with a ‘8’ superscript). The overall identity between MADD-4A and its orthologs, as well as the Blastp score between MADD-4A and its orthologs is reported on the right of the three orthologs. Louis Barbier performed the madd-4 mapping experiments. I determined the percentage of identity between the different MADD-4 domains and the corresponding domains of its orthologs shown in 2.3C. This figure has been adapted from Seetharaman et al. 2011.
Figure 2.4. A Multiple Sequence Alignment of MADD-4 and its *Drosophila* and Human Orthologs. Residues are blackened if identical in 50% or more of the orthologs. The domains of MADD-4A are underlined and may not correspond precisely with the domain boundaries of the orthologs. The boundaries of the domains are those defined by the SMART protein database. The additional IG type 2 domains of the ADAMTSL proteins are underlined in yellow and are not present in either the worm or fly ortholog. The mutations of the five *madd-4* alleles are indicated. *madd-4*(tr182) is a W488 nonsense mutation (g1463a, with respect to the DNA sequence and initiator codon of *madd-4a*). *madd-4*(tr185) is a M1I mis-sense mutation in the only *madd-4B*-specific exon (g3a mutation, with respect to the DNA sequence and initiator codon of *madd-4b*). The *madd-4B*-specific exon encodes the signal peptide plus one additional residue (i.e., MLPLLILSAPLGVSAF) and is conjoined with the sequence after the orange arrow indicating the site of the tr185 mutation. *madd-4*(tr158) is a splice site mutation in the third intron of *madd-4b* (g3116a wrt *madd-4b*, g7767a wrt *madd-4a*). *madd-4*(tr158) results in a stop codon three codons into the preceeding intron. *madd-4*(ok2854) is a 962 bp out-of-frame deletion made by the *C. elegans* knock-out consortium and is common to *madd-4a* and *madd-4b*. *madd-4*(ok2862) is a 631 bp out-of-frame deletion also made by the *C. elegans* knock-out consortium and is also common to *madd-4a* and *madd-4b*. The beginning of the ok2862 deletion is indicated with a red arrow. The four residues of ADAMTSL3 found mutated specifically in colorectal tumors (Sjoblom et al., 2006), including the conserved residue R587, are indicated with blue circles. Aside from the *C. elegans* MADD-4A protein (wormpep CE36859), the sequences used are from Uniprot- human ADAMTSL1 (Q8N6G6); human ADAMTSL3 (P82987); *Drosophila* CG31619 (Q0EBN3). All the analysis shown in this figure were performed by Ashwin Seetharaman and Dr. Peter Roy. Figure adapted from Seetharaman et al. 2011.
MADD-4 is orthologous to two mammalian paralogs named ADAMTSL1 and ADAMTSL3 [Figure 2.3C]. ADAMTS-like proteins are homologues to the ADAMTS (a disintegrin and metalloprotease with thrombospondin domains) proteases, but lack the disintegrin and metalloprotease domains (Porter et al. 2005). Both ADAMTSL1 and 3 are secreted and localize to the extracellular matrix of COS cells in a punctate pattern, resulting in their alternative monikers, punctin-1 and 2, respectively (Hall et al. 2003; Hirohata et al. 2002). There also exists a Drosophila ortholog of MADD-4 called CG31619 [Figure 2.3C]. By performing multiple sequence alignments between MADD-4 and its orthologs, I determined that the domain organization of MADD-4 is largely conserved among vertebrates and flies [Figure 2.3C and Figure 2.4]. However, very little is known about the function of any of MADD-4’s orthologs.

2.3.2 MADD-4B is Necessary for Muscle Arm Extension

The madd-4(tr185) mutation is within a madd-4B-specific exon and changes the initiator methionine codon of MADD-4B to an isoleucine codon [Figure 2.3A]. When tr185 is placed in trans to large deletions that remove the entire madd-4 locus and more (i.e., nDf23 and nDf29) or smaller deletions that remove only a part of the locus (i.e., ok2854 and ok2862), the resulting muscle arm extension defects are no worse than in tr185 homozygotes [Figure 2.2I]. This genetic test indicates that tr185 is null for madd-4’s role in muscle arm extension and that MADD-4B is necessary for this activity. It should be noted that although the three madd-4 alleles (tr185, ok2854 and ok2862) behave as genetic nulls with respect to muscle arm extension, it remains possible that these alleles retain activity for other madd-4 activities. Consistent with this idea, since the publication of our work on MADD-4, another group has
shown that MADD-4 also has an important role in specifying the identities of the excitatory and inhibitory neuromuscular junctions in *C. elegans* (Pinan-Lucarre et al. 2014).

**2.3.3 MADD-4 Likely Functions from the Midline Motor Neurons to Guide Muscle Arm Extension**

To identify the cells that express *madd-4*, Dr. Guillermo Selman (a former research associate in our lab) generated several constructs that use *madd-4* enhancer and promoter elements to drive the expression of fluorescent proteins *in vivo* [Figure 2.5]. Relative to more complex animals, the typical gene structure of *C. elegans* is simple in that, the elements that control gene expression exist within ~3 kb directly upstream of the gene’s initiator methionine (Dupuy et al. 2007).

The *madd-4A* reporter (*madd-4Ap::RFP*) consists of 3.2 kb of DNA sequence upstream of the MADD-4A initiator methionine that drives the expression of red fluorescent protein (RFP). I generated transgenic animals carrying the *madd-4A* reporter and analyzed the *madd-4A* expression pattern in detail. I found that *madd-4Ap::RFP* is expressed mainly in the commissural cholinergic motor neurons [Figure 2.6A-C]. All commissural motor neurons extend processes in both the ventral and dorsal cords and their cell bodies reside exclusively in the ventral cord (White et al. 1986). *madd-4Ap::RFP* expression is weak in hatchlings and is seen in both the GABAergic and cholinergic motor neurons. During the second larval stage, RFP expression increases dramatically in only the cholinergic commissural motor neurons [Figure 2.6A and B], which is coincident with the period of larval muscle arm extension (Dixon & Roy 2005). This pattern of cholinergic motor neuron-specific expression persists into adulthood [Figure 2.6C] and no other cells or stages express RFP from the *madd-4A* promoter.
Figure 2.5. Constructs Designed to Investigate madd-4’s Expression Pattern. Shown here are four constructs used to investigate madd-4’s expression pattern. The madd-4 genomic locus is shown and intron 9 highlighted in blue. The regions corresponding to the madd-4A and madd-4B promoter regions are highlighted in green and purple respectively. Dr. Peter Roy designed and Dr. Guillermo Selman constructed the constructs shown in this figure. I performed all the injections and generated transgenic lines with the constructs shown here. This figure is adapted from Seetharaman et al. 2011.
**Figure 2.6. madd-4’s Post-Embryonic Expression Pattern.** Shown here is the expression pattern from extrachromosomal arrays of the transcriptional reporters pPR681(madd-4Ap::RFP) and an integrated version of pPR731(madd-4Bp::RFP) (i.e. trls80). The expression pattern of an extra-chromosomal array of pPR725, which is a fosmid clone carrying a functional (see Figure 2.2J) bi-cistronically-tagged madd-4 locus within the madd-4 fosmid WMR0626cA02 is similar to that generated by pPR731(madd-4Bp::RFP) (See Figure 2.9). The purple box on the mCherry (red box) cistron indicates a nuclear localization signal. (A-C). An analysis of the expression pattern of madd-4Ap::RFP (on the left) and madd-4Bp::RFP (on the right). The animals on the left harbour an extrachromosomal transgenic array that contains pPR681(madd-4Ap::RFP) and a YFP marker for the array that is expressed in the pharynx (black asterisks). The animals on the right harbour the trls80 integrated transgenic array that contains the pPR731(madd-4Bp::RFP) construct and no other markers. In addition, all of the animals shown have the trls8 chromosomally integrated transgenic array. trls8 drives expression of YFP in all 26 gabaergic neurons from the unc-25 promoter, including the 19 motor neurons in adults (and the six in young larvae) (Jin et al., 1999), and CFP in only select cholinergic motor neurons (from the unc-129 neural specific promoter) (Colavita et al., 1998), and RFP in the excretory canal (marked with white asterisks). ‘A’ shows typical L1 hatchlings. The RFP channel on the left (i.e. madd-4Ap::RFP) is increased in relative intensity in order to visualize the RFP-expressing cells. ‘B’ shows typical L2 larvae. ‘C’ shows typical adults. The white arrowheads indicate overlap between RFP-expressing cells, and CFP or YFP-expressing cells. In contrast to madd-4Bp::RFP expression, which is consistently high in all classes of commissural motor neurons in all larval stages and adults, animals carrying madd-4Ap::RFP express low levels of RFP in the first larval stage in both gabaergic and cholinergic motor neurons. madd-4Ap::RFP expression then increases dramatically after the L1 stage, but only in cholinergic cells. The scale bar represents 25 μm. All images shown in this figure were captured by me. Figure adapted from Seetharaman et al. 2011.
In addition, I also determined that the same 3.2 kb of upstream DNA sequence when used to drive the expression of the MADD-4B cDNA can rescue the muscle arm defects of the *madd-4*(tr185) mutant [Figure 2.2J]. This indicates that the 3.2 kb sequence likely contains all of the spatio-temporal regulatory elements that are necessary for *madd-4* function.

To examine the expression pattern of *madd-4B*, Guillermo Selman conjoined the intronic sequence that flank the *madd-4B*-specific exon that harbors the 5’UTR and initiator codon of MADD-4B (see the yellow and green highlighted sequence in Figure 2.3A) and used it to drive YFP and RFP expression [See Figure 2.5]. We define this sequence as the *madd-4B* promoter (or *madd-4Bp*). I next generated several transgenic lines of animals bearing the *madd-4B* reporters as extrachromosomal arrays and with help from Rachel Puckrin (our former lab manager) we generated two chromosomally integrated strains of animals harboring either *madd-4Bp::YFP* or *madd-4Bp::RFP* (called trIs79 and trIs80, respectively). I then analyzed the *madd-4B* expression pattern from the trIs79 and trIs80 strains in detail. I found that both strains expressed fluorescent protein in all commissural motor neurons (GABAergic and cholinergic) and in no other cell types within the body (as opposed to the head) [Figures 2.7A, B and Figure 2.6A-C].

Through a detailed mosaic analysis of animals harboring an extra-chromosomal array of *madd-4Bp::YFP*, I found that the *madd-4B* promoter drives expression in several head neurons including the RIA, RIC, lateral IL1s, lateral IL2s, OLLs, RMEs, and SABs [Figure 2.7C-I]. YFP may also be expressed in AVH, AVKL, and ASG, and AIZ. All of these head neurons extend an axon into the major neuropil of the head, called the nerve ring (White et al., 1986). Given that the head and neck muscles extend muscle arms into the nerve ring, this expression pattern suggests that MADD-4 may also play a role in the development of the head and neck muscle
Figure 2.7. The Expression Pattern Driven by the madd-4b Promoter Elements. (A). An L2 animal expressing madd-4bp::RFP from the trls80 chromosomally integrated transgenic array. The animal also has the trls8 expression pattern driven by the promoter elements. (A'). An L2 animal expressing madd-4bp::RFP + cholinergic motor neurons (CFP). (A''). An L2 animal expressing madd-4bp::RFP + gabaergic motor neurons (YFP). The overlap between RFP-expressing neurons and CFP (A') or YFP (A'')-expressing neurons is indicated with white arrows. The scale bar in 'A' represents 25 μm. (B). The madd-4B promoter drives the expression of YFP in all commissural motor neurons. Shown is the right side of an adult animal that harbours the trls79(madd-4bp::YFP) chromosomally integrated transgenic array. Anterior is to the right in A & B. The scale bar in 'B' represents 50 μm. (C-I). Adult animals that are mosaic for an extra-chromosomal array harbouring the madd-4bp::YFP transgene. The white arrowhead indicates the cell body of the indicated neuron. The white arrow points anteriorly and indicates the centre of the nerve ring. The scale bar in ‘C’ represents 25 μm and is the same for micrographs D-I. The trls79 and trls80 integrated strains were generated with the help of Rachel Puckrin. I captured all images shown in this figure. Figure obtained from Seetharaman et al. 2011.
arms. I also observed madd-4B-driven YFP expression during embryogenesis in the blast cells and corresponding terminally differentiated ventral cord motor neurons and head neurons [Figure 2.8]. Finally, we also generated a bicistronic madd-4 reporter containing an RFP cassette that is inserted immediately 3’ of the madd-4 stop codon within a fosmid harbouring madd-4 [see methods and Figure 2.5]. I determined that the resulting construct rescues the muscle arm extension defects of madd-4(tr185) [Figure 2.2J] and expresses RFP faintly in neurons of the ventral cord [Figure 2.9]. Together, these results suggest that MADD-4 functions from commissural motor axons to attract muscle arms.

2.3.4 MADD-4 localization to the Dorsal Midline is Dependent on the UNC-104 Anterograde Kinesin

One of the first pieces of evidence to suggest that the motor neurons express a secreted cue to attract muscle arms came from an investigation of the UNC-104 anterograde kinesin. UNC-104 is required to transport vesicles along the commissural motor axons to the dorsal cord from the cell bodies that reside within the ventral cord (Hall & Hedgecock, 1991). In unc-104 mutants, vesicles accumulate within the motor neuron cell bodies of the ventral cord, and dorsal muscle arms occasionally extend to the sites of vesicle accumulation (Hall & Hedgecock, 1991). Hence, we hypothesized that if MADD-4 is the muscle arm attractant, its localization to the dorsal cord should be dependent on unc-104. To investigate this possibility, we tagged MADD-4B with YFP and expressed it from commissural motor neurons using the unc-119 promoter. The chromosomally integrated version of this transgene is called trls57.
Figure 2.8. The Embryonic Expression Pattern Driven by the madd-4b Promoter Elements. The promoter and enhancer elements of madd-4B, but not madd-4A drive YFP expression in embryos. The left-most column in show DIC micrographs of a surface plane of the embryo and the corresponding YFP channel in the next column. The third and fourth columns show a deeper focal plane of the same embryo that is depicted in the first two columns for A-E. A single focal plane is shown for the two-fold stage embryo in F and the three-fold stage embryo shown in G. (A). An embryo at about 220 minutes post-fertilization. No YFP expression is observed. (B). An embryo at about 320 minutes post-fertilization at the onset of dorsal hypodermal intercalation. YFP expression is faintly observed in two ventral hypodermal blast cells (blue arrows) and a neuroblast (green arrow). (C). An embryo at about 350 minutes post-fertilization during dorsal intercalation. YFP expression is similar to earlier time points except that it is evident in more anterior neuroblast cells. The arrows indicate the same cells as in B and D. (D). An embryo at about 400 minutes post-fertilization at the onset of elongation. YFP expression is very prominent in the anterior neurons (green arrows). (E). An embryo at about 430 minutes post-fertilization during elongation. Expression in the ventral hypodermal blasts is maintained (blue arrows), as is the expression in the anterior neuroblasts and neurons (green arrows). (F). A two-fold stage embryo (~450 minutes post-fertilization) showing prominent YFP expression in anterior neurons (green arrows). (G). A three-fold stage embryo (~520 minutes post-fertilization) showing YFP expression in the ventral cord commissural motor neurons (green arrows). Anterior is to the right in all micrographs. I captured all the images shown in this figure. The scale bar in ‘A’ is 10 μm and is representative of all micrographs. Figure obtained from Seetharaman et al. 2011.
Figure 2.9. Expression From A madd-4 Fosmid Reporter. The Expression Pattern of the bicistronically-tagged madd-4 locus within the madd-4 fosmid WMR0626cA02 is similar to that generated by pPR731(madd-4Bp::RFP) shown in Figure 2.6. Anterior is to the left and dorsal is up. White arrowheads indicate faint expression of RFP among neuronal cell bodies located along the ventral nerve cord. Dr. Guillermo Selman generated the madd-4 fosmid reporter construct and I performed the injections and generated the transgenic lines used in this study.
Together with Eric Wong (a former undergraduate thesis student in our lab), I determined that trls57 is able to rescue the muscle arm extension defects of madd-4 mutants [Figure 2.2J] and therefore expresses functional MADD-4B::YFP. In an otherwise wild type background, MADD-4B::YFP is localized to both the ventral and dorsal nerve cords [Figure 2.10A]. By contrast, MADD-4B::YFP localization to the dorsal cord is undetectable in more than 90% of unc-104 mutants [Figure 2.10B and C]. This is not a secondary consequence of commissural axons failing to reach the dorsal cord because commissural axons extend normally in unc-104 mutants (Hall and Hedgecock, 1991). Even in unc-40 mutants in which some commissural axons fail to reach the dorsal cord, MADD-4::YFP can still be observed along the dorsal cord and/or tracts of misguided axons [Figure 2.10C].

We previously reported that unc-104 mutants have dorsal muscle arm extension defects (Dixon & Roy 2005). If the muscle arm extension defects of the unc-104 mutant are solely a consequence of its inability to transport MADD-4, then a mutation in unc-104 should not be able to enhance the Madd phenotype of madd-4(tr185). Indeed, the Madd phenotype of the madd-4; unc-104 double is no worse than either single mutant [Figure 2.10D]. Together, these results are consistent with the idea that MADD-4 is a muscle arm attractant that UNC-104 transports to the dorsal nerve cord.

### 2.3.5 Ectopic Expression of MADD-4 Redirects Muscle Arm Extension in an UNC-40-Dependent Manner

The results described above support the idea that MADD-4 is a muscle arm chemoattractant. We reasoned that if this is true, then ectopically expressing MADD-4 along the lateral side of the animal should redirect muscle arm extensions laterally. Dr. Peter Roy
Figure 2.10. The UNC-104 Kinesin is Required for MADD-4 Localization to the Dorsal Nerve Cord. (A). Functional MADD-4B::YFP is localized as puncta along the ventral and dorsal cords when expressed pan-neuronally from the *unc-119* promoter from the *tris57* chromosomally integrated transgenic array (see Figure 2.2J). The scale bar, representative of both A and B, represents 25 μM. (B). In the background of *unc-104(rh43)*, which is a strong loss-of-function mutation, MADD-4B::YFP is localized to the ventral cord, but not the dorsal cord. (C). The quantification of the observed defects in the localization of MADD-4B::YFP in the indicated genetic background. (D). Muscle arm extension defects of the indicated genetic background. The muscle arm extension defects of *madd-4* is not enhanced by *unc-104(rh43)*. Muscle arm extension of ventral left muscle 11 and dorsal right muscle 15 (Dixon and Roy, 2005) was quantified. Standard error of the mean is shown. Statistical significance (*p* < 0.001) is indicated with a filled asterisk whose color is matched with a dot above the data point to which the comparison was made. A lack of significant difference (*p* > 0.05) is indicated with an open asterisk whose color is matched with a dot above the data point to which the comparison was made. Dr. Peter Roy captured the images and performed all the analysis shown in this figure. This figure is adapted from Seetharaman et al. 2011.
conceived of the following experiments, generated most of the transgenic animals and performed the phenotypic characterization with help from Dr. Guillermo Selman who generated all the constructs used in this study and Rachel Puckrin who helped build strains.

