A MOLECULAR GENETIC ANALYSIS OF THE ROLE OF THE GUANINE NUCLEOTIDE EXCHANGE FACTOR TRIO DURING AXON PATHFINDING IN THE EMBRYONIC CNS OF DROSOPHILA MELANOGASTER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
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By

David J. Forsthoefel, B.S.

* * * * *

The Ohio State University
2005

Dissertation Committee:
Dr. Mark. A. Seeger, Advisor
Dr. Helen Chamberlin
Dr. Michael C. Ostrowski
Dr. Harald E. F. Vaessin

Approved by

Advisor
Graduate Program in Molecular Genetics
ABSTRACT

The central nervous system of the *Drosophila melanogaster* embryo is an ideal model system in which to study the basic problem of axon guidance, because of the relative simplicity of the nervous system and the evolutionary conservation of the molecules utilized during axon pathfinding.

The Abelson tyrosine kinase is a cytoplasmic kinase that regulates actin cytoskeletal dynamics in flies, mice, and humans. In flies, *Abl* is expressed in the developing central and peripheral nervous systems. In a genetic screen for enhancers and suppressors of the semilethality phenotype in homozygous *Abl* mutant flies, we identified *trio*, a cytoplasmic guanine nucleotide exchange factor that regulates actin dynamics through Rho GTPases. *trio* is also expressed widely in the CNS, and mutations in *Abl* and *trio* interact genetically, leading to dramatic disruption of axon pathways at the CNS midline choice point of the developing embryo.

Building upon these initial observations, we analyzed interactions between *Abl*, *trio*, and the attractive Netrin receptor *frazzled (fra)/Deleted-in-Colorectal-Cancer (DCC)* during axon guidance across the CNS midline. In *fra; Abl* and *fra; trio* double mutants, very few axons crossed the midline, similar to the phenotype in *trio,Abl* mutants. Furthermore, mutations in *Abl* and *trio* suppressed the inappropriate midline crossover phenotype in embryos expressing the chimeric Robo-Fra receptor, consistent with an *in vivo* role for these molecules as Fra effectors. Additionally, Fra bound Abl and
Trio in coimmunoprecipitation and GST pulldown experiments, and Abl overexpression in cultured *Drosophila* cells led to tyrosine phosphorylation of both Fra and Trio. Mutations in *enabled (ena)*, another Abl substrate originally identified as a genetic suppressor of *Abl* semilethality, suppressed the loss-of-commissure phenotype in *fra, Abl*, and *trio* mutant combinations, as well as the inappropriate crossover phenotype in neurons expressing Robo-Fra. Taken together, these results suggest that Abl and Trio are important effectors for multiple attractive receptors at the CNS midline, and that Ena may function downstream of both attractive as well as repulsive signaling.

Finally, a functional dissection of the requirement for Trio’s various domains during midline guidance has been initiated. The only Trio domain required to rescue the loss-of-commissure phenotype in *trio,Abl* mutants was the GEF1 domain. Conversely, deletion of the SH3 domain led to more effective rescue than full-length, wild-type Trio. Consistently, coexpression of wild-type or SH3-deleted *trio* with the chimeric Robo-Fra receptor led to synergistic axon crossing and collapse at the midline, while coexpression of GEF1-deleted *trio* suppressed the Robo-Fra phenotype. Overexpression experiments in various other tissues and in cultured cells indicate that the N-terminal domain, the spectrin repeats, and the SH3 domain may regulate GEF activity in the context of the full-length molecule.

Together, these observations are consistent with a model in which Trio, and possibly Abl and Ena, are recruited by the Fra receptor during attractive signaling at the CNS midline. Future experiments must determine the mechanistic details of cytoskeletal control downstream of Trio and Fra, and the identity of additional attractive receptor(s).
DEDICATION

To my parents, David and Rose.

For their love, support, patience, and perspective, I am deeply grateful.
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I would like to acknowledge my advisor, Dr. Mark Seeger, for his generosity, insight, encouragement, trust, and patience during my graduate education. Mark has allowed me to succeed, to fail, and to learn independently, but he was always present, guiding. I am indebted for everything Mark has taught and shared through the years, and I am grateful to have such an excellent mentor.

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A number of other professors and colleagues in the Department of Molecular Genetics, as well as other Departments, Programs, and Institutions have contributed to my intellectual and professional development. I would like to thank Dr. Christine Beattie, Dr. David Bisaro, Dr. Anthony Brown, Dr. Tien-Hsien Chang, Dr. Dorothy Engle, Dr. Tsonwin Hai, Dr. Paul Henion, Dr. Paul Herman, Dr. Russell Hill, Dr. Lee Johnson, Dr.
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VITA

August 20, 1972............................................................... Born – Celina, Ohio, USA

1990-1994................................................................. B.S. Natural Sciences
Xavier University, Cincinnati, Ohio

1994-1996................................................................. College of Medicine,
The Ohio State University

1997................................................................. Research Assistant,
Progenitor, Inc., Columbus, Ohio

1998-present............................................................... Teaching and Research Associate,
The Ohio State University,
Columbus, Ohio.

PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular Genetics
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<tr>
<td>Abl</td>
<td>Abelson tyrosine kinase</td>
</tr>
<tr>
<td>AC</td>
<td>anterior commissure</td>
</tr>
<tr>
<td>Arp</td>
<td>actin-related protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CA</td>
<td>constitutively active</td>
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<tr>
<td>c-Abl</td>
<td>cellular Abl</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>c-Src</td>
<td>cellular Src</td>
</tr>
<tr>
<td>Cy</td>
<td>curly</td>
</tr>
<tr>
<td>Dbl</td>
<td>diffuse B-cell lymphoma</td>
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<tr>
<td>DH</td>
<td>Dbl homology</td>
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<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EMS</td>
<td>ethyl methane sulfonate</td>
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<tr>
<td>Ena</td>
<td>enabled</td>
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<td>EST</td>
<td>expressed sequence tag</td>
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<tr>
<td>EVH</td>
<td>ena/VASP homology</td>
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<td>EVL</td>
<td>ena/VASP-like</td>
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<tr>
<td>FasII</td>
<td>fasciclin II</td>
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<tr>
<td>Fra</td>
<td>frazzled</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDI</td>
<td>guanosine dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanosine (or guanine nucleotide) exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine triphosphatase</td>
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<tr>
<td>HDA</td>
<td>haploinsufficiency dependent on Abl</td>
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<tr>
<td>ISN</td>
<td>intersegmental nerve</td>
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<tr>
<td>Kb</td>
<td>kilobase</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MG</td>
<td>Department of Molecular Genetics</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NetA</td>
<td>netrin A</td>
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<tr>
<td>NetB</td>
<td>netrin B</td>
</tr>
<tr>
<td>NTM</td>
<td>Trio conserved N-terminal domain</td>
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N-WASP  neuronal Wiscott-Aldrich syndrome protein
OH    go bucks!
ORF   open reading frame
PC    posterior commissure
PCR   polymerase chain reaction
PDGF  platelet-derived growth factor
PH    pleckstrin homology
PNS   peripheral nervous system
pTyr  phosphotyrosine
pY    phosphotyrosine
RACE  rapid amplification of cDNA ends
RNA   ribonucleic acid
RTK   receptor tyrosine kinase
Sb    stubble
SH2   Src-homology 2 domain (phosphotyrosine-binding)
SH3   Src-homology 3 domain (polyproline-binding)
SN    segmental nerve
SPR   Trio region of spectrin-like repeats
Src   Rous sarcoma virus tyrosine kinase
Tb    tubby
v-Abl viral Abl
v-Src viral Src
VASP  vasodilator-stimulated protein
WASP  Wiscott-Aldrich syndrome protein
WT    wild-type
CHAPTER 1

