A Dissertation

titled
Uncoupling of UNC5C with Polymerized TUBB3 in Microtubules is Required in Netrin-1-Mediated Axonal Repulsion

by
Qiangqiang Shao

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Biological Sciences

_________________________________________
Dr. Guofa Liu, Committee Chair

_________________________________________
Dr. Deborah Chadee, Committee Member

_________________________________________
Dr. Jianyang Du, Committee Member

_________________________________________
Dr. Scott C. Molitor, Committee Member

_________________________________________
Dr. Joshua J. Park, Committee Member

_________________________________________
Dr. Robert Steven, Committee Member

_________________________________________
Dr. Amanda Bryant-Friedrich, Dean
College of Graduate Studies

The University of Toledo

December 2017
An Abstract of

Uncoupling of UNC5C with Polymerized TUBB3 in Microtubules is Required in Netrin-1-Mediated Axonal Repulsion

by

Qiangqiang Shao

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biological Sciences

The University of Toledo
December 2017

Modulation of microtubule (MT) dynamics is a key event of cytoskeleton remodeling in the growth cone (GC) during axon pathfinding. Here, we show that uncoupling of polymerized TUBB3 with netrin-1 repulsive receptor Uncoordinated-5C (UNC5C) is involved in netrin-1-mediated axonal repulsion. TUBB3 directly interacted with UNC5C and partially colocalized with UNC5C in the peripheral area of the GC of primary neurons from P2 mouse cerebellar external granule layer (EGL). Netrin-1 treatment reduced this interaction as well as the colocalization of UNC5C and TUBB3 in the GC. Data from in vitro co-sedimentation assay indicated that UNC5C interacted with polymerized TUBB3 in MTs and netrin-1 decreased this interaction. Knockdown of either TUBB3 or UNC5C blocked Netrin-1-promoted axonal repulsion in vitro and caused defective axonal projection of dorsal root ganglion (DRG) towards the spinal cord in vivo. Live-cell imaging of end-binding protein 3 tagged with EGFP (EB3-GFP) in primary EGL cells showed that netrin-1 asymmetrically altered MT dynamics in the GC with more MT growth in the distal than the proximal region of the GC during repulsion and knockdown of either UNC5C or TUBB3 abolished the netrin-1 effect. Altogether,
these data indicate that the disengagement of UNC5C with polymerized TUBB3 is crucial in netrin-1-mediated axonal repulsion.
This thesis is dedicated to my love, Yanmei, who has made life worth living for. This work is also dedicated to my parents, who have always loved me unconditionally and whose examples have taught me to work hard for what I am after.
Acknowledgements

I would also like to give my sincere gratitude to my advisor Prof. Guofa Liu for the continuous support of my Ph.D. study and research. His guidance helped me in all the time of research and writing of this thesis. Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Deborah Chadee, Dr. Jianyang Du, Dr. Scott C Molitor, Dr. Joshua J. Park, Dr. Robert Steven for their insightful comments and questions. Special thanks to Dr. Richard Komuniecki, an emeritus professor and a former committee member). Also my good friends Tao and Huai, who have been a constant source of support and encouragement during the challenges of graduate school. I am truly grateful to have you in my life.
# Table of Contents

Abstract iii

Acknowledgements v

Table of Contents vii

List of Figures x

List of Abbreviations xii

1. Introduction 1

1.1. The Vertebrate Nervous System 1

1.2. Axon Guidance 1

1.3. Netrins 4

1.4. Netrin Receptors 8

1.4.1. Deleted in Colorectal Cancer (DCC) 8

1.4.2. Down Syndrome Cell Adhesion Molecule (DSCAM) 10

1.4.3. Uncoordinated-5 (UNC5) 11

1.5. The Developing Mouse Cerebellum 12

1.6. Netrin-1-Mediated Axonal Repulsion in UNC5 Expressing Granule Cells 14

1.7. The Developing Dorsal Root Ganglion (DRG) 15

1.8. Netrin-1-Mediated Axonal Repulsion of DRG Neurons 15

1.9. The Cytoskeleton 16

1.9.1. Actins 16

1.9.2. Microtubules (MTs) 17

1.9.3. The Growth Cone Cytoskeleton 18
<table>
<thead>
<tr>
<th>Section Number</th>
<th>Section Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9.4</td>
<td>TUBB3 in Axon Guidance</td>
<td>21</td>
</tr>
<tr>
<td>1.9.5</td>
<td>TUBB3 in Netrin-1 Signaling</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Significance</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>Material and Methods</td>
<td>26</td>
</tr>
<tr>
<td>3.1</td>
<td>Plasmids and Constructs</td>
<td>26</td>
</tr>
<tr>
<td>3.2</td>
<td>Antibodies and Reagents</td>
<td>26</td>
</tr>
<tr>
<td>3.3</td>
<td>Primary Neuron Dissection and Cultures</td>
<td>27</td>
</tr>
<tr>
<td>3.4</td>
<td>Protein Purification</td>
<td>28</td>
</tr>
<tr>
<td>3.5</td>
<td>Immunoprecipitation and Immunoblotting</td>
<td>29</td>
</tr>
<tr>
<td>3.6</td>
<td>MT Cosedimentation Assay</td>
<td>29</td>
</tr>
<tr>
<td>3.7</td>
<td>Immunofluorescence</td>
<td>30</td>
</tr>
<tr>
<td>3.8</td>
<td>Axonal Projection of Chicken DRG Neurons <em>in vivo</em></td>
<td>31</td>
</tr>
<tr>
<td>3.9</td>
<td>Dunn Chamber Axon Guidance Assay</td>
<td>31</td>
</tr>
<tr>
<td>3.10</td>
<td>Time-Lapse Recording of EB3-GFP in Primary Cerebellar EGL Neurons</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>Results</td>
<td>33</td>
</tr>
<tr>
<td>4.1</td>
<td>UNC5C Interacts with TUBB3 in a Netrin-1-Dependent Manner</td>
<td>33</td>
</tr>
<tr>
<td>4.2</td>
<td>Netrin-1 Regulates Colocalization of UNC5C with TUBB3</td>
<td>37</td>
</tr>
<tr>
<td>4.3</td>
<td>Netrin-1 Inhibits the Interaction of UNC5C with Dynamic TUBB3 in MTs</td>
<td>39</td>
</tr>
<tr>
<td>4.4</td>
<td>TUBB3 is Required for Netrin-1/UNC5C-Mediated Axonal Repulsion</td>
<td>43</td>
</tr>
<tr>
<td>4.5</td>
<td>Netrin-1 Differentially Modulates MT Dynamics in the GC during Axonal Repulsion</td>
<td>50</td>
</tr>
<tr>
<td>4.6</td>
<td>TUBB3 is Specifically Involved in Netrin-1/UNC5C-Regulated MT Dynamics in the GC</td>
<td>55</td>
</tr>
</tbody>
</table>
4.7. TUBB3 is Required for Axonal Projection of DRG Neurons in vivo. 58

5. Discussion 65

5.1. Disengagement of UNC5C with Polymerized TUBB3 in Netrin-1 Repulsion 66

5.2. The Working Model of Direct Modulation of MT Dynamics in Netrin-1 Signaling 68

5.3. TUBB3 is a Key Downstream Component in Netrin-1 Signaling 71

5.4. Future Direction 72

References 75
List of Figures

Figure 1 Axon Guidance Ensures Neurons to Reach Proper Targets ..................................2
Figure 2 UN5C Interacts Directly with TUBB3 .................................................................34
Figure 3 Netrin-1 Reduces the Interaction of UNC5C with TUBB3 ..................................35
Figure 4 UN5C Interacts Specifically with TUBB3 .............................................................36
Figure 5 Netrin-1 Reduces the Subcellular Overlap of UNC5C with TUBB3 in the
  GC of Primary Neurons .................................................................................................38
Figure 6 Taxol and Nocodazole (Noc) Inhibit the Netrin-1-Induced UNC5C/TUBB3
  Dissociation..................................................................................................................41
Figure 7 Netrin-1 Inhibits the Interaction of UNC5C with Dynamic TUBB3 in MTs..41
Figure 8 Knockdown of TUBB3 Abolishes the Effect of Netrin-1 on
  Cosedimentation of UNC5C with Polymerized MTs ..................................................42
Figure 9 Netrin-1 Mediates Axonal Repulsion of P2 EGL Neurons .................................45
Figure 10 Knockdown of Endogenous UNC5C in Primary Neurons .................................46
Figure 11 UNC5C is Required for Netrin-1-Mediated Axonal Repulsion of P2 EGL
  Neurons .......................................................................................................................47
Figure 12 TUBB3 is Required for Netrin-1/UNC5C-Mediated Axonal Repulsion of P2
  EGL Neurons. ...............................................................................................................48
Figure 13 Quantification of Axon Turning of P2 EGL Neurons. ........................................49
Figure 14 Quantification of Initial Axon Length of P2 EGL Neurons ...............................49
Figure 15 Visualization of MT Dynamics in the GC via Time-lapse Recording of EB3-
  GFP in Primary Neurons during Axon Turning ..........................................................53
Figure 16  TUBB3 is Specifically Involved in Netrin-1/UNC5C-Regulated MT Dynamics in the GC of P2 EGL Neurons.................................................................57
Figure 17  Schematic Showing DRG Axonal Projection toward the Chick Spinal Cord after Electroporation .................................................................61
Figure 18  Overexpression of Netrin-1 in the Dorsal and Intermediate Regions of the Spinal Cord at Stage 12-15 Increases DREZ Size...............................................61
Figure 19  Overexpression of Netrin-1 in the Dorsal and Intermediate Regions of the Spinal Cord at Stage 16 Increases DREZ Size .......................................62
Figure 20  TUBB3 is Essential for DRG Axonal Projection in vivo..........................63
Figure 21  A Working Model of Direct Involvement of MT Dynamics in Netrin-1-Mediated Axonal Repulsion. .................................................................70
List of Abbreviations

AOE ....................... Area of Effect
cAMP .......................... Adenosine-3’ , 5’ -cyclic Monophosphate
ANOVA ........................ Analysis of Variance

C. elegans .................. Caenorhabditis elegans
cGMP .......................... Guanosine-3’ , 5’ -cyclic Monophosphate
CNS ............................ The Central Nervous System

DBD ......................... DCC Binding Domain
DCC ........................... Deleted in Colorectal Cancer
DD ............................... Death Domain
DRG ........................... Dorsal Root Ganglion
DSCAM ........................ Down Syndrome Cell Adhesion Molecule

EGL .......................... External granule layer
EGF ........................... Epidermal Growth Factor

FAK ........................... Focal Adhesion Kinase
FBS ........................... Fetal Bovine Serum
FNIII .......................... Fibronectin III Repeats

GC ............................ The Growth Cone

ICD ........................... Intracellular Domain
Ig ............................... Immunoglobulin
IGL ............................. Inner Granule Layer

JNK 1 .......................... c-Jun N-terminal Kinase 1

MAPs .......................... Microtubule Associate Proteins
ML ............................... Molecular Layer

NGF ........................... Nerve Growth Factor

PAK ........................... p21-Activated Kinase
PCL ............................. Purkinje Cell Layer
PLL ............................. Poly-L-lysine
PNS ............................ The Peripheral Nervous System

SH3 ........................... SRC Homology 3 Domain motifs
TSP..........................Thrombospondin
TUBB3 .....................Tubulin beta-3

UNC5 .......................Uncoordinated-5
UNC6 .......................Uncoordinated-6
uRL ........................Upper Rhombic Lip

WM ........................White Matter
WT ..........................Wild Type

MTOC .......................Microtubule-Organizing Center

VZ ..........................Ventricular Zone

γ-TuRC ......................γ-Tubulin Ring Complex
Chapter One

Introduction

1.1 The Vertebrate Nervous System

The nervous system is a complex neural network transmitting neuronal signals among different parts of the body. The vertebrate nervous system is composed of the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is the processing unit of the nervous system while the PNS functions to receive information from the environment and transmit CNS signaling outputs. The neuron is the basic functional unit of the nervous system. A typical neuron includes three parts: dendrites, soma, and the axon. Dendrites receive incoming signals while the axon carries the signals to other neurons or cells. By estimation, the human brain contains 100 billion neurons and 100 trillion synapses to achieve everyday cognition, learning, memory and so on.

1.2 Axon Guidance

Axon guidance, as known as axon pathfinding, is critical for proper nervous system development and function. During the elaboration of the nervous system, neurons and axons may migrate for a long distance before reaching to their targets. Observations of developing axonal projections reveal that axons extend to the vicinity of their proper target in a highly stereotype and directed manner. The process named axon guidance ensures axons reach their proper targets (Fig. 1).
Studies on axon guidance have indicated that axons respond to the coordinate actions of two types of guidance cues: long-range and short-range guidance cues, which can be either attractive or repulsive.

Axons are guided by short-range guidance cues through contact-mediated mechanisms involving nondiffusible cell surface and extracellular matrix molecules. Axons need a suitable physical substrate that is both adhesive and permissive for growth. Contact attraction allows axons to project freely to an axon-free environment at the very beginning of the embryo development or project in an environment that crossed by a scaffold of earlier projecting axons. Later axons are likely to travel along preexisting axons tracts. Contact repulsion, on the other hand, akin to the contact inhibition of cell migration. Therefore, axon growth can be limited by a corridor of a permissive substrate.
bounded with repulsive cues. Local repulsive cues can also block the forward progression of axons or even retract axons (Tessier-Lavigne and Goodman., 1996).

The concept of long-range axon guidance was first proposed in the late nineteenth century. The GC in the developing mammalian brain appears as a swelling at the end of the growing nerve fibers. During the extension of an axon, the morphology of the GC changes. The active involvement of the GC in steering the direction of axon growth in response to an unknown force. It was not until the 1940s that the first breakthrough in axon guidance occurred. Work on the organization of regenerating optic nerve and motor neurons found that the correct reconnection of these topological arranged neurons is not mediated by mechanical or functional factors, but “particular affinities” within neurons (reviewed in Kozulin and Richards., 2016). The theory was developed further in the 1950s and 1960s with advances in the understanding of the biochemical properties of neuronal development (Levi-Montalcini., 1950; Levi-Montalcini., 1964; Levi-Montalcini and Booker., 1960). Transplanting mouse sarcoma tissue into chicken embryos resulted in innervation of embryo’s internal organs, which provided leads to the later discovery of nerve growth factor (NGF) (Levi-Montalcini., 1975). In 1979, evidence was discovered to support NGF as a chemoattractant for chick dorsal root ganglion axons in vivo (Cohen et al., 1979). These discoveries laid the foundation of GC and axon guidance.