To ectopically express MADD-4 along the lateral side of the animal, we used the ceh-23 promoter (Forrester et al. 1998) to express MADD-4 from the CAN neurons, which are a bilaterally symmetrical pair of neurons that extend an anterior and posterior axon along the lateral line [Figure 2.11A and B]. We observed a dramatic redirection of muscle arm extension laterally in these transgenic animals [Figure 2.11C]. MADD-4A and B have equivalent activity in this assay. Muscle arm extension to the CAN is enhanced in response to CAN-expressed MADD-4 in madd-4(tr185) mutants [Figure 2.11D]. In these animals, there is also a simultaneous reduction in muscle arm extension to the dorsal and ventral nerve cords [Figure 2.11E]. We infer that the spatial distribution of MADD-4 is shifted from the midlines to the lateral lines when MADD-4 is expressed from the CAN and is further shifted in a madd-4 mutant background.

To investigate the possibility that MADD-4 attracts muscle arm extensions through an interaction with cues that guide migrations along the dorsal-ventral axis, we asked whether CAN-expressed MADD-4 can redirect muscle arm extension in the background of null mutations in unc-6, unc-129 andslt-1. We found that MADD-4 does not rely on any of these three guidance cues to attract muscle arms [Figure 2.11D]. These results are consistent with our previous observations that unc-6 null mutants and animals with slt-1 or unc-129 mutations have no primary defects in muscle arm extension [See Figure 2.2K] (Alexander et al. 2009; Dixon et al. 2006), but cannot formally rule out the possibility that MADD-4 interacts with an as yet unidentified component to guide migrations.
We previously established that the UNC-40 receptor and the MADD-2 adaptor protein functions in muscles to direct arm extension (Alexander et al. 2009; Alexander et al. 2010; Hao et al. 2010). We tested whether mutations in madd-2 and unc-40 could suppress the extension of redirected arms and found that they could [Figure 2.11D]. These results are consistent with the idea that MADD-4 functions through an UNC-40 pathway to attract muscle arms to the dorsal and ventral nerve cords.

2.3.6 A Domain Analysis of MADD-4 Indicates that TSR4 and the IG Domain are both Necessary and Sufficient for Activity

As a first step towards understanding the function of MADD-4 domains, we investigated which MADD-4 domains are necessary and sufficient for its activity. We made a series of deletions in the MADD-4B::YFP construct that is expressed from the CAN neurons and then quantified their ability to attract muscle arms to the CANs [Figure 2.11F]. This assay is more sensitive to the perturbation of MADD-4 activity than a rescue assay for muscle arm extension (i.e. there is only a two-fold loss of normal muscle arms in madd-4 nulls [Figure 2.2I], but there is a nine-fold increase in the number of laterally-projected muscle arms when MADD-4 is expressed in the CAN [Figure 2.11F]. We found that the deletion of the C-terminal PLAC, and thrombospondin repeats (TSR) 5, 6, 7, 8, and 9, have only mild effects on the activity of MADD-4. By contrast, removal of TSR4 dramatically reduces MADD-4 activity and removal of the IG domain eliminates it [Figure 2.11F]. Expression of TSR4 (together with TSRs 5, 6, and 7) has no activity, nor does the expression of only the IG domain. However, the expression of a TSR4::IG fusion protein exhibits strong activity [Figure 2.11F]. These results suggest that MADD-4’s TSR4 and IG domains are both individually necessary and together are sufficient to attract muscle arms.
Figure 2.11. Ectopic Expression of MADD-4 from the CAN neuron Attracts Muscle Arms to the Lateral Line. (A). A schematic of the worm showing the CAN neuron (yellow) on the left side with the body muscles above and below it outlined in black. (B). A control animal expressing a muscle plasma membrane marker in the body muscles (the dorsal and ventral muscle quadrants are indicated with an asterisk) and YFP in the CAN neuron (yellow arrowhead). The axon of the CAN neuron is indicated with yellow arrows. (C). An animal carrying the same markers as in (B), but also expressing MADD-4B::YFP from the CAN neuron. Muscle arms extend towards the CAN (red arrows). For B and C, anterior is to the left and the inset shows a magnified view around the CAN cell body. The scale bar represents 50 μM for both B and C. (D). The redirection of muscle arms in response to CAN-expressed MADD-4B::YFP (shown as CAN-MADD-4) is dependent on madd-2 and unc-40, but not madd-4, unc-6, slit-1 or unc-129 (E). The number of (normal) muscle arms extended to the nearest nerve cord decreases upon expressing MADD-4B::YFP ectopically from the CAN neuron (CAN-MADD-4) and decreases further in this background upon the removal of endogenous madd-4. The body muscles examined are ventral left 11 (Vl11) and dorsal right 15 (Dr15). The loss-of-function alleles used in D and E are madd-2(tr129), unc-40(n324), madd-4(tr185), unc-6(ev400), slit-1(ok255) and unc-129(ev554). (F). Animals carrying the muscle plasma membrane marker (trIs30) were made transgenic for constructs expressing the indicated proteins (fused to YFP) and the number of lateral membrane projections is reported. Construct names are reported on the left. Two independent transgenic lines were examined for each construct and each behaved similarly. For simplicity, amalgamated counts of the left and right sides of the two characterized lines are shown. The orange box at the C-terminus of the second protein indicates a fusion to the transmembrane domain of the β-integrin PAT-3. Statistical significance (p < 0.001) is indicated with a filled asterisk whose color is matched with a dot above the data point to which the comparison was made. A lack of significant difference (p > 0.05) is indicated with an open asterisk whose color is matched with a dot above the data point to which the comparison was made. Dr. Peter Roy captured the images and performed the analysis shown in this figure. Dr. Guillermo Selman generated all the MADD-4 deletions depicted in this figure. I generated the transgenic line containing the MADD-4 (R398H) mutation shown in 2.11F. This figure is adapted from Seetharaman et al 2011.
2.3.7 MADD-4 is a Secreted Cue that Diffuses

MADD-4 has a putative signal peptide and can attract muscle arms when expressed at a distance from muscles. Hence, MADD-4 is likely secreted and can diffuse. We performed three experiments to further investigate this idea. First, we removed MADD-4’s putative signal peptide and tested whether the mutant protein could attract muscle arms when expressed from the CAN neurons and found that it could not [Figure 2.11F]. Second, we fused MADD-4 to a transmembrane domain to tether it to the plasma membrane and expressed this fusion protein from the CAN neurons. The membrane-tethered MADD-4 dramatically reduced lateral arm extension [Figure 2.11F], suggesting that MADD-4 must diffuse to function. Third, I discovered that when YFP-tagged MADD-4 is ectopically expressed from only the dorsal muscles, it diffuses and accumulates at the ventral midline [Figure 2.12]. The same promoter was then used to drive the expression of plasma membrane-anchored YFP in control animals, however I did not detect YFP localizing to the ventral midline in these animals [Figure 2.12]. Together, these findings indicate that MADD-4 is secreted and diffuses to attract muscle arms.

2.3.8 MADD-4 Cooperates with UNC-6 to Guide Ventral Muscle Arm Extension

Given that UNC-40 directs muscle arm extension and that its canonical cue is UNC-6/netrin, it was surprising that unc-6 null mutants have no muscle arm extension defects to the ventral midline [Figure 2.2G and (Alexander et al. 2009; Hedgecock et al. 1990)]. The observation that madd-4 mutants have dramatic defects in muscle arm extension to the dorsal midline, but not the ventral midline, raised the question of whether MADD-4 and UNC-6 function redundantly to direct ventral muscle arm extension. To test this hypothesis, I, along with and Eric Wong, examined the ventral muscle arm extension defects in madd-4; unc-6
Figure 2.12. Dorsally-Expressed MADD-4 Diffuses to the Ventral Midline. A&B. Shown are animals that express YFP-tagged MADD-4 from the *unc-129* muscle-specific promoter (Colavita et al., 1998), which drives expression in only the dorsal body wall muscles (green arrows in ‘A’ and ‘C’) and vulval epithelium (yellow arrow in ‘B’ and ‘D’). MADD-4::YFP accumulates at both the dorsal (A) and ventral (B) midlines (white arrows). C&D. Shown is a dorsal (C) and ventral (D) view of two animals that express membrane-anchored YFP from the *unc-129* muscle-specific promoter. YFP is not observed at the ventral midline in any animal examined (n>20). The scale bar in ‘A’ represents 40 μm and is the same for all four. I captured all the images shown in this figure. This figure was obtained from Seetharaman et al. 2011.
double mutants and found that they indeed displayed synthetic ventral muscle arm extension defects that were as dramatic as the dorsal muscle arm extension defects of the madd-4(tr185) single mutant [See Figure 2.2H and K]. Next, I investigated whether null alleles of other prominent secreted ligands like slt-1/slit and unc-129/ TGF-β could enhance madd-4’s muscle arm extension defects and found that they did not [See Figure 2.2I and K]. These results are consistent with the aforementioned results that removal of slt-1 and unc-129 does not disrupt muscle arms redirected towards ectopically expressed MADD-4 [see Figure 2.11]. Hence, we conclude that MADD-4 is sufficient to guide larval muscle arm extension to the dorsal cord and that it cooperates with UNC-6 to guide muscle arm extension to the ventral cord during larval development.

2.3.9 MADD-4 Guides Axons along the Dorsal-Ventral Axis

Given the cooperativity of midline signals to orient migrations along the dorsoventral axis, we wondered whether MADD-4 plays any role in guiding other UNC-40-dependant migrations along this axis. To answer this question, we investigated the guidance of the AVM and PVM mechanosensory axons [Figure 2.13A]. Dr. Peter Roy performed the following work with the help of Rachel Puckrin and Dr. Guillermo Selman.

The AVM and PVM neurons express the UNC-40 receptor, which directs axon extension towards the UNC-6 ligand at the ventral midline (Chan et al. 1996; Wadsworth et al. 1996). The AVM and PVM neurons also express the SAX-3 receptor, which mediates repulsion from the dorsally expressed SLT-1 (Hao et al. 2001; Zallen et al. 1998). Null mutations in either the netrin or slit pathways result in mild AVM and PVM axon guidance defects (Hedgecock et al. 1990; Zallen et al. 1998). However, the ventrally directed extension of the AVM and PVM
Figure 2.13. Expression of MADD-4 from the Dorsal Muscles Redirects the AVM and PVM Mechanosensory Axons Dorsally. (A). A schematic of the worm showing the six mechanosensory axons. Dorsal is up and anterior is to the right. At the forefront are the three axons on the right side of the animal, PLMR, ALMR and AVM. On the other side of the worm are the PLML, PVM, and ALML. (B). A chart showing that the madd-4(ok2854) null allele can enhance the AVM guidance defects of the slt-1(ok255) and unc-6(ev400) null mutants, but not those of the unc-40(n324) null. (C). A fluorescent micrograph of a young adult that express GFP in the six mechanosensory neurons, including the AVM (shown) from the muls32 chromosomally integrated transgenic array (Ch’ng et al., 2003). (D). The same as in C except MADD-4::YFP is expressed from the tris78 chromosomally integrated transgenic array, which drives the expression of both MADD-4A::YFP and MADD-4B::YFP in only the dorsal muscles from the unc-129 muscle-specific promoter (Colavita et al., 1998). The direction of the green arrows in C and D show the trajectory of the AVM axon. The scale bar in C represents 25 μM and is the same for both micrographs. (E). Charts showing the percentage of AVM (left chart) and PVM (right chart) guidance errors in the indicated mutant background when MADD-4 is expressed from the dorsal muscles (D-MADD-4) or not. Standard error of the mean is shown in B and E. Statistical significance (p < 0.001) is indicated with a filled asterisk whose color is matched with a dot above the data point to which the comparison was made. A lack of significant difference (p > 0.05) is indicated with an open asterisk whose color is matched with a dot above the data point to which the comparison was made. Dr. Peter Roy captured the image shown in 2.13-C. I captured the image shown in 2.13-D. Dr. Peter Roy performed all the analysis shown in this figure. Rachel Puckrin helped generate all the madd-4 double mutants shown in this figure. This figure is adapted from Seetharaman et al 2011.
axons is dramatically reduced in animals with both pathways knocked out [Figure 2.13B], (Hao et al. 2001).

We found that madd-4 null animals have no defects in AVM and PVM axon guidance [Figure 2.13B]. However, the madd-4 null allele enhanced the AVM guidance defects of unc-6, and slit-1 null mutants, but not that of unc-40 null mutants [Figure 2.13B]. It is unclear why the guidance defects of the PVM axon in slit-1 mutants is not enhanced by madd-4, but others have also noted that the PVM behaves differently than the AVM (Fujisawa et al. 2007). Others have also noted that AVM guidance in unc-6 null mutants is more defective than in unc-40 null animals (Chang et al. 2004; Fujisawa et al. 2007), suggesting that UNC-6 plays other roles in guiding the AVM aside from signaling through UNC-40. Regardless, these results indicate that MADD-4 plays an ancillary role in guiding the AVM axon and are consistent with the idea that MADD-4 signals through an UNC-40 complex.

To further explore MADD-4’s ability to direct sensory axon guidance, we examined axon trajectories in animals that ectopically express MADD-4 from the dorsal muscles. The AVM and PVM axons often extend toward the dorsal midline in these animals [Figure 2.13C-E]. This is in contrast to the trajectories of the misguided AVM and PVM axons of netrin and slit mutant animals that invariably extend along the anterior-posterior axis. We found that the unc-40 null mutation nearly abolishes the ability of MADD-4 to redirect the AVM and PVM axons dorsally [Figure 2.13E]. As expected, the removal of the UNC-6 and SLT-1 cues that guide axons ventrally enhances the ability of dorsally expressed MADD-4 to redirect axons dorsally [Figure 2.13E]. These results are consistent with the model that MADD-4 attracts extending processes through a direct or indirect interaction with the UNC-40 receptor. Intriguingly, we found that dorsally expressed MADD-4 enhances the lateral AVM and PVM axon guidance defects of the
unc-40 null [Figure 2.13E]. This suggests that MADD-4 may also act through a non-UNC-40 receptor to influence the guidance of the AVM and PVM axons.

To determine which MADD-4 domains are required for its ability to redirect the trajectories of sensory axons dorsally, we generated a series of MADD-4 deletions and quantified their ability to redirect the axon of the AVM neuron dorsally when expressed from the dorsal muscles. Our results indicate that the removal of the MADD-4 IG domain abolishes the dorsal redirection of the AVM axon [Figure 2.14A]. Furthermore, TSR4, which is central to MADD-4’s ability to attract muscle arms, is dispensable for its ability to attract the AVM axon [Figure 2.14A]. Finally we found that a combination of TSRs 6, 8 and 9 together with the IG and PLAC domains can robustly redirect the AVM axon extension dorsally [Figure 2.14A]. However, immunoprecipitation analysis indicate that the different dorsally-expressed MADD-4 truncations are not expressed at similar levels [Figure 2.14B]. Hence, the findings from this study need to be further validated by data indicating that the different dorsally expressed MADD-4 deletion proteins are expressed at similar levels.

2.3.10 Determining the influence of Heparan Sulfate Proteoglycan Core Proteins on MADD-4’s Ability Direct Migrations Along the Dorsoventral Axis

Previous work suggested that members of both the ADAMTS and ADAMTSL family of proteins are capable of associating with a vast array of ECM molecules including heparan sulfate proteoglycans (HSPGs) through their TSRs (de Wit & Verhaagen 2007; Kubota et al. 2008; Baeg et al. 2000; Tsutsui et al. 2010; Adams & Tucker 2000). These findings, together with our observation that different TSR domains may mediate MADD-4’s ability to direct muscle arm extension vs. sensory axon guidance inspired us to investigate the influence of
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**Negative Control**

### B

**IP:** Anti GFP Polyclonal Antibody  
**Blot:** Anti GFP Monoclonal Antibody
Figure 2.14. Determining the Domains Required to Mediate MADD-4s Ability to Direct Sensory Axon Guidance. (A). Shown here is a structure-function analysis to determine how the different MADD-4 domains contribute towards its ability to redirect the AVM sensory axon dorsally. The different dorsally-expressed MADD-4 truncations are presented on the left along with the construct name and an assessment of their ability to redirect AVM axons dorsally is shown on the right. Positive effects are denoted by a (+) while negative effects are shown by a (-). For each construct shown, two independent transgenic lines were analyzed. (B). An immunoprecipitation analysis conducted on several MADD-4 deletion constructs described in 2.14A. Note: trls78 refers to the integrated transgenic strain expressing MADD-4A::YFP and MADD-4B::YFP under the control of an unc-129 muscle-specific promoter which drives expression in the dorsal muscles. Dr. Guillermo Selman generated most of the constructs used in this study and Dr. Peter Roy performed all the analysis shown in 2.14A and I performed the immunoprecipitation experiments shown in 2.14B.
ECM components such as HSPGs on MADD-4’s ability to direct muscle arm extension and sensory axon guidance along the dorsoventral axis during development.

I initiated a pilot candidate gene analysis where I examined the ability of three distinct HSPG core proteins (SDN-1/syndecan, LON-2/glypican and UNC-52/perlecan) to modulate the redirection of muscle arms and the AVM sensory axon towards lateral and dorsal sources of MADD-4 (from the tris65 and tris78; muls32 integrated strains respectively). I found that the genetic removal of SDN-1 has little to no impact on MADD-4’s ability to attract muscle arms or the AVM sensory axon [Figure. 2.15]. On the other hand, I found that the removal of LON-2 suppresses both MADD-4’s ability to attract muscle arms and the AVM axon, suggesting that LON-2 may have a key role in both ECMS [Figure. 2.15]. Finally, the removal of UNC-52 suppresses only the ability of MADD-4 to attract muscle arms but not the AVM axon [Figure. 2.15]. Together, these results highlight a role for HSPGs in modulating MADD-4’s ability to attract cell-extensions along the dorsoventral axis. Further work is required to explore UNC-52 and LON-2’s involvement in modulating MADD-4’s guidance function along the dorsoventral axis.