INTRODUCTION

1.1. A brief history of axon guidance

Hippocrates (460-377 B.C.) was probably the first physician to propose that the brain, not the heart, as suggested by Aristotle, was the primary seat of the mind, responsible for learning and emotion (Finger, 2000). While Herophilus subsequently distinguished between blood vessels and nerves, it was not until the first century A.D. that the Greek physician Galen proposed based on experimental evidence that the brain was the source of sensation, voluntary motion, and thought (Rocca, 2003; Taylor, 1922). In his dissections of humans and animals, Galen found that most nerves originated in the brain and spinal cord, and Galen extensively studied the cranial nerves, confirming the work of a contemporary anatomist, Marianus (Finger, 2000). In vivisections of pigs and apes, Galen demonstrated that severing the laryngeal nerve caused loss of voice, and that sectioning the spinal cord resulted in muscle paralysis (Finger, 2000; Taylor, 1922). Many of Galen’s ideas held sway for over a thousand years, but were questioned and disproven (especially those concerning the physiology of the brain) by European physicians during the Renaissance or later (Rocca, 2003). However, Galen’s observations concerning the organization of the nervous system were accurate, and were upheld and
refined by later anatomists, beginning with Vesalius in the 1500’s (Finger, 2000). In the 1700’s, Luigi Galvani discovered that electrical stimulation of a nerve could induce the contraction of a frog leg, showing convincingly that nerves carry information to the periphery, and revealing for the first time the identity of the “spirit” transmitted by nerves (Finger, 2000).

However, the origin of the nervous system was not seriously investigated until the late 1800’s, when improvements in microscopy, tissue fixation, and staining procedures made it possible to study the nervous system in developing organisms. Santiago Ramón y Cajal utilized and improved upon the “black reaction” developed by Camillo Golgi to disprove the “reticular” theory, definitively showing that the tips of nerve processes did not fuse with other nerves (Cajal, 1897-1899 (1999 English translation); Finger, 2000). Cajal (who wrote over 100 articles after he won the Nobel Prize) also extensively described the highly ordered nervous system in a variety of adult and embryonic organisms, especially the chick and rodents (Cajal, 1897-1899 (1999 English translation)). He was the first to describe and name the “growth cone,” the expanded, club-like tip of an extending axon, which he hypothesized was a “battering ram” that pressed aside other nerves and cell bodies on the way towards its target (Fig. 1.1) (Cajal, 1897-1899 (1999 English translation)). Cajal also proposed that the growth cone migrated chemotactically, that is, in response to cues secreted by target cells (Cajal, 1897-1899 (1999 English translation)). Ross Granville Harrison, who first developed the technique of tissue culture, verified the existence of the growth cone by observing an axon
extending from a single frog spinal ganglion neuron in culture, while Speidel observed living growth cones in the tadpole tail (Gahwiler, 1999; Goodman and Tessier-Lavigne, 1997; Landis, 1983).

Although the question of how growing axons located their targets briefly fell out of favor, landmark experiments by Roger Sperry in the 1940’s revived interest in the mechanism of neural connectivity (Goodman and Tessier-Lavigne, 1997; Meyer, 1998). Taking advantage of recent observations that frog and newt could regain vision after severing of the optic nerve, Sperry showed that rotating the newt eye 180° before allowing the severed optic nerve to regrow caused the newt to respond abnormally when prey was presented. For example, if prey was presented above the animal, it would turn downward (Meyer, 1998). Similar results were obtained by surgically uncrossing the optic nerve within the optic chiasm before regeneration, causing the animal to turn left when prey was offered on the right, and vice versa (Meyer, 1998). Based on these results, Sperry revived the idea of “chemoattraction,” and proposed that the growth cone was able to detect specific markers during migration and target recognition – the “chemoaffinity” hypothesis (Goodman and Tessier-Lavigne, 1997; Meyer, 1998).

In the 1970’s, investigators turned to the analysis of single neuron projections in early grasshopper and Drosophila embryos (Goodman et al., 1984; Goodman and Tessier-Lavigne, 1997). In these and other organisms such as chick and zebrafish, it was discovered that individual growth cones follow specified pathways to their targets, selectively fasciculating with some cells or axons and not others, leading to the proposal of the “labeled pathway” hypothesis (Goodman et al., 1984; Goodman and Tessier-Lavigne, 1997). With the availability of immunohistochemical techniques, it was found
that subsets of axons expressed specific surface markers that presumably allowed them to fasciculate with each other, supporting a molecular basis for axonal recognition (Goodman and Doe, 1993; Goodman and Tessier-Lavigne, 1997). Since these studies in the 1970’s and 1980’s, a major field of study has blossomed around the identification of the surface molecules that regulate axon guidance and the mechanisms by which they function.

### 1.2. Guidance molecules and their receptors

Using genetic and biochemical approaches, a diverse variety of guidance molecules and their receptors have been identified, and many of these are conserved across species (Fig. 1.2). These molecules can be grouped into classes either by similarity of structure, for example, whether the molecules are secreted, membrane-tethered, or transmembrane, or whether they contain immunoglobulin-like or fibronectin-like repeats (Goodman and Tessier-Lavigne, 1997). Guidance molecules can also be grouped into ligand-receptor classes based on whether they mediate growth cone/axon attraction, repulsion, or adhesion/fasciculation (Dickson, 2002; Kiryushko et al., 2004).

For example, the Deleted-in-Colorectal Cancer (DCC) family of molecules are transmembrane receptors for the Netrins, which are secreted, laminin-related molecules (Goodman and Tessier-Lavigne, 1997). In culture, application of a point source of Netrins causes DCC-expressing *Xenopus* axons to turn and extend up the diffusing Netrin gradient (Fig. 1.3A) (de la Torre et al., 1997). The turning behavior and increased axon outgrowth rate are characteristic of growth cone attraction induced by other diffusible ligands, such as brain-derived neurotrophic factor (BDNF) (Stein and Tessier-Lavigne,
DCC’s homologs in flies (Frazzled), worms (UNC-40), and mice (DCC) also mediate growth cone attraction (Huber et al., 2003) (reviewed in section 1.8 and Chapter 3’s Introduction). In the presence of Netrin’s other receptor, Unc-5, DCC-mediated attraction is converted to repulsion; such a mechanism exists in worms, flies, and vertebrates (Guan and Rao, 2003; Hong et al., 1999). Therefore, Netrin is actually a bifunctional cue, and the specificity of the response it induces depends on the combination of DCC and Unc-5 receptors expressed on the growth cone’s surface.