More recently, however, a compelling proof of chemotaxis was provided by the discovery of netrins and their homologs in C. elegans and mice (Hedgecock et al., 1990; Kennedy et al., 1994), which were the first molecules purified based on their chemotactic axon guidance activity. Since this discovery, more and more guidance molecules and their receptors and downstream signaling pathways have been discovered.
The key event involved in axon guidance is the interaction of the GC with guidance molecules. Each developing axon has a highly motile structure at the very end of the growing tip named the GC. The GC plays a primary role in guiding axons to their proper target field. Axon guidance is achieved by the GC detecting guidance molecules in the extracellular environment and initiating specific downstream signaling to alter GC morphology to determine their direction of growth and motility (Guan and Rao, 2003; Dent et al., 2011; Kolodkin and Tessier-Lavigne, 2011). These guidance molecules include guidance cues and others. Guidance cues can be either fixed in particular tissues or diffusible, functioning to attract or repel axons. The four canonical guidance cues are netrins (Colamarino and Tessier-Lavigne, 1995; Kennedy, 2000; Merz and Culotti, 2000), semaphorins (Kolodkin and Ginty, 1997; Raper, 2000), ephrins (Flanagan and Vanderhaeghen, 1998; O’Leary and Wilkinson, 1999) and Slits (Wong et al., 2002; Wu et al., 1999). In addition, many other classes of extracellular molecules are involved in GC navigation, such as 1) developmental morphogens, such as, BMPs, Wnts, Sonic Hedgehog, and FGFs; 2) extracellular matrix and adhesion molecules, such as, laminin, tenascins, proteoglycans, N-CAM, and L1; 3) growth factors, such as, NGF and BDNF; 4) neurotransmitters and modulators, such as GABA (Connors et al., 2016).

1.3 Netrins

Netrin family is one of the most well-studied canonical guidance cue families. The first netrin family member Unc 6 was described in C. elegans in 1990 (Hedgecock et al., 1990). The Unc6 gene encoded a signaling molecule (Hedgecock et al., 1990). Mutations of Unc 6 gene cause an uncoordinated deficiency in C. elegans due to disruption of axon pathfinding, indicating that Unc 6 was involved in the pioneer axon
guidance and cell migration along the body wall in *C. elegans* (Hedgecock et al., 1990). In 1992, the *Unc 6* gene was cloned, and the deduced amino acid sequence from the *Unc6* cDNA showed that the *Unc6* gene encoded a novel laminin-related protein with 591 amino acids novel laminin-related protein (Ishii N et al., 1992). The vertebrate homologs of *Unc 6* proteins were named after “The netrins,” meaning one who guides in Sanskrit. The first two vertebrate homologs of *Unc 6*, netrin-1 and netrin-2, were identified from embryonic rat dorsal spinal cord explants and through biochemical purification of growth-promoting factors for commissure axons in the spinal cord from chick embryo in 1994 (Kennedy et al., 1994). The amino acid sequence of chicken netrin-1 and netrin-2 were found to be 72% identical to each other and 50% identity with *Unc 6* protein (Kennedy et al., 1994). Vertebrate netrins include netrin 1-4, which are secreted proteins of the laminin family, and netrin G1-G2, which are glycopathidylinositol (GPI)-linked plasma membrane proteins.

Netrins are related to the laminin family of glycoprotein regarding protein structure. The N-terminal portion of netrins contains three repeats of the epidermal growth factor (EGF) domain V and a globular domain VI. Netrin N-terminal domains V1-3 resemble laminin γ subunits while netrin-4 and netrin-Gs resemble the β laminin subunits. Domain VI and V bind to Deleted in Colorectal Cancer (DCC) and the Uncoordinated-5 (UNC5) family receptors, respectively (Rajasekharan and Kennedy, 2009).

Netrin-1 is by far the best-characterized molecule among all the Netrin family proteins. It has a unique spatial and chromatic expression pattern and plays a crucial role during the development of nervous system. Netrin-1 is highly expressed in the ventral
midline of the neural tube, playing a critical role in axonal migration and pathfinding during the development of the nervous system (Tessier-Lavigne et al., 1988; Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Kolodziej et al., 1996). For example, netrin-1 secreted from cells in the floor plate directs commissural axons to reach the midline (Kennedy et al., 1994; Kolodziej et al., 1996). Netrin-1 knockout mice exhibit several axon guidance defects, including a reduction in the number of spinal cord commissural axons, as well as lack of the corpus callosum and hippocampal commissure, size reduction of the anterior commissure and absence of the pontine nuclei in the brain stem (Serafini et al., 1996). Netrin-1 is also weakly expressed in the developing dorsal spinal cord and is involved in primary sensory axon guidance (Serafini et al., 1996). Another study indicated that netrin-1 is transiently expressed in the dorsal spinal cord at early stages during development to prevent DRG axons from entering the mantle layer. The netrin-1 expression is then down-regulated at later stages to allow DRG axons to enter the dorsal spinal cord (Watanabe et al., 2006). In contrast to the general model of netrin-1 functioning as a long-range diffusible guidance cue, recent studies suggested that netrin-1 may instead act locally by promoting growth cone adhesion. Upon selective removal of netrin-1 from the floor plate of spinal cord and hindbrain, commissural axons normally cross the CNS midline (Dominici et al., 2017; Varadarajan et al., 2017). Moreover, netrin1 protein accumulates on the pial surface adjacent to the path of commissural axon extension, instead of being present in a gradient (Varadarajan et al., 2017). However, selective removal of netrin-1 from ventricular zone (VZ) neural progenitors in the spinal cord caused defects of axon guidance crossing the CNS midline, suggesting that expression of netrin-1 in VZ neural progenitors dorsal to the floor plate is
critical for commissural axon guidance to the CNS midline. (Dominici et al., 2017; Varadarajan et al., 2017). These findings suggested that the diffusible source of netrin-1 at the CNS midline is dispensable to direct axon trajectory towards the midline. Netrin-1 from neural progenitors, however, directs axon guidance by providing the GC an adhesive axon growth substrate. Future investigations on netrin-1 may reveal new mechanisms underlying the neural circuit formation during development.

Netrin-1 is capable of attracting or repelling axonal projection depending on the assembly of appropriate receptor complexes (Colamarino and Tessier-Lavigne, 1995; Alcaíntara et al., 2000; Quinn and Wadsworth, 2008; Lai Wing Sun et al., 2011; Liu and Dwyer, 2014). Recent studies propose a couple of hypotheses to explain the mechanism of its bi-functionality. For example, a recent crystal structure study proposed that the bi-functionality of netrin-1 resulted from its special structure (Dun and Parkinson, 2017). This study discovered that netrin-1 has a rigid molecular architecture with little interdomain flexibility due to the formation of a disulfide bond network throughout the molecule as well as the short liners between the individual netrin-1 domains. The crystal structure of chicken, human and mouse netrin-1 (LN domain and three EGF repeats) show similar head-to-stalk arrangement structure in which the globular shaped LN domain forms the head, and the three rod-like consecutive EGF repeats make up the stalk. It is an elongated molecule with a length of 150 Angstroms. This elongated structure allows different binding sites on netrin-1 to be distant, so at least two receptors can bind to netrin-1. In this way, netrin-1 receptors can coordinate utilizing different binding sites to cause the structural change to initiate distinct signaling. Netin-1 binding sites also
accommodate different receptors negatively charged molecules such as heparan sulfate, which could also determine which signaling to be initiated.

1.4 Netrin Receptors

Three mammalian netrin-1 receptors are DCC, Down Syndrome Adhesion Molecule (DSCAM) and UNC5. DCC together with DSCAM mediate netrin-1-induced axon outgrowth, branching, and attraction (Keino Masu et al., 1996; Fazeli et al., 1997; Ly et al., 2008; Liu et al., 2009; Huang et al., 2015), although DSCAM knock-out mice do not exhibit guidance defects of spinal cord commissural axonal projections (Palmesino et al., 2012). In contrast, UNC5, alone or interacting with either DCC or DSCAM, is involved in axonal repulsion (Keino-Masu et al., 1996; Kolodziej et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997; Hong et al., 1999; Finger et al., 2002; Purohit et al., 2012).

1.4.1 Deleted in Colorectal Cancer (DCC)

The human DCC gene is located at 18q21.3, containing a total of 57 possible exons and 43 possible introns. There are 13 possible alternatively sliced DCC proteins in theory. The typical DCC protein has one signal peptide motif and 12 domains, which include four immunoglobulins (Ig), and six fibronectin type III (FNIII) domains in the extracellular region, a transmembrane domain and an intracellular domain with three large cytoplasmic motifs termed P1-3 (Huber et al., 2003). The fourth and fifth FNIII repeats are needed for netrin-1 binding (Bennett et al., 1997; Geisbrecht et al., 2003; Kruger et al., 2004). In the intracellular domain, P1-3 regions contain sites required for signal protein binding and phosphorylation. The P2 domain is a proline-rich region containing four SRC Homology 3 Domain motifs (SH3), and the P3 domain includes
possible phosphorylation sites. DCC homodimerizes upon netrin-1 stimulation, through P3-P3 domain binding leading to axonal attraction (Stein and Tessier-Lavigne., 2003).

The DCC gene was expressed in many normal tissues but was absent in most colorectal carcinomas. Early studies indicated that DCC was indicated as a putative tumor suppressor gene (Fearon et al., 1990).

A role for DCC functioning as a netrin receptor in axon guidance was suggested in 1997 when both DCC and netrin-1 knockout mice showed a similar deficiency in the developing nervous system (Fazeli et al., 1997; Serafini et al., 1997). The role of DCC in commissural axonal projection has been well-studied. Dorsal commissural axons project ventrally towards the floor plate during development. DCC is expressed on the surface of commissural axons to mediate netrin-1 attraction. A gradient of netrin-1 promotes axonal projections towards the midline (Fazeli et al., 1997). During this process, complex downstream signaling is triggered after interaction of netrin-1 and DCC. The involved signaling molecules include adenosine-3’,5’-cyclic monophosphate (cAMP)/guanosine-3’,5’-cyclic monophosphate (cGMP), the Rho family of small GTPases, Rac and Cdc42, focal adhesion kinase (FAK), Fyn, Pak1, P130CAS, TRIO, Src, DOCK180 and c-Jun N-terminal Kinase 1 (JNK 1) (Shekarabi et al., 2005; Li et al., 2002; Li et al., 2004; Meriane et al., 2004; Li et al., 2006; Liu et al., 2004; Causeret et al., 2004, Qu et al., 2013). Phosphoinositide breakdown, by phosphatidylinositol-3-kinase, through phospholipase C activation, releases intracellular calcium that is needed for GC turning (Ming et al., 1999; Hong et al., 2000).

DCC can also interact with translation machinery, including EIFs, ribosomal 40S and 60S subunits, and 80S ribosomes, in a netrin-1 dependent manner. The interaction of
DCC with protein synthesis machinery is involved in local protein synthesis (Tcherkezian et al., 2010). This research was consistent with previous discoveries that netrin-1 regulates local protein translation in chemoattractant (Campbell and Holt, 2001; Ming et al., 2002). These researches suggested that DCC play a crucial role in regulating translation with spatial precision in netrin-1 mediated axonal attraction.

1.4.2 Down Syndrome Cell Adhesion Molecule (DSCAM)

The human *DSCAM* gene was initially identified as a gene associated with Down syndrome (Yamakawa et al., 1998). DSCAM maps to chromosome 21q22, and it is one of the 275 genes on chromosome 21. It encodes a transmembrane protein containing ten Ig domains, six FN III domains, one transmembrane domain, and one intracellular domain (Zhu et al., 2011).

*Drosophila* DSCAM plays an essential role in the development of the fly nervous system and possesses massive molecular diversity (Zipursky et al., 2006). The *Drosophila* genome has four *DSCAM* genes, but one of them could generate up to 38,016 different mRNA through alternative splicing (Schmucker et al., 2000). These DSCAM isoforms are involved in neuronal connections including axon guidance and branching (Chen et al., 2006; Hughes et al., 2007; Millard et al., 2007; Schmucker et al., 2000; Zhan et al., 2004; Zhu et al., 2006).

The two mammalian DSCAMs, however, show little alternative splicing (Schmucker et al., 2000). DSCAM is involved in neuronal self-avoidance for dendrite arborization (Soba et al., 2007; Fuerst et al., 2008). Retinal ganglion cells (RGCs) have defects in neuronal spacing and dendritic arborization patterns in DSCAM knockout mice (Fuerst et al., 2009). DSCAM strongly expresses in commissural axons and interacts with
netrin both in the presence or absence of DCC (Ly et al., 2008). A DCC function-blocking antibody failed to abolish the turning of commissural axons within the dorsal spinal cord (Ly et al., 2008). The absence of DSCAM results in defects of commissural outgrowth and turning (Andrews et al., 2008; Liu et al., 2009; Ly et al., 2008). These findings suggest that DSCAM can function independently as a netrin receptor in the axonal attraction.

DSCAM also strongly expresses in cerebellar neurons. The absence of DSCAM results in loss of netrin induced growth cone collapse. DSCAM interacts with multiple netrin downstream signaling components, including PAK1, FAK, and Fyn. Netrin can induce these interactions. Moreover, netrin can increase the tyrosine phosphorylation of DSCAM, together with PAK1, FAK, and Fyn. These findings suggest that DSCAM is also part of axonal repulsion (Purohit et al., 2011).

1.4.3 Uncoordinated-5 (UNC5)

The UNC5 gene was first identified in *C. elegans* (Leung-Hagesteijn et al., 1992). UNC5 is required for guiding pioneer axons (Leung-Hagesteijn et al., 1992). Vertebrate UNC5 has four homologs, UNC5 A-D, also known as UNC5 1-4. The UNC5 extracellular region contains two Ig domains and two thrombospondin (Tsp) domains, while its intracellular domain contains a ZU-5, DCC binding domain (DBD), and death domain (DD). The ZU-5 domain assembles the Zona Occludens-1 scaffolding protein found in tight junctions (Itoh et al., 1997). UNC5 alone can function in short-range netrin-1 repulsion. Binding with DCC through DBD or DSCAM allows UNC5 to function in netrin-1 long-range repulsion (Keino-Masu et al., 1996; Kolodziej et al., 1996;
Ackerman et al., 1997; Leonardo et al., 1997; Hong et al., 1999; Finger et al., 2002; Purohit et al., 2012)

In *C. elegans*, the ectopic UNC5 expression in axons in the CNS promotes both short-range and long-range repulsion away from the midline, where netrin strongly expresses (Colamarino and Tessier-Lavigne, 1995; Leonardo et al., 1997). In *Drosophila*, UNC5 is expressed in motor neurons to avoid their axons to reach netrin-expressing midline cells or muscles. UNC5 mediated repulsion contributes to guidance axons to project out of the CNS and arrive at their target location in the peripheral (Keleman and Dickson, 2001).