**2.3.11 An ADAMTSL3 Mutation implicated in Colorectal Cancer Disrupts MADD-4 Activity**

Mutations in ADAMTSL3, which is one of the two MADD-4 orthologs in humans, is associated with colorectal cancer (Sjoblom et al. 2006). Of the four missense mutations in ADAMTSL3 that are specifically associated with colorectal cancer, only one (R587H) disrupts a conserved residue (see Figure 2.3 and (Sjoblom et al. 2006)). This residue is part of the fourth thrombospondin repeat (TSR4) of both ADAMTSL3 and MADD-4, which we have shown to be one of two domains that are necessary for MADD-4 activity (above). To test whether mutation
Figure 2.15. Determining the Ability of Select HSPG Mutants to Modulate MADD-4’s Guidance Function. (A). Fluorescent micrograph images showing the lateral sides of young adult animals expressing the muscle marker (trls30) and MADD-4::YFP from the CAN neuron (from the trls65 integrated strain) in the indicated genetic backgrounds. A yellow arrowhead indicates the cell body of the CAN and red arrowheads indicate muscle arm projections towards the lateral sources of MADD-4. The anterior is to the left and dorsal is up. The scale bar shows 50 μM. (B). A quantification of the ability of the candidate ECM mutants to redirect muscle arm extensions laterally in response to CAN-expressed MADD-4::YFP. (C). A quantification of the ability of the candidate ECM mutants to redirect AVM axons dorsally in response to dorsal-muscle expressed MADD-4::YFP (from the trls78 integrated strain). The standard error of the mean is shown. Statistical significance (p < 0.001) is indicated with a filled asterisk whose color is matched with a dot above the data point to which the comparison was made. A lack of significant difference (p > 0.05) is indicated with an open asterisk whose color is matched with a dot above the data point to which the comparison was made. I captured all the images shown in this figure and performed the data analysis.
of the corresponding residue (R398H) affects MADD-4 activity, I generated transgenic lines of animals carrying the (R398H) missense mutation in MADD-4B expressed under the CAN-promoter. We found that this missense mutation disrupts MADD-4B activity to a similar extent as removing the TSR4 domain [See Figure 2.11F], suggesting that MADD-4 and ADAMTSL3 have a related biochemical function.
2.4. Discussion

Most migrations along the dorsoventral axis of *C. elegans* are asymmetrical. Two examples of this asymmetry include the extension of the AVM and PVM mechanosensory axons towards the ventral midline and the extension of the commissural motor axons towards the dorsal midline (Hedgecock et al. 1990). The asymmetric expression pattern of the UNC-6 and SLT-1 guidance cues can largely account for the proper guidance of these migrations. By contrast, muscle arm extension to both the dorsal and ventral midlines is a symmetrical guidance event. Hence, the guidance cues that originate at dorsal or ventral midlines cannot fully account for the guidance of muscle arm extension. These observations suggest the existence of another guidance cue that is expressed from both midlines. In this chapter I have provided several lines of evidence that indicate that MADD-4 is this symmetrically expressed guidance cue.

2.4.1 MADD-4 Attracts Cell-Extensions in an UNC-40-Dependent Fashion

We previously showed that the UNC-40 transmembrane receptor functions in muscles to direct muscle arm extension to the motor axon targets (Alexander et al. 2009). The work discussed in this chapter suggests that MADD-4 may signal through UNC-40 to attract muscle arms and other cell extensions. First, MADD-4’s ability to attract membrane extensions along the dorsoventral axis is dependent on UNC-40. Second, the ability of ectopic MADD-4 to attract AVM and PVM axons to the dorsal midline is suppressed by the removal of UNC-40, but enhanced by the removal of other components that guide the mechanosensory axons to the ventral midline, including UNC-6 and SLT-1. Third, MADD-4 functions redundantly with UNC-6, the canonical ligand for UNC-40 to attract ventral muscle arm extensions and the axons of the
AVM and PVM neurons towards the ventral midline. In the next chapter, I provide evidence that indicates MADD-4 interacts with an UNC-40-EVA-1 co-receptor complex to direct midline-oriented migration events.

2.4.2 MADD-4 Acts Locally to Attract UNC-40-Dependent Migrations

The existence of a second guidance cue that attracts migrating membrane extensions in an UNC-40-dependent fashion raises an apparent paradox. Why does the expression of endogenous MADD-4 at the dorsal midline not interfere with the ventral extension of other UNC-40-dependent process such as the AVM axon? One possibility is that there may be more MADD-4 at the ventral midline than at the dorsal midline. This idea is consistent with the fact that all cell bodies belonging to dorsal cord motor axons reside in the ventral cord (White et al. 1986). A second possibility is that the cumulative attractive signal that reaches the AVM from ventrally expressed UNC-6 and MADD-4 may simply out-compete any MADD-4-mediated attractiveness that emanates from the dorsal side of the animal. Finally, relative to UNC-6, MADD-4 may be expressed at lower levels or be secreted less efficiently in order to restrict its potency to only nearby cells. Dorsally expressed MADD-4 may attract processes from cells that are relatively close, like the dorsal muscles, but fail to interfere with the guidance of other cells that are further away, like the AVM neuron.

Muscle arms extend towards their midline motor axon targets, which express MADD-4. Once the muscle arms reach their target destinations, they likely remain in place indefinitely (Dixon & Roy, 2005). However, two lines of evidence suggest that it is unlikely that MADD-4 serves as both a muscle arm chemoattractant and cell adhesion molecule to anchor the muscle arm termini in place. First, I have not observed any wayward muscle arm termini in madd-4
null mutants. All remaining muscle arms in madd-4 mutant animals appear to be in close contact with the nerve cords. Second, an examination of the muscle membrane that extends towards CAN-expressed MADD-4 suggests that the redirected processes do not adhere to the CAN neuron, but instead adhere to other redirected muscle arms. By contrast, the few processes that extend towards CAN-expressed membrane-tethered MADD-4 appear to adhere to the CAN neuron. These observations suggest that MADD-4 may not play a role in the adherence of muscle arm termini to the nerve cords but may instead function strictly as an attractant.

### 2.4.3 Distinct TSR Domains May Mediate Distinct Biological Activities of MADD-4

Previous work has shown that members of both the ADAMTS and the ADAMTSL family of proteins can associate with many distinct ECM components through their TSR domains (Kubota et al. 2008; Kuno & Matsushima, 1998; Tsutsui et al. 2010; Adams & Tucker, 2000). Our work on MADD-4 has shown that the shorter MADD-4B isoform is both necessary and sufficient for directing the migration of membrane extensions such as muscle arms and sensory axons along the dorsoventral axis in C. elegans. MADD-4B lacks the first three N-terminal TSRs found in the longer MADD-4A/C isoforms, but is otherwise identical to MADD-4A/C.

As a first step towards elucidating the biochemical mechanism of MADD-4’s activity, we investigated which domains of MADD-4 were necessary and sufficient for its guidance function during development. Through a systematic analysis of MADD-4’s domain functions we found that the signal sequence is essential for MADD-4’s activity, consistent with the idea that MADD-4 is a secreted protein. Similarly, we found that removing the IG domain abolishes MADD-4’s ability to attract muscle arms and sensory axons, indicating that the IG domain is fundamental
to MADD-4’s guidance function. Next, we found that the TSR4 domain is critical while other the N-terminal (TSRs 1,2,3,5,6 & 7) and C-terminal (TSRs 8 & 9) TSR domains together with the PLAC domain are dispensable for MADD’s ability to attract muscle arms. Finally, we found that a TSR4::IG domain fusion protein exhibits strong activity towards attracting muscle arm extensions. This indicates that the TSR4 domain likely harbors most of the functional elements required for MADD-4’s ability to attract muscle arm extensions. Preliminary evidence suggests that a distinct set of TSR domains (TSRs6, 8 & 9) together with the IG and PLAC domains may be involved in directing sensory axon guidance along the dorsoventral axis. Given that TSR domains are known to interact with diverse ECM molecules, we speculate that the different TSR domains of MADD-4 may function in a combinatorial manner with the IG and PLAC domains to mediate interaction with distinct ECM components.

Through a small-scale candidate gene analysis, I determined that the HSPG core protein LON-2/glypican is required for both MADD-4’s ability to direct muscle arm extension and sensory axon guidance while UNC-52/perlecan is required only for MADD-4’s ability to direct muscle arm extension. Furthermore, I found that SDN-1/syndecan is dispensable for MADD-4’s ability to attract cell-extensions along the dorsoventral axis [see Chapter 4, section 4.5 for a more detailed discussion on the ability of HSPGs to modulate MADD-4’s guidance function]. Although a more in-depth investigation is warranted to better understand the role of UNC-52 and LON-2 in modulating MADD-4’s guidance function, the results obtained so far highlight a role for ECM proteins in modulating MADD-4’s ability to attract cell-extensions along the dorsoventral axis.

A recently published study from Jean-Louis Bessereau’s group has identified an important role for MADD-4 at the C. elegans neuromuscular junctions as an organizer of the
postsynaptic domains (Pinan-Lucarre et al. 2014). It was observed that the long and short isoforms of MADD-4 have distinct roles in the specification of the excitatory and inhibitory postsynapses. The longer MADD-4A/C isoforms were found to be both necessary and sufficient for the proper localization of levamisole-sensitive acetylcholine receptor (L-AChR) clusters at the cholinergic neuromuscular junctions while the shorter MADD-4B isoform was dispensable for this activity (Pinan-Lucarre et al. 2014). By contrast, MADD-4B was found to play a critical role in clustering GABA\textsubscript{A} receptors at the GABAergic neuromuscular junctions (Pinan-Lucarre et al. 2014). These observations suggest that the three N-terminal TSR domains found in longer MADD-4A/C isoforms may have a critical role in localizing L-AChR clusters at the cholinergic neuromuscular junctions. Further investigation will be required to get a better understanding of the contribution of the different domains of MADD-4 contribute towards its different biological activities.

Collectively, the findings from our group together with that of Jean-Louis’s highlight an important role for the different TSR domains of MADD-4 in regulating distinct biological activities during development.

2.4.3 Mammalian MADD-4 Orthologs are Widely Expressed

MADD-4’s expression is likely restricted to the nervous system of the worm. RNA-Seq data from modENCODE indicates that the expression pattern of the fly’s ortholog is also likely restricted to the nervous system (Roy et al. 2010). In mammals, however, MADD-4 orthologs are more widely expressed. Isoforms of ADAMTSL1 are expressed at higher levels in skeletal muscle, while ADAMTSL3 is widely expressed (Hirohata et al. 2002; Koo et al. 2007). These expression patterns suggest that MADD-4’s mammalian orthologs may have roles beyond axon
guidance. Whether the ADAMTSs play a role in axon guidance or guiding cells that are associated with epithelial development or maintenance remains to be determined.
2.5 Materials and Methods

2.5.1 Nematode Strains, Counts and Microscopy

All strains were cultured and maintained at 20°C according to standard protocol (Brenner, 1974). All muscle arm counts were performed in the background of the chromosomally integrated transgenic array trls30 I or trls70 II. Both arrays express membrane-anchored YFP from the him-4 promoter in select distal body wall muscles along with other markers of commissural motor axons (Dixon & Roy 2005). Muscle arms were counted as previously described (Dixon & Roy, 2005), except for in deficiency mapping experiments where full dorsal (dorsal right muscles 9, 11, 13, and 15- see (Dixon & Roy 2005)) counts were performed on a minimum of 15 individual young adults. AVMs and PVMs were visualized using the fluorescent reporter background mulIs32 (Ch'ng et al. 2003). The direction of axon extension of 50 AVMs and PVMs from at least three separate populations of the same strain was counted on standard culture plates using an epifluorescent MZ16 dissection scope (Leica Inc.) with a 2X objective. Redirected muscle arms were counted from 20 left and right sides of worms for each strain. For transgenic strains, muscle arm redirection was characterized in two lines harbouring independently derived extra-chromosomal transgenic arrays. All counts of muscle arms and axon extensions are from worms paralyzed with 2-10 mM levamisole (Sigma) in M9 solution (Lewis and Fleming 1995) and photographed as previously described (Dixon & Roy 2005). The alleles of all double mutant combinations whose genotype cannot be verified through phenotype alone (madd-4; slt-1 for example) are genotyped using PCR and/or sequence of 5 or more individual progeny of a single cloned double mutant candidate. Statistical differences were investigated using the Student’s T-test.
2.5.2 The Genetic Screen for Madd Mutants

A genetic screen for *C. elegans* muscle arm development defective (Madd) mutants was performed by incubating a mixed stage population of RP112 trls25 [pPRRF138.2(him-4p::MB::YFP), pPRZL47(F25B3.3p::DsRed2), pRF4(rol-6(su1006)]; rrf-3(pk1426) animals in 50 μM ethyl methanesulfonate (EMS) for 4 hours. pRF4, which induces a rolling phenotype, was used to facilitate the observation of muscle arm extension in the living animals, and rrf-3(pk1426) was used to decrease the visual noise imparted by the pPRRF138.2 transgene. Resulting F1s were synchronized as L1s (Lewis and Fleming, 1995) and grown on 6 cm plates (~3000 per plate). A COPAS Biosort (Union Biometrica, Inc) was used to place 3 L3-stage F1 worms per well in 12-well plates, and the F2 progeny were screened 4 days later for mutants with muscle arm extension defects using a Leica MZFLIII epifluorescence dissection microscope with a 2X objective.

2.5.3 Molecular Biology and Transgenics

The following chromosomally integrated transgenic arrays were made using standard techniques (Mello et al.1991; Mitani 1995) and are available either from the *C. elegans* Genetic Centre or from us: trls8 [pPR4.1(B0285.6p::dsRed2)(50ng/ul); pPR17(unc-129nsp::CFP) (15ng/ul); pPR15(ceh-23p::YFP) (15ng/ul), pPR18(unc-25p::YFP) (15ng/ul), pKS(55ng/ul)]; trls25 (described above); trls57 [pPREW507(unc-119p::MADD-4B::YFP) (50ng/ul); pPRGS382 (myo-2p::mCherry) (20 ng/ul)] X; trls63 [pPRGS630(ceh-23p::MADD-4B::YFP) (50 ng/ul); pPRGS629(ceh-23p::YFP) (50 ng/ul); pPRGS382 (myo-2p::mCherry) (5 ng/ul)] X; trls70 [pPRZL138.2(him-4p::Mb::YFP) (5 ng/ul); pPR16(unc-25p::CFP) (20 ng/ul); pKS (75 ng/ul)] II; trls78 [pPRGS698(unc-129msp::MADD-4A::YFP) (12.5 ng/ul); pPRGS699(unc-129msp::MADD-
The constructs described above, along with the following constructs, were built using standard protocols. The constructs, maps and the details about the methods used to make them are available upon request. 

- pPRGS657(ceh-23p::MADD-4B(no TSR4)::YFP);
- pPRGS658(ceh-23p::MADD-4B(no PLAC)::YFP);
- pPRGS659(ceh-23p::MADD-4B(no IG)::YFP);
- pPRGS669(ceh-23p::MADD-4B(no TSR8, TSR9, PLAC)::YFP);
- pPRGS670(ceh-23p::MADD-4B(TSR4 to TSR7-only)::YFP);
- pPRGS683(ceh-23p::MADD-4B(IG only)::YFP);
- pPRGS684(ceh-23p::MADD-4B(IG- TSR9)::YFP);
- pPRGS706(ceh-23p::MADD-4B(TSP4+IG only)::YFP);
- pPRGS681(madd-4Ap::mCherry);
- pPRGS695(ceh-23p::MADD-4B(no signal sequence)::YFP);
- pPRGS704(him-4p::MADD-4B::YFP);
- pPRGS725 (madd-4 tagged bicistronically with mCherry within the context of the madd-4 fosmid WMR0626ca02);
- pPRSAD744(ceh-23p::MADD-4B (R398H)::YFP);
- pPRGS751(ceh-23p::MADD-4B::TM::YFP).

See section 2.5.6 below for additional information on the spatiotemporal control of the promoters used in this analysis.

For the analysis of MADD-4 domain function [shown in Figure 2.11F], we injected the experimental construct at a concentration of 50 ng/µL together with co-injection markers pPRGS629(ceh-23p::YFP) at 50 ng/µL and pPRGS382 (myo-2p::mCherry) at 4 ng/µL. To determine which head neurons express YFP from the madd-4B promoter [i.e. Figure 2.7C-I], we injected pPRGS721(madd-4Bp::YFP) at 50 ng/µL (together with pPRGS382 (myo-2p::mCherry) at 2 ng/µL and 50 ng/µL of pKS) into wild type N2 animals, and established several lines that
maintained the resulting extra-chromosomal transgenic array. We characterized the YFP expression pattern in animals that lost the extra-chromosomal array from all but a few cells.

2.5.4 Mapping madd-4(tr185)

madd-4(tr185) was mapped to a 0.2 map unit interval using snip-SNP mapping (Wicks et al., 2001). The map location of tr185 was further refined through complementation tests with deficiencies whose end points are genetically defined, an example of which follows: The chromosome I deficiencies nDf23 and nDf29 are each balanced on the left by unc-13(e1091) and on the right by lin-11(n566) in the MT2180 and MT2138 strains, respectively. We built the strain unc-13(e51) madd-4(tr185) trIs30 I and then heterozygous males from this strain were crossed into the deficiency strains balanced by unc-13(e1091) and lin-11(n566). Any fluorescent cross progeny inheriting the unc-13(e1091) lin-11(n566) balancer chromosome would be Unc, since mutant unc-13 is also being provided by the parental male. All non-Unc fluorescent cross progeny should be madd-4(tr185)/nDfXX. Counting the dorsal muscle arms of these cross-progeny and comparing to tr185, tr185/+, and Df controls unambiguously revealed whether or not the deficiency uncovers madd-4’s locus. A similar approach was used for the qDf9 complementation test.