The Slit family of leucine-rich repeat ligands, on the other hand, signal growth cone repulsion through the Roundabout (Robo) family of receptors (Guan and Rao, 2003). Application of a point source of Slit causes Robo-expressing Xenopus axons in culture to turn away from the gradient of ligand (Fig. 1.3B) (Stein and Tessier-Lavigne, 2001). Similarly, Slit repels cultured spinal motor axons and olfactory bulb axons, even causing olfactory bulb axons to collapse, withdrawing filopodia and lamellipodia (Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999). The Slit-Robo ligand-receptor system also mediates growth cone repulsion 	extit{in vivo} in flies, worms, mice, and zebrafish (Huber et al., 2003; Long et al., 2004). Growth cone turning, collapse, and/or retraction have been observed in response to other repulsive cues in culture as well, for example Ephrins and Semaphorins (Huber et al., 2003; Raper, 2000).

A third class of molecules are the cell adhesion molecules (CAMs), so named because many of the members of this group can mediate homophilic or heterophilic adhesion between cultured cells (Tessier-Lavigne and Goodman, 1996). These molecules regulate axon-axon, axon-cell, or axon-substrate adhesion (Fig. 1.3C), and include members of the Ig (immunoglobulin) CAMs and the cadherins. In the 	extit{Drosophila}
embryonic nervous system, the IgCAM *fasciclin II* (*fasII*) regulates the fasciculation (axon-axon adhesion) of both longitudinally-projecting CNS axons and peripheral motoneurons. Decreasing levels of *fasII* leads to defasciculation of axons in the CNS, while increasing *fasII* expression levels causes axon bundles to fasciculate or course together inappropriately (Lin et al., 1994). In the periphery, segmental nerve b axons overexpressing *fasII* fail to defasciculate, instead bypassing their muscle targets and continuing to project with other intersegmental nerve axons (Lin and Goodman, 1994). In the chick embryo, disruption of the interaction between the IgCAMs axonin-1 (expressed on commissural neurons) and NrCAM (expressed on floor plate cells) causes dramatic failure of axons to cross the CNS midline, demonstrating that adhesive interactions can provide directional information (Stoeckli and Landmesser, 1995). Vertebrate N-Cadherin mediates calcium-dependent, homophilic cell-cell adhesion, and promotes neurite outgrowth; *Drosophila N-cadherin* regulates embryonic axon fasciculation and photoreceptor pathfinding (Iwai et al., 1997; Prakash et al., 2005; Tepass et al., 2000; Ting et al., 2005).

Axon guidance and outgrowth are also modulated by various extracellular matrix molecules or substrates, including laminin, fibronectin, tenascins, heparan sulfate proteoglycans (HSPGs), and chondroitin sulfate proteoglycans (Goodman and Tessier-Lavigne, 1997; Holt and Dickson, 2005; Kiryushko et al., 2004). These molecules may be adhesive, as in the case of laminin or fibronectin, but in this case are often described as “permissive” or allowing axons to project properly along pathways in response to other cues (Goodman and Tessier-Lavigne, 1997). For example, *in vitro*, laminin promotes neurite outgrowth; axons extending on laminin-coated coverslips will not cross the
laminin boundary onto uncoated surface, and axons change speed and behavior at
laminin-fibronectin borders (Fig. 1.3D) (Gomez and Letourneau, 1994; Gundersen, 1987;
Turney and Bridgman, 2005). Integrins, a large family of receptors for laminin and
fibronectin, modulate signaling by a number of axon guidance receptors, including Robo
and DCC (Nakamoto et al., 2004). Proteoglycans are extracellular molecules composed
of a protein core such as syndecan or glypican that can be glycosylated in an immense
number of combinations (Holt and Dickson, 2005). Although the mechanism is not clear,
HSPGs have been implicated in the modulation of Slit- and Semaphorin-mediated
repulsion, and could provide an additional level of combinatorial specificity (the
hypothetical “sugar code”) during axon pathway selection (Holt and Dickson, 2005).

To summarize, as axons grow, they encounter a variety of cues in their
environment. These signals can be attractive or repulsive, acting at long or short range to
cause a growth cone to turn towards or away from a particular cue. They can also be
adhesive, causing axons to prefer one substrate over another, or permissive, merely
promoting or modulating axon extension that is imbued with directionality by other cues.
A host of receptors are expressed on the surface of the growth cone to detect and
integrate these environmental signals, but how do they regulate growth cone migration?
Ultimately, the signals transduced by these receptors regulate the dynamics of the actin
and microtubule cytoskeletons.

1.3. Guidance effectors regulate cytoskeletal dynamics

In migrating growth cones, the “leading edge” is the distal-most peripheral region,
farthest from the base or “central region” of the growth cone (Fig. 1.4). This region is
very thin, composed essentially of a collection of cytoskeletal proteins sandwiched by two sheets of membrane (Figs. 1.4C, 1.5, 1.6). The leading edge in growth cones (and in migrating cells) is filled with actin filaments, helical molecules composed of polymerized actin monomers (Figs. 1.4C, 1.5, 1.6). These polymers are generally arranged into either a mesh-like, cross-linked network of filaments, or into thick bundles of parallel filaments (Fig. 1.4, 1.5). Actin polymerization is thermodynamically favored at its “plus” or “barbed” end, while depolymerization is favored at its “minus” or “pointed” end (Fig. 1.6A). (The ends are named after their appearance in vitro when coated with myosin heads (Pollard and Borisy, 2003)). The actin cytoskeleton, long thought to be static in nature, is now known to be a very dynamic “machine,” constantly polymerizing at its plus ends (at the surface or periphery of the cell), and depolymerizing at its minus ends (inside the cell or growth cone, this is called the “central” region) (Pollard and Borisy, 2003). In images of live cells or growth cones expressing fluorescently-tagged actin, the entire cytoskeleton “treadmills” from the leading edge towards the central region, driven by the activity of myosin motors (Brown and Bridgman, 2003; Brown and Bridgman, 2004). A variety of molecules regulate the rate of actin polymerization/depolymerization, the geometry of polymerization (i.e., bundled or cross-linked filaments), and whether the actin cytoskeleton can be regulated by signaling of surface receptors, or physically linked to the substrate (Fig. 1.4, 1.5, and 1.6). The sum of these influences ultimately leads to directed migration. In general, attractive cues promote polymerization and stabilization of actin filaments, leading edge extension, and transport of membrane to the expanding leading edge (Dent and Gertler, 2003; Lee and Van Vactor, 2003). Conversely, repulsive cues inhibit polymerization/promote depolymerization, promote the retraction or
treadmilling of actin away from the leading edge, and stimulate endocytosis of membrane from the withdrawing surface (Gallo and Letourneau, 2004; Lee and Van Vactor, 2003). Adhesive cues promote the assembly of actin-linked “focal complexes” arranged around receptors that bind the substrate (Fig. 1.4C) (Lee and Van Vactor, 2003; Petit and Thiery, 2000).