In mammals, four UNC5 receptors are UNC5A, UNC5B, UNC5C, and UNC5D. All four isoforms of UNC5 express in the developing CNS to mediate axonal repulsion. In mouse embryos, hindbrain-associated trochlear motor axons expressing UNC5 project away from netrin source (Burgess et al., 2006). UNC5 is also required for DRG axons to precisely project into the dorsal spinal cord in a netrin-1 dependent manner (Watanabe et al., 2006; Masuda et al., 2008). Moreover, UNC5C is involved in repulsive migratory events to establish of the rostral cerebellar boundary during the development of the nervous system (Ackerman et al., 1997; Przyborski et al., 1998). The expression of UNC5A or UNC5B in *Xenopus* spinal axons converts DCC-dependent netrin attraction into repulsion *in vitro* (Hong et al., 1999), suggesting that UNC5 can also function as a switch to shift axonal attraction to repulsion.

**1.5 The Developing Mouse Cerebellum**

The postnatal mouse cerebellum system is a typical model to study neuronal migration due to the development of cell-specific layers (Donkelaar et al., 2003).
The isthmus organizer at the midbrain-hindbrain boundary, arising from cells fated by secreting specific fate-deciding factors, initiates the growth of the mouse cerebellum. The organizing center plays a crucial role in the development of the mesencephalon and the metencephalon. A constriction forms from the caudal mesencephalon and the rostral metencephalon, which eventually develop into the anterior boundary of the cerebellum. At the caudal portion of the metencephalon, the hindbrain is segmented into rhombomeres 1 and 2 (r1 and 2). The r1 eventually develops into the rhombic lip, a structure formed between the dorsal neuroepithelium and the expanding fourth ventricle roof plate (the source of netrin-1) (Donkelaar et al., 2003). The edge of the ventricle roof plate (the r1 region or the upper rhombic lip), and the hindbrain boundary (the lower rhombic lip) define the rhombic lip. The upper rhombic lip (uRL) will generate immature migrating neuronal progenitors and give rise to the cerebellar primordium and lateral pons (Przyborski et al., 1998). The lower rhombic lip (lRL) will generate the pontine and reticulotegmental nuclei and the inferior olive (Gilthorpe et al., 2002).

Some mitotic cells in the uRL migrate to a rostromedial direction to form the cerebellar external granule layer (EGL). The EGL cells migrate away from the fourth ventricle, where the netrin-1 is expressed in E13 mouse embryos. Mitotically active EGL cells then migrate inward to the cerebellar primordium to create the whole cerebellum (Przyborski et al., 1998).

During the first two postnatal weeks, EGL cells undergo mitosis every three days and then migrating tangentially to the deeper EGL. The rate of migration then slows and neuronal processes elongate. In the molecular layer (ML), granule cells switch from the
tangential to radial migration and migrate vertically downward via attachment to Bergmann glial fibers. Granule cells detach from glial fibers after reaching the Purkinje cell layer (PCL) and detach from glial fibers. Granule cells then come across brief stationary phase. In this stage, granule cells round up and halt migration. The somas and vertical processes elongate again and migrate into the inner IGL. Granule cell migration is eventually arrested near the IGL-WM border. The parallel fibers in the ML connect to synapses in Purkinje cell dendrites (Donkelaar et al., 2003).

1.6 Netrin-1 Mediates Axonal Repulsion in UNC5C Expressing Granule Cells

Netrin-1 is a chemorepellent for axons of *Xenopus* spinal neurons and rodent trochlear motoneurons, which express UNC5 homologs (Colamarino and Tessier-Lavigne, 1995), suggesting that UNC5 is a netrin-1 receptor to mediate axonal repulsion. During development of the vertebrate cerebellum, cells from the uRL migrate over the anlage around E9.5 in response to the netrin-1 source confined to the floor plate area (Przyborski et al., 1998). This finding suggested that netrin-1 regulates EGL neurons during development in vertebrates. UNC5C is enriched in developing cerebellum, and UNC5C deficient mice demonstrated aberrant migration phenotypes during the cerebellar development. Moreover, postnatal EGL explant axons are repelled by netrin-1 (Alcantara et al., 2000). EGL cells are also described to be repelled by netrin-1 in a Dunn Chamber axonal turning assay *in vitro* (Shao et al., 2017). These studies indicated that UNC5C mediates netrin-1 repulsion in UNC5C expressing granule cells during cerebellar development.
1.7 The Developing Dorsal Root Ganglion (DRG)

A DRG is a cluster of neurons in the dorsal root of the spinal cord. These neurons derived from neural crest cells in the developing spinal cord are (pseudo) unipolar, containing an axon-like process that bifurcates with one branch extending toward the periphery and the other branch heading toward the grey matter of the spinal cord. Establishment of connectivity between peripheral and the CNS is essential for sensory processing by DRG neurons. DRG neurons can be divided into three groups: nociceptive, mechanoreceptive and proprioceptive. Nociceptive afferent neurons penetrate into the dorsal horn of the spinal cord and terminate in laminae I and II. Mechanoreceptive neuron afferents (axons) invade the gray matter of the spinal cord, then turn and enter the dorsal horn ventrally to terminate in laminae III and IV of the dorsal horn. Proprioceptive afferents (axons) pass through the medial part of the dorsal horn without branching and reach the ventral spinal cord (Honma Y et al., 2010).

1.8 Netrin-1 Mediated Axonal Repulsion of DRG Neurons.

Proprioceptive central afferents (axons) do not branch in the dorsal horn and can invade the ventral spinal cord (Sharma and Frank, 1998). Also, the axon terminals of nociceptive neurons are strictly confined to the dorsal horn and do not enter the ventral spinal cord (Perrin et al., 2001). These findings suggested that guidance cues residing in the dorsal horn and/or the ventral spinal cord guide each afferent to the proper region. However, the identity(s) of molecules involved in the ventral spinal cord-derived guidance is not well understood. Early DRG axons are sandwiched between the dermomyotome and the ventral spinal cord, both of which express netrin-1, and DRG neurons express the netrin receptor UNC5C (Masuda et al., 2008). These expression
patterns suggest that netrin-1/UNC5C signaling could play a role in early DRG neuron development. Explant assays showed that netrin-1 repelled DRG axonal projection and that netrin-1-deficient ventral spinal cord explants lost their repulsive influence on DRG axons. In embryonic day 10 (E10) netrin-1 mutant mice, DRG axons exhibit momentary disorientation (Masuda et al., 2008). Ectopic expression of netrin-1 in the dorsal and intermediate spinal cord prevents DRG axons from being directed toward the dorsal spinal cord (Masuda et al., 2008). Together, these results indicate that netrin-1 is possibly involved in DRG axon development as a guidance cue.

1.9 The Cytoskeleton

1.9.1 Actins

Actin is a subunit of microfilaments, one of the main components of the cytoskeleton. Actins participate in various cellular processes including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape. Many of these processes are achieved by extensive and intimate interactions of actin with cellular membranes. In vertebrates, three main groups of actin isoforms, α, β, and γ have been identified. The α-actin in muscle tissues is a major constituent of the contractile apparatus. The β and γ actins co-exist in most cell types as components of the cytoskeleton and mediators of internal cell motility (Doherty, 2008).

Actin polymerization and depolymerization are critical in chemotaxis and cytokinesis. Nucleating factors are required to stimulate actin polymerization. One such nucleating factor is the ARP complex which functions as a barbed end of actin to boost the nucleation of G-actin (or monomeric actin). The Arp2/3 complex can bind to actin
filaments at 70 degrees to form new actin branches off of existing actin filaments. Also, ATP-bound actin polymerizes, while hydrolysis of this ATP-bound filaments stimulates destabilization of the polymer. The growth of actin filaments can be regulated by thymosin and profilin. Thymosin binds to G-actin to buffer the polymerizing process while profilin binds to G-actin to exchange ADP for ATP promoting the monomeric addition to the plus end (Cristian Suarez and David R. Kovar, 2016).

1.9.2 Microtubules (MTs)

MTs are 25 nm hollow cylindrical structures that can grow as long as 25 µm and are highly dynamic. MTs are ubiquitous cytoskeletal elements serving a variety of structural and functional roles including maintaining cell structure, providing platforms for intracellular transport and forming the spindle during mitosis (Peter Kozulin, Linda J. Richards, 2003).

The MT is not stable and undergoes the process of growing or shrinking. Dynamic instability refers to the coexistence of assembly and disassembly at the plus end of an MT. During polymerization, both α- and β-subunits are bound to GTP. While the GTP bound to α-tubulin is stable, the GTP bound to β-tubulin is hydrolyzed to GDP shortly after assembly. The kinetics of GDP-tubulin is different from those of GTP-tubulin: GDP-tubulin is prone to depolymerization. A GDP-bound tubulin subunit at the tip of an MT will fall off, though a GDP-bound tubulin in the middle of an MT cannot spontaneously pop out. Since tubulins add to the end of the MT only in the GTP-bound state, there is a cap of GTP-bound tubulin at the tip of the MT, protecting it from disassembly. When hydrolysis catches up to the tip of the MT, it begins a rapid depolymerization and shrinkage. This switch from growth to contraction is called a
catastrophe. GTP-bound tubulins start adding to the tip of the MT again, providing a new cap and protecting the MT from shrinking (Rescue). MTs are often nucleated at an MT-organizing center (MTOC). \( \gamma \)-tubulin, which is distinct from the \( \alpha \) and \( \beta \) subunits is one of key MTOC components. The \( \gamma \)-tubulin combines with several other associated proteins to form a circular structure known as the "\( \gamma \)-tubulin ring complex" (\( \gamma \)-TuRC). This complex serves as a scaffold for \( \alpha/\beta \) tubulin dimers to begin polymerization; it acts as a cap of the minus end while MT growth continues away from the MTOC in the plus direction. Notably, there is no such MTOC in mature neurons, and the regulation of MT dynamics in the GC remains poorly understood (Borisy G et al., 2016).

In the axon shaft, MTs are bundled by MT-associated proteins (MAPs), whereas in the growth-cone central domain, they are spread apart, and could extend through the transition zone and as far as the filopodia in the peripheral-domain. MTs in GCs exhibit dynamic instability, alternating between relatively slow growth from their plus end and rapid plus-end disassembly (catastrophe), which might be followed by recovery of plus-end assembly (rescue).

### 1.9.3 The Growth Cone Cytoskeleton

The GC is a “fan-shaped” structure located at the terminus of a developing axon and is the major structure involved in guiding the axon to their target field. GCs vary in shape and size, functioning to explore the environment by continually extending and retracting membrane protrusions. The finger-like protrusions are called filopodia, while the flat sheet-like protrusions are called lamellipodia. The key feature that ensures the GC to maintain its sensitivity to guidance cues is the dynamic nature of the morphology. It can form, extend or withdraw within seconds to minutes. This level of motility is
achieved by the non-stop reconstructing of the two primary components of the GC: F-actin and MTs (Buck and Zheng, 2002; Dent and Gertler, 2003; Kalil and Dent, 2005; Lowery and Van Vactor, 2009; Dent et al., 2011; Vitriol and Zheng, 2012; Liu and Dwyer, 2014).

The GC protrusion and motility involve cyclical dynamics of actin filaments. Actin microfilaments locate at the peripheral domain of the GC where they present as bundles in the filopodia or as meshwork in the lamellipodia. These structures are dynamic and are regulated by at least two simultaneous processes, including polymerization of filamentous actin (F-actin) microfilaments at the distal tip of the filopodia, depolymerization of the lamellipodia actin meshwork and the retrograde transport of F-actin from the leading edge to the C-domain of the GC. Disassembly of the centrally located actin allows F-actin subunits to be recycled to the distal end of the filopodia microfilaments. In this way, actin meshwork distal to the area of effect (AOE) continues to depolymerize and provide F-actin subunits to the microfilaments, resulting in the retraction of distal filopodia and extension of the GC along the AOE. Many studies, both in culture (Marsh and Letourneau 1984; Lafont et al. 1993; Dent and Kalil 2001) and in model organisms (Bentley and Toroian-Raymond 1986; Chien et al. 1993; Kaufmann et al. 1998) have indicated that neurons treated with agents which depolymerize F-actin are still capable of elongation but cannot respond to guidance cues. As a result, their axons lose the capability of changing direction once they start turning and become misrouted in vivo or for axonal loops in culture. Also, F-actin forms a dynamic balance between the rate of polymerization and retrograde flow, which determines if the GC extends or withdraws its protrusions. If the polymerization rate exceeds into the retrograde flow,
then the membrane of the GC protrudes the extracellular environment, forming a GC protrusion. Also, driven by dynamic remodeling of the actin cytoskeleton, lamellipodial and filopodial protrusion is regulated in GC motility and guidance. In these process, F-actin accumulates on the side of the GC closest to the source of the gradient (Marsick et al., 2010).

Actins play an important role in GC dynamics. However, it has been brought to attention that MT cytoskeleton is in the game as well (Tanaka and Kirschner, 1995; Challacombe et al., 1997; Buck and Zheng, 2002; Dent et al., 2004, 2011; Qu et al., 2013a; Huang et al., 2015). The majority of MTs form large bundles and extend along the axonal shaft. These MT bundles provide structural support for the axon and a substrate for the intracellular transportation of vesicles and organelles to the GC. The idea that MTs are involved in axonal pathfinding was first implicated by the discovery that MTs were capable of exploring the GC periphery and the orientation of the MTs often predicted the orientation of the GC protrusion (Buck and Zhang, 2002). A hypothesis came on board that actin filaments initiated the orientation of GC and MTs may play a role followed by their leading. However, application of MT-specific drugs on only one side of a GC was sufficient to induce GC steering, indicating that just asymmetrically altering MT dynamics is sufficient for GC steering (Buck and Zhang, 2002). This discovery led to a new perspective that MTs can play an instructive role in GC guidance. Later on, netrins were shown to induce changes in MTs, resulting in increased splaying of MTs in the GC (Dent et al., 2004). Bath application of Wnt3a caused MTs to lose directionality and polymerize perpendicular to the direction of GC translocation (Purro et al., 2008). These findings suggested that MTs are directly sensitive to guidance cues.
1.9.4 TUBB3 in Axon Guidance

MT behavior varies according to isotype composition, suggesting each isotype may have properties necessary for specific cellular functions (Chao et al., 2016). β-tubulin isotype III (TUBB3) is one of the tubulin subunits found in mammals. It is distinct because the TUBB3 expression is primarily limited to neurons and it is a highly dynamic β-tubulin subunit in the nervous system; therefore, TUBB3 is widely used as a neuronal marker to differentiate neurons from glial cells.