2.5.5 Investigating Commisural Axon Guidance in the madd-4 Null Mutant

We investigated whether madd-4(ok2854) animals have commisural axon guidance defects by passing in the trIs79 integrated transgenic array that express YFP in all commisural motor axons into the mutant. An examination of the right side of madd-4(ok2854) animals
showed 23 (+/-0.3) commissures compared to the 23 (+/-0.5) for controls. *madd-4(ok2854)* animals had on average 1 (+/- 0.1) errant commissural axon compared to 0.5 (+/- 0.1) errant commissural axons in control animals. Standard error of the mean is reported.

2.5.6 The Spatiotemporal Control of the Promoters Used to drive Transgene Expression

The following promoters were used to drive expression in the indicated tissues:

*B0285.6p* (the *B0285.6* promoter drives expression in the excretory cell beginning in early larval development (see wormbase and (Bamps & Hope 2008)); *ceh-23p* (the *ceh-23* promoter drives expression in the CAN neuron in larvae and adults, among other cells in the head as described in (Wang et al. 1993)); *F25B3.3p* (the *F25B3.3* promoter drives expression in nearly all neurons as described in (Altun-gultekin et al. 2001)); *him-4p* (the *him-4* promoter drives expression in select distal body wall muscles as described in (Dixon & Roy 2005; Vogel & Hedgecock 2001)); *myo-2p* (the *myo-2* promoter drives expression in the pharynx as described in (Okkema et al. 1993)); *unc-25p* (the *unc-25* promoter drives expression in all 26 gabaergic neurons, including the 19 gabaergic commissural motor neurons(Jin et al. 1999)); *unc-119p* (the *unc-119* promoter drives expression in nearly all neurons in the worm (Maduro & Pilgrim 1996)); *unc-129nsp* (the *unc-129* neural-specific promoter drives expression in select DA and DB commissural motor neurons muscles from mid to late-embryogenesis onward as described in (Colavita et al. 1998)); *unc-129msp* (the *unc-129* muscle-specific promoter drives expression in the dorsal body muscles from mid-embryogenesis onward as described in (Colavita et al. 1998)).
2.5.7 Calculation of Percent Identity between the Domains of MADD-4 and its Orthologs

The percentage Identity between the different domains of MADD-4A and its orthologs was calculated using Jalview, a multiple sequence alignment editor and analysis tool. The amino acid sequences for domains of MADD-4A and DM-CG31619 were obtained from the SMART protein domain database and the amino acid sequences for H-ADAMTSL1 and H-ADAMTSL3 were obtained from the UniProt protein database. A fasta file containing the protein sequences of the domains of MADD-4A and its orthologs was used as the input in Jalview. Next, pair-wise alignments were generated for the different protein sequences and the percentage identity between the sequences was calculated.
Chapter Three: MADD-4 Attracts Midline-Oriented Migrations through an EVA-1-UNC-40 co-Receptor Complex in Caenorhabditis elegans

A substantial fraction of the data presented in this chapter is derived from the following published research article:


* Equal Contribution

The work discussed in this chapter was a collaborative effort between myself and other members of our lab, particularly Kevin Chan (another graduate student in our lab). James Kim (a former summer student in our lab) isolated the eva-1(tr301) allele through a forward genetic screen for suppressors of muscle arms directed towards ectopic sources of MADD-4. Dr. Guillermo Selman was the first to show that MADD-4 can bind EVA-1 and UNC-40 through a cell culture based assay. Jenny Zhang (a former research technician in our lab) performed additional repeats of the MADD-4-EVA-1-UNC-40 interactions in cell culture and carried out further experiments using the cell culture platform. Jenny Zhang also generated most of the EVA-1 constructs used in this study. Rachel Puckrin provided key technical support by generating several strains used in this study. Dr. Peter Roy performed all the analysis shown in Figures 3.1 and 3.9. Kevin Chan and I together performed many of the analysis and counts presented in this chapter. For more specific information on the contribution of different members of our group to this study, please refer to the figure legends.
3.1 Abstract

How migrating cells integrate spatial information from multiple guidance cues to extend a single stereotypical trajectory is not fully understood. In the previous chapter, I described the discovery of MADD-4, a novel secreted guidance cue that diffuses and acts in parallel to the UNC-6/netrin and Slt-1/slit cues to direct migrations along the dorsal-ventral axis in C. elegans. MADD-4 is a member of the poorly understood non-enzymatic ADAMTSL family of proteins and is well conserved among most complex animals including humans. We also showed that MADD-4’s activity is dependent on the UNC-40/DCC, receptor whose canonical ligand is UNC-6/netrin. In this chapter I describe a second receptor called EVA-1, which is a conserved transmembrane protein with predicted extracellular galactose-binding lectin domains that functions cell autonomously to mediate MADD-4 attraction. Our work demonstrates that MADD-4 can interact with both EVA-1 and UNC-40. We also show that EVA-1 interacts with UNC-40 and this interaction is likely central to EVA-1’s ability to mediate attraction towards MADD-4. Finally, we demonstrate that the binding of EVA-1 to UNC-40 increases UNC-40’s sensitivity to MADD-4. Taken together, the work described in this chapter supports a model whereby an EVA-1-UNC-40 co-receptor complex increases the precision by which migrating cells and membrane extensions reach their target.
3.2 Introduction

During animal development, migrating cells and membrane extensions like axons confront a diverse array of spatial cues as they navigate towards their target sites. For example, upon exiting the spinal column in the developing spinal cord, spinal motor axons encounter well above six distinct spatial cues (Bonanomi et al. 2014). How a migrating axon or cell responds to different guidance cues depends upon the receptors expressed on their cell surface. However, many guidance receptors have been shown bind multiple guidance cues, and likewise several guidance cues have been shown to interact with multiple guidance receptors within the same spatial environment (Ahmed et al. 2011; Haddick et al. 2014). Thus, how migrating cells or membrane extensions are able to modulate their responses to distinct cues such that they are able to generate stereotypical trajectories is not well understood. The tiny nematode *Caenorhabditis elegans* provides an ideal platform to tackle this question due to its amenability to genetic manipulations and the presence of a limited repertoire of paralogous guidance cues and receptors.

As mentioned in the previous chapters, our group specializes in the study of plasma membrane extensions from the body wall muscles of *C. elegans*, known as muscle arms, as a model system to better understand the genetic mechanisms governing directed migrations during development (Dixon & Roy 2005). Muscle arm extension has proven to be a useful model of guidance because its study has led to the discovery of novel guidance molecules that also have roles in axon guidance. In the previous chapter, I described the discovery of MADD-4, which is the sole member of the poorly understood non-enzymatic ADAMTS family of proteins in *C. elegans*. MADD-4 is the diffusible guidance cue that is secreted by motor neurons to attract extending larval muscle arms (Seetharaman et al. 2011). The UNC-40/DCC
guidance receptor and the MADD-2 (C1-family TRIM protein) adaptor protein, are both required in muscles to direct muscle arm extension towards sources of MADD-4 (Seetharaman et al. 2011; Alexander et al. 2009; Alexander et al. 2010). The UNC-40 receptor is well known for its role in directing membrane extensions towards the UNC-6/netrin guidance cue, which is considered to be its canonical ligand. Previous work has shown that UNC-6 is expressed by the motor neurons along the ventral nerve cord in *C. elegans* and is thought to form a ventral-dorsal gradient with a maximum at the ventral midline (Wadsworth et al. 1996). Although *unc-6* null mutants on their own have no noticeable muscle arm extension defects to the ventral nerve cord, we found that the genetic removal of UNC-6 in a *madd-4* null background enhances the muscle arm extension defects of the *madd-4* null mutants, suggesting that both ligands might function through UNC-40 to attract ventral muscle arms (Seetharaman et al. 2011).

In addition to directing muscle arm extension, MADD-4 also has a role in directing the axon of the AVM mechanosensory neuron towards the ventral midline (Seetharaman et al. 2011). The cell body of the AVM neuron is located anteriorly towards the lateral midline on the right side of the animal and extends a single pioneer axon that is directed towards the ventral midline (Yu et al. 2002). Previous work has shown that the extension of the AVM axon towards the ventral midline is achieved through the coordinated activities of at least two genetic pathways. In one pathway, the UNC-40 receptor functions cell-autonomously to direct the migration of the AVM axon towards the source of UNC-6 at the ventral midline (Chan et al. 1996; Hedgecock et al. 1990). In a second parallel genetic pathway, the SAX-3/robo receptor together with a co-receptor called EVA-1 also functions cell-autonomously to mediate the repulsion of the AVM axon from the dorsally-expressed SLT-1/slt guidance cue (Zallen et al. 1996).
1998; Fujisawa et al. 2007; Hao et al. 2001). EVA-1 is a conserved single pass transmembrane protein with a short cytoplasmic region and an extracellular portion that encodes two predicted lectin-like galactose-binding domains (Fujisawa et al. 2007). Work from our group suggests that in addition to functioning in an UNC-6 pathway, the UNC-40 receptor also acts within a MADD-4 genetic pathway to ensure the AVM axon reaches its ventral midline target (Seetharaman et al. 2011).

Previous work has demonstrated that UNC-40’s activity can be modulated in at least two ways resulting in distinct outcomes. First, the association of the UNC-5 receptor with UNC-40 has been shown to convert UNC-40’s response to UNC-6 from attraction to a repulsion (Hamelin et al. 1993, Hong et al. 1999). Second, through what is known as a set-point mechanism, the SAX-3 receptor is thought to somehow dampen UNC-40’s sensitivity and/or response to UNC-6 (Fujisawa et al. 2007). Work from Joseph Culotti’s group suggest that as SAX-3-expressing cells migrate away from the dorsal maximum of the SLT-1 gradient, SAX-3 is thought to become increasingly free from SLT-1 to antagonize UNC-40 pathway activity. SAX-3’s antagonism of UNC-40’s sensitivity to UNC-6 is thought to prevent signal saturation of the netrin pathway as the migrating AVM axon confronts increasing concentrations of UNC-6 as it navigates towards the ventral midline (Fujisawa et al. 2007).

In this chapter, I describe a third mechanism by which UNC-40’s activity is modulated. We found that EVA-1 also functions as part of an UNC-40 co-receptor complex to sensitize UNC-40 to the MADD-4 guidance cue. Hence, through this mechanism, EVA-1 counteracts UNC-6’s interference of UNC-40-mediated attraction towards MADD-4-expressing targets. This mechanism may enhance the ability of cell extensions to reach MADD-4-expressing targets within a field of the more globally distributed UNC-6 guidance cue.
3.3 Results

3.3.1 EVA-1 Functions in a MADD-4 Pathway

To identify novel components that mediate MADD-4 attraction, James Kim (a former summer student in our lab) performed a forward genetic screen for mutants that disrupt the ability of ectopically expressed MADD-4 to attract muscle arms. James used a previously described strain (Seetharaman et al. 2011) that expresses MADD-4::YFP from a pair of bilaterally-symmetrical CAN neurons [Figure 3.1A-C]. In this strain, muscle arms are redirected towards the lateral source of MADD-4 in an UNC-40-dependent fashion (Seetharaman et al. 2011). A madd-4 null mutation was incorporated into the screening strain because it increases the number of processes directed towards the laterally expressed ectopic MADD-4 and therefore creates a sensitized background [see Figure 3.1F for example].

James screened approximately 22,000 randomly mutagenized genomes and identified over 44 penetrant mutants that suppressed redirected muscle arms without obviously diminishing the expression of the transgenes from the CAN neuron [Table 3.1]. Nine of these mutants likely represent new alleles of unc-40 or unc-73 based on their characteristic uncoordinated phenotype and commissural axon guidance defects, and fifteen mutants with mutations in madd-2 [Table 3.1]. Given our previous characterization of these gene classes (Alexander et al. 2009; Alexander et al. 2010), we did not pursue these mutants further. Among the remaining twenty mutants, one represented a novel allele of eva-1 that we call tr301 [Table 3.1]. tr301 mutates eva-1’s Q86 codon to non-sense. We subsequently determined that all known eva-1 alleles are suppressors of muscle arm extension towards ectopic MADD-4 [Figures 3.1D and 3.1F].
A. [Image of a worm]

B. YFP expressed in CAN neuron

C. MADD-4::YFP expressed in CAN neuron

D. eva-1(ok1133); MADD-4::YFP expressed in CAN neuron

E. eva-1(ok1133); unc-6(ev400); MADD-4::YFP expressed in CAN neuron

F. Bar graph showing lateral arms per muscle for different genotypes:

- Wild-type
- unc-6(ev400)
- madd-4(a101)
- madd-4(d151)
- eva-1(ok1133)
- eva-1(ju131)
- eva-1(ju135)
- un-c-6(ev400)
- un-c-6(d151)
- un-c-6(ju131)
- un-c-6(ju135)
- madd-4(a101)
- madd-4(d151)
- madd-4(ju131)
- madd-4(ju135)

The bars represent the number of lateral arms in each condition, with error bars indicating standard error.
Figure 3.1. EVA-1 Functions with UNC-40 to Mediate Muscle Arm Attraction to MADD-4. (A). A schematic of the worm indicating the area shown in B-E. Anterior is to the left and dorsal is up. (B-E). Lateral views of worms expressing a muscle membrane marker (tris30) along with the indicated transgenic protein from the CAN neuron (yellow arrowhead). Examples of muscle arms extending into the lateral space are indicated with a red arrowhead. Anterior is to the left and dorsal is up. (F), Quantification of muscle arm extension into the lateral space in response to CAN-expressed MADD-4::YFP (CAN-MADD-4). The pound sign (#) next to the x-axis labels indicate that these animals are also mutant for madd-4(tr185), which increases the sensitivity of the assay. All of the alleles used are predicted to be null for the indicated gene, except for tr185, which is a null allele of the madd-4b isoform. Statistical significance (p<0.01) is indicated with a solid asterisk that is matched in colour with a dot above the data point to which the comparison was made. An outlined asterisk indicates a lack of significance (p>0.01). CAN expression is driven by the ceh-23 promoter for all transgenes presented here. The scale bar shows 50 micrometers. Standard error of the mean is shown in all graphs. Dr. Peter Roy captured all the images shown in this figure and performed the muscle arm counts. Figure adapted from Chan et al. 2014.
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Table 3.1. A Screen for Suppressors of MADD-4 Redirected Muscle Arms. Shown in the table are lists of mutants that are suppressed for their ability to redirect muscle arm extensions towards lateral sources of MADD-4 at the CAN neuron. The mutants are classified as strong, medium or unc-40/unc-73 like based on their phenotypes. Mutants classified as either strong or medium indicate that the mutation strongly or mildly suppresses the lateral-redirection of muscle arm extensions, respectively. Mutants classified as unc-40/unc-73 display phenotypes consistent with those reported for either of these genes. Note that bolded alleles have all been found to harbor mutations in the madd-2 gene based on sequence data obtained with the help of Rachel Puckrin. The asterisk denotes a novel allele of eva-1. James Kim performed the forward genetic screen and isolated the mutants presented in this table.
Next, I examined the role of eva-1 in normal muscle arm extension towards the midline motor neurons and found that significant reduction compared to wild type [Figure 3.2A-C]. eva-1 mutants have more dorsal muscle arm defects than ventral muscle arm defects, which is similar to madd-4 mutants (Seetharaman et al. 2011). I next used double mutant analysis to investigate whether EVA-1’s role in muscle arm extension might be restricted to the MADD-4 pathway. madd-4 eva-1 double mutants have muscle arm extension defects that are no more severe than the strongest defect of either single mutant alone [Figure 3.2D]. Given that muscle arm extension defects can be more severe than that observed in the madd-4 eva-1 double mutant, this result indicates that EVA-1 and MADD-4 function in the same pathway to guide muscle arms.

To further explore the genetic relationship between eva-1 and madd-4, we constructed additional double mutants with other genes known to regulate muscle arm extension. The near absence of muscle arm extension towards the midline in the unc-40 null mutant precludes its use in double mutant analyses to reliably determine whether a second gene product likely functions with UNC-40. However, we previously demonstrated that mutations in unc-6, which do not elicit ventral muscle arm defects on their own, enhance the ventral muscle arm extension defects of the madd-4 null mutant, suggesting that UNC-6 plays a role in attracting muscle arms to the ventral midline in MADD-4’s absence (Seetharaman et al. 2011) [Figure 3.2D]. I found that the unc-6 null mutant also enhances the ventral muscle arm defects of eva-1 [Figure 3.2D], consistent with the idea that MADD-4 and EVA-1 function together in a pathway that operates in parallel to UNC-6.
Figure 3.2. EVA-1 Functions in a MADD-4 Pathway to Counteract UNC-6 Interference. (A). A schematic of the extension of muscle arms from the ventral left distal body wall muscles. Anterior is to the left and dorsal is up. (B & C). Fluorescent micrograph images showing the ventral view of wild-type and an eva-1 null mutant animal. The yellow arrowhead indicates the ventral cord and white and green arrows indicate the VL11 and VL19 muscle cells respectively. The red arrowheads indicate the muscle arms of VL11 and VL19. The scale bar shows 50 μM. (D). Quantification of muscle arm extension from VL11 and dorsal right muscle number 15 (DR15) in young adult animals of the indicated genotype. Dorsal muscle arm extension data is not shown for strains carrying the unc-6 mutation because they lack motor neurons within the dorsal cord, which consequently confounds any interpretation of the resulting muscle arm phenotypes. (E). The average distance of the mid-proximal face of the indicated muscle to the ventral nerve cord in wild type animals (that also harbor the trls30 muscle arm and neuronal markers). (F). Quantification of VL19 muscle arm extension in young adults of the indicated genotype. Statistical significance (p<0.01) is indicated with a solid asterisk that is matched in color with a dot above the data point to which the comparison was made. An outlined asterisk indicates a lack of significance (p>0.01). Standard error of the mean is shown in all graphs. Kevin Chan and Ashwin Seetharaman performed all the analysis shown in this figure. Figure adapted from Chan et al. 2014.
Finally, we also found that over-expression of MADD-4 from dorsal muscles or all neurons induces muscle arm extension defects in a dose-dependent manner [Figure 3.3A and 3.3B]. I hypothesized that excessive MADD-4 might saturate its signaling pathway and consequently interfere with the muscle's ability to extend muscle arms. One prediction of this hypothesis is that removing the receptor with which MADD-4 interacts should suppress these muscle arm extension defects. *unc-40* nulls have few-to-no muscle arms, precluding their analysis in this assay. However, I found that an *eva-1* null mutation was able to suppress the muscle arm extension defects that are induced by pan-neuronal over-expression of MADD-4 from the *trls66* integrated transgenic array and dorsal-muscle over-expression of MADD-4 from the *trls78* integrated transgenic array respectively [Figure 3.3C]. As a potential negative control, I examined *slt-1* and *sax-3* null mutants and neither mutant suppressed the defects that are induced by MADD-4 over-expression [Figure 3.3C]. These results further support the idea that MADD-4’s activity is mediated by the EVA-1 receptor.