In order to migrate, a cell or growth cone first extends filopodia—thin, spike-like extensions of bundled actin filaments (Figs. 1.4 and 1.7A) (Dent and Gertler, 2003). These processes, which can be up to several growth cone diameters in length, are believed to be sensory structures that probe the environment for instructive or adhesive cues, possibly mediating selective interactions with the growth cone’s surroundings (Dickson, 2002). The actin cytoskeleton constitutively extends and retracts filopodia, but external cues regulate the length or number of these extensions. For example, Netrin engagement of the DCC receptor has been shown to promote the formation of filopodia, while in Drosophila, mutations in the robo receptor lead to an increase in length of filopodia (Murray and Whittington, 1999; Shekarabi and Kennedy, 2002).

Next, lamellae (or lamellipodia, the terms are interchangeable) are extended (Dent and Gertler, 2003). These are broad, fan-like sheets of membrane composed of a meshwork of cross-linked actin filaments that spread over the substrate, often between filopodia, if they have been extended (Figs. 1.4 and 1.7B). Lamellae are also dynamic, constantly extending and retracting. If a permissive substrate is present and the appropriate receptors are active, adhesive contacts (“focal adhesions”) are formed between the lamella and its substrate, nucleated by receptors such as integrins which bind the substrate (Fig 1.4C) (Giniger, 2002; Petit and Thiery, 2000). If a permissive substrate
is not present, the lamellae retract back towards the growth cone unproductively (Brown and Bridgman, 2003; Giniger, 2002; Small et al., 2002). As with filopodia, extracellular cues also regulate lamellipodia extension, often measured as an increase or decrease in the surface area covered by a cell or growth cone. For example, Netrin-DCC signaling promotes dramatic increases in the surface area of neuroblastoma cells (Shekarabi and Kennedy, 2002), and increases in the surface area of cultured rat commissural growth cones (Shekarabi et al., 2005). By contrast, Slit-Robo or Semaphorin-Plexin signaling can inhibit lamellipodia extension, cause localized retraction, or even complete collapse (withdrawal of all filopodia and lamellipodia) (Gallo and Letourneau, 2004; Jurney et al., 2002; Nguyen Ba-Charvet et al., 1999).

Finally, the cell must translocate, or the growth cone must advance. In cells, this process is achieved by the formation and contraction of actin-myosin contractile filaments (“stress fibers”) between a stable, adherent lamellipodium at the leading edge and contacts at the rear of the cell which detach from their substrate (not shown). Contraction of the filament “pulls” the rear of the cell forward, in the direction of the stable leading edge, and the cycle repeats (Raftopoulou and Hall, 2004).

In growth cones, stress fibers have not been observed, but it is believed that the retrograde flow of the actin cytoskeleton (anchored to the substrate through adhesions) is somehow harnessed in stable lamellae, leading to forward migration (Fig. 1.7C) (Brown and Bridgman, 2003; Giniger, 2002). Myosins, the same molecular motors that control the contractility of stress fibers, have been implicated in the control of retrograde flow rate in the growth cone (Brown and Bridgman, 2003; Brown and Bridgman, 2004; Lin et al., 1996). The current consensus is that the generation of traction forces by myosin
motors can result in either axon extension or retraction, depending on the presence of adhesive contacts with the substrate (no contacts would lead to retraction), and the rates of actin polymerization and depolymerization (decreased overall polymerization would lead to retraction) (Brown and Bridgman, 2003; Brown and Bridgman, 2004; Gallo and Letourneau, 2004). In *Drosophila*, expression of constitutively active myosin light chain kinase promotes attraction across the CNS midline in a Fra/DCC-dependent manner (Kim et al., 2002). Conversely, both RhoA and its effector, RhoA kinase (ROCK), which function upstream of myosins (see below), have been implicated in repulsive signaling by Ephrin and Semaphorin receptors (Aurandt et al., 2002; Dickson, 2001; Driessens et al., 2001; Hu et al., 2001; Perrot et al., 2002; Shamah et al., 2001).

Recently, evidence has accumulated that microtubules also play a role during axon extension (Dent and Gertler, 2003). In both migrating cells and growth cones, a number of molecules that regulate microtubule stability or polymerization rate, or track along microtubules have been identified (Dent and Gertler, 2003; Fukata et al., 2003; Raftopoulou and Hall, 2004). It also seems that microtubules and actin interact and regulate each others’ dynamics in a number of ways (Etienne-Manneville, 2004; Rodriguez et al., 2003). In neurons, microtubules are confined to central region of the growth cone (only occasionally probing the leading edge) and to the axon shaft, providing rigidity as the region behind the migrating growth cone is converted into the axon shaft (Fig. 1.4) (Dent and Gertler, 2003; Dickson, 2002). Microtubules may play a role during growth cone turning, since pharmacological stabilization of microtubules on one side of a growth cone causes the axon to extend in the direction of stabilization, and conversely, destabilization of microtubules causes axon extension in the opposite
direction (Zhou and Cohan, 2004). Based on this observation, it has been hypothesized that attractive guidance cues stabilize the transient exploration of the periphery by microtubules, while repulsive cues would destabilize or inhibit this process, thus microtubule assembly and stabilization is favored in the direction of permissive or attractive cues (Dent and Gertler, 2003).

1.4. Rho GTPases: Critical cytoskeletal regulators in the growth cone

Summarizing the Introduction to this point, extracellular cues at the surface of the growth cone are detected by receptors that signal changes in the dynamics and geometry of the actin- and microtubule-based cytoskeletons (Dent and Gertler, 2003). An important family of molecules that act downstream of these receptors to modulate cytoskeletal dynamics are the Rho GTPases.

GTPases are molecular switches that are active when bound to GTP, but inactive when bound to GDP. This GTP- or GDP-bound status is regulated by three classes of molecules, the GEFs (guanine nucleotide exchange factors), the GAPs (GTPase activating proteins), and the GDIs (guanine nucleotide dissociation inhibitors) (Fig. 1.8) (Rossman et al., 2005). GEFs activate GTPases by promoting the exchange of GTP for GDP. GAPs promote the intrinsic hydrolytic activity of the GTPases, converting GTP to GDP, thereby inactivating the GTPase. GDIs inhibit GTPase activity by sequestering the GDP-bound GTPases in the cytosol, preventing GEF binding and the release of GDP for GTP (Rossman et al., 2005).

Downstream, all three GTPases influence actin dynamics, by mechanisms which are only partially understood, through numerous effectors, including myosins, myosin
light chain kinase and phosphatase, LIM kinase, p21 activated kinase (Pak),
Scar/WAVEs, N-WASP, the Arp2/3 complex, formins, coflin, profilin, and many others
(Fig. 1.9) (Dickson, 2001; Govek et al., 2005; Huber et al., 2003). Rho GTPases also
influence the stability and polymerization of the microtubule-based cytoskeleton,
although the mechanisms by which this occurs are currently much less understood than
GTPase-mediated control of actin dynamics (Fukata et al., 2003; Raftopoulou and Hall,
2004). Nonetheless, a few putative microtubule-regulating effectors have been identified,
including the formin mDiaphanous, the microtubule-destabilizing protein stathmin, and
the atypical protein kinase C\(\zeta\) (Fukata et al., 2003; Raftopoulou and Hall, 2004). Since
this aspect of GTPase signaling was not explored during the investigations described in
this Dissertation, GTPase regulation of the microtubule cytoskeleton will not be
discussed further.