The TUBB3 expression is at its highest during periods of axon guidance and maturation, while it decreases in the adult CNS but remains high in the PNS. The unique dynamic properties and spatiotemporal expression patterns of TUBB3 suggest that it has a specific function for neuronal development and axon maintenance.

Congenital fibrosis of the extraocular muscles type 3 (CFEOM3) is a rare ocular motility disorder in which affected individuals are born with blepharoptosis and restricted eyeball movement (Tischfield et al., 2010). CFEOM3 was studied for gene mutations that regulate human nervous system development and functions. A total of 29 Patients from unrelated families indicated 8 different heterozygous missense mutations in TUBB3 that can lead to a spectrum of human nervous system disorders named TUBB3 syndromes, including hypoplasia of oculomotor nerves, dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts (Tischfield et al., 2010). A knock-in disease mouse model was then made to reveal the mechanisms of these nervous system defect in human (Tischfield et al., 2010). For example, TUBB3R262/R262 mice showed defects in the guidance of commissural axons and cranial nerves. In comparison to WT mice, these knock-in mice have significant thinning and/or absence of commissural axon midline
crossing. Also, these knock-in mice often have aberrant fiber projections at the midline (Tischfield et al., 2010). Whole-mount neurofilament staining showed that various defects in axon guidance and branching of cranial nerves. The oculomotor nerve often mistakenly projected towards the position of the superior oblique muscles which is normally innervated by the trochlear nerve. These studies indicate that mutation of TUBB3 mutations cause major defects in the axon guidance. What's interesting is that the expression level of TUBB3 is significantly lower in TUBB3R262/R262 than WT mice. The expression level of TUBB3 decreases, however, causes an approximate 30% increase in de-tyrosinated α-tubulin, indicating an increase of stability of MTs (Tischfield et al., 2010). A follow-up study discovered six mutations in human TUBB3 gene from 12 patients bearing cortical disorganization and axonal abnormalities associated with pontocerebellar hypoplasia but not CFEOM3. Interestingly, the spectrum of these mutation-related defects is broader than previously reported including malformations of cortical development (MCD) associated with neuronal migration and differentiation as well as axon guidance defects (Poirier et al., 2010). This study also indicated that various TUBB3 mutations can alter MT instability in different ways (Poirier et al., 2010). These findings hypothesize the requirement for a neuronal β-tubulin and MT dynamic in axon guidance and brain development.

1.9.5 TUBB3 in Netrin-1 Signaling

Our recent study suggests that TUBB3 plays a role in netrin-regulated MT dynamics (Chao et al., 2013). Netrin-1/DCC mediated axonal attraction is a widely studied axon guidance model. TUBB3 colocalizes with DCC in the peripheral region of both developing spinal cord commissural neurons and cortical neurons (Chao et al., 2013).
It was then revealed that TUBB3 directly interacts with DCC and netrin-1 can induce this interaction in primary neurons (Chao et al., 2013). Disruption of MT dynamics by either MT stabilizer Taxol or de-stabilizer nocodazole abolishes this induction, suggesting that the induction of TUBB3/DCC interaction by netrin-1 involves the MT dynamics (Chao et al., 2013). It was further indicated that DCC not only binds to monomer TUBB3 as protein-protein form interaction but also to polymer TUBB3 in MTs (Chao et al., 2013). The knockdown of TUBB3 in primary neurons caused defects in commissural outgrowth and pathfinding, suggesting that TUBB3 is involved specifically in netrin-1 promoted attraction (Chao et al., 2013).

These discoveries lead to a possible working model based on the idea that TUBB3 directly binds netrin-1 receptor DCC in axonal attraction by altering the MT stability in the GC. The initiation of netrin-1 signaling is the interaction between netrin-1 and DCC. DCC can also directly interact with TUBB3 in MTs, and this interaction can be induced by the presence of netrin-1. As a result, netrin-1 signaling increases the stability of MTs that are interacting with DCC. Since the netrin-1 present as a gradient, different area of the GCs is responding to various concentration of netrin-1, causing an asymmetrical MT growth and stabilization in the GC. On the proximal side of the GC, netrin-1-dependent initial local stabilization of MTs increases MT growth and number of MT-plus-ends, which could influence actin dynamics and facilitate MT to protrude into this side, thus stabilize lamellipodia and filopodia on the proximal side. On the distal side, however, lamellipodia and filopodia are less stable and have a higher chance to collapse. Eventually, GC turns towards the netrin-1 source. In this model, the capture of dynamic
MTs by DCC is a critical step because it could stabilize filopodia with these MTs within against retraction and promote axonal outgrowth and turning.

DSCAM, another netrin-1 receptor, plays a rule in netrin-1-regulated MT dynamics in axonal branching and guidance (Huang et al., 2016). A recent study indicated TUBB3 colocalizes with DSCAM in the peripheral area of the GC including lamellipodia and filopodia. DSCAM also directly interacts with TUBB3. Moreover, TUBB3 colocalizes with DSCAM and DCC in the axon branching points and branches of developing cortical neurons and presence of netrin-1 can increase the interaction. Disruption of MT dynamics by either MT stabilizer Taxol or de-stabilizer nocodazole abolishes the induction. DSCAM can also interact with monomer TUBB3 and/or polymer TUBB3 in MTs.

What's interesting is both DCC and DSCAM can interact with TUBB3 in primary neurons, and the presence of netrin-1 can increase the interaction (Huang et al., 2016). The knockdown of DCC or use of an anti-DCC function-blocking antibody can cause the loss of netrin-1 induction on TUBB3/DSCAM interaction (Huang et al., 2016). The knockdown of DSCAM can also cause the loss of netrin-1 induction on TUBB3/DCC (Huang et al., 2016). These findings suggested that both DCC and DSCAM are involved in regulation of MT dynamics in netrin-1-mediated axon guidance. These findings suggested that the collaboration of DCC and DSCAM is required to recruit polymerized TUBB3 and MTs in the axon branching point to stabilize newly generated axon branches or filopodia against retraction and promote branch outgrowth in response to netrin-1 (Huang et al., 2016).
Chapter Two

Significance

During the development of nervous system, axonal pathfinding is a key event to ensure neurons to establish a precise connection. Axonal pathfinding is achieved through regulation of the GC navigation. The GC is a highly dynamic structure, and MTs play a crucial part within. Among all subunits of MTs, TUBB3 is distinct since its expression is primarily limited to neurons and it’s a highly dynamic β-tubulin subunit in the nervous system. Mutations of TUBB3 in patients lead to a spectrum of human nervous system disorders named TUBB3 syndromes, including hypoplasia of oculomotor nerves, dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts, suggesting that TUBB3 is involved in axonal pathfinding.

TUBB3 and MT dynamics have been recently discovered involved in netrin-1 induced axonal attraction and branching through direct binding of TUBB3 and DCC or DSCAM. However, whether modulation of MT dynamics is required for netrin-1-mediated axonal repulsion is unclear. Here, we show that disengagement of UNC5C and polymerized TUBB3, the most dynamic β-tubulin isoform in neurons, is essential for netrin-1/UNC5C-promoted axonal repulsion and axon pathfinding of DRG neurons. These results provide not only a working model of direct modulation of MTs by guidance cues in GC navigation but also increase our understanding of molecular mechanisms underlying developmental brain disorders associated with TUBB3 mutations.
Chapter Three

Material and Methods

3.1 Plasmids and Constructs

The cDNA encoding intracellular and transmembrane domains of human UNC5C (UNC5C-ICD) tagged with GST was subcloned into p-Dest 565 vectors via Gateway technology (Invitrogen), and recombinant UNC5C-ICD was purified from BL21 competent Escherichia coli. EB3-GFP constructs were gifts from Dr. Niels Galjart. TUBB3-FLAG was modified from TUBB3-V5, a generous gift from Dr. Elizabeth C. Engle. TUBB3 shRNA and control shRNA are gifts from Dr. David L. Turner. Other constructs include UNC5C control shRNA, UNC5C shRNA, full-length human UNC5C-HA, full-length human TUBB3-FLAG, and Venus-YFP (Liu et al., 2009; Purohit et al., 2012; Qu et al., 2013).

3.2 Antibodies and Reagents

The following antibodies were used: rabbit anti-FLAG (Abcam catalog #ab124462, RRID:AB_11000959), rabbit anti-UNC5C (Abcam catalog #ab89938, RRID:AB_2050439), rabbit anti-TUBB2 (Abcam catalog #ab80326, RRID:AB_1604033), rabbit anti-His (Abcam catalog #ab14923, RRID:AB_443105), rabbit anti-TUBB3 (Abcam catalog #ab2276-1, RRID:AB_1267370), rabbit anti-hemagglutinin (HA) (Santa Cruz Biotechnology catalog #sc-805, RRID:AB_631618), mouse anti-TUBB3 (Covance catalog #MMS-435P, RRID:AB_2313773), mouse anti-Myc (Calbiochem catalog #OP10F-100UG, RRID:AB_564474), rabbit anti-TUBB1 (Abiocode catalog #R0742-vp, RRID:AB_2631111), mouse BEN antibody (DSHB,
RRID:AB_2313998), rabbit anti-GST (Cell Signaling Technology catalog #2622, RRID:AB_331670), bovine anti-mouse IgG-HRP (Santa Cruz Biotechnology catalog #sc-2371, RRID:AB_634824), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology catalog #sc-2004, RRID:AB_631746), donkey anti-goat IgG-HRP (Santa Cruz Biotechnology catalog #sc-2020, RRID:AB_631728), AlexaFluor-488 goat anti-mouse IgG (Invitrogen catalog #A-21121, RRID:AB_141514), and AlexaFluor-647 goat anti-rabbit IgG (Invitrogen catalog #A-21244, RRID:AB_141663).

Taxol and nocodazole were obtained from MP Biochemicals. Purified TUBB3 was purchased from Atgen. Netrin-1 was either obtained from R&D Systems or purified with anti-Myc tag affinity matrix from the conditioned media of HEK cells stably secreting netrin-1. The control was made by sham purification from the conditioned media from HEK cells without expression of Myc-tagged netrin-1.

### 3.3 Primary Neuron Dissection and Cultures

The cerebellum from the postnatal day 2-4 (P2-P4) mice was dissected in cold HBSS and tissues were dissociated using 0.25% Trypsin at 37°C for 15 min. DNaseI was added and incubated for the last 5 min. Cells were then triturated 5 times with ice-cold DMEM culture medium containing 10% Fetal Bovine Serum (FBS) and then centrifuged at 1000 RPM for 5 min. The pellet was resuspended in cold culture media and centrifuged at 1000 RPM for 5 min 3 times. The washed cells were then plated on PLL-coated 35 mm tissue culture dishes for biochemical analysis (cell number: 4x10⁶) or PLL-coated glass coverslips for the immunostaining assays (cell number: 4x10⁴).
For nucleofection, dissociated neurons from P4 mouse cerebella or P2 external granule layer (EGL) were mixed with desired constructs and nucleofection solution. Nucleofection program is G-013(Amaxa).

For MT cosedimentation assay, transfected cerebellar neurons were cultured on poly-L-lysine (PLL, 200 µg/ml)-coated Petri dishes for 2 d and cell lysates then analyzed by immunoblotting.

For the Dunn chamber axon guidance assay, P2 cerebellar EGL neurons after nucleofection were plated onto PLL-coated coverslips and cultured in DMEM + B27 + 20 U/ml of penicillin/streptomycin at 37°C with 5% CO₂ for 2–3 d.

### 3.4 Protein Purification

Construct of UNC5C-ICD were transformed into BL21 (DE3) cells, and plated on ampicillin (100 mg/ml) selection plate and cultured for 14-16 h at 37°C. A single colony was picked and inoculated in 250 ml of LB broth with ampicillin (100 mg/ml) at 37°C overnight. When the OD reached 0.8, bacteria were induced by 100 mM IPTG at 37°C for 3 hours. The culture was centrifuged at 7k rpm at 4°C for 10 min, and the bacterial pellets were collected and re-suspended by 10 ml chilled lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% NP-40), followed by a 20 min incubation on ice. The lysed bacteria were sonicated at an output of 30% for 140 sec (20 sec sonication with 40 sec break) and centrifuged at 15k rpm for 20 min, and the supernatant was subjected to a GST column for purification. The columns were washed by the wash buffer (50 mM Tris, pH 7.5) 3 times, and the purified proteins were eluted by 2 ml of elution buffer (50 mM Tris, pH 7.5) 6 times. The eluted proteins were stored in the nitrogen tank for future use.
3.5 Immunoprecipitation and Immunoblotting

HeLa cells transfection was done using the PEI method and cells were cultured in 10% FBS culture media at 37°C for 24 h after transfection. P4 mouse cerebellar neurons were dissociated, nucleofected, and then cultured. Before netrin-1 stimulation, transfected HeLa cells and primary neurons were starved for 6 h in serum-free DMEM media and then incubated with either netrin-1-conditioned media or purified netrin-1 protein (200 ng/ml) up to 20 min. The control was made by either sham purification from the conditioned media from HEK cells or conditioned media from regular HEK293 cells not transfected with a cDNA expressing the Myc-tagged netrin-1. Primary neurons or HeLa cells were lysed and cell lysates incubated with antibodies and protein A/G-agarose beads (Santa Cruz Biotechnology) at 4°C overnight. The A/G-agarose beads were added for 3 hours, washed 3 times in cold MLB, and boiled. Protein extracts were separated with 7.5% SDS-PAGE and immunoblotted with specific antibodies. The membrane was blocked with 5% non-fat milk in PBST (1% Tween in PBS) after transfer and incubated with primary antibodies. After washing in PBST (1% Tween in PBS) 10 min 3 times, the membrane was incubated with secondary antibodies. The membrane was then washed in PBST (1% Tween in PBS) 15 min 3 times. Western blots were visualized with the enhanced chemiluminescence kit (Fisher).

3.6 MT Cosedimentation Assay

Primary cerebellar neurons from P4 mice were dissociated and cultured with culture medium (DMEM + 10% FBS + 20 U/ml of penicillin/streptomycin) for 16 h. Neurons were then stimulated with purified netrin-1 (200 ng/ml) or sham-purified control for 5 min. Primary neurons were lysed in the RIPA buffer (25 mm Tris, 150 mm NaCl,
0.1% SDS, 5 mm EDTA, 0.5% sodium deoxycholate, 1% NP-40, protease inhibitor mixture, pH 8) and cell lysates centrifuged at 100,000 × g for 1 h at room temperature. To stabilize MTs, the supernatant was incubated with 40 µm Taxol or DMSO in PEMG buffer (100 mm PIPES, 1 mm EGTA, 1 mm MgSO4, 1 mm GTP, pH 6.8) at room temperature for 30 min. MTs were pelleted by centrifugation through a 10% sucrose cushion at 50,000 × g for 30 min at 25°C. After resuspension of pellets in tubulin buffer (50 mm HEPES, 1 mm MgCl2, 1 mm EGTA, 10% glycerol, 150 mm KCl, 40 µm taxol, 1 mm GTP, 5 mm Mg-ATP, 1 mm PMSF, 1× protease inhibitor mixture), proteins in the supernatant and the pellet were analyzed by Western blotting.