### 3.3.2 EVA-1 is a MADD-4 Receptor

Previous work showed that *eva-1* is likely expressed in several tissues, including body wall muscles (Fujisawa et al. 2007). Together with Kevin Chan, I investigated whether *eva-1* functions cell-autonomously by expressing an EVA-1::CFP fusion protein specifically in the body wall muscles and found that it could rescue the muscle arm defects of *eva-1* mutants [Figure 3.4A]. These results indicate that, like UN Carolina-40 (Alexander et al. 2009), EVA-1 functions cell-autonomously to direct muscle arm extension. Next, I carried out a systematic analysis of the functional requirement of EVA-1’s domains. With help from Jenny Zhang, we generated several muscle-expressed EVA-1::CFP deletion constructs, where the different domains of
Figure 3.3. EVA-1 Mediates MADD-4 Activity. (A). Over-expression of MADD-4 from either the CAN neuron (using the ceh-23 promoter), all neurons (using the unc-119 promoter) or the dorsal muscles (using the unc-129 muscle-specific promoter) from the indicated chromosomally-integrated and repetitive transgenic arrays (trIsXX) reduces the number of muscle arms extended to a degree that is roughly correlated with the level of MADD-4 expression. The muscle arm defects are reduced upon halving the number of MADD-4 transgenes through heterozygosity. All transgenically expressed MADD-4 is tagged with YFP. (B). A western blot of lysates of five strains harbouring the indicated transgenic arrays probed and with anti-MADD-4 antibodies. The MADD-4A and MADD-4B isoforms are indicated with arrowheads. b-tubulin is used as a loading control. Only trIs66 (neuronally-expressed MADD-4B) and trIs78 (MADD-4A and MADD-4B expressed from the dorsal muscles) are of relevance here. A quantification of the relative abundance of the proteins (normalized to tubulin) is shown above the blot (C). An analysis of the ability of neuronally-expressed MADD-4 (trIs66) or dorsal muscle-expressed MADD-4 (trIs78) to induce muscle arm extension defects upon loss- or reduction-of-function of the indicated gene. Statistically significance (p<0.001) is indicated with a solid asterisk which is color matched with a solid dot above the data point to which the comparison was made. An outlined asterisk indicates a lack of significance (p>0.001). Standard error of the mean is shown in all graphs. Muscle arm counts for all strains shown in Figure 3.3A except the trIs30; trIs66 strain was performed by Ashwin Seetharaman and Kevin Chan. Serena D’ Souza performed the muscle arm counts for the trIs30; trIs66 strain. Kevin Chan performed the western blot analysis shown in Figure 3.3 B. Figure adapted from Chan et al 2014.
Figure 3.4. EVA-1 Functions Cell-Autonomously in Muscles and Interacts with MADD-4. (A). Muscle-expressed EVA-1::CFP rescues the muscle extension defects of eva-1 mutants. (B). A summary of EVA-1 domain function that is fully detailed in Figure 3.5. (C&D). FLAG-tagged receptors were expressed from HEK293 cells, bathed in conditioned media from other HEK293 cells that express HA- and Gaussia luciferase-tagged MADD-4 or SLT-1 ligands, and immunoprecipitated to determine the relative amounts of ligand that co-immunoprecipitates with the receptor (see methods for more details). (C). The western blot on the left shows the five immunoprecipitated FLAG-tagged receptors. The western blot on the right shows the two HA- and Gaussia luciferase-tagged ligands that were collected from cell culture. (D). The normalized relative levels of luciferase signal that immunoprecipitated with each potential ligand-receptor complex. (E-I). Shown are animals harbouring one of of three different transgenes that drive the expression of either neuronally-expressed MADD-4::YFP (from the trls66 transgenic array) (E), muscle-expressed MADD-4::YFP (from the trls78 transgenic array) (F), or muscle-expressed EVA-1::CFP (from the trls89 transgenic array) (G), or animals harbouring two of the transgenes; trls66 and trls89 (H) and trls78 and trls89 (I). The relative levels of MADD-4::YFP expression from trls66 and trls78 is shown in Figure 3.3B. Images show either the CFP channel (top), YFP channel (middle) or a merged view (bottom). Arrows in ‘H’ indicate the localization of MADD-4::YFP to EVA-1::CFP expressing muscles; arrows in ‘I’ indicate the vesicularization of MADD-4::YFP and EVA-1::CFP in the muscle cells. (J). The quantification of neuronally-secreted MADD-4::YFP localization to muscles over-expressing the indicated receptor. (K). The quantification of CFP vesicles in animals that over-express the indicated CFP-tagged receptors (x-axis) in muscles in either the presence of MADD-4::YFP expressed from dorsal muscles (mMADD-4) or pan-neuronally (nMADD-4). The colocalization of MADD-4 and EVA-1 with the RAB-11 and RAB-5 endosomal markers are shown in Figure S2b and S2c. (L&M). MADD-4::YFP fails to induce obvious vesicularization of UNC-40::CFP in a wild type background (L), but YFP-CFP vesicles are obvious in animals that lack UNC-6 (M). In A, J, and K, statistical significance (p<0.05) is indicated with a solid asterisk which is matched with a dot above the data point to which the comparison was made. In all micrographs, the scale bar represents 50 micrometers. In all graphs, standard error of the mean is shown. All analysis shown in this figure was performed by Kevin Chan except for Figure 3.4B, which was performed by myself with help from Jenny Zhang. I generated the EVA-1 construct containing the PAT-2 transmembrane domain shown in Figure 3.4B and Jenny Zhang generated the rest of the EVA-1 deletion constructs shown in Figure 3.4B. Figure adapted from Chan et al. 2014.
EVA-1 were removed either individually or in tandem with the neighboring domain [Figure 3.4B]. Next, I generated transgenic lines of eva-1 null mutant animals harboring different EVA-1 truncations and investigated the ability of the EVA-1 truncations to rescue the muscle arm development defective (Madd) phenotype of the eva-1 null mutant [Figures 3.4B, 3.5A] when expressed from the muscles. Through this study, I found that that each of EVA-1’s two predicted galactose-binding lectin-like domains, its transmembrane domain, and its short cytoplasmic region are required for muscle arm extension [Figures 3.4B and 3.5A]. Given that: i) EVA-1 is a single-pass transmembrane protein that functions in muscle to direct muscle arm extension, and that ii) our genetic analyses indicate that MADD-4 and EVA-1 function in the same pathway, we hypothesized that EVA-1 may be a receptor for MADD-4. We therefore investigated whether MADD-4 can physically interact with EVA-1. Dr. Guillermo Selman and Jenny Zhang performed the following experiments and Kevin Chan analyzed the data.

We expressed luciferase-tagged MADD-4 and SLT-1 (as a control) from HEK293 cells and collected the conditioned medium [Figure 3.4C]. We overlaid this conditioned medium onto cells expressing FLAG-tagged EVA-1, UNC-40, SAX-3, UNC-5, PAT-2 (α-integrin), or control cells not expressing any C. elegans proteins [Figure 3.4C]. We then immunoprecipitated the receptors and measured luciferase activity in the washed immunoprecipitate. We found that MADD-4 had obvious affinity for EVA-1-expressing cells, some affinity for UNC-40 and UNC-5-expressing cells, and negligible affinity for SAX-3 and PAT-2-expressing cells [Figures 3.4D]. By contrast, SLT-1 had obvious affinity for SAX-3-expressing cells, some affinity for EVA-1-expressing cells, and negligible affinity for UNC-40, UNC-5, and PAT-2-expressing cells [Figures 3.4D]. Consistent with the functional necessity of both of EVA-1’s galactose-binding lectin-like
Figure 3.5. Analyses of EVA-1 Domain Function. (A). Quantification of VL11 muscle arm extension in young adults of the indicated genotype. Statistical significance ($p<0.001$) is indicated with a solid asterisk which is matched in colour with a dot above the data point to which the comparison was made. An outlined asterisk indicates a lack of significance. (B). Analysis of the requirement of EVA-1’s predicted galactose-binding lectin-like domains in binding luciferase-tagged MADD-4. The indicated version of FLAG-tagged EVA-1 was expressed in HEK293 cells, incubated with luc-tagged MADD-4-conditioned media, immunoprecipitated using antibodies against FLAG, and the resulting co-immunoprecipitated luciferase signal was measured (see methods for more details). Statistical significance ($p<0.01$) is indicated with a solid asterisk which is matched in colour with a dot above the data point to which the comparison was made. An outlined asterisk indicates a lack of significance. (C). Representative images of the ability of the indicated versions of muscle-expressed EVA-1::CFP to recruit neuronally-expressed (from the trls66 transgenic array) MADD-4::YFP to muscle cells. The orange arrows indicate MADD-4::YFP recruitment to EVA-1::CFP-expressing muscle cells. The red asterisks indicate gut auto-fluorescence. The scale bar represents 50 micrometers. Jenny Zhang performed the experiments shown in Figure 3.5B. All the EVA-1::CFP constructs used in this study contain a C-terminal MYC and 3XFLAG epitopes. I generated the EVA-1 construct containing the PAT-2 transmembrane domain and Jenny Zhang generated all other the EVA-1 deletion constructs shown in this figure. I captured all the images shown in this figure and performed all the analysis shown in 3.5A. Figure adapted from Chan et al. 2014.
domains [Figures 3.4B and 3.5A], EVA-1 requires both of these domains to be able to co-immunoprecipitate (coIP) MADD-4 [Figures 3.4B and 3.5B].

We next analyzed MADD-4’s interaction with EVA-1 and UNC-40 in vivo. When expressed from the nervous system, MADD-4::YFP localization appears restricted to neurons [Figure 3.4E]. However, when expressed from the nervous system of animals that also express EVA-1::CFP in muscles, MADD-4::YFP localizes to the EVA-1::CFP-expressing muscles [Figures 3.4E-3.4H and 3.4J]. The ability of muscle-expressed EVA-1::CFP to relocalize neuronally-secreted MADD-4::YFP to muscles is independent of UNC-40 [Figure 3.4J]. I performed a systematic domain analysis of EVA-1 to determine which domains of EVA-1 are required for its relocalize neuronally-secreted MADD-4::YFP to the muscles. Consistent with the findings from our coIP analyses, I found that both of EVA-1’s galactose-binding lectin-like domains are required for muscle-expressed EVA-1 to recruit neuronal-expressed MADD-4 [Figures 3.4B & 3.5C]. We observe negligible recruitment of neuronally-expressed MADD-4::YFP to muscles over-expressing UNC-40 [Figure 3.4J].

In animals expressing MADD-4::YFP from neurons and EVA-1::CFP in muscles, we also observe the coincidental vesicularization of YFP and CFP [Figure 3.4K]. When MADD-4::YFP is expressed from the same EVA-1::CFP-expressing muscles, more MADD-4::YFP/EVA-1::CFP vesicles are apparent [Figures 3.4I and 3.4K]. The increase in vesicles when MADD-4::YFP is expressed from muscles is coincidental with a higher level of expression of MADD-4::YFP in this strain [See Figure 3.3B] and increased proximity to the EVA-1::CFP expressed on muscles. An early endosomal marker (RAB-5) and a recycling endosomal marker (RAB-11), (Lorenowicz et al. 2014) coincide with the MADD-4::YFP/EVA-1::CFP-containing vesicles [Figures 3.6A and 3.6B], suggesting that the MADD-4-EVA-1 complex is internalized. These results provide further
Figure 3.6. MADD-4 Induces EVA-1 Endocytosis. (A). A strain expressing muscle specific MADD-4::YFP (from the trls78 array), EVA-1::CFP (from the trls89 array) and mCherry::RAB-11 (from the huls97 array). Arrows indicate vesicles in which all three markers co-localize. (C). A strain expressing muscle specific MADD-4::YFP (from the trls78 array), EVA-1::CFP (from the trls89 array) and mCherry::RAB-5 (from the huls91 array). Arrows indicate vesicles in which all three markers co-localize. Kevin Chan captured all the images and performed all the analysis shown in this figure. Adapted from Chan et al. 2014.
support for the idea that MADD-4 interacts with EVA-1 in vivo. We observe negligible vesicularization of MADD-4::YFP with UNC-40::CFP [Figures 3.4k and 3l]. Together, our genetic, transgenic, cell biological, and coIP analyses support a model whereby EVA-1 acts as a receptor for MADD-4.

3.3.3 EVA-1 Functions within an UNC-40 Complex

So far, the work discussed above indicates that both EVA-1 and UNC-40 are required for muscle arm extension towards MADD-4 [Figure 3.1F]. Additionally, Dr. Alexandra Byrne (a former graduate student in our lab) has observed that transgenic EVA-1::CFP and UNC-40::YFP fusion proteins co-localize with one another in muscle cells [Figure 3.7A]. Of note, the absence of EVA-1 does not affect the subcellular localization UNC-40 and vice versa [Figure 3.7B and 3.7C]. Hence, we hypothesized that the two transmembrane proteins might function in a co-receptor complex to mediate MADD-4 activity. We then investigated whether EVA-1 and UNC-40 could be found in the same complex. Kevin Chan expressed functional epitope-tagged EVA-1 and UNC-40 in C. elegans muscles and tested whether they coIP and found that they do [Figure 3.8A]. By contrast, neither UNC-40 nor EVA-1 co-IP with PAT-2/α-Integrin, an unrelated transmembrane protein (negative control) [Figures 3.8B and 3.8C].

Next, I performed a systematic domain analysis to determine which domains of EVA-1 are required for its ability to coIP with UNC-40. Through this analysis, I found that both extracellular GAL lectin domains and the cytoplasmic region of EVA-1 are dispensable for its ability to coIP UNC-40 [Figure 3.8D]. This suggested that the EVA-1-UNC-40 interaction maybe mediated via the EVA-1 transmembrane domain. To test the hypothesis that the EVA-1-UNC-40 interaction is mediated by the EVA-1 transmembrane domain, I designed and generated a
Figure 3.7. Characterizing the Sub-Cellular Localization and Interaction Specificity of EVA-1 and UNC-40. (A-A’’). Functional muscle-expressed EVA-1::CFP (expressed from an extra-chromosomal array) is enriched at muscle arm termini (arrowhead) and co-localizes with functional UNC-40::YFP (expressed from the tris34 array). The white arrow in the left-hand panels indicates the muscle arm termini. The area that is boxed is magnified in the right-hand panels. The arrow in the right-hand panels indicates one area of co-localization. (B). Muscle-expressed EVA-1::CFP (from an extrachromosomal array) remains localized to muscle arm termini (arrows) in the unc-40(n324) null mutant. (C). Muscle-expressed UNC-40::YFP (from the tris34 transgene) remains localized to muscle arm termini (arrows) in the eva-1(tr301) null mutant. Dr. Alexandra Byrne captured the images shown in Figure 3.7(A-A’’). Kevin Chan captured the images shown in Figure 3.7B&C. Adapted from Chan et al. 2014.
Figure 3.8. Analysis of the EVA-1–UNC-40 interaction. (A). A western blot showing that full-length EVA-1 and full-length UNC-40 coIP (B) A control blot showing that EVA-1 fails to coIP PAT-2, an unrelated TM protein. (C). A control blot showing that UNC-40 fails to coIP PAT-2. (D). An EVA-1 domain analysis to determine the domains of EVA-1 required to coIP UNC-40 shows that both extracellular GAL Lectin domains and the cytoplasmic region of EVA-1 are dispensable for its ability to coIP UNC-40. (E). A western blot showing that UNC-40::YFP can co-immunoprecipitate the wild-type version of EVA-1::CFP but not a version of EVA-1 where its transmembrane domain has been swapped for that of PAT-2. Note that all proteins used in this study were over-expressed from worm muscles under the control of a him-4 promoter. All the EVA-1::CFP constructs used in this study also contain a C-terminal MYC and 3XFLAG epitopes. Kevin Chan generated the blots shown in 3.8A, B & C. I generated the EVA-1 construct containing the PAT-2 transmembrane domain and Jenny Zhang generated all other the EVA-1 deletion constructs shown in this figure. I generated the blots shown in 3.8D & E. Figure adapted from Chan et al. 2014
muscle-expressed FLAG-tagged EVA-1::CFP construct where the EVA-1 transmembrane domain was swapped with the transmembrane domain of PAT-2 [Figure 3.8E and see Figure 3.4B].

Next, I investigated the ability of the EVA-1(PAT-2TM)::CFP fusion protein to coIP UNC-40::YFP from worm muscles. I found that swapping out EVA-1’s transmembrane domain for that of PAT-2 abolishes its ability to coIP UNC-40 [Figure 3.8E], suggesting that EVA-1’s transmembrane domain is necessary for its interaction with UNC-40.

Next, I determined that the version of EVA-1 with its transmembrane domain swapped for that of PAT-2 fails to rescue the muscle arm defects of the eva-1 null mutant [see Figure 3.4B and 3.5A], despite being subcellularly localized to the plasma membrane of muscles, and being able to recruit neuronally-secreted MADD-4::YFP to muscles [see Figures 3.4B and 3.5C]. This suggests that the interaction between EVA-1 and UNC-40 is functionally relevant. Together, these results support the idea that EVA-1 functions within an UNC-40 complex.

3.3.4 UNC-6 Interferes with UNC-40’s Interaction with MADD-4

We previously showed that UNC-40 is localized to the tips of muscle arms (Alexander et al. 2009); is required for muscle arm extension towards normal and ectopic sources of MADD-4 [see Figure 3.1F] (Seetharaman et al. 2011), and can coIP MADD-4 when expressed from HEK293 cells [see Figure 3.4D]. However, we did not observe an obvious in vivo association between MADD-4::YFP and muscle-expressed UNC-40::CFP in worms [see Figures 3.4J-3.4I]. Given that UNC-6 is UNC-40’s canonical ligand, we tested the idea that MADD-4’s ability to interact with UNC-40 is restricted by UNC-6. Kevin Chan performed the following experiments with the help of Rachel Puckrin and Jenny Zhang. Upon the genetic removal of UNC-6, we observed an obvious increase in the vesicularization of muscle-expressed UNC-40::CFP in a
MADD-4 dependent manner [see Figures 3.4K and 3.4M], suggesting that MADD-4 and UNC-40 are capable of interacting in vivo. Together, these results suggest that UNC-40 can serve as a MADD-4 receptor and that UNC-6 can interfere with the MADD-4-UNC-40 interaction.