In migrating cells and growth cones, Cdc42 and Rac GTPases promote actin
polymerization, leading to the generation of sensory filopodial microspikes (Cdc42) and
fan-shaped, protrusive lamellipodia (Rac) (Gallo and Letourneau, 2004; Govek et al.,
2005). In migrating cells, Rho promotes the formation of contractile actin-myosin stress
fibers, and all three GTPases promote the formation of cell-substrate focal contacts,
allowing cells to generate the traction force necessary for “crawling” over or through
substrates (Raftopoulou and Hall, 2004). In neurons, stress fibers have not been observed,
but bipolar myosin II “minifilaments” do form in growth cones (Bridgman, 2002; Gallo
and Letourneau, 2004). Rho and its effectors increase myosin II activity in growth cones
(as in migrating cells), and myosins likely regulate retrograde flow of the actin
cytoskeleton (Brown and Bridgman, 2004; Gallo and Letourneau, 2004; Lin et al., 1996).
Based on many studies in cultured vertebrate neurons using pharmacological inhibitors of GTPases, expression of active or inactive GTPase isoforms, and analysis of GTPase activity in the presence of stimulators or inhibitors of neurite outgrowth, Cdc42 and Rac have been generally thought to promote axon extension, while Rho and its effectors mediate growth cone collapse and axon retraction (Govek et al., 2005; Luo, 2000). Studies of guidance receptor signaling partially support this view. For example, the attractive DCC receptor promotes neurite outgrowth, filopodia and lamellipodia formation by activating Cdc42 and Rac1, while repulsive Plexin and Ephrin receptors activate RhoA (Huber et al., 2003). Additionally, the repulsive Robo receptor inhibits Cdc42 by recruiting srGAPs, and PlexinB1 sequesters active GTP-bound Rac from its effector Pak (Huber et al., 2003).

Recent discoveries, however, indicate that the role of GTPases during axon guidance and outgrowth is not so simple. For example, the N-terminal GEF of Kalirin, a Trio-like, alternatively-spliced GEF expressed in the vertebrate nervous system, restricts cortical neurite length in a Rac1-specific manner, while Kalirin GEF2 promotes axon extension in a RhoA-dependent manner (Penzes et al., 2001). Similarly, expression of dominant negative Rac1 or pharmacological inhibition of Rac1 function interferes with semaphorin 3A- and ephrin A2-mediated growth cone collapse, respectively, in chick neurons (Jin and Strittmatter, 1997; Jurney et al., 2002). While Rac1 activity is initially downregulated in response to these repulsive signals, it returns to normal levels prior to collapse and is required for ligand-induced endocytosis (but not F-actin depolymerization) during growth cone collapse (Jurney et al., 2002). In Drosophila, expression of constitutively active Rac1 (and to a lesser extent, Cdc42) in embryonic
motoneurons results in a severe failure of these axons to extend out of the CNS towards their peripheral muscle targets, illustrating that, in vivo, general elevation of Cdc42 and Rac1 activity does not promote axon extension, but rather, the activities of these GTPases must be precisely regulated by upstream signals for proper outgrowth and guidance to occur (Kim et al., 2003; Luo et al., 1994). Similarly, at the Drosophila embryonic CNS midline, expression of either dominant-negative or constitutively active Rac1 or RhoA causes inappropriate crossing of this boundary by axons (Fan et al., 2003; Fritz and VanBerkum, 2002; Giniger, 2002; Matsuura et al., 2004). The current confusion regarding GTPase function at the Drosophila CNS midline will be discussed more thoroughly in the Chapter 4 Introduction. But it can be said unequivocally that in all organisms studied to date, the Rho GTPases play critical and essential roles during cytoskeletal changes induced by axon guidance receptors.

1.5. An ideal system: the Drosophila embryo

Drosophila melanogaster, the “fruit fly” (D. melanogaster does not eat fruit, but the yeast which grows on rotting fruit) has been a model organism for biologists since Thomas Hunt Morgan and his colleagues began to study the inheritance of mutant traits in the early 1900’s (Chudley, 2000; Sturtevant, 2001). Since those early days, D. melanogaster (or simply “Drosophila”) has become one of the major systems in which to study developmental biology in general. Drosophila reproduces rapidly and robustly in the laboratory environment, and has many of the same tissues and undergoes similar developmental processes as higher organisms, including humans. A host of powerful genetic techniques have been developed through the years, including the ability to
generate and map random mutations through a variety of methods, to maintain mutations over marked “balancer” chromosomes (allowing simple genotyping of progeny and effortless maintenance of mutant lines), and more recently, to generate targeted molecular lesions (Venken and Bellen, 2005). *Drosophila melanogaster*’s genome has been completely sequenced, leading to the stunning discovery that many human disease genes have homologs in *Drosophila* and allowing the application of genomic-scale approaches to a variety of developmental problems, many with medical significance (Adams et al., 2000; Bier, 2005; Burdett and van den Heuvel, 2004; Matthews et al., 2005; Venken and Bellen, 2005).

The *Drosophila* embryo is ideal for the study of axon guidance. In contrast to the daunting complexity of vertebrate nervous systems with billions of neurons, the *Drosophila* embryo has only a few thousand neurons, and only a few hundred in each segment of the thoracic and abdominal nerve cord (Campos-Ortega, 1993; Goodman and Doe, 1993). The anatomy of the axonal tracts and positions of neurons at early stages is very similar to the grasshopper embryo, in which many neurons were first identified and named, a fact that allowed grasshopper neurobiologists to build on their discoveries in the more genetically amenable fruit fly (Goodman and Doe, 1993; Thomas et al., 1984). In addition to powerful genetic approaches for identifying axon guidance genes and probing their function, a variety of molecular genetic and immunohistochemical reagents have been developed for the analysis of specific axon tracts in the embryo (Araujo and Tear, 2003). This has allowed an increasingly precise understanding of gene function, since the effect of mutations can be studied at specific times or locations during the course of axon pathfinding by individual neurons or subsets of neurons.
1.6. *Early embryogenesis and neurogenesis*

[The following description has been summarized from Volker Hartenstein’s “Atlas of *Drosophila* Development” (Hartenstein, 1993) and (Campos-Ortega, 1993).]

130-180 minutes (at 25°C) after fertilization (stage 5), the *Drosophila* embryo cellularizes (Fig. 1.10A,F). Shortly thereafter, gastrulation begins. Endoderm invaginates both anteriorly and mid-dorsally; these tissues will eventually meet and fuse during later stages to become the gut (Fig. 1.10B). Mesodermal progenitors invaginate from the ventral furrow, forming a flattened tube by stage 8 (Fig. 1.10G). The mesoderm reorganizes into a monolayer at stage 9 (Fig. 1.10C,H), and retains its connection to the ventral ectoderm through two rows of mesectodermal cells until stage 11, when the mesoderm begins to differentiate and disperse into different organs, including the somatic musculature. The mesectodermal cells eventually differentiate into median neuroblasts and midline glial cells.