### 3.7 Immunofluorescence

Primary EGL cells from P2 mouse cerebella were grown on PLL-coated coverslips and treated with either sham purified control or purified netrin-1 (200 ng/ml) for 5 or 10 min. Neurons were fixed in pre-warmed 4% PFA in DMEM at 37°C for 30 min and permeabilized with PBST (0.5% Triton X-100 in PBS) for 15 min. Cells were blocked with 0.25% BSA + 0.1% Triton in PBS at room temperature for 30 min and then incubated with primary antibody solution containing rabbit anti-UNC5C and mouse anti-TUBB3 antibodies at 4°C overnight. Neurons were incubated with fluorescent secondary antibodies (AlexaFluor-488 donkey anti-mouse IgG and AlexaFluor-647 donkey anti-rabbit IgG), and images of GCs were taken sequentially using a confocal microscope (Leica Microsystems, TCS, SP8). Fluorescent signals were acquired in a photon counting mode using Leica Microsystems HyD detector with the same setting, and the Pearson correlation coefficient (PCC) of each ROI (the peripheral region of GCs, including lamellipodia and filopodia) was calculated using the colocalization module of Leica.
Microsystems confocal software (RRID: SCR_013673). PCC values from different groups were analyzed by a one-way ANOVA with Tukey's test for post hoc comparisons.

3.8 Axonal Projection of Chicken DRG Neurons in vivo

Fertilized White Leghorn chicken eggs were incubated and embryonic development was staged according to methods described by Hamburger and Hamilton. At stages 12–16, either Venus YFP only or Venus YFP plus specific plasmids or siRNAs were injected into the neural tube of chicken embryos, and the in ovo electroporation was performed with the following program: 25 V, 5 ms, 5 pulses (BTX ECM830). At stages 23–25, chicken embryos were collected and lumbosacral segments of the chick spinal cords labeled with YFP fluorescence were isolated under a fluorescent microscope. Transverse 200 µm sections of chicken spinal cords were prepared, fixed in 4% PFA in 1 × PBS, permeabilized with PBST (1% Triton X-100 in 1 × PBS), and stained with BEN antibody (SC1/DM-GRASP protein, 1:10 in 1 × PBS). The fluorescence images of DRG axons obtained under a confocal microscope (Leica Microsystems, TCS, SP8) and the size of the dorsal root entry zone (DREZ) was measured using the Leica imaging software (Leica Microsystems, Application Suite X). Data were analyzed with a one-way ANOVA with Tukey's test for post hoc comparisons.

3.9 Dunn Chamber Axon Guidance Assay

P2 cerebellar EGL neurons were grown on appropriately coated square #3D coverslips (Erie Scientific, Portsmouth, NH) at low density. The Dunn chamber assembly protocol was adapted from Yam et al. (2009). Briefly, The Dunn chambers were pre-washed with Neurobasal media and then with neuron culture media twice before assembly. The assembly process was done in 5 min. Neuron culture media containing
sham purified control was added to fill the inner and outer wells. A coverslip with neurons was inverted over the Dunn chamber, leaving a narrow slit at the edge. Excess media was removed by blotting with filter paper, and three sides of the Dunn chamber were sealed with hot wax. Media in outer well was drained using a filter paper and replaced by neuron culture media containing purified netrin-1 (200 ng/ml) before the last side was sealed. Time-lapse images were then acquired every 5 min for 90 min using a confocal microscope (Leica, TCS, SP8). Multiple fields of view on a same coverslip were imaged sequentially using the “Split channel” function from the Leica Imaging software (Leica Application Suite X). Most GCs of EGL neurons showed significant repulsion within 40–55 min in response to a netrin-1 gradient, which was defined as “the turning stage”.

3.10 Time-Lapse Recording of EB3-GFP in Primary Cerebellar EGL Neurons

P2 cerebellar EGL neurons were nucleofected with EB3-EGFP plus desired plasmids or siRNAs and cultured for 48 h before live imaging. The Dunn chamber axon guidance assay was performed in a 37°C chamber using a confocal microscope (Leica Microsystems, TCS, SP8). Images were taken every 1–3.5 s over 60 frames per GC and corrected for photo-bleaching and background subtraction using Fiji. Images were passed through a bandpass filter before particle detection and trajectories of EB3-GFP comets in the GC were tracked using the Fiji (RRID:SCR_002285) with MTrackJ plug-in (http://www.imagescience.org/meijering/software/mtrackj/). Only comets that could be tracked for >3 frames were considered moving comets. Movies acquired at 10, 40, and 60 min were used to analyze EB3-GFP comet behavior in the GC at the pre-turning, turning, and post-turning stages, respectively.
Chapter Four

Results

4.1 UNC5C Interacts with TUBB3 in a Netrin-1-Dependent Manner.

To determine if TUBB3 is required in netrin1-mediated repulsion, we examined the potential interaction of TUBB3 with netrin-1 receptor UNC5C. FLAG-tagged human TUBB3 (TUBB3-FLAG) and HA-tagged human UNC5C (UNC5C-HA) were expressed in HeLa cells. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by Western blotting with anti-FLAG and anti-HA antibodies. TUBB3 was readily detected in the lysates immunoprecipitated with anti-HA antibody (UNC5C) (Fig. 2A). This result suggested that TUBB3 and UNC5C may interact. To further determine if UNC5C directly interacts with TUBB3, a truncated protein containing only the intracellular and transmembrane domains of UNC5C (UNC5C-ICD-GST) was purified and incubated with purified TUBB3 protein. Immunoblotting shows that TUBB3 interact directly with UNC5C-ICD (Fig. 2B). These findings suggest that TUBB3 directly interacts with UNC5C.

Secondly, to determine if endogenous TUBB3 interacts with UNC5C, we performed co-immunoprecipitation on the lysates of dissociated cerebellar neurons from postnatal day 4 (P4) mice. Previous studies indicated cerebellar neurons at this stage have a high expression of UNC5C and are repelled by netrin-1 (Bartoe et al., 2006; Purohit et al., 2012). Anti-UNC5C antibody co-immunoprecipitated TUBB3 (Fig. 3 A). The interaction of endogenous UNC5C with TUBB3 was confirmed by the reverse co-immunoprecipitation (Fig.3A). Treatment of dissociated neurons with netrin-1 decreased
the interaction of UNC5C with TUBB3 within 3 min, and the reduction lasted up to 10 min after netrin-1 stimulation (Fig. 3 B, quantification in lower panel). The interaction of endogenous UNC5C with TUBB3 in P4 cerebellar neurons was decreased in a netrin-1 dose-dependent manner (Fig. 3 C, quantification in lower panel). These data suggest that TUBB3 interacts with UNC5C in primary P4 cerebellar neurons in a netrin-1 dependent manner.

UNC5C, however, did not interact with endogenous TUBB1 and TUBB2, two other β-tubulin subunits, either in the presence or absence of netrin-1 stimulation (Fig. 4). These data suggest that TUBB3 is the specific MT β-subunit to interact with UNC5C in primary P4 cerebellar neurons.

Fig 2. UN5C Interacts Directly with TUBB3. A, UNC5C interacted with TUBB3 in HeLa cells. B, Direct interaction of TUBB3 with UNC5C. Purified TUBB3 was incubated with purified intracellular domain of UNC5C tagged with GST in vitro. The anti-GST antibody was used to immunoprecipitate proteins, and the blot was analyzed
Fig 3. Netrin-1 Reduces the Interaction of UNC5C with TUBB3. A, Netrin-1 decreased the interaction of endogenous TUBB3 with UNC5C. Dissociated neurons from P4 mouse cerebella were stimulated with either the control or netrin-1-conditioned media (netrin-1 concentration was ~300 ng/µl) for 5 min. Cell lysates were immunoprecipitated with either anti-UNC5C (left) or with anti-TUBB3 (right), and the immunoblot was analyzed with anti-TUBB3 and anti-UNC5C. Right panel, quantification of A from three independent experiments. ***p < 0.001 (two-tailed Student's t test). B and C, Upper panel, Netrin-1 regulated the interaction of endogenous UNC5C with TUBB3 in a time-dependent (B) and dose-dependent manner (C). Primary neurons from P4 mouse
cerebella were stimulated with either netrin-1-conditioned media (netrin-1 concentration was \( \sim 300 \) ng/\( \mu l \)) for 0–20 min (B) or purified netrin-1 for 5 min (C). Bottom, Quantification. *\( p < 0.05 \) (one-way ANOVA and Tukey's test for post hoc comparisons). ***\( p < 0.001 \) (one-way ANOVA and Tukey's test for post hoc comparisons).

Fig 4. UN5C Interacts Specifically with TUBB3. Endogenous UNC5C interacted with TUBB3, not TUBB1 and TUBB2, in response of netrin-1 treatment. Primary neurons from P4 mouse cerebella were stimulated with either the control or netrin-1-conditioned media (netrin-1 concentration was \( \sim 300 \) ng/\( \mu l \)) for 5 min. Cell lysates were immunoprecipitated with anti-UNC5C and followed by probing with anti-TUBB1, anti-TUBB2, or anti-TUBB3.
4.2 Netrin-1 Regulates Colocalization of UNC5C with TUBB3.

UNC5C is highly expressed in the axon GC of EGL neurons from developing mouse cerebella and involved in netrin-1-mediate GC collapse and axonal repulsion (Bartoe et al., 2006; Purohit et al., 2012). To determine if endogenous UNC5C is colocalized with TUBB3 in the GC, primary neurons from the P2 mouse EGL were cultured and stimulated with netrin-1. Neurons were then fixed and immunostained with anti-UNC5C antibodies and anti-TUBB3 antibodies. Immunofluorescent signals of UNC5C and TUBB3 were collected using a confocal microscope in “counting mode.” Counting mode allows users to acquired signals of images without any amplification. Quantitative colocalization analysis of confocal images was performed using Fiji, and the result showed the partial overlap of immunofluorescent signals of UNC5C and TUBB3 in the peripheral region of GCs, including lamellipodia and filopodia (Fig. 5A–C, quantification in J). These data suggested that UNC5C partially colocalizes with TUBB3 in the GC. In addition, netrin-1 decreased the signal correlation of UNC5C with TUBB3 in the GC of P2 EGL neurons (Fig. 5D–I, quantification in J): the PPC was 0.59 ± 0.02 in the control group (sham-purified control) and reduced to 0.49 ± 0.02 in the 5 min netrin-1 stimulation group, and further reduced to 0.37 ± 0.02 in the 10 min netrin-1 treatment group. An analysis was performed with only one fluorescent channel of the same confocal image rotated 90 degrees to test if random signals affect the colocalization analysis (Fig. 5K). The PCC in all groups reduced to approximated zero after one channel rotation, indicating that real signals from these two channels had a high degree of correlation, not an artificial overlap of random signals (Tcherkezian et al., 2010; Dunn et
al., 2011; Horn et al., 2016). Altogether, these data suggest that netrin-1 decreases UNC5C colocalization with TUBB3 in the GC of primary P2 EGL cells.

Fig 5. Netrin-1 Reduces the Subcellular Overlap of UNC5C with TUBB3 in the GC of Primary Neurons. A–C, Overlap of immunofluorescent signals of UNC5C (A) and TUBB3 (B) in the GC of mouse EGL cells. P2 mouse EGL neurons were cultured for 20 h and stimulated with a sham-purified control. C, Merged image of A, B. A–C, The value of PCC is 0.59 ± 0.02. D–I, Overlap of endogenous UNC5C with TUBB3 in the GC of EGL neurons was reduced after netrin-1 stimulation for either 5 min (D–F) or 10 min (G–I). F, I, Merged images of D, E and G, H, respectively. D–F and G–I, The value of PCC of UNC5C and TUBB3 is 0.49 ± 0.02 and 0.37 ± 0.02, respectively. Scale bar, 10 µm. J, Quantitative analysis of PCC in the control and netrin-1 groups. A total of 37 GCs in each group were analyzed. ***p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons). K, Quantification of PCC after a 90-degree counterclockwise rotation of one fluorescence channel. ***p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons). R, Rotation.
4.3 Netrin-1 Inhibits the Interaction of UNC5C with Dynamic TUBB3 in MTs.

Heterodimers of α- and β-tubulin polymerize into dynamic MTs. Our previous studies have indicated that dynamic MTs play a critical role in the interaction of TUBB3 with DCC or DSCAM during axon outgrowth, branching, or attraction (Qu et al., 2013a; Huang et al., 2015). To examine the potential role of MT dynamics in the interaction of TUBB3 and UNC5C, MT dynamics-disruption drugs were applied to primary P4 cerebellar neurons. Neurons were dissociated, cultured and then treated with either the MT-stabilizing drug paclitaxel (Taxol) or the MT-destabilizing drug nocodazole to disrupt MT dynamics before coimmunoprecipitation. UNC5C was coimmunoprecipitated with TUBB3, and netrin-1 decreased this interaction (Fig. 6). However, either pretreatment with Taxol or nocodazole abolished netrin-1 effect on the reduction of the interaction of TUBB3 and UNC5C (Fig. 6). Given that both Taxol and nocodazole disrupt MT dynamics, this result suggests that polymerized TUBB3 in MTs may be involved in the netrin-1-mediated reduction of UNC5C/TUBB3 interaction. These results indicate that MT dynamics are involved in the binding of TUBB3 to UNC5C.

To determine if UNC5C interacts with polymeric TUBB3 in MTs, cell lysates from P4 mouse cerebellar neurons were treated with or without Taxol in vitro, and an MT cosedimentation assay was carried out. In the absence of Taxol, MTs depolymerized in vitro and yielded monomerized TUBB3 subunits in the soluble supernatant and polymerized TUBB3 in the pellet (Fig. 7). The addition of Taxol to cell lysates stabilized the polymerized MTs, resulting in more sedimentation of TUBB3 in the pellet than the supernatant (Fig. 7). When netrin-1 was utilized for stimulation of cells, it further increased pellet/supernatant fractionation of TUBB3 either with or without Taxol (Fig.
These data suggested that netrin-1 promotes MT polymerization in P4 cerebellar neurons. In the absence of Taxol, most of the endogenous UNC5C remained in the supernatant with or without netrin-1 (Fig. 7). Taxol treatment increased cosedimentation of UNC5C with MTs with more UNC5C in the pellet than in the supernatant (Fig. 7). Netrin-1 reduced UNC5C in the MT-sedimented pellets (Fig. 7). These data indicate that netrin-1 decreases the interaction of endogenous UNC5C with polymerized MTs.