3.3.5 EVA-1 Sensitizes UNC-40 to the MADD-4 Cue

The results mentioned in the above sections demonstrated that: i) Both EVA-1 and UNC-40 are required for muscle arm extension towards endogenous and ectopic sources of MADD-4; ii) EVA-1 likely functions within an UNC-40 co-receptor complex; iii) In the absence of MADD-4, UNC-6 has demonstrable activity in attracting muscle arms to the ventral nerve cord; and iv) UNC-6 is capable of interfering with the MADD-4-UNC-40 interaction. Together, these observations led us to hypothesize that EVA-1’s role might be to counteract the UNC-6 interference by sensitizing UNC-40 to the MADD-4 cue. We tested this hypothesis in several ways.

First, we returned to our assay in which we redirect arms laterally by ectopically expressing MADD-4 from the CAN neuron [See Figure 3.1]. If EVA-1’s role in mediating MADD-4 attraction is to counteract UNC-6 interference, then the reason why EVA-1’s absence suppresses muscle arm extension towards CAN-expressed MADD-4 is because ventral sources of UNC-6 are interfering with UNC-40-mediated attraction to the CAN-expressed MADD-4. If true, then removal of UNC-6 should restore muscle arm extension towards CAN-expressed MADD-4 in the eva-1; unc-6 double null mutant background. Dr. Peter Roy performed the following experiment and analysis with help from Rachel Puckrin. Consistent with our hypothesis, we observed the restoration of lateral muscle arm extension towards ectopic MADD-4 in the eva-1; unc-6 double null mutants [See Figures 3.1E-F]. In addition, lateral muscle arm extension
remains dependent on UNC-40 in the eva-1; unc-6 double mutants [See Figure 3.1F]. These observations support the model whereby EVA-1 sensitizes UNC-40 to MADD-4 and this sensitization is necessary to counteract UNC-6’s interference.

Next, with help from Kevin Chan, I investigated whether our also model holds true for muscle arms that extend towards the endogenous source of MADD-4 at the ventral nerve cord. We already knew that genetic removal of UNC-6 does not restore muscle arm extension towards the ventral nerve cord in eva-1 null mutants and instead enhances the defect [See Figure 3.2D]. Although this provides evidence that UNC-6 and EVA-1 function in parallel to guide arms to the ventral cord, this observation is seemingly incongruent with the idea that UNC-6 interferes with the MADD-4 UNC-40 interaction. However, we reasoned that endogenous levels of MADD-4 from the ventral nerve cord might not be high enough to engage UNC-40 without EVA-1 present on the body wall muscle cell that we normally study, which is called ventral left muscle number 11 (VL11) (Dixon & Roy, 2005 and See Figure 3.2B). Consistent with this idea, I found that when more MADD-4 is expressed from the nervous system (from the trls66 transgenic array), the VL11 muscle arm defects of the eva-1 unc-6 double null mutant are rescued [See Figure 3.2D]. I next examined muscle arm extension from a muscle (VL19) that is located ~10 micrometers closer to the ventral nerve cord than VL11 [See Figure 3.2E] and is consequently likely to encounter more endogenous MADD-4. I found that the pattern of genetic interactions exhibited by VL19 is the same as the pattern that we uncovered in the ectopic assay; the genetic removal of UNC-6 suppresses the muscle arm defects of the eva-1 null mutant [See Figure 3.2F]. Hence, EVA-1 counteracts UNC-6’s interference of UNC-40-mediated guidance towards MADD-4 from either ectopic or endogenous sources.
Notably, in animals lacking UNC-6, I found that endogenous MADD-4 is sufficient to attract muscle arms from the muscle that is closer to it (VL19), but requires EVA-1 to attract muscle arms from the muscle that is further from it (VL11) [See Figures 3.2D-F]. This suggests that EVA-1’s ability to sensitize UNC-40 to MADD-4 is independent of UNC-6.

### 3.3.6 EVA-1 and UNC-40 Direct Axon Extension Towards MADD-4

We were curious to know whether EVA-1 might play a similar role in directing axons towards MADD-4 as it does in guiding muscle arms. Dr. Peter Roy performed the following work with help from Rachel Puckrin.

We previously demonstrated that a madd-4 null mutation can enhance the AVM mechanosensory axon guidance defects of a slt-1 or unc-6 null mutant, but not those of an unc-40 null mutant (Seetharaman et al. 2011), [Figures 3.9A-D]. Here, we found that madd-4 also failed to enhance the AVM guidance defects of an eva-1 mutant [Figures 3.9E], suggesting that EVA-1 and UNC-40 may function together in a third pathway that is independent of SLT-1 and UNC-6 to mediate attraction towards MADD-4. Note that the AVM guidance defects reported in Figure 3.9E are invariably lateral extensions like that shown in Figure 3.9C.

We next examined the requirement for these factors in guiding the AVM axon towards heightened MADD-4 expression from the dorsal muscles, which attracts the AVM axon from its normal ventral trajectory to lateral and dorsal trajectories (Seetharaman et al. 2011) [Figures 3.9C and 3.9D]. Upon considering only the lateral guidance errors, the eva-1 null is enhanced by the unc-40 null, and not enhanced by the slt-1 null [Figure 3.9F]. These interactions are entirely consistent with a previous report that shows that EVA-1 can act as SAX-3’s co-receptor.
Figure 3.9. MADD-4’s Ability to Attract AVM Axons is Dependent on EVA-1 and UNC-40. (A). A schematic illustrating the area of the worm shown in B-D. Anterior is to the right and dorsal is up. (B-D). Three worms expressing MADD-4::YFP from the dorsal muscles (from the tris78 transgenic array) in which the AVM axon (green arrowhead) extends ventrally (B), laterally (C) or dorsally (D). The ALMR neuron (yellow arrowhead) is also seen in all three panels. The muIs32 transgene is used to visualize the AVM and ALMR neurons. (E). A genetic analysis of the AVM axon guidance errors in the indicated genetic background without transgenic expression of MADD-4. In all of these loss-of-function mutant backgrounds, the misguided axons invariably extend laterally. (F & G). An analysis of laterally (F) or dorsally (G) -directed AVM axon extension in response to dorsally-expressed MADD-4::YFP. Shown is the percentage of animals in which the AVM extends laterally or dorsally, respectively. Note that in the background of dorsally-expressed MADD-4, the AVM axon already extends dorsally in more than half of the eva-1; unc-6 double mutant animals and leaves no room for the expected enhancement of eva-1’s lateral axon guidance defects by unc-6. Statistical significance is documented as described for Figure 3.1F. The alleles used in this analysis are madd-4(ok2854); unc-40(n324), eva-1(ok1133), slt-1(ok255), and sax-3(ky200). Standard error of the mean is shown in all graphs. Dr. Peter Roy captured all the images and performed all the analysis shown in this figure. Rachel Puckrin helped generate most of the strains shown in this figure. This figure is adapted from Chan et al. 2014.
for the SLT-1 ligand and that this pathway acts in parallel to one that includes UNC-40 and UNC-6 (Fujisawa et al. 2007).

A distinct set of relationships between EVA-1, UNC-40, UNC-6 and SLT-1 become evident upon considering only the dorsal guidance errors of the AVM axon like that shown in Figure 3.9D. We previously found that unc-40 is required for the dorsal redirection of the AVM axon towards dorsally-expressed MADD-4 (Seetharaman et al. 2011). We found that eva-1 is also required for this dorsal redirection and that the unc-40 eva-1 double mutant is as compromised in dorsal redirection of the AVM axon as the single unc-40 mutant [Figure 3.9G]. By contrast, eliminating the dorsal repulsive cue (SLT-1) or the ventral attractive cue (UNC-6) dramatically enhances AVM axon extension towards dorsally expressed MADD-4 (Seetharaman et al. 2011) [Figure 3.9G]. These results are consistent with the idea that EVA-1 also functions with UNC-40 in a pathway that is distinct from SLT-1 and UNC-6 to mediate the extension of the AVM axon towards MADD-4.

Finally, we tested whether UNC-6 interferes with the UNC-40-mediated AVM axon extension towards MADD-4, and concurrently, whether EVA-1 counteracts UNC-6’s interference. Indeed, we found that AVM axon guidance towards dorsally expressed MADD-4 is suppressed in animals lacking EVA-1, but is restored in these eva-1 null mutants upon removing UNC-6 [Figure 3.9G]. We tested two other allelic combinations of eva-1 and unc-6 and found similar results. Genetic removal of UNC-40 in the background of the eva-1; unc-6 double reduces AVM axon attraction to the levels seen in the eva-1 single mutant, demonstrating that guidance towards MADD-4 remains dependent on UNC-40 in the absence of EVA-1 and UNC-6 [Figure 3.9G]. As a control, we tested whether genetic removal of SLT-1 is able to restore AVM axon guidance towards dorsally-expressed MADD-4 in the eva-1 mutant.
background and found that it cannot [Figure 3.9G]. These results show that UNC-6 interferes with UNC-40’s interaction with MADD-4 with not only in the context of muscle arm extension, but during axon guidance as well. In both cell types, EVA-1’s ability to sensitize UNC-40 to MADD-4 is needed to overcome UNC-6’s interference of the UNC-40-MADD-4 interaction.
3.4. Discussion

In this chapter, I along with my collaborators have provided several lines of evidence to support three main conclusions: The first main conclusion is that the EVA-1 transmembrane protein interacts with the MADD-4 cue. Numerous lines of evidence support of this conclusion, including the following: i) EVA-1 functions cell-autonomously; ii) EVA-1 localizes to the plasma membrane of muscle arm extensions; iii) EVA-1 is necessary and sufficient to enable UNC-40-mediated attraction towards MADD-4 in the presence of UNC-6; iv) EVA-1 functions in the same genetic pathway as MADD-4; v) muscle-expressed EVA-1 is sufficient to recruit neuronally-expressed MADD-4; vi) EVA-1 co-IPs MADD-4; and vii) EVA-1 interacts with MADD-4 via its two galactose binding lectin-like domains.

The second main conclusion is that EVA-1 and UNC-40 function within a co-receptor complex to mediate attraction towards MADD-4. In the above sections, I along with my collaborators have outlined numerous lines of evidence in support of this second conclusion, including: i) Both EVA-1 and UNC-40 are necessary to mediate attraction towards MADD-4; ii) In the presence of UNC-6, neither EVA-1 nor UNC-40 alone is sufficient to mediate attraction towards MADD-4; iii) EVA-1 and UNC-40 likely function in the same pathway; iv) EVA-1 co-immunoprecipitates with UNC-40 and does so likely via its transmembrane domain; v) EVA-1’s transmembrane domain cannot be substituted with another transmembrane domain and preserve EVA-1 function, suggesting that EVA-1’s interaction with UNC-40 via its transmembrane domain is functionally relevant.

The third main conclusion is that EVA-1 sensitizes UNC-40 to the MADD-4 cue and that this heightened sensitivity is necessary to counteract UNC-6’s interference of UNC-40-mediated attraction towards MADD-4. Again, we have numerous lines of evidence in support
of this conclusion. First, we found that EVA-1 is necessary to prevent UNC-6’s interference of UNC-40-mediated muscle arm and axon extension towards endogenous and/or ectopic sources of MADD-4. Second, we determined that EVA-1 is also sufficient to enable UNC-40-mediated attraction towards MADD-4 in the presence of UNC-6 in vivo.

We examined EVA-1’s ability to modulate an UNC-40-dependent cell migration that normally doesn’t require EVA-1 (Chan et al. 2014). The expansion of the bi-lobed somatic gonad during *C. elegans* development is led by the migratory distal tip cell at the leading edge of each gonad lobe (Hedgecock et al. 1987). The distal tip cell leads a ventral to dorsal gonad expansion via the expression of UNC-40 and UNC-5 co-receptors that mediate a repulsive migration away from the ventral source of UNC-6 (Hedgecock et al. 1987). Genetic removal of any of these components results in a gonad that often fails to reach the dorsal body wall and instead expands ventrally, which in turn, results in a white patch on the animal’s ventral side (Chan et al. 2014). EVA-1 is not obviously expressed in the distal tip cells (Fujisawa et al. 2007), and loss of EVA-1 has no consequence on the migration of the distal tip cell (Chan et al. 2014), suggesting that EVA-1 plays no major role in distal tip cell migration. Given that the distal tip cells express UNC-40 (Chan et al. 1996), we tested whether dorsally-expressed MADD-4 could rescue the distal tip cell migration defect of the netrin pathway mutants.

If our model of EVA-1/UNC-40/MADD-4/UNC-6 interactions are true, then dorsal expression of MADD-4 should only be able to rescue distal tip cell migration defects in the absence of UNC-6. Indeed, dorsally-expressed MADD-4 does significantly rescue the distal tip cell migration defects of animals that lack UNC-6, but not those that lack UNC-40 or UNC-5 (Chan et al. 2014). However, we found that expressing EVA-1 ectopically in the distal tip cell of *unc-5* mutants allows the migrating cell to respond to dorsally-expressed MADD-4 despite the
presence of endogenous UNC-6 (Chan et al. 2014). Furthermore, as expected, the ability of
distal tip cell-expressed EVA-1 to confer sensitivity to the dorsally-expressed MADD-4 cue is
dependent on UNC-40 indicating that EVA-1 is both necessary and sufficient to sensitize UNC-
40 to the MADD-4 cue (Chan et al. 2014). Finally, we also determined that EVA-1 can
counteract UNC-6’s interference of UNC-40’s interaction with MADD-4 in HEK293 cell culture
experiments (Chan et al. 2014).

A model by which EVA-1 may counteract UNC-6’s interference of the MADD-4-UNC-40
interaction emerges upon considering all of the data: EVA-1, UNC-40 and MADD-4 form a
ternary complex that limits the ability of UNC-6 to engage UNC-40. It is unlikely that EVA-1
allosterically modulates UNC-40 to limit its interaction with UNC-6. If this were true, then
removing EVA-1 should suppress some of the ventral muscle arm defects observed in MADD-
4’s absence because UNC-6 would then be free to engage UNC-40. However, suppression is
not observed in the eva-1 madd-4 double mutant, making it unlikely that EVA-1 antagonizes
UNC-6’s interaction with UNC-40 in a MADD-4-independent fashion. Furthermore, our
observation that ectopically expressing EVA-1 in the distal tip cells in an otherwise wild type
background fails to induce distal tip cell migration defects, provides further evidence that it is
unlikely that EVA-1 reduces UNC-40’s sensitivity to UNC-6 in any direct way. Instead, we infer
that EVA-1 may limit UNC-6’s access to UNC-40 by increasing the local concentration of MADD-
4 and presenting it to UNC-40 within a ternary complex. This idea is consistent with our
observation that in animals lacking UNC-6, EVA-1 facilitates MADD-4 function in muscles that
are further away from the ventral nerve cord (and the source of endogenous MADD-4), but is
superfluous in muscles that are closer to the ventral nerve cord.
EVA-1’s role within the complex is unlikely to be limited to the presentation of MADD-4 to UNC-40 because an EVA-1 mutant protein lacking its cytoplasmic tail is non-functional, despite being able to bind UNC-40 and MADD-4. We speculate that EVA-1’s cytoplasmic tail is needed to recruit or engage factors or processes that facilitate UNC-40-mediated signal transduction.

We have shown that EVA-1 functions with UNC-40 to direct muscle arm and AVM axon extensions towards the MADD-4 guidance cue. However, upon comparing lateral AVM axon guidance errors to dorsal AVM guidance errors in response to dorsally-expressed MADD-4, it is evident that EVA-1 plays two distinct roles in AVM axon guidance. Considering only the dorsal guidance of the AVM axon in response to dorsally-expressed MADD-4, EVA-1 functions with UNC-40 and independent of the SLT-1 pathway to mediate extension towards MADD-4. By contrast, when we consider only lateral AVM axon guidance errors, EVA-1 functions with SLT-1 in a pathway that is distinct from UNC-40. Together, these results suggest that EVA-1 can play simultaneous roles in distinct guidance pathways within the AVM neuron during axon extension.

Why are multiple pathways needed to guide migrations along a single trajectory? One answer might be to provide increased precision. UNC-6 can be considered a global cue because ventral sources of UNC-6 can guide migrations along the entire circumference of the animal (Wadsworth et al. 1996). For example, UNC-6 guides circumferential motor axons from the ventral cord all the way to the dorsal cord (Hedgecock et al. 1990). By contrast, MADD-4’s distribution must be more locally restricted than UNC-6 because ventral sources of MADD-4 do not interfere with dorsal muscle arm extension and vice versa (Seetharaman et al. 2011). Given that UNC-6 is a global cue and MADD-4 acts locally, the MADD-4-EVA-1-UNC-40 pathway may increase the precision by which cell processes reach their target. For example, as the
ventral muscles extend arms towards the ventral cord within a field of UNC-6 ligand, EVA-1 may ensure that UNC-40 directs muscle arm extension to the source of MADD-4 expression at the ventral cord and is not confounded by the surrounding field of UNC-6 molecules. In dorsal muscles, EVA-1 might ensure that UNC-40 promotes muscle arm extension to the source of MADD-4 at the dorsal cord, and that UNC-40’s effect is not diluted by ventrally-expressed UNC-6. In this way, migrating cells and cell extensions may capitalize upon multiple sources of spatial information while at the same time, guarding themselves against this information leading them astray.
3.5 Materials and Methods

3.5.1 Nematode Strains, Counts, and Microscopy

All strains were cultured and maintained at 20°C according to standard protocol (Brenner 1974). All muscle arm counts were performed in the background of the chromosomally integrated transgenic array trIs30I or trIs70II, both of which express membrane-anchored YFP from the him-4 promoter in select distal body wall muscles along with other markers of commissural motor axons (Dixon & Roy 2005). See below for details on the trIs integrated transgenes. For transgenic strains, muscle arms were visualized either in the background of trIs30I or trIs70II, or directly from fluorescence associated with the expression of the transgenic array. To visualize YFP-tagged proteins, we used the Leica 513867 filter set; to visualize CFP-tagged proteins, we used the Leica 513866 filter set; to visualize RFPs, we used Leica’s 513868 filter set. Muscle arms were counted as previously described (Dixon & Roy 2005). AVMs and PVMs were visualized using the fluorescent reporter background muIs32II (Ch’ng et al. 2003). The direction of axon extension of 50 AVMs and PVMs from at least three separate populations of the same strain was counted from living animals on standard culture plates using an epifluorescent MZ16 dissection scope (Leica Inc.) with a 2X objective. All counts of muscle arms are from worms anaesthetized with 2-10 mM levamisole (Sigma) in M9 solution and photographed as previously described (Dixon & Roy 2005). During strain construction, genotypes like madd-4(ok2862) or slt-1(ok255), which cannot be followed using obvious behavioral phenotypes, were instead followed using PCR and/or sequence of 5 or more individual progeny from a single cloned animal.
3.5.2 Statistical Analyses

All statistical analyses were performed using a one-tailed, two-sample equal variance Student’s t-test. We used a one-tailed test because for all analyses we hypothesized that the effect would be unidirectional. For example, when comparing muscle arm extension in mutants with wild type worms, we hypothesize that the mutant mean would be smaller than the wild type mean. Lastly, we chose a test that compared two independent samples of equal variance because all counts performed fall under normal distributions.