Beginning at stage 9 (230 minutes post-fertilization), neurons begin to delaminate from the ventral neuroectoderm, doing so in three waves in the ventral neurogenic region (Fig. 1.10C,H). Delamination of neuroblasts is completed during stage 11 (Fig. 1.10D,I). Neuroblasts divide assymetrically during eight waves of mitosis from stages 9 to 13, giving rise to smaller ganglion mother cells which are positioned between the neuroblasts and the overlying monolayer of mesoderm (Fig. 1.10I). Ganglion mother cells, in turn, divide equally to produce two daughter neurons. This process of delamination and neuroblast mitosis occurs during the process of germ band extension and retraction, during which the layer of ectoderm, neuroblasts, and mesoderm extends over the dorsal
surface of the embryo during stages 7-11 (inset in Fig. 1.10D), and then retracts ventrally
during stage 12 (440-580 minutes), so that by the beginning of stage 13, the entire cord is
located ventrally (inset in Fig. 1.10E). Neuronal differentiation and axonogenesis begins
in stage 12, and nascent longitudinal and commissural axon tracts have all formed by
stage 13 (Figs. 1.10J, 1.11A-C, and 1.12B).

Although beyond the scope of this Dissertation, the molecular genetics of
neurogenesis have been studied extensively, and a diverse array of molecules regulating
the proliferation and specification of neurons have been identified (Campos-Ortega,
1993; Skeath and Thor, 2003). The study of how these molecules function, in particular
the transcriptional control of neuronal differentiation, remains an area of intensive
investigation (Shirasaki and Pfaff, 2002; Thor and Thomas, 2002).

1.7. Axonogenesis

During stage 12, the newly-formed neurons begin to extend axons. The axons can
be divided into two categories: those that cross the ventral midline boundary
(contralaterally-projecting or “commissural” axons, named after the large bundles in
which they eventually reside, the commissures), and those which project ipsilaterally, on
the same side of the embryo (also referred to as “longitudinal” axons, because they
extend in tracts that run longitudinally for the entire length of the nerve cord) (Figs.
1.11B,C, 1.12, 1.13) (Goodman and Doe, 1993).

These axons can be visualized by immunohistochemical staining methods in fixed
embryos. Two of the most widely used antibodies are mAb BP102, which recognizes an
unidentified surface antigen on nearly all axons in the central nervous system (Fig. 1.12),
and mAb 1D4/anti-Fasciclin II (FasII), which recognizes a subset of longitudinally-projecting axons in the central nervous system as well as efferent motoneurons that exit the CNS to innervate the musculature of the outer body wall (Fig 1.13) (Grenningloh et al., 1991; Klambt et al., 1991; Seeger et al., 1993).

In the CNS, the first axons to extend across the midline boundary, the commissural “pioneers,” extend from anterior and posterior neurons in both sides of each segment. These axons extend towards the midline, fasciculate with each other in a “bowtie” shape at early stages, and then cross to the other side of the midline before proceeding on to their final targets (Fig. 1.12A). During late stage 12/early stage 13, midline glial cells interdigitate between commissural axons and enwrap them, dividing them into an anterior and posterior commissure (the “rungs” of the ladder-like CNS architecture), in which all future “follower” CNS axons will project across the midline (Fig. 1.11C, 1.12B) (Goodman and Doe, 1993). Longitudinal pathways are also intimately associated with a population of lateral glia (Fig. 1.11C). In addition to pathfinding by follower axons, the nerve cord also begins to condense after germ band retraction, beginning around stage 14 (Fig. 1.12C). Accordingly, the CNS axon tracts (the “neuropil”) also condense, shortening along the anterior-posterior axis and narrowing in width through stage 17 (Fig. 1.12F).

Longitudinal pathways are initially pioneered by two sets of neurons in each segment on either side of the midline (Fig. 1.13) (Goodman and Doe, 1993). The pCC and vMP2 neurons extend axons anteriorly, while the MP1 and dMP2 neurons extend axons posteriorly (Fig. 1.13A,B and not shown). These axons then fasciculate with each other, and project into adjacent segments to form the nascent longitudinal tract (Fig.
1.13B,C). Follower neurons project along this pathway, and during middle and late stages of axonogenesis, FasII-positive axons (a small set of the hundreds of axons that project in each segment of the longitudinal tract) reassort into three distinct bundles of axons on either side of the CNS midline (Fig. 1.13D-F) (Hidalgo and Brand, 1997).

1.8. The CNS midline, a “choice point”

As axons extend, at times they encounter important cues at specific locations, called “choice points,” in their environment and must respond appropriately before proceeding on to their next intermediate target. The ventral midline of the embryonic CNS in *Drosophila* is one such choice point at which a binary decision is made: CNS axons either cross or do not cross this boundary. The axons of some interneurons (~10%) in the embryo never cross the midline (Kidd et al., 1998b). Most (~90%), however, cross the midline once, and thereafter project ipsilaterally on either side of the midline, never recrossing this boundary (Kidd et al., 1998b).

Glial cells positioned at the CNS midline (Fig. 1.11C, Fig. 1.14A-C) secrete the attractive ligands Netrin A and Netrin B, as well as the repulsive ligand Slit (Battye et al., 1999; Harris et al., 1996; Kidd et al., 1999; Mitchell et al., 1996; Rothberg et al., 1988). Two receptors expressed in the growth cone that control an axon’s decision whether to cross the midline are the Netrin receptor Fra and the Slit receptor Robo1. In *fra* mutants (as well as *Netrin* mutants, see Chapter 3), commissural axons fail to cross the midline in ~20-25% of segments, because they are no longer attracted to the midline (Fig. 1.14B) (Kolodziej et al., 1996). This phenotype is relatively mild, implying the presence of another receptor(s) that guides axons across the CNS midline. In *robo1* mutants, axons
inappropriately cross and re-cross the CNS midline, or partially collapse onto the midline, because their ability to perceive Slit as a repellent is reduced (Fig. 1.14C) (Kidd et al., 1998a; Seeger et al., 1993). In slit mutants or robo1;robo2 mutants, axons collapse severely onto the midline, because they no longer perceive it as repulsive (not shown) (Kidd et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000b).

Why aren’t all axons repelled by the midline? The answer to this lies in the fact that in wild-type embryos, Robo expression is initially downregulated on commissural axons by axonal Commissureless (Comm) protein, allowing them to respond to attractive cues (Georgiou and Tear, 2002; Keleman et al., 2002; Kidd et al., 1998b; McGovern and Seeger, 2003). Once these axons have crossed, however, Robo expression is quickly upregulated (presumably because Comm expression is rapidly downregulated), allowing commissural axons to leave the midline and proceed towards their targets, without being drawn back towards the midline by Fra and/or other attractants (Georgiou and Tear, 2002; Keleman et al., 2002; Kidd et al., 1998b). Thus, precise temporal and spatial regulation of the expression levels of guidance molecules is a crucial “precondition” for axons to reach their correct targets.