To further determine if the netrin-1 effect depends on TUBB3, we transfected neurons with either control TUBB3 shRNA or TUBB3 shRNA (Fig. 8 A). After stimulating such neurons with netrin-1, a cosedimentation assay was then performed in the presence of Taxol (Fig. 8 B). Data indicated that netrin-1 stimulation yielded a significant amount of TUBB3 and UNC5C in the MT-sedimented pellets in the control shRNA group (Fig. 4-3 B). Knockdown of TUBB3 abolished the effect of netrin-1 on cosedimentation of UNC5C with polymerized MTs (Fig. 8 B). These findings indicate that netrin-1 reduces the interaction of endogenous UNC5C with polymerized TUBB3 in MTs.
Fig 6. Taxol and Nocodazole (Noc) Inhibited the Netrin-1-Induced UNC5C/TUBB3 Dissociation. Dissociated P4 mouse cerebellar neurons were treated with purified netrin-1 in the presence of 1 µm Taxol, 3 µm nocodazole, or DMSO. Right panel shows the quantification of A from three independent experiments showing relative binding of UNC5C to TUBB3. ***p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons).

Fig 7. Netrin-1 Inhibits the Interaction of UNC5C with Dynamic TUBB3 in MTs. Primary P4 mouse cerebellar neurons were stimulated with netrin-1 or sham-purified control, and a cosedimentation assay of cell lysates was performed in the absence or presence of Taxol. UNC5C and TUBB3 in the pellet (P) and supernatant (S) fractions were examined by immunoblotting using anti-UNC5C and anti-TUBB3 antibodies, respectively. Right Panel shows the quantification of three independent experiments showing P/S ratio of UNC5C and TUBB3. *p < 0.05 (one-way ANOVA and Tukey's test for post hoc comparisons). ***p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons).
Fig 8. Knockdown of TUBB3 Abolished the Effect of Netrin-1 on Cosedimentation of UNC5C with Polymerized MTs. A, TUBB3 shRNA specifically knocked down endogenous TUBB3. P4 mouse cerebellar neurons were transfected with control shRNA, TUBB3 shRNA, or TUBB3 shRNA plus wild-type human TUBB3. B, Dissociated P4 mouse cerebellar neurons were transfected with either TUBB3 shRNA or control shRNA and stimulated with purified netrin-1. The cosedimentation assay was conducted with Taxol to stabilize MTs in vitro as above. Right panel shows the quantification of P/S ratio of B from three independent experiments. ***p < 0.001 (two-tailed Student's t test). Ctl, Control shRNA; R, wild-type human TUBB3.
4.4 TUBB3 is Required for Netrin-1/UNC5C-Mediated Axonal Repulsion.

The Dunn chamber allows generating a stable gradient of guidance cues that can last for hours and has been used to study chemotaxis (Wells and Ridley, 2005) and long-term axon growth from chick neurons (Maden et al., 1998). The Dunn chamber has two concentric annular wells (Fig. 9A). In this assay, the outer well was filled with either the sham-purified control or netrin-1 and P2 EGL neurons immersed in the gradient right over the bridge area were examined (Fig. 9B). The angle turned was evaluated by measuring the angle between the initial and final position of an axon. Only the neurons with net extension during the 1.5 h observation period were analyzed. In a control gradient, the direction of axonal growth remained unchanged (Fig. 9C, D). In contrast, when the neurons were placed in a netrin-1 gradient, the axons turned away from the gradient (Fig. 9C, E, F). These results indicate that netrin-1 mediates axonal repulsion of P2 EGL neurons.

To study the role of UNC5 in netrin-1-mediated axonal repulsion, primary EGL neurons from P2 mouse cerebella were transfected with Venus YFP only with or Venus YFP with UNC5C control shRNA, UNC5C shRNA, and UN5C shRNA plus WT human UNC5C plasmids before being applied to the Dunn chamber assay. As expected, in neurons transfected with Venus YFP alone or in combination with UNC5C control shRNAs, netrin-1 repelled the axon (Fig. 11, Quantification in Fig. 13). We use UNC5C shRNA to significantly reduce the level of endogenous UNC5C expression in the primary neuron. Expression of UNC5C shRNA in primary P2 EGL neurons abolished netrin-1-induced axonal repulsion (Fig. 10). Importantly, expression of WT human UNC5C rescued netrin-1-promoted axonal repulsion in neurons treated with UNC5C shRNA,
respectively (Fig. 11, Quantification in Fig. 13). These results indicate that UNC5C is required for the netrin-1-mediated axonal repulsion of P2 EGL neuron.

To examine the involvement of TUBB3 in netrin-1/UNC5C-mediated axonal repulsion, primary EGL neurons from P2 mouse cerebella were transfected with Venus YFP only or Venus YFP with TUBB3 control shRNA, TUBB3 shRNA, and TUBB3 shRNA plus WT human TUBB3 (Fig. 12, Quantification in Fig. 13). Our previous studies indicated that TUBB3 shRNAs significantly reduce the level of endogenous TUBB3 in primary neurons (Fig. 8A). Similar to UNC5C knockdown, knockdown of TUBB3 abolished netrin-1 induced axonal repulsion, while the control shRNA treated neurons still responded to netrin-1 and turned away from the netrin-1 gradient. Moreover, expression of WT human TUBB3 rescued the deficiency and allowed neurons to be repelled by netrin-1 at the presence of TUBB3 shRNAs (Fig. 12, Quantification in Fig. 13). These data indicated that TUBB3 is indeed specifically involved in netrin-1/UNC5C-mediated repulsion.

To determine if nucleofection affects axonal extension, the initial length of axons was measured. The initial axon length of transfected neurons in all transfected conditions was comparable to neurons not transfected. Also, no significant difference in axon length was suggested between control shRNA, shRNA or shRNA plus WT human rescue construct groups in either UNC5C or TUBB3 targeted group (Fig. 14). These findings suggest that knockdown of either UNC5C or TUBB3 doesn’t affect the outgrowth of P2 EGL neurons but abolishes netrin-1-mediated axonal repulsion.
Fig 9. Netrin-1 Mediates Axonal Repulsion of P2 EGL Neurons. A, A schematic of the Dunn chamber. B, Schematic diagram of axon turning of P2 mouse EGL cells in a control (left) or netrin-1 (right) gradient using a Dunn chamber axon guidance assay. C, Live-cell phase imaging showing axon turning of WT P2 EGL cells. A left-to-right netrin-1 gradient was established as shown in B. Scale bar, 10 µm. D and E, Scatter plot of the angle turned versus the initial angle for P2 EGL axons in a control (D) and netrin-1 (E) gradient. F, Quantification of axon turning of P2 EGL neurons. Data are mean ± SEM. ***p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons). The numbers on the top of each bar indicate the numbers of GCs analyzed in the corresponding groups.

Fig 10. Knockdown of Endogenous UNC5C in Primary Neurons. P4 mouse cerebellar neurons were nucleofected with control shRNA, UNC5C shRNA, or UNC5C shRNA plus wild-type human UNC5C. Ctl, Control shRNA; R, wild-type human UNC5C.
Fig 11. UNC5C is Required for Netrin-1-Mediated Axonal Repulsion of P2 EGL Neurons. Live-cell phase imaging showing axonal turning of P2 EGL neurons transfected with Venus-YFP only or combination of Venus-YFP with other indicated constructs. Nucleofection of Venus-YFP into neurons allowed visualization of axonal projection. A left-to-right netrin-1 gradient was established as shown in Fig 5-1 B. Scale bar, 10 μm.
Fig 12. TUBB3 is Required for Netrin-1/UNC5C-Mediated Axonal Repulsion of P2 EGL Neurons. Live-cell phase imaging showing axonal turning of P2 EGL neurons transfected with Venus-YFP only or combination of Venus-YFP with other indicated constructs. Nucleofection of Venus-YFP into EGL neurons allowed visualization of axonal projection. A left-to-right netrin-1 gradient was established as shown in Fig 9 B. Scale bar, 10 µm.
Fig 13. Quantification of Axon Turning of P2 EGL Neurons. Data are mean ± SEM. ***p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons). The numbers on the top of each bar indicate the numbers of GCs analyzed in the

Fig 14. Quantification of Initial Axon Length of P2 EGL Neurons. Data are mean ± SEM. ***, p < 0.001 (one-way ANOVA and Tukey's test for post hoc comparisons).
4.5 Netrin-1 Differentially Modulates MT Dynamics in the GC during Axonal Repulsion.

To examine MT dynamics in the GC during netrin-1-mediated axonal repulsion, plasmids encoding GFP-tagged end-binding protein 3 (EB3-GFP), a marker of MT plus end, were transfected into P2 mouse cerebellar EGL cells. After culturing for 2-3 d, the Dunn chamber assay was performed with either a sham-purified control gradient (Fig. 15A, B) or a netrin-1 gradient (Fig. 15C, D). The activity of EB3-GFP in the GC was recorded by live-cell imaging to indicate MT dynamics. Kymographs were generated using Fiji for each live-cell imaging to track the movement of EB3-GFP comets. Kymographs can represent the dynamic feature on images and often used to monitor a moving organelle and characterize its motion. Here, kymographs were generated for the proximal (P) (close to the gradient) and the distal (D) (distal to the gradient) regions of the GC as an x-t scan. Time points of 10, 40, and 60 min were selected to present the status of MT dynamics of the pre-turning, turning, and post-turning stages of the GC, respectively (Fig. 15B, D). The velocity (Fig. 15E–G, left panels) and travel distance (Fig. 15E–G, right panels) of the moving EB3-GFP comets were selected as indexes for MT dynamics and analyzed to assess MT dynamics in the P and D regions. The velocity of neurons in the control gradient was 0.23 ± 0.01 µm/s (P) and 0.21 ± 0.02 µm/s (D). However, the velocity was increased to 0.29 ± 0.01 µm/s (P) and 0.33 ± 0.02 µm/s (D) in the presence of netrin-1 gradient. The travel distance was increased from 1.02 ± 0.04 µm (P) and 1.15 ± 0.06 µm (D) for neurons in a control gradient in comparison to 1.30 ± 0.06 µm (P) and 1.39 ± 0.03 µm (D) in a netrin-1 gradient, respectively. In the sham-purified control group, the velocity or travel distance of moving EB3 comets during the turning or
post-turning stages was compatible to those in the pre-turning stage. This result indicates that sham-purified control gradient doesn't alter either the velocity or travel distance of moving EB3 comets in the GC at turning or post-turning stages (Fig. 15A, B, E, F). Netrin-1, in contrast, dramatically increased both the velocity and travel distance of EB3 comets in both P and D regions of the GC at the turning stage in comparison to the sham-purified control group (Fig. 15C–G). However, netrin-1 did not alter the velocity and travel distance of EB3 comets in either P or D regions at the pre-turning or post-turning stage (Fig. 15E–G). Moreover, the velocity and travel distance of moving EB3 comets in the P and D regions were affected asymmetrically by netrin-1 during GC turning. When the GC was exposed to a netrin-1 gradient, the velocity and travel distance of moving EB3 comets gained a significant increase (from 0.29 ± 0.01 µm/s to 0.33 ± 0.02 µm/s and from 1.30 ± 0.06 µm to 1.39 ± 0.03 µm in the P and D regions, respectively). To further analyze the asymmetric effect of netrin-1, the P/D ratio was introduced. In the control group, the P/D ratios of comet velocity and travel distance at the pre-turning were 1.03 ± 0.13 and 0.89 ± 0.04, respectively. The P/D ratios of comet velocity and travel distance at the post-turning stage were 1.01 ± 0.13 and 0.87 ± 0.03, respectively. The P/D ratios of comet velocity and travel distance at the turning stage were 1.11 ± 0.5 and 0.88 ± 0.04, respectively. The statistical analysis indicated that both P/D ratio of velocity and travel distance are comparable in all three stages in the control group. In the presence of a netrin-1 gradient, the P/D ratios of comet velocity and travel distance at the pre-turning were 0.98 ± 0.04 and 0.95 ± 0.02, respectively. The P/D ratios of comet velocity and travel distance at the post-turning stage were 0.99 ± 0.03 and 0.98 ± 0.04, respectively. Similar to the control gradient group, the statistical analysis indicated no significant
difference between P/D ratio of either velocity or travel distance. However, at the turning stage, the P/D ratios of velocity and travel distance were 0.77 ± 0.09 and 0.83 ± 0.05, respectively. Netrin-1 dramatically reduced both the velocity and travel distance P/D ratios. (Fig. 15E–G). Altogether, these findings show that netrin-1 asymmetrically increases MT dynamics in the GC during axonal repulsion with more dynamic MT in the D than the P region.
A. EB3-GFP

B. 10 min | 40 min | 60 min

C. EB3-GFP

D. 10 min | 40 min | 60 min

E. Comet Velocity (µm/s) vs. Time
   - D region (Control)
   - P region (Control)
   - D region (Netrin-1)
   - P region (Netrin-1)

F. P/D Ratio of Comet Velocity vs. Time
   - Control
   - Netrin-1

G. Comet Velocity (µm/s) and Travel Distance vs. Region
   - Control
   - Netrin-1
Fig 15. Visualization of MT Dynamics in the GC via Time-lapse Recording of EB3-GFP in Primary Neurons during Axon Turning. A–D, Trajectories of EB3-GFP comets in the GC of P2 mouse EGL neurons. P2 mouse EGL neurons were dissociated and nucleofected with EB3-GFP. Primary neurons were cultured for 2 d, and a Dunn chamber axon guidance assay was performed in the absence (A, B) or presence of a netrin-1 gradient (C, D). B, D, Kymographs of EB3-GFP comet movements in the proximal (upper) and distal regions (lower) of the GCs in A and C, respectively. Scale bar, 10 µm. White triangle represents either a control or netrin-1 gradient. E, F, Quantification of velocity (E, left) and travel distance (E, right) as well as ratios of velocity (F, left) and travel distance (F, right) of moving EB3-GFP comets in proximal and distal regions of the GC of P2 EGL cells (A–D). G, Bar graph comparisons of the velocity (left) and travel distance (right) of moving EB3-GFP comets in the GC of P2 EGL cells at 40 min after image capture from A–D. Data are mean ± SEM (3 GCs in each group). *p < 0.05 (one-way ANOVA with Tukey's test for post hoc comparisons). ***p < 0.001 (one-way ANOVA with Tukey's test for post hoc comparisons).
4.6 TUBB3 is Specifically Involved in Netrin-1/UNC5C-Regulated MT Dynamics in the GC.