3.5.3 A Forward Genetic Screen for Mutants that Suppress Muscle Arm Redirection towards CAN-expressed MADD-4

A forward genetic screen for mutants that suppress muscle arm redirection towards CAN-expressed MADD-4 was performed by incubating a mixed stage population of RP1912 madd-4(ok2854); trls70II; trls63X animals in 50 μM ethyl methanesulfonate (EMS) for 4 hours. 

trls63 drives expression from the ceh-23 promoter in the bisymmetrical pair of CAN neurons that extend axons along the lateral length of the worm. See below for details on the trls integrated transgenes. Resulting F1s were synchronized as L1s and grown on 6 cm plates (~3000 per plate) for 3 days, and then transferred manually to each well of a 24-well plate. F2 progeny were screened 4-7 days later for mutants with defects in redirected muscle arm extensions using a Leica MZFLIII epifluorescence dissection microscope with a 2X objective. A total of ~17,500 haploid genomes were screened clonally as described, and a further ~2,400 haploid genomes were screened non-clonally by batch screening of F2 progeny. 51 suppressor mutants were isolated from these screens.
3.5.4 Molecular Biology and Transgenics

The following chromosomally-integrated transgenic arrays were made using standard techniques (Mello et al. 1991) and are available either from the C. elegans Genetic Centre or from The constructs were built using standard protocols. Note that expression of MADD-4 and EVA-1 at relatively high concentrations can induce dominant muscle arm extension defects.

The constructs, maps and the details about the methods used to make them are available upon request.

\[\text{trls30} [pPRRF138.2(him4p::Mb::YFP)(10ng/ul);pPRZL44(hmr1bp::DsRed2)(80ng/ul);pPR2.1(unc129np::DsRed2)(40ng/ul)];\text{trls34} [pPRKC294(him4p::UNC40::YFP)(2ng/ul);pRF4(rol6(su1006))(100ng/ul)];\text{trls63} [pPRGS630(ceh23p::MADD4B::YFP)(50ng/ul);pPRGS629(ceh23p::YFP)(50ng/ul);pPRGS382(myo2p::mCherry)(5ng/ul)];\text{trls65} [pPRGS655(ceh23p::MADD4B::YFP)(50ng/ul);pPRGS629(ceh23p::YFP)(50ng/ul);pPRGS382(myo2p::mCherry)(5ng/ul)];\text{trls66} [pPRGS628(unc119p::MADD4B::MYC::3XFLAG::YFP)(50ng/ul);pPRGS382(myo2p::mCherry)(5ng/ul)];\text{trls69} [pPRKC404(him4p::PAT2::CFP)(100ng/ul);pRF4(rol6(su1006))(100ng/ul)];\text{trls70} [pPRZL138.2(him4p::Mb::YFP)(5ng/ul);pPR16(unc25p::CFP)(20ng/ul);pKS(75ng/ul)];\text{trls73} [pPRKC638.2(him4p::UNC40::MYC::3XFLAG::YFP)(100ng/ul)];\text{trls77}[pPRGS698(unc129msp::MADD4A::YFP)(12.5ng/ul);pPRGS699(unc129msp::MADD4B::YFP)(12.5ng/ul);pPRGS382(myo2p::mCherry)(2ng/ul)];\text{trls78}[pPRGS698(unc129msp::MADD4A::YFP)(12.5ng/ul);pPRGS699(unc129msp::MADD4B::YFP)(12.5ng/ul);pPRGS382(myo2p::mCherry)(2ng/ul)];\text{trls89}[pPRKC793(him4p::EVA1::MYC::3XFLAG::CFP)(50ng/ul);pPRGS382(myo-2p::mCherry)(2ng/ul);pKS(50ng/ul)].

The constructs were built using standard protocols. Note that expression of MADD-4 and EVA-1 at relatively high concentrations can induce dominant muscle arm extension defects.
3.5.5 Co-immunoprecipitation and Western Analysis

500mL of mixed stage packed worms were washed thrice with M9 solution and once with PBS, and re-suspended in 1mL ice cold solubilization buffer (25mM Tris pH7.4, 100mM NaCl, 1mM EDTA, 0.25% NP40, 1mM PMSF, 1mM Na3VO4, 2.5mg/ml Pepstatin-A, 10mM NaF and 1 protease inhibitor cocktail tablet (Roche) per 10ml of solution). The samples were incubated on ice for 30 minutes, and lysed through three cycles of flash freezing in liquid nitrogen, partial thawing and sonnicaeton for 5 seconds at 8 Watts. Next, the samples were incubated at 4°C with agitation for 30 minutes and then centrifuged (13000 rpm, 30 min, 4°C). A BCA assay (Thermo Scientific) was used to measure the protein concentration in the supernatant. 6mg/ml of protein lysate was incubated with either 30ml of packed Antiflag M2 agarose coupled antibody (Sigma) or 30ml of packed Protein A/G agarose beads (SantaCruz) along with 2-4mg of rabbit polyclonal anti-GFP antibody (GenScript) for 3 hrs with agitation at 4°C. The agarose beads were centrifuged and washed 5 times with 1ml of cold solubilization buffer. Samples were resuspended in 2X Laemllli buffer and analyzed by western blot. The FLAG immunoprecipitated samples were analyzed using rabbit anti-GFP polyclonal antibodies (Genscript), and the GFP immunoprecipitated samples were analyzed using mouse anti-GFP monoclonal antibodies (SantaCruz).

Normalization of luciferase signal to protein level expression was performed by first subtracting control luciferase signal values from experimental luciferase signal values to obtain relative luciferase signal values. Next, protein level expression was measured by quantifying the number of pixels in a given protein band as visualized on film, using the publicly available software ImageJ (http://imagej.nih.gov/ij/). Normalized relative luciferase signal was obtained by expressing relative luciferase signal values as a fraction of protein level expression. The
integrated transgenes used in the coIP experiments are as follows:

trls41[pPRKC294(him4p::UNC40::YFP)(125ng/ul);pRF4(rol6(su1006))(100ng/ul)];trls69[pPRKC404(him4p::PAT2::CFP)(100ng/ul);pRF4(rol6(su1006))(100ng/ul)];trls73[pPRKC638.2(him4p::UNC40::MYC::3XFLAG::YFP)(100ng/ul)];trls89[pPRKC793(him4p::EVA1::MYC::3XFLAG::CFP)(50ng/ul)];pPRGS382(myo2p::mCherry)(2ng/ul);pKS(50ng/ul)]. The ability of EVA-1 truncations to co-immunoprecipitate UNC-40 was performed by establishing extrachromosomal transgenes in the trls41 integrated strain, and by following the aforementioned co-IP protocol:

pPRGS382(myo2p::mCherry), pPRKC793
(him4p::EVA1::MYC::3XFLAG::CFP),pPRJZ866(him4p::EVA1(GAL1+2)::MYC::3XFLAG::CFP),pPRJZ867(him4p::EVA1(CYTO)::MYC::3XFLAG::CFP),pPRJZ868(him4p::EVA1(GAL1)::MYC::3XFLAG::CFP),pPRJZ869(him4p::EVA1(GAL2)::MYC::3XFLAG::CFP), pPRAVS927.5(him-4p::EVA-1(PAT-2-TM)::MYC::3XFLAG::CFP). EVA-1 truncation constructs were all injected at 10ng/ul together with pPRGS382 at 2ng/ul.

For protein level analysis of MADD-4 transgenes, total protein was obtained by sonnication and flash freezing worm lysates of respective integrated strains as described above. 20ul containing 50ng of total protein in 2X Laemmlsi sample buffer was analyzed by western blot. Blots were analyzed using rabbit anti-MADD-4 antibodies (which we made in conjunction with Genscript Inc. by injecting rabbits with MADD-4 peptides) and mouse anti-tubulin antibodies (Sigma) for the loading control.

### 3.5.6 HEK293T Cell Surface Binding Experiments

The constructs for expressing the five receptors in HEK293T cells are as follows:

pPRGS861(CMV8-SS::3XFLAG::EVA-1); pPRGS831(CMV8-SS::3XFLAG::UNC-5(no death domain);
pPRGS757(pCDNA3-SS::3XFLAG::PAT-2). CMV3-SS:SAX-3::3XFLAG and CMV3-SS::3XFLAG::UNC-40 were obtained from Joe Culotti (Fujisawa et al. 2007). The constructs for expressing the MADD-4B and SLT-1 from HEK293T cells are as follows: pPRGS827(pCDNA3-SS::HA::MADD-4B::Gaussia luciferase (Gluc)); and pPRGS854(pCDNA3-SS::HA::SLT-1::Gluc)). All expression plasmids have been sequence verified, and details of the constructs are available upon request.

HEK293T cells were maintained and transfected in Dulbecco’s Modified Eagle’s Medium (Gibco) supplemented with 10% FBS (DMEM-10% FBS). All transfections were performed using JetPrime transfection reagent (Polyplus Transfection).

To obtain concentrated conditioned culture media containing HA::MADD-4B::Gluc or Gluc::SLT-1::HA, 10ug of respective expression plasmid was transfected into HEK293T cells, and culture media was changed to DMEM-0.2% FBS. After 24 hours of culture, the conditioned media was collected and centrifuged to remove cellular debris, and subsequently concentrated 10 times using Amicon Ultra-15 centrifugal filter units (Millipore). When necessary, ligands were immunoprecipitated from transfected cells by using an anti-HA affinity matrix (Roche) and analyzed by western blot using rabbit anti-HA antibodies (Sigma).

For the cell surface binding assays, 2ug of individual plasmids expressing the various C. elegans receptors (previously described) were transfected into HEK293T cells. 48 hours post-transfection, culture media was removed and cells were incubated with concentrated conditioned media for 4 hours at 4°C. Cells were then harvested in a solution containing PBS, 0.2% BSA and protease inhibitors (Roche), and lysed in TNTE buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 10mM NaF, 1mM Na3VO4, 1mM PMSF, 0.5% Triton X-100) containing protease inhibitors. C. elegans receptors were immunoprecipitated using mouse anti-FLAG antibodies (SIGMA), and were washed once in lysis buffer, and then in TNTE buffer containing 0.1% Triton
X-100 plus protease inhibitor; five times for HA::MADD-4B::Gluc and 3 times for Gluc::SLT-1::HA ligands. One third of the immunoprecipitate was taken for Gaussia luciferase assays by using the BioLux Kit (NEB) and MicroLumat Plus LB96V luminometer (BERTHOLD). The remainder of the immunoprecipitates was analyzed by western blot using rabbit anti-Flag antibodies (NEB).

3.5.7 MADD-4-EVA-1 Interaction Analysis in vivo

To assay the ability of MADD-4::YFP (from the trls66 array) to localize to EVA-1::CFP expressing muscles (from the trls89 array), transgenic muscles were first identified by fluorescence under the CFP filter, and then re-examined under the YFP filter for presence of MADD-4::YFP. To assay the presence of MADD-4/EVA-1 vesicles, muscles were first identified for co-expression of both MADD-4::YFP (from the trls78 array) and EVA-1::CFP (from the trls89 array). These muscles were then examined for the presence of vesicles and were considered a positive score if containing more than two vesicles. Colocalization studies between MADD-4/EVA-1 vesicles and endosomal markers were performed by creating strains that harbor trls89, trls78 and either huls91 or huls97. huls91 is a marker for early endosomes and huls97 is a marker for recycling endosomes; both transgenes were obtained from Rik Korswagen.

Integrated transgenes used in these co-localization studies are as follows:

\textit{trls66[pPRGS628(unc119p::MADD4B::MYC::3XFLAG::YFP)(50ng/ul];pPRGS382(myo2p::mCherry)(5ng/ul)]X;trls78[pPRGS698(unc129msp::MADD4A::YFP)(12.5ng/ul];pPRGS699(unc129msp::MADD4B::YFP)(12.5ng/ul];pPRGS382(myo2p::mCherry)(2ng/ul)]IV;trls89[pPRKC793(him4p::EVA1::MYC::3XFLAG::CFP)(50ng/ul];pPRGS382(myo2p::mCherry)(2ng/ul];pKS(50ng/ul);huls91[myo3p::mCherry::RAB5];huls97[myo-3p::mCherry::RAB-11].}
3.5.8 Characterizing Sub-Cellular Localization of EVA-1 and UNC-40

Localization of EVA-1::CFP and UNC-40::YFP were examined under 63X magnification. Muscle expressed EVA-1::CFP was from an extrachromosomal array harboring pPRGS394(him-4p::EVA-1::CFP)(10ng/ul) and pPRGS382(myo-2p::mCherry)(2ng/ul). Muscle expressed UNC-40::YFP came from the trls34 transgene.
Chapter 4: Discussion

Some sections of this chapter are adapted from the following published research articles:


* Equal Contribution
4.1 MADD-4 is a Novel Guidance Cue Required for Midline-Oriented Guidance

Pioneering studies of directed membrane extension in *C. elegans* lead to the suggestion that motor axons may secrete a chemoattractant to attract muscle arms towards the dorsal and ventral midlines (Hall & Hedgecock, 1991; Hedgecock et al. 1990). This early work also suggested that the UNC-6/netrin guidance cue was unlikely to be the sole muscle arm chemoattractant because muscle arms extended to the motor axons devoid of UNC-6 (Hedgecock et al. 1990; Ishii et al. 1992). Later work from our group suggested that other characterized guidance cues were also unlikely to be the primary muscle arm chemoattractant (Dixon et al. 2006; Alexander et al. 2009).

Through a forward genetic screen aimed at uncovering key genetic regulators of muscle arm extension, we discovered a previously uncharacterized gene product called MADD-4, a member of the poorly understood ADAMTSL family of proteins (Seetharaman et al. 2011).

MADD-4 is homologous to two mammalian proteins, ADAMTSL1 & ADAMTSL3. ADAMTSL1 is known to have at least two secreted isoforms, the larger isoform is expressed exclusively in the skeletal muscles while the smaller isoform is expressed in multiple tissues including skeletal muscles, heart, liver and the kidneys (Hirohata et al. 2002). ADAMTSL3 on the other hand, has multiple isoforms that are broadly expressed (Hall et al. 2003). ADAMTSLs 1 & 3 are also referred to as “punctins” because they localize to the extracellular matrix (ECM) in a punctate pattern (Hirohata et al. 2002; Hall et al. 2003). Intriguingly, secreted MADD-4::YFP also adopts a similar punctate localization pattern around the tissue from where it is expressed (see Figures 2.10A, B and 2.12 A, B for example). Previous studies have linked ADAMTSL3 to the progression of colorectal cancers (Sjöblom et al. 2006). Furthermore, ADAMTSL3 has also been recognized as a candidate gene for schizophrenia (Dow et al. 2011).
However, outside of MADD-4, very little is known about the biological role of any ADAMTSL family member.

My work on MADD-4 alongside other members of our group has demonstrated that MADD-4 exhibits all of the expected hallmarks of a muscle arm chemoattractant and is well conserved among animals (Seetharaman et al. 2011). MADD-4 is secreted by the target cells of extending muscle arms and attracts muscle arms from a distance. Like most other components that are involved in the regulation of muscle arm extension to the midlines (Alexander et al., 2009; Alexander et al., 2010; Hao et al., 2010), MADD-4 also has a role in directing the migration of sensory axons along the dorsoventral axis (Seetharaman et al. 2011). MADD-4’s ability to direct midline-oriented migrations along the dorsoventral axis is dependent on an EVA-1 – UNC-40 co-receptor complex (see section 4.3 below) (Chan et al. 2014). Given that little is known about the mechanism of action of any ADAMTSL family member outside of MADD-4, it is highly likely that my work on MADD-4 may offer key insights into the broader role of the poorly understood ADAMTSL family of proteins.

4.2 Human Orthologs of MADD-4 and UNC-40 May Share a Common Function

Our work has demonstrated that MADD-4 and UNC-40 share a common function of directing muscle arms towards their motor neuron targets in C. elegans. Intriguingly, the human orthologs of MADD-4 and UNC-40 may also share a common function. ADAMTSL3, which is one of two MADD-4 orthologs in humans, is one of 69 genes that are frequently mutated in colorectal cancers (Sjöblom et al. 2006). DCC, which is one of two UNC-40 orthologs in humans, is so named because the chromosomal region in which it resides is frequently deleted in colorectal cancers (Fearon et al. 1990). Whether DCC is a true tumour
suppressor is contentious because mouse knockouts of DCC do not develop colorectal tumours (Fazeli et al. 1997). However, there is evidence to suggest that a stepwise disruption of the netrin receptors UNC5C and DCC is strongly associated with the progression of colorectal cancer in humans (Shin et al. 2007). Hence, it is possible that both ADAMTSL3 and DCC function as tumour suppressors.

The mechanism by which DCC may suppress colorectal cancer is distinct from its role in axon guidance. Within the epithelium of the gastro-intestinal tract, DCC is thought to mainly function as a dependence receptor (Mehlen et al., 1998), whereby when engaged by netrin at the base of the colorectal microvillus (i.e. the crypt), DCC likely promotes the survival of the epithelial cells (Mazelin et al., 2004). As the cells proliferate and move away from the source of netrin within the crypt, DCC dissociates from netrin and is proteolytically cleaved within its cytoplasmic domain to reveal a motif that promotes apoptosis (Mazelin et al., 2004). The deletion of DCC within colorectal tumours may therefore facilitate the survival of metastatic cells beyond the niche of the colorectal crypt.