The CNS midline in Drosophila has been, and continues to be, a powerful tool for understanding the signaling pathways regulating axon guidance. The conclusions drawn in this Dissertation are based largely on genetic interactions between molecules in this system.
1.9. A molecular understanding of growth cone attraction

When I began my graduate work in Mark Seeger’s laboratory in the spring of 1999, Frazzled, the Netrins, Slit, and one Robo receptor had been identified in *Drosophila*, but the mechanisms by which these receptors regulated directional axon extension were completely unknown (Goodman and Tessier-Lavigne, 1997). Furthermore, only a few cytoplasmic molecules involved in cytoskeletal dynamics were known to function during axon pathfinding in *Drosophila*. One of these was the Abelson non-receptor tyrosine kinase (Abl) (Lanier and Gertler, 2000; Van Etten, 1999). Working in Mark Seeger’s laboratory, in collaboration with Eric Liebl’s laboratory at Denison University (Granville, Ohio), I began my graduate career helping to characterize a novel genetic enhancer of the *Abl* mutant semilethality phenotype, *Drosophila trio*. *trio* and its orthologs in other organisms encode guanine nucleotide exchange factors (GEFs) that activate members of the Rho GTPase family (Bateman and Van Vactor, 2001). In this study, we discovered that double mutant *trio,Abl* embryos had severe defects in axon guidance, including a striking loss of commissural axon pathways (Chapter 2) (Liebl et al., 2000). This observation led us to explore further the role of Abl and Trio at the midline. Our experiments led to the discovery that Abl and Trio interacted genetically and biochemically with the attractive Netrin receptor Frazzled, implicating these molecules (and another, Enabled) as effectors of Fra signaling to the growth cone cytoskeleton (Chapter 3) (Forsthoefel et al., 2005). Finally, my recent efforts have been to functionally dissect Trio with the goal of determining which protein domains are required for axons to cross the CNS midline, and to begin to understand how Trio’s domains collaborate with each other to regulate cytoskeletal dynamics during axon
guidance signaling in the growth cone (Chapter 4). Since a rather large body of research exists for each of these molecules in *Drosophila* and other organisms, relevant information on each will be presented in the Introduction to each Chapter and as appropriate elsewhere.

**1.10. Significance of this work**

A molecular genetic understanding of axon guidance in the *Drosophila* embryo is important for several reasons. First, and most fundamentally, axon guidance in *Homo sapiens* is dauntingly complex: by some estimates over 100 billion neurons make over a trillion connections with each other in the human brain (Goodman and Tessier-Lavigne, 1997). How such complexity is created and orchestrated by a genome of less than 100,000 genes is still one of the most fascinating and poorly understood problems in biology. The *Drosophila* embryo has only a few thousand neurons, is amenable to genetic manipulation, has a short life cycle enabling rapid discovery, and utilizes many of the same genes and gene families during development of the nervous system (Dickson, 2002; Goodman and Tessier-Lavigne, 1997). Discoveries made in *Drosophila* have contributed greatly to a basic understanding of the molecular mechanisms of axon guidance and of human disease; for example, recently a mutation was identified in the human Rig1/Robo3 gene in patients with horizontal gaze palsy with progressive scoliosis (Jen et al., 2004; Vulliemoz et al., 2005). The insights gained from *Drosophila* may inform an understanding of neurobiology that may one day even enable central nervous system regeneration in humans (McDonald, 1999).
Second, numerous recent studies have led to a surprising realization: guidance molecules are utilized in numerous contexts outside of the nervous system (Hinck, 2004). Guidance molecules such as Slit, Netrin, Semaphorin, and Ephrin have been implicated in the development of the lung, heart, and kidney (Hinck, 2004). During development, blood vessels also utilize many of the same ligand-receptor systems as migrating axons, and endothelial tip cells adopt growth-cone like morphologies (Carmeliet and Tessier-Lavigne, 2005; Eichmann et al., 2005). Guidance molecules, their receptors, and effectors including Netrin, DCC, Abl, and even Trio have been found to be mutated, upregulated, or downregulated in various types of cancer (Arakawa, 2004; Wong and Witte, 2004; Zheng et al., 2004). The actin and microtubule cytoskeletons are actively organized in every cell type or tissue which moves or changes shape, but a complete understanding of the mechanisms by which this occurs during wound healing, angiogenesis, metastatic cancer cell migration, or immune cell homing has yet to be achieved. Clearly, understanding of signaling pathways in one process has and will continue to lead to advances and insights in another.
Figure 1.1. Anatomy of a neuron. At the tip of the growing axon is a dynamic, motile structure termed the “growth cone.” The axon is the efferent projection of the neuron – after development, once the axon has formed a synapse with its target, usually a muscle cell or another neuron, electrical signals are sent away from the cell body towards the target through the axon. Afferent dendrites also extend from the soma, but receive signals from other neurons.
Figure 1.2. **Major classes of guidance receptors.** Representative examples of ligands, receptors, CAMs, and others are shown. Ligand-receptor systems are shown above, while IgCAMs, N-Cadherin, and receptor phosphatases are shown below. Figure was adapted from (Tessier-Lavigne and Goodman, 1996) and (Dickson, 2002).
Figure 1.3. Axon guidance cues regulate growth cone behavior. (A) Cartoon of a *Xenopus* spinal axon turning towards a diffusible gradient of Netrin in a DCC-dependent manner. (B) Cartoon of a *Xenopus* spinal axon turning away from a diffusible gradient of Slit in a Robo-dependent manner. (C) Examples of fasciculation. At top, axons fasciculate together for a time because they perceive each other as more permissive than the surrounding environment. However, the lower axon soon becomes less adherent to the axon bundle, defasciculating towards a more attractive ligand at a distance. This type of behavior is similar to that seen in segmental nerve b motor axons in *Drosophila*, which leave the intersegmental nerve to innervate their target muscles. The bottom axons express FasII/NCAM, which bind homophilically and mediate axon-axon interaction, as with SNb axons in *Drosophila*. In (D), a migrating axon reaches the laminin boundary and migrates along its edge, rather than crossing onto uncoated glass. (A), (B), and (C), are partially based on figures in (Goodman and Tessier-Lavigne, 1997; Guan and Rao, 2003; Huber et al., 2003).
Figure 1.4. The growth cone’s leading edge. (A) and (B), cartoons of a turning growth cone. Filopodia are enriched in parallel bundles of actin filaments (red), while lamellipodia are composed of a veil-like meshwork of cross-linked filaments. Microtubules are mainly localized to the axon shaft and central region of the growth cone. However, they also explore the periphery, and are stabilized in one or more filopodia in the direction of turning. As the growth cone turns and extends, microtubules polymerize in the direction of turning, and the growth cone is converted into axon shaft on the side opposite the direction of turning as filopodia and lamellae are disassembled. (C), a side view of a lamellipodium filled with cross-linked filaments towards the rear (left) and parallel bundles at the leading edge (right). Two stylized focal contacts are shown (yellow), which anchor the cytoskeleton to the substrate. (A) and (B) were adapted from (Dickson, 2002), while (C) was adapted from (Brown and Bridgman, 2003) and (Alberts et al., 2002).
Figure 1.5. **Control of actin dynamics.** Cartoon of the actin cytoskeleton at the leading edge. Actin can be organized into a lattice-like meshwork or parallel bundled filaments. Polymerization occurs at the membrane at the plus-ends and depolymerization occurs deep in the cell or growth cone at the minus-ends. Major control points for actin-binding and actin-regulating proteins are indicated, along with some of the best understood molecules that function at each point. Figures in (Small et al., 2002) and (Pollard and Borisy, 2003) were utilized as references for this figure.
Figure 1.6. Overview of actin dynamics. (A) The plus and minus ends are indicated. Actin polymerization is thermodynamically favored at the plus end, while depolymerization is favored at the minus end. Profilin binds G-actin monomers and is thought to promote polymerization, although evidence exists for an inhibitory role as well (see Chapter 2 Introduction). Thymosin β also binds actin monomers, reducing the pool of available monomers for polymerization. (B) Examples of modulators of polymerization and depolymerization. Capping protein binds plus-ends of actin filaments, preventing polymerization, but is antagonized by Ena/VASP proteins. Formins are processive actin cappers that promote polymerization. The Arp2/3 complex nucleates new filaments off the sides of existing filaments (“branches”), while ADF/Cofilin destabilizes and severs filaments at the minus end. (C-E) Examples of actin-binding proteins that regulate the geometric organization and movement of actin filaments. All figures were adapted and modified from (Alberts et al., 2002).
Figure 1.6. Continued.
Figure 1.7. Growth cone extension. (A) The growth initially extends filopodia (arrow) to sense environmental cues and/or establish selective contacts with the environment. (B) Lamellae (arrow, green) are extended in the direction of the permissive or attractive cues. (C) If focal contacts with the substrate have been established (red blocks) and are linked to the actin cytoskeleton, traction force will be generated, allowing the growth cone to extend forward as unproductive lamellae and/or filopodia are retracted. (D) The cycle repeats.
Figure 1.8. Regulation of Rho GTPases. Rho GTPases are active in their GTP-bound form (left). GTPase activating proteins (GAPs) promote the intrinsic hydrolytic activity of the GTPase, releasing a phosphate group and deactivating the GTPase. Guanine nucleotide dissociation inhibitors (GDI’s) sequester GDP-bound GTPases and inhibit the release of GDP. GEFs (guanine nucleotide exchange factors) promote the exchange of GTP and the release of GDP, activating the GTPase.
Figure 1.9. Schematic of GTPase effectors. Rho GTPases effect changes in actin cytoskeletal polymerization, depolymerization, and contractility through a variety of effectors. Well-characterized pathways include Cdc42/IRSp53/Ena (filopodia), Cdc42/Rac/Pak inhibition of MLCK, Rac/Pak/LIMK inhibition of cofilin, Cdc42/Rac pathways through N-WASP and SCAR to Arp2/3 (filopodia, lamellipodia), and Rho/ROCK inhibition of MLCP, leading to myosin activation (stress fibers/contractility/retrograde flow). This is only a partial list and schematic. Adapted from (Dickson, 2001) and (Govek et al., 2005).
Figure 1.10. Embryogenesis and neurogenesis in *Drosophila melanogaster*. (A-E) Side views of embryos. Anterior is to the left and dorsal is to the top in all panels. (F-J) Cross sections of embryos. Top, dorsal. (A,F) Stage 5, cellularization. Green bracket in (F) denotes mesodermal precursors. Magenta brackets indicate the ventral neurogenic region, which ventralizes during gastrulation. (B,G) Stage 8, gastrulation. Endoderm (arrows, brown arrow, hatched) invaginates anteriorly and dorsally (B), while mesoderm (green arrows in G) has invaginated along the ventral furrow, forming a flattened tube above the ventral neuroectoderm (magenta brackets in (G)). Mesectodermal cells are indicated by the blue arrowhead. This cross section is at about 2/3 of embryo length, at the level of invaginating dorsal endoderm, which is seen as the developing dorsal tube (brown arrow). (C,H) Stage 9, the beginning of neuroblast delamination during germ band elongation. Neuroblasts in the process of delaminating from the ventral neuroectoderm are shown in magenta, while neuroblasts that have already delaminated are positioned between the flattened mesodermal monolayer (green arrows) and the ventral ectoderm. Blue arrowheads indicate mesectodermal cells at the ventral midline. (D,I) Stage 11, germ band-extended embryo. Inset highlights the position of the nerve cord. Neuroblasts have begun to divide, giving rise to ganglion mother cells (magenta arrows) which are positioned mainly between the neuroblasts and the mesoderm. (E,J) Stage 13, neurogenesis and germ band retraction have completed, and differentiation is beginning. Inset in (E) highlights the position of the nerve cord, showing the completion of germ band retraction. Nascent longitudinal axon tracts are indicated by the arrow in (J). Other tissue in (J) has been excluded for clarity. Images in this figure were adapted and modified from Hartenstein’s “Atlas of *Drosophila* Development” (Hartenstein, 1993). 