To examine if UNC5C is involved in netrin-1-mediated MT dynamics in the GC, EB3-GFP was co-transfected with control shRNA (Fig. 16A), UNC5C shRNA (Fig. 16B), or UNC5C shRNA plus wild-type human UNC5C (Fig. 16C) into primary P2 EGL neurons. Live-cell imaging of GFP-EB3 was recorded and analyzed to indicate the MT dynamics of the GC in a netrin-1 gradient. In EGL cells transfected with the control shRNA, netrin-1 caused more MT growth in the D region than the P region (Fig. 16A, quantification in F), indicating that netrin-1 granted the turning GC an asymmetrically MT growth. When endogenous UNC5C was knocked down by shRNA, both netrin-1-increased MT growth in the GC and the differential induction of MT dynamics in the D region were abolished (Fig. 16B, quantification in F). The velocity of moving GFP-EB3 comets in the P and D regions was decreased from 0.29 ± 0.01 µm/s and 0.32 ± 0.02 µm/s in the control shRNA group to 0.26 ± 0.01 µm/s and 0.27 ± 0.01 µm/s in the UNC5C shRNA group, respectively. The travel distance of EB3 comets in the P and D regions was decreased from 1.31 ± 0.03 µm and 1.41 ± 0.03 µm in the control shRNA group to 0.97 ± 0.04 µm and 0.99 ± 0.04 µm in the UNC5C shRNA group, respectively. The deficiency caused by UNC5C knockdown on netrin-1-induced MT dynamics was rescued by the expression of wild-type human UNC5C (Fig. 16C, quantification in F). These results indicate that UNC5C is required for netrin-1-induced MT dynamics in the GC of primary EGL neurons. On the other hand, to determine if TUBB3 is required for netrin-1-regulated MT dynamics during axonal repulsion, mouse P2 cerebellar EGL cells were transfected with EB3-GFP along with either control shRNA, TUBB3 shRNA, or TUBB3
shRNA together with wild-type human TUBB3. The Dunn chamber assay was performed to track the movement of GFP-EB3 comets in the GC responding to a netrin-1 gradient. In neurons transfected with control shRNA, netrin-1 asymmetrically induced MT growth in the P region and D region of the GC (Fig. 16A, quantification in F). However, expression of TUBB3 shRNA abolished netrin-1-induced MT growth in both D and P regions of the GC (Fig. 16D, quantification in F). The velocity of moving GFP-EB3 comets in the P and D region was decreased from 0.29 ± 0.01 µm/s and 0.32 ± 0.02 µm/s in the control shRNA group to 0.26 ± 0.01 µm/s and 0.27 ± 0.02 µm/s in the TUBB3 shRNA group, respectively. The travel distance of EB3 comets in the P and D region was decreased from 1.31 ± 0.03 µm and 1.41 ± 0.03 µm in the control shRNA group to 1.07 ± 0.07 µm and 1.14 ± 0.04 µm in the TUBB3 shRNA group, respectively. When wild-type TUBB3 plasmid was expressed along with TUBB3 shRNA in neurons, the deficiency of MT dynamics in the GC was rescued (Fig. 16E, quantification in F). Quantification indicated that the velocity of moving GFP-EB3 comets was 0.29 ± 0.01 µm/s in the P region and 0.33 ± 0.01 µm/s in the D region, while the travel distance of EB3 comets was 1.31 ± 0.02 µm in the P region and 1.44 ± 0.07 µm in the D region. Collectively, these findings indicated that TUBB3 is particularly involved in netrin-1/UNC5C-mediated MT dynamics in the GC during axonal repulsion.
Fig 16. TUBB3 is Specifically Involved in Netrin-1/UNC5C-regulated MT Dynamics in the GC of P2 EGL Neurons. A–E, P2 mouse EGL neurons were cotransfected EB3-GFP with control shRNA (A), UNC5C shRNA (B), UNC5C shRNA plus wild-type human UNC5C (C), TUBB3 shRNA (D), or TUBB3 shRNA plus wild-type human TUBB3 (E), respectively, and a Dunn chamber axon guidance assay was performed. A, D, E, Left, Live-cell imaging tracking EB3-GFP comet movements in the GC. Right, Kymographs of EB3-GFP comets in the proximal (upper) and distal (lower) regions of the GC. B, C, Kymographs of EB3-GFP comet movements in the proximal (upper) and distal (lower) regions of the GC of P2 cerebellar EGL cells transfected with UNC5C shRNA (B) or UNC5C shRNA plus wild-type human UNC5C (C). Scale bar, 10 µm. White triangle represents either a control or netrin-1 gradient. F, Quantification of the velocity (left) and travel distance (right) of moving EB3-GFP comets in the GC of P2 EGL cells (A–E) at 40 min after image capture. Data are mean ± SEM (3 GCs in each group). *p < 0.05 (one-way ANOVA with Tukey's test for post hoc comparisons). ***p < 0.001 (one-way ANOVA with Tukey's test for post hoc comparisons).
4.7 TUBB3 is Required for Axonal Projection of DRG Neurons in vivo.

Netrin-1-mediated axonal repulsion plays a crucial role in DRG axonal projection. During spinal cord development, a ventral-to-dorsal gradient of netrin-1 is formed from the floor plate. This netrin-1 gradient repels DRG axons preventing the DRG axon from entering aberrantly into the intermediate and ventral regions of the spinal cord and guides them to project precisely to the spinal cord through the DREZ region (Kennedy et al., 2006; Masuda et al., 2008, 2009). Studies also indicated that a transient expression of netrin-1 in the dorsal spinal cord could act as a short-range inhibitory cue which elaborates a “waiting period” for extension of sensory afferents. Premature DRG axons are prevented from entering into the mantle layer of the spinal cord (Watanabe et al., 2006). We use the chicken embryos to study the DRG axonal projection in vivo. DRG neurons are derived from neural crest cells. The neural crest cells are a population of migratory cells, locating at the dorsal part of the spinal cord. These neural crest cells begin to migrate and give rise to DRG at around stage 12-16 (Bronner-Fraser and Fraser, 1989; Serbedzija et al., 1989; Ruffins et al., 1998). Firstly, to confirm the role of netrin-1 in DRG axon pathfinding in vivo, Venus YFP was introduced either alone or with AP-netrin-1 into the chick neural tube at stage 12-15 (Fig. 18A-B’). YFP-labeled embryos at stages 22-25 were collected. In embryos expressing Venus YFP only (Fig. 18A-A’), DRG axons normally projected toward and eventually entered the dorsal spinal cord at the DREZ (Fig. 18A-B’). Overexpression of netrin-1 in the dorsal and intermediate regions of the spinal cord (Fig. 18B-B’) increased DREZ size, suggesting that DRG axons may enter aberrantly into the spinal cord. However, netrin-1 in this assay was expressed in half of the spinal cord, and developing spinal cord contains various cell
types other than the DRG neurons. Venus YFP, a widely used fluorescent protein, doesn’t specifically label DRG neurons. Therefore, the size-increase DREZ may not only result from DRG axonal misguidance. Accordingly, the TUJ-1 antibody was applied as a neuron marker for a double staining of the Venus-YFP spinal cord. Venus YFP was still introduced either alone or with AP-netrin-1 into the chick neural tube at stage 12-15 and embryos were collected at stage 22-25 to confirm that the size-increase DREZ resulted from neurons responding to disruption of netrin-1 signaling. Afterwards, embryos were labeled with the TUJ-1 antibody (Fig. 19A-H). DREZ size of Venus YFP labeled and TUJ-1 labeled DRG axons were quantified. Size of the TUJ-1 labeled DREZ was increased when netrin-1 was overexpressed in the spinal cord. The quantitative analysis showed that the DREZ size in the Venus YFP group and TUJ-1 antibody staining group was comparable (Fig. 19I). To further increase the specificity of tracking DRG axonal projection, BEN antibody, a specific marker for DRG, motor neurons and FP cells (Avraham O et al., 2010), was used. Transverse sections of the lumbosacral segments of the spinal cords after electroporation were prepared and immunostained with the BEN antibody. BEN labeled DREZ again confirmed that netrin-1-overexpressing embryos dramatically increased the size of DREZ, but the Venus YFP only expressed embryos did not (Fig. 20A-B, quantification in Fig. 8-4 I). These results suggest that overexpression of netrin-1 in the dorsal and intermediate regions of the spinal cord causes abnormal pathfinding of DRG axon, increasing the size of DREZ.

To determine if UNC5C is required for DRG axonal projection in vivo, Venus YFP was electroporated either alone or with UNC5C control shRNA, UNC5C shRNA, and UNC5C shRNA plus wild-type human UNC5C into the chick neural tube at Stage
12-15. As introduced before, neural crest cells emigrate from the chick spinal cord to DRG during the stage 12-16 (Bronner-Fraser and Fraser, 1989; Serbedzija et al., 1989; Ruffins et al., 1998). UNC5C shRNA (Fig. 20D-D”), but not UNC5C control shRNA (Fig. 20C-C”), significantly increased the DREZ size (quantification in Fig. 20I). Expression of wild-type human UNC5C rescued the effect of UNC5C knockdown on DRG axonal projection toward the spinal cord, resulting in a DREZ size comparable to the control (Fig. 20E-E”, quantification in Fig. 20I). These data indicate that netrin-1/UNC5C signaling is required for proper DRG axon pathfinding in vivo. Lastly, to study the role of TUBB3 in DRG axonal projection, Venus YFP was introduced into the chick spinal cord by in ovo electroporation with TUBB3 control shRNA, Venus YFP with TUBB3 shRNA, or Venus YFP with TUBB3 shRNA plus wild-type human TUBB3 at stage 12-15. TUBB3 control shRNA did not affect DRG axonal projection toward the spinal cord, and the DREZ size was compared with the Venus YFP group (Fig. 20F – F”, quantification in J). However, knockdown of TUBB3 dramatically increased DREZ size (Fig. 20G-G”, quantification in J). The phenotype of TUBB3 knockdown on DRG axonal projection was rescued by cotransfecting wild-type human TUBB3 plasmids (Fig. 20H-H”, quantification in J). Altogether, these results demonstrate that TUBB3 is required for the DRG axonal projection and pathfinding in the developing spinal cord.

Fig 18. Overexpression of Netrin-1 in the Dorsal and Intermediate Regions of the Spinal Cord at Stage 12-15 Increases DREZ Size. A-B', The chick neural tube was electroporated with Venus YFP only (A-A’) and Venus YFP plus AP-netrin-1 (B-B’). The scale bar is 50 µm. C, Quantification of the DREZ size. ***, p < 0.001 (one-way ANOVA with Tukey's test for post-hoc comparisons). The numbers on the top of each bar indicate the numbers of samples tested in the corresponding groups. R, rescue.
Fig 19. Overexpression of Netrin-1 in the Dorsal and Intermediate Regions of the Spinal Cord at Stage 16 Increases DREZ Size. A-H, The chick neural tube was electroporated with Venus YFP only (A-D) and Venus YFP plus AP-netrin-1 (E-H). The scale bar is 50 µm. I, Quantification of the DREZ size. ***, p < 0.001 (one-way ANOVA with Tukey's test for post-hoc comparisons). The numbers on the top of each bar indicate the numbers of samples tested in the corresponding groups.
The image depicts a series of panels labeled A, A', A'', B, B', B'', C, C', C'', D, D', D'', E, E', E'', F, F', F'', G, G', G'', H, H', H'', and I. Each panel contains multiple images with various colors and markers. The panels are arranged in a grid format with titles at the top indicating groups such as 'Vein Only', 'Netrin-1', 'UNC5c shRNA', 'UNC5c shRNA+R', and 'TUBB3 shRNA'. Below the image, there is a bar graph with the x-axis labeled '31, 32, 28, 30, 29, 31, 31' and the y-axis labeled 'DREZ Size (μm)'. The graph includes error bars and statistical significances marked with asterisks. The overall layout suggests a comparison of different experimental conditions on DREZ size.
Fig 20. TUBB3 is Essential for DRG Axonal Projection *in vivo*. A-B″, The chick neural tube at stage 16 was electroporated with Venus YFP only (A-A″) or Venus YFP plus AP-netrin-1 (B-B″), and transverse sections of the spinal cord were stained with BEN antibody (red). C-H″, The chick neural tube at stages 12-15 was electroporated with Venus YFP plus UNC5C control shRNA (C-C″), Venus YFP plus UNC5C shRNA (D-D″), Venus YFP plus UNC5C shRNA and wild-type human UNC5C (E-E″), Venus YFP plus TUBB3 control shRNA (F-F″), Venus YFP plus TUBB3 shRNA (G-G″), Venus YFP plus TUBB3 shRNA and wild-type human TUBB3 (H-H″). Transverse sections of the Venus YFP-labeled spinal cords were stained with BEN antibody. A-H, Overlay confocal images of green (Venus YFP) and red (BEN staining) fluorescence. Insets, Corresponding low-magnification images of A – H. A’-H, A″-H, Venus YFP images and images of BEN antibody immunostaining of the region of interest in B-I (dashed lines), respectively. Scale bar, 50 μm. I, Quantification of DREZ size. ***p < 0.001 (one-way ANOVA with Tukey's test for post hoc comparisons). Both Venus YFP and BEN antibody staining consistently demarcates DRG axonal projections with no statistically significant difference in DREZ size in each group. The numbers on the top of each bar indicate the numbers of samples tested in the corresponding groups. R, Rescue constructs.
Chapter Five

Discussion

Netrin family is one of the canonical axon guidance cue families. It plays a crucial role in axonal pathfinding during the development of the nervous system. Netrin-1 is a well-studied netrin family member and can function as either a chemoattractant or a chemorepellent. This dual-functional feature is achieved by activating particular downstream signaling through different receptors, including DCC, DSCAM, and UNC5 (Colamarino and Tessier-Lavigne, 1995; Fazeli et al., 1997; Serafini et al., 1997; Hong et al., 1999; Kennedy, 2000; Merz and Culotti, 2000; Keleman and Dickson, 2001; Killeen et al., 2002; Huber et al., 2003; Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009;)

MTs are one of the major cytoskeleton structure in the GC. MTs are a highly dynamic hollow cylindrical structure and continuously switch between phases of growth and shortening. MTs are required for cellular transport, forming the spindle during physiological activities like mitosis et al. (Desai and Mitchison, 1997). Studies have reviewed that MTs are responsive to guidance cues. For example, bath application of Wnt3a causes MT to lose directionality and polymerize perpendicular to the direction of GC translocation. Moreover, netrins can induce changes in MTs, resulting in increased splaying of MTs in the GC. These findings suggested that in the developing GC, MT dynamic may function as a direct sensor to control GC steering (Tanaka and Sabry, 1995; Suter and Forscher, 2000; Buck and Zheng, 2002; Dent et al., 2004; Purro et al., 2008; Conde and Cáceres, 2009; Dent et al., 2011; Liu and Dwyer, 2014).
MTs are composed of tubulin dimers. Studies indicate that MTs serving in different physiological activities vary in isotype composition, suggesting that these isotypes may be the determination of MT function in specific cellular activities. β-tubulin isotype III (TUBB3) is one of the tubulin subunits found in mammals. It is distinct because the expression of TUBB3 is primarily limited to neurons and the most dynamic β-tubulin subunit in the nervous system. Therefore, TUBB3 is highly used as a neuronal marker to differentiate neurons from glial cells. It was reported that patients bearing mutations of TUBB3 suffer a spectrum of human nervous system disorders named TUBB3 syndromes, including hypoplasia of oculomotor nerves, dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts, suggesting that TUBB3 plays a crucial role in axon guidance and brain development (YQ Jiang et al., 1992; Tischfield et al., 2010).