One possible mechanism by which ADAMTSL3 may function as a tumour suppressor is by changing the nature of DCC’s interaction with netrin. By interfering with the netrin-DCC interaction that promotes proliferation of epithelial cells, ADAMTSL3 might promote DCC’s pro-apoptotic signaling function. Disabling ADAMTSL3 would therefore promote the proliferation of colorectal tumour cells. An important question to address going forward would be to determine whether the relationship that we have uncovered between MADD-4 and UNC-40 is indeed conserved in higher animals.
4.3 An EVA-1-UNC-40 Co-Receptor Complex Mediates Attraction Towards the MADD-4 Guidance Cue

The EVA-1 transmembrane protein was first identified as a co-receptor for the SAX-3/robo receptor within the SLT-1/slit signaling pathway that functions to direct the axons of the AVM and PVM mechanosensory neurons towards the ventral midline in *C. elegans* (Fujisawa et al. 2007). EVA-1 has been shown to co-immunoprecipitate (coIP) with SAX-3 when co-expressed in cell culture, and SLT-1 has been shown to bind both EVA-1 and SAX-3-expressing cells in culture (Fujisawa et al. 2007). We were able to independently verify these findings although in our hands SLT-1 binds with EVA-1-expressing cells to a lesser degree (Chan et al. 2014). Compelling genetic analyses, which we replicated, indicate that EVA-1 functions within a SLT-1-SAX-3 pathway that operates in parallel to the UNC-6-UNC-40 pathway to direct the ventral migration of AVM/PVM sensory axons (Fujisawa et al. 2007). Furthermore, recent work from our group presented in this thesis has revealed that apart from functioning as a SAX-3 co-receptor in the SLT-1 pathway, EVA-1 also functions as a co-receptor for UNC-40 to mediate attraction towards the MADD-4 guidance cue (Chan et al. 2014).

My investigations into the role of EVA-1 alongside my collaborators have led to three important conclusions. (i) EVA-1 binds MADD-4 both *in vitro* and *in vivo* (ii) EVA-1 binds UNC-40 and functions in a co-receptor complex with the UNC-40 to mediate the MADD-4 response (iii) The binding of EVA-1 to UNC-40 increases UNC-40’s sensitivity to MADD-4 thereby counteracting UNC-6’s interference of UNC-40-mediated attraction towards MADD-4.

A recent publication from Jean-Louis Bessereau’s group has shown that apart from functioning as a guidance cue, MADD-4 also plays a key role at the *C. elegans* neuromuscular junctions as an organizer of the postsynaptic domains (Pinan-Lucarre et al. 2014). Surprisingly, EVA-1 does not seem to be required for MADD-4’s role at the synapses (Jean-Louis Bessereau
and Peter Roy, personal communication). These observations coupled with the fact that a role for EVA-1 outside of guidance is yet to be revealed, suggest that EVA-1 may strictly function as a co-receptor within multiple guidance pathways to enhance the sensitivity of different guidance receptors to distinct guidance cues during development.

Through a systematic analysis of EVA-1’s domain functions, we found that distinct domains mediate EVA-1’s interaction with MADD-4 and UNC-40. EVA-1 is a conserved single pass transmembrane protein with a short cytoplasmic region that is rich in serine and arginine (SR) residues and an extracellular portion that is comprised of two predicted lectin-like galactose-binding (or GAL lectin) domains (Fujisawa et al. 2007). Through cell biological and coIP analyses we have determined that both extracellular GAL lectin domains of EVA-1 are required for its ability to interact with MADD-4 while the EVA-1 transmembrane domain is required for its ability to coIP UNC-40 from worm muscles (Chan et al. 2014). The cytoplasmic region of EVA-1 is dispensable for its interaction with both MADD-4 and UNC-40.

EVA-1 also shares homology with a human protein (referred to as C21orf63) encoded in the Down syndrome region of chromosome 21 (James et al. 2013; Fujisawa et al. 2007). The biological role of EVA-1’s human homolog is presently unknown. However, a recent study exploring the role of EVA-1’s ortholog in mice called EVA-1C, has shown that EVA-1C is expressed by several axons in the developing mouse spinal cord and forebrain (James et al. 2013). Given that ADAMTSL3, one of MADD-4’s mammalian orthologs is widely expressed in several tissues including the central nervous system (Hall et al. 2003) and is implicated in schizophrenia (Dow et al. 2011), it will be interesting to further explore the role of EVA-1C in the developing mouse nervous stem and determine whether there is any relationship between
EVA-1C and ADAMTLS3 or DCC (the mammalian ortholog of UNC-40) towards the establishment of neural circuits during development.

4.4 The EVA-1 Cytoplasmic Region is Critical for Muscle Arm Extension Towards MADD-4

Previous work has shown that a version of EVA-1 lacking its cytoplasmic region can rescue the AVM sensory axon guidance defects of an \textit{eva-1} null mutant, suggesting that the EVA-1 cytoplasmic region is dispensable for its role in mediating sensory axon guidance (Fujisawa et al. 2007). However, through my domain analysis study of EVA-1, I have uncovered a potentially functionally relevant role for EVA-1’s cytoplasmic region. I found that EVA-1’s cytoplasmic region is required for its ability to direct muscle arm extension towards MADD-4 (Chan et al. 2014). This raises the possibility that the EVA-1 cytoplasmic region may contain functional elements required for MADD-4 signal transduction.

Several lines of evidence indicate that EVA-1’s role in transducing the MADD-4 signal is entirely dependent on UNC-40. First, in the absence of UNC-40, both the extension of muscle arms and sensory axons towards MADD-4 is abolished, suggesting that EVA-1 cannot mediate attraction towards MADD-4 without UNC-40. Second, the suppression of muscle arm extension and axon guidance towards ectopic MADD-4 in animals that lack UNC-40 cannot be further suppressed by removing EVA-1. This suggests that EVA-1 and UNC-40 function together to mediate attraction towards MADD-4. Third, in normal circumstances, MADD-4 activity is dependent on both EVA-1 and UNC-40 (Chan et al. 2014). Hence, if EVA-1 had an UNC-40-independent role in muscle arm extension, then genetic removal of EVA-1 would enhance the muscle arm defects of the \textit{madd-4} null mutant. Because the \textit{eva-1} null mutation fails to enhance the \textit{madd-4} mutant, EVA-1 must not have a role in muscle arm extension that
is independent of UNC-40. Fourth, in special circumstances (i.e. in animals without UNC-6), MADD-4 attraction can be mediated by UNC-40 in an EVA-1-independent manner. This suggests that UNC-40 contains all of the signaling elements necessary to transduce the MADD-4 signal. Together, these observations suggest that EVA-1’s role in mediating MADD-4 attraction must be UNC-40-dependent (Chan et al. 2014).

My discovery that a version of EVA-1 lacking its cytoplasmic region is non-functional with respect to muscle arm extension, despite being able to interact with both UNC-40 and MADD-4 raises the interesting possibility the EVA-1 cytoplasmic region may be required to either recruit or engage factors or processes that facilitate UNC-40-mediated signal transduction in response to MADD-4. Potential avenues to further investigate the role of EVA-1’s cytoplasmic region in muscle arm extension would be to determine whether the EVA-1’s cytoplasmic region is required for engaging factors such as the Rho GEF UNC-73 and other downstream components like members of the WASP/WAVE family that we have previously shown (Alexander et al. 2009, Alexander et al. 2010) to function downstream of UNC-40 to direct muscle arm extensions towards the midlines. Previous work from our lab has demonstrated that muscle-expressed UNC-40::YFP and UNC-73::CFP fusion proteins co-localize at the muscle arm termini at the dorsal and ventral midlines (Alexander et al. 2009). In addition, we have also shown that UNC-40 can coIP UNC-73 when over-expressed from worm muscles (Alexander et al. 2010). Hence, it would be interesting to see if the removal of EVA-1’s cytoplasmic region disrupts the midline co-localization pattern of the UNC-40::YFP and UNC-73::CFP fusion proteins and UNC-40’s ability to coIP UNC-73 from worm muscles. Additionally, it would be worthwhile to carry out a detailed investigation towards identifying EVA-1 binding partners via mass spectrometry. This could potentially offer many new insights into the
biological role of EVA-1 in directed cell migration events during development.

4.5 Exploring the Role of ECM Components in Regulating MADD-4 Mediated Guidance

The ECM is a dynamic network comprised of a variety of diverse macromolecules including collagens, heparan sulfate proteoglycans (HSPGs), laminins, fibronectins, elastins, fibrillins and thrombospondins (Myers et al. 2011; Adams & Tucker 2000). Previous studies conducted by several groups have demonstrated that HSPGs and components of the basement membrane play a prominent role in regulating directed migration events during development (Sarrazin et al. 2011; de Wit & Verhaagen 2007; Kubota et al. 2008; Kramer & Yost 2003). The proteoglycan superfamily is comprised of molecules that are characterized by the presence of one or more glycosaminoglycan (GAG) chains covalently attached to a central protein core (Sarrazin et al. 2011). HSPGs have been shown to modulate the activities of several signaling pathways by regulating the distribution of secreted guidance cues and facilitating their interaction with cell surface receptors (Baeg & Perrimon 2000; de Wit & Verhaagen 2007). Additionally, previous work suggests that the TSR domains of members of both the ADAMTS and ADAMTSL family of proteins can mediate interactions with many distinct ECM components including HSPGs (Kuno & Matsushima 1998; Kubota et al. 2008; Tsutsui et al. 2010; Adams & Tucker 2000; Guo et al. 1992).

As a first step towards understanding the contribution of the different MADD-4 domains to its guidance function, we generated several truncated versions of MADD-4 where the different MADD-4 domains were removed either individually or in tandem with the neighboring domains (Seetharaman et al. 2011). We then analyzed the ability of the different MADD-4 truncations to redirect muscle arms and sensory axons when expressed ectopically
from the lateral and dorsal sides of the animals respectively. The results from our MADD-4 domain analysis suggest that distinct TSR domains may be involved in mediating MADD-4’s ability to attract muscle arms and sensory axons. This led us to hypothesize that interaction between distinct ECM components and different TSR domains of MADD-4 may lie at the root of MADD-4’s ability to attract muscle arms and sensory axons along the dorsoventral axis.

To further probe this idea, I initiated a pilot candidate gene analysis to determine the ability of three distinct HSPG core proteins (SDN-1/syndecan, LON-2/glypican and UNC-52/perlecan) to modulate MADD-4’s ability to redirect muscle arms and the AVM sensory axon towards lateral and dorsal sources of MADD-4. The findings from this study suggest that (i) both LON-2 and UNC-52 are required for MADD-4’s ability to redirect muscle arm extensions laterally while UNC-52 is dispensable for MADD-4’s ability to redirect the axon of the AVM neuron dorsally (ii) SDN-1 is entirely dispensable for MADD-4’s guidance function.

Previous work from our lab has shown that unc-52 loss-of-function mutants display a Madd phenotype (Dixon et al. 2006), suggesting that UNC-52’s activity is required for normal muscle arm extension towards the dorsal and ventral nerve cords (Dixon et al. 2006). UNC-52 is typically seen at the basement membrane between the hypodermis and the body wall muscles in C. elegans and is required for the attachment of the myofilament lattice to the muscle cell membrane (Rogalski et al. 1993). UNC-52 has also been shown to play an important role towards the establishment of the muscle cell polarity (Muriel et al. 2006). Hence, it is conceivable that the genetic removal of UNC-52 may affect the proper localization of receptors that are required for the MADD-4 signaling response on the plasma membrane. Consistent with idea, Serena D’Souza (another graduate student in our lab) has determined that the localization of muscle-expressed EVA-1::CFP on the plasma membrane is dramatically
diminished in *unc-52* loss-of function mutant backgrounds (Serena D’Souza, personal communication). Therefore, it is possible that *unc-52* mutants may be disrupted in their ability to extend muscle arms towards endogenous and ectopic sources of MADD-4 as a secondary consequence of their body wall muscles being disorganized.

*lon-2* loss-of-function mutants on the other hand, do not display any defects in muscle arm extension towards the dorsal and ventral nerve cords (Dixon & Roy 2005). This is intriguing because it suggests that LON-2 activity is required for the redirection of muscle arms towards ectopic sources of MADD-4 along the lateral sides of the animal but not towards endogenous sources of MADD-4 at the nerve cords. It is possible that LON-2 may function redundantly with other ECM components to regulate muscle arm extension towards the nerve cords. A more detailed investigation is required to tease out the role of UNC-52 and LON-2 in modulating MADD-4’s guidance function a definitive manner. Furthermore, it will be worthwhile to investigate if other HSPG core proteins like GPN-1/glypican, CLE-1/ collagen XVIII and AGR-1/agrin have a role in regulating MADD-4’s ability to attract cell-extensions along the dorsoventral axis.

Previous work from our lab has also identified a role for the ECM laminins in mediating muscle arm extension to the nerve cords (Dixon et al. 2006). Hence, the candidate gene analysis could be further expanded to include additional ECM components such as *nid-1*/nidogen, *let-2*/α2 type-IV collagen, *fbl-1*/Fibulin and *lam-1*/Laminin β-subunit, lam-2/Laminin γ-subunit.

As an alternative approach to identify ECM components that uniquely interact with different MADD-4 TSR domain combinations, one can look into employing a cell culture based assay such as BioID (Roux et al. 2012), to identify ECM proteins that interact with MADD-4 in
mammalian cells. Briefly, the BioID technique is as follows: The first step involves the generation of a bait protein whereby a protein of interest is fused to the biotin ligase (BirA). The bait protein is then introduced into mammalian cells. Upon the addition of free biotin to the cell culture medium, the bait protein would promiscuously biotinylate vicinal or proximal proteins. The biotinylated proteins are then selectively isolated using Streptavidin, a protein that binds to biotin with high affinity. Finally, the interacting proteins are then analyzed by mass spectrometry (Roux et al. 2012). A key advantage of BioID over other standard protein-protein interaction detection methods is that it has the ability to detect interactions with insoluble cellular proteins (Roux et al. 2012). Given that a large fraction of ECM proteins are insoluble, BioID could be a useful method to identify ECM proteins that uniquely interact with MADD-4 TSR domain combinations that show robust activity in guiding muscle arms vs. sensory axons. However, whether or not the BioID technique could be adapted for the study of worm proteins remains to be tested. Finally, an unbiased mass spectrometry approach can also be used to identify binding partners of MADD-4.

4.6 Extracellular Sugar Modifications May Regulate Muscle Arm Extension Towards MADD-4

There is mounting evidence that modifications to heparan sulfate (HS) side chains such as the epimerization of hexuronic acid residues and the sulfation of sugar residues have a major impact on the regulation of directed migration events during development (Bülow et al. 2006). Previous studies have demonstrated a critical role for HS modifications in modulating the interaction between the slit guidance cue and the robo receptor (Hussain et al. 2006). Similarly, genetic experiments conducted by Oliver Hobert’s group reveal that the HS modifying enzyme HS 60-Sulfotransferase, encoded by hst-6 modulates the ability of the EVA-1
receptor to mediate the SLT-1 response during axon guidance along the dorsoventral axis in *C. elegans* (Bülow et al. 2008). Finally, studies conducted in mice show that HS 6-O-Sulfotransferase-1 (Hs6st-1) which regulates the 6-O sulfation of HS plays a key role in regulating the migration of axon trajectories at the optic chiasm and the corpus callosum (Pratt et al. 2006; Conway et al. 2011). Together, these studies highlight an important role for extracellular sugar modifications in determining how migrating cells interpret spatial information towards the directed extension of a plasma membrane.

In this context, it will be meaningful to investigate whether mutations in genes, which encode HS modifying enzymes, also affect muscle arm extension to the midlines in *C. elegans*. Recent work from Hannes Bülow’s group has shown that two HS 3-O- Sulfotransferases, encoded by *hst-3.1* and *hst-3.2* respectively, play an important role in regulating neurite branching in *C. elegans* (Tecle et al. 2013). Interestingly, the *hst-3.1* expression pattern shows that it is expressed in multiple tissues including the body wall muscles and muscle arms (Tecle et al. 2013). Therefore, it will be worthwhile to investigate whether *hst-3.1* mutants harbor any muscle arm extension defects. Furthermore, it will be interesting to determine whether the HS 3-O-Sulfation activity is required for MADD-4’s ability to attract cell-extensions along the dorsoventral axis during development.

### 4.7 Concluding Remarks

Directed cell migration is a biological process that is central to both normal animal development and the progression of numerous congenital diseases and tumor metastasis. The Identification of key genetic regulators of directed migration events, organizing them into genetic pathways and elucidating their mechanism of action in detail is vital towards getting a
deeper understanding and appreciation of the role of directed migrations during development. The work that I carried out towards my doctoral research project has helped identify a previously uncharacterized gene product called MADD-4 as a novel guidance cue required for midline-oriented migrations in *C. elegans*.

MADD-4 is a member of the poorly understood ADAMTS family of proteins and exhibits all the expected traits of a muscle arm guidance cue. Our results demonstrate that MADD-4 is likely secreted by the motor neurons to attract muscle arms to the dorsal and ventral nerve cords. When ectopically expressed, MADD-4 can robustly redirect the trajectories of both muscle arms and sensory axons.

Together with Kevin Chan, I have shown that attraction towards MADD-4 is mediated through an EVA-1 – UNC-40 co-receptor complex. Importantly, my work along side my colleagues, has demonstrated that the interaction between EVA-1 and UNC-40 enhances UNC-40’s sensitivity to the MADD-4 cue. This enhancement becomes especially meaningful in the presence of other UNC-40 ligands, such as UNC-6. In EVA-1’s absence, UNC-6 interferes with UNC-40’s ability to respond to MADD-4. In the absence of UNC-6, UNC-40’s responsiveness to MADD-4 becomes less dependent on EVA-1. Hence, by modulating UNC-40’s responsiveness to MADD-4, EVA-1 may increase the precision by which UNC-40-directed processes can successfully navigate towards target cells expressing the MADD-4 guidance cue.

Given that we are aware of only a handful of guidance cues that direct the migration of cells and membrane extensions during development, the cloning and characterization of MADD-4 represents a major advance in our understanding of animal development.

Finally, given that no biological role has been assigned to any MADD-4 ortholog, it is very likely that the findings from my work on MADD-4 will shed light on the biological role of
the poorly understood ADAMTS family of proteins.

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