Continued on next page
Figure 1.10. Continued.
Figure 1.11. Axon tracts in the embryonic CNS. (A) Mid-abdominal section of a stage 17 embryo, demonstrating the position of the nerve cord and the peripheral axon tracts (arrows), in which motor axons exit the CNS, innervating the muscles of the outer body wall, and afferent axons project from sensory neurons in the body wall into the CNS. (B) The axon tracts and glia (together referred to as the neuropil) of the CNS lie dorsally to the neuronal cell bodies in the nerve cord. (C) Position of glial cells along the axon tracts. MGA and MGM are positioned around the anterior commissure, while MGP glia enwrap the posterior commissure. There are three pair of longitudinal glia (LG) per hemisegment. SBC (segment boundary cell) is the most proximal glial cell of the intersegmental nerve, composed of both afferent and efferent axons. Panel (A) was adapted from (Hartenstein, 1993). Panel (C) was adapted from (Jacobs and Goodman, 1989).
Figure 1.12. Axonogenesis in the embryonic CNS. Anterior is to the top in each panel. Axon tracts were visualized immunohistochemically with mAb BP102, which recognizes nearly all CNS axons. Stages are indicated. Note in (A), commissures (the “bowtie”) have not yet separated, and in (B), longitudinal tracts are relatively thin. From stage 14 onward, the axon tracts condense with the nerve cord. The entire nerve cord is ~75μm wide.
Figure 1.13. Longitudinal pathfinding. Anterior is to the top in each panel. A subset of longitudinally-projecting axons was visualized immunohistochemically with mAb 1D4 (anti-FasII). In (A) and (B), individual axons that pioneer the longitudinal tract can be seen extending both anteriorly and posteriorly. Individual growth cones of the anteriorly-projecting pCC axons can be seen in the four lower segments in (A). By stage 14 (C), other axons have begun to extend along this pathway. At stage 15 (D), FasII-positive axons begin to sort into distinct fascicles. Note also that a few FasII-positive axons cross the midline in the posterior commissure, but that FasII staining is rapidly lost by these axons by stage 16. (E) At stage 16, two distinct fascicles have formed, while the lateral fascicle is still incomplete. (F) Only at stage 17 are all three longitudinal fascicles on either side of the midline continuous