Recent studies demonstrate that direct coupling of DCC and DSCAM to MT dynamics via TUBB3 is required for netrin-1-promoted axon outgrowth, branching, and attraction (Qu et al., 2013a; Huang et al., 2015). Both DCC and DSCAM are netrin-1 receptors in mammals, suggesting that all netrin-1 receptors could be involved in modulation of MT dynamics via TUBB3. Therefore, we hypothesize that uncoupling of UNC5C with polymerized MTs via TUBB3 plays an important role in netrin-1-mediated repulsion.

### 5.1 Disengagement of UNC5C with Polymerized TUBB3 in Netrin-1 Repulsion.

In this study, we first discovered that UNC5C interacts with TUBB3 in Hela cells. Purified UNC5C-ICD interacts with purified TUBB3 in vitro, suggesting a direct biochemical binding between UNC5C and TUBB3. The endogenous protein binding was
confirmed using P4 cerebellar cells. Netrin-1 can decrease UNC5C/TUBB3 interaction in both time-dependent and dose-dependent manner. However, UNC5C doesn’t interact with other tubulin subunits such as TUBB1 and TUBB2, suggesting that TUBB3 is the specific MT β-subunit binding UNC5C (Fig. 2-4). Moreover, TUBB3 distribution has a correlation with that of UNC5C in the GC of developing cerebellar EGL cells, suggesting TUBB3 binds UNC5C in the developing GC. Since netrin-1 can reduce this correlation, netrin-1 is confirmed to impact UNC5C/TUBB3 binding (Fig. 5). Altogether, TUBB3 is the specific MT subunit to directly interact with UNC5C in the GC.

On the other hand, we found that MT dynamics play a role in altering UNC5C/TUBB3 binding. Our data indicated that after disruption of MT dynamics with either Taxol or nocodazole treatment, the netrin-1-reduced interaction of endogenous UNC5C with TUBB3 was abolished. This finding suggests that MT dynamics are required for the netrin-1-regulated UNC5C/TUBB3 interaction (Fig. 6). UNC5C not only bind monomer TUBB3 protein but also interact with polymeric TUBB3. The tubulin cosedimentation assay showed that UNC5C cosedimented with polymerized MTs from primary cerebellar neuron lysates. Furthermore, netrin-1 also reduces this cosedimentation, resulting in less UNC5C in the pellet than in the supernatant. Netrin-1 stimulation increased the ratio of polymerized TUBB3 in the pellet versus those in the supernatant, suggesting that netrin-1 may promote MT dynamics through the UNC5C/TUBB3 interaction (Fig. 7). Knockdown of TUBB3 abolished the netrin-1-reduced cosedimentation of UNC5C with polymerized MTs, suggesting that this process relies on TUBB3 (Fig. 8). Our functional data indicate that TUBB3 is specifically involved in netrin-1-mediated axonal repulsion both in vitro and in vivo (Fig. 9-14 and
Fig. 17-20). Live-cell imaging data showed that netrin-1 differentially increased MT dynamics in the GC of cerebellar EGL neurons. More MT growth is induced in the distal region than the proximal region. Knockdown of either UNC5C or TUBB3 abolished the netrin-1-promoted differential MT growth in the GC during axon turning (Fig. 15 and 16). These results indicate that differential dissociation of UNC5C with polymerized TUBB3 in the GC result in asymmetric MT dynamics in netrin1-mediated axonal repulsion.

Altogether, UNC5C specifically and directly interacts with TUBB3, and the interaction is down-regulated by netrin-1 stimulation. The dissociation of TUBB3 with UNC5C is involved in regulation of asymmetric MT dynamics in the GC in netrin-1-mediated axonal repulsion.

5.2 The Working Model of Direct Modulation of MT Dynamics in Netrin-1 Signaling.

Previous studies on MT dynamics suggested that MTs are involved in axon guidance. MTs are oriented and stabilized in the way that is consistent with the direction of GC turns (Tanaka and Kirschner, 1995). Moreover, GC can be induced to turn towards a particular direction where local stabilization of MTs is applied to that side of the GC (Buck and Zheng, 2002). Also, axon outgrowth can be increased by stabilizing MTs and promoting their polymerization at plus ends in vitro and in vivo (Sengottuvel et al., 2011). Wnt5a-promoted cortical axon outgrowth and repulsion requires asymmetric redistribution of dynamic MTs toward the far side of the GC (Li et al., 2014).

Our recent studies showed a model for axonal attraction that netrin-1 signaling directly regulates MT dynamics through coupling its receptor DCC and DSCAM to
TUBB3 in axon outgrowth, branching, and attraction (Qu et al., 2013a; Huang et al., 2015). In this model, netrin-1 is hypothesized to differentially promote MT dynamics and local stabilization of MTs in the proximal region of the GC through the interaction of DCC and/or DSCAM with polymerized TUBB3, resulting in GC protrusion on the proximal side. In contrast, the distal region of the GC would be less stable would eventually get out-grown by the proximal side, resulting in the axon turning toward the netrin-1 source (Liu G and Dwyer T, 2014).

This study provides a working model that uncoupling of UNC5C with polymerized TUBB3 in MTs mediates netrin-1 repulsion (Fig. 21). In this model, netrin-1 asymmetrically induces MT dynamics in the GC to have more MTs to polymerize in the D region in comparison to the P region. Meanwhile, netrin-1 promotes dissociation of polymerized TUBB3 (MT) with UNC5C in the GC area, causing less polymerized MTs binding with UNC5C on the proximal side than on the distal side of the GC. As a consequence, lamellipodia and filopodia in the proximal region become less stable and finally retract.

Altogether, the interaction of guidance receptors with dynamic MTs in the GC may function as a molecular switch. Engaging of receptors with dynamic MTs could stabilize and promote MT growth in the P region of the GC to trigger axon outgrowth, branching, and attraction, while disengagement of polymerized MTs with guidance receptors may induce retraction and collapse in the P region of the GC to cause axonal repulsion.
Fig 21. A Working Model of Direct Involvement of MT Dynamics in Netrin-1-Mediated Axonal Repulsion. A, A typical GC of developing neurons with lamellipodia and finger-like filopodia. In the peripheral region of the GC, UNC5C interacts with TUBB3 in “pioneer polymerized MTs” in the absence of netrin-1 gradient. B, Binding of netrin-1 to UNC5C on the side of GC close to the netrin-1 gradient differentially reduces the interaction of UNC5C with polymerized TUBB3 in MTs and triggers GC collapse on that side resulting in axonal repulsion, despite the fact that netrin-1 induces MT polymerization/dynamics in the GC.
5.3 TUBB3 is a Key Downstream Component in Netrin-1 Signaling.

In this study, we made a netrin-1 gradient in a Dunn chamber and found that the axons of primary P2 cerebellar EGL neurons were repelled by netrin-1 in vitro (Fig. 9-14), and knockdown either UNC5C or TUBB3 abolished netrin-1-induced GC repulsion (Fig. 9-14). However, expression of WT UNC5C or TUBB3 along with shRNA rescued the deficiency caused by UNC5C or TUBB3 knockdown on netrin-1-promoted GC repulsion, respectively (Fig. 9-14). These results indicate that TUBB3 is specifically involved in netrin-1/UNC5C-induced axonal repulsion in vitro.

To investigate the role of TUBB3 in axonal repulsion in vivo, we applied in ovo electroporation studies with chick spinal cords. Previous studies suggested that a ventral-to-dorsal gradient of netrin-1 derived from the floor plate repels DRG axonal projection, preventing the DRG axons from entering aberrantly into the intermediate and ventral regions of the spinal cord, and guides them to project precisely toward the DREZ (Kennedy et al., 2006; Masuda et al., 2008, 2009). We over-expressed netrin-1 in the dorsal and intermediate regions of the spinal cord to disrupt the netrin-1 gradient, which, surprisingly, increased DREZ size (Fig. 18-19). Three possible mechanisms could cause this guidance defect: (1) ectopic expression of netrin-1 in the dorsal and intermediate regions of the spinal cord disrupts the ventral-to-dorsal gradient of netrin-1, allowing axons enter into the spinal cord more ventrally; (2) overexpression of netrin-1 in the dorsal spinal cord could extend the “waiting period” of DRG axonal projection into mantle layer of the spinal cord, increasing DREZ size; and (3) overexpression of netrin-1 in the spinal cord could de-sensitize DRG axons to netrin-1 (adaptation), resulting in the axonal projection defect. Knockdown of either UNC5C or TUBB3 affected the normal
DRG axonal projection, resulted in significantly increased DREZ size, which is consistent with the phenotype of overexpression of netrin-1. Expression of WT UNC5C or TUBB3 rescued the effect of knockdown on DRG axonal projection toward the spinal cord, respectively (Fig. 17-20). Results from in ovo electroporation studies with chick spinal strongly supporting the idea that TUBB3 is required for netrin-1/UNC5C-mediated axonal repulsion in the developing nervous system.

5.4 Future Directions

Mammalian UNC5 has four homologs: UNC5A-UNC5D. UNC5A (Bartoe et al., 2006) and UNC5B (Hata et al., 2009) were reported to mediate netrin-1-induced axon GC collapse of primary hippocampal and cortical neurons, respectively. Our results indicate that UNC5C specifically interacts with TUBB3, not TUBB1 or TUBB2, indicating that TUBB3 is the specific MT β-subunit involved in netrin-1/UNC5C signaling. However, it is unknown that whether other UNC5 homologs, such as UNC5A, UNC5B, and UNC5D, can functionally associate with these β-tubulin isoforms in netrin-1-mediated repulsion.

In addition, UNC5 coordinates with either DCC or DSCAM to initiate netrin-1 mediated axonal repulsion (Keino-Masu et al., 1996; Kolodziej et al., 1996; Leonardo et al., 1997; Hong et al., 1999; Finger et al., 2002; Purohit et al., 2012), whereas collaboration of DCC with DSCAM mediates chemoattraction (Ly et al., 2008; Liu et al., 2009; Qu et al., 2013b; Huang et al., 2015). Our previous studies suggested that DSCAM coordinates with DCC in coupling netrin-1 signaling to MT dynamics via dynamic TUBB3 to promote axon outgrowth, branching, and attraction (Qu et al., 2013a; Huang et
al., 2015). However, it is unknown that whether UNC5 coordinates with DSCAM or DCC in coupling netrin-1 repulsive signaling with MT dynamics via dynamic TUBB3.

On the other hand, TUBB1, TUBB2, and TUBB3 are major β-tubulin subunits of the MT. They are well conserved across species. The major difference of amino acid sequences of these β-tubulins locates at the C terminus, which is the region associating with MT-associated proteins (MAPs) (Sullivan and Cleveland, 1986). These β-tubulin isotypes display differential tissue expression patterns: TUBB1 is ubiquitous, whereas TUBB2 and TUBB3 are restricted to neuronal tissues (Sullivan et al., 1986; Wang et al., 1986; Cleveland, 1987; Leandro-García et al., 2010). MT dynamics could be affected by the tubulin isotype composition. Among its subunits, TUBB3 was thought to be more dynamic than other β-tubulin isotypes (Panda et al., 1994). Mutations of TUBB1, TUBB2, or TUBB3 cause developmental disorders with different phenotypic spectra (Jaglin et al., 2009; Guerrini et al., 2012; Cushion et al., 2013). These results suggest that these β-tubulin isotypes may be differentially involved in tubulin heterodimer formation and MT stability in developing neurons. The direct interaction of UNC5C with TUBB3, but not TUBB1 or TUBB2, may rely on the difference between their sequences, protein conformation, expression patterns, and isotype-engaged MT dynamics. However, it is still unknown if TUBB1 and/or TUBB2 play a role in netrin-1-mediated axonal repulsion guidance by means other than direct binding with UNC5C.

Furthermore, it was reported that patients bearing mutations of TUBB3 suffer a spectrum of human nervous system defects in axonal projection and neuronal migration, including agenesis or hypoplasia of commissural and cranial axons, misorientation and dysgenesis of the corticospinal tract, basal ganglia dysmorphisms, malformation of
cortical development associated with neuronal migration defects, hypoplasia of hippocampi, thalami, and brainstem, and cerebellar dysplasia (Poirier et al., 2010; Tischfield et al., 2010; Whitman et al., 2016). The disease-associated TUBB3 mutations were suggested to impair tubulin heterodimer formation, disrupt kinesin-MT interactions, and alter MT instability (Poirier et al., 2010; Tischfield et al., 2010; Whitman et al., 2016), but it is still unclear that how these mutations cause specific defects in axon guidance and neuronal migration. Further studies will be necessary to determine whether disease-related TUBB3 mutations affect the interaction with UNC5C, impair netrin-1-regulated MT dynamics in the GC, and disrupt netrin-1-mediated chemoattraction and chemorepulsion in the developing nervous system.
References


Finger, J.H., Bronson, R.T., Harris, B., Johnson, K., Przyborski, S.A., and Ackerman, S.L. (2002). The netrin 1 receptors Unc5h3 and Dcc are necessary at multiple choice points for the guidance of corticospinal tract axons. J Neurosci 22, 10346-10356.


discrete subdomains of DCC and UNC5 and mediates interactions between DCC


Symmetric polymicrogyria and pachygyria associated with TUBB2B gene

Gundersen RW, Barrett JN (1979) Neuronal chemotaxis: chick dorsal-root axons turn
toward high concentrations of nerve growth factor. Science (New York, NY)
206:1079-1080.

LARG to mediate the action of repulsive guidance molecule. J Cell Biol 184, 737-
750.

genes guide circumferential migrations of pioneer axons and mesodermal cells on

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M., and Stein, E.
(1999). A ligand-gated association between cytoplasmic domains of UNC5 and
DCC family receptors converts netrin-induced growth cone attraction to


and Src family kinases for axon outgrowth and attraction. Nat Neurosci 7, 1222-1232.


87


Sharma K, Frank E (1998) Sensory axons are guided by local cues in the developing dorsal spinal cord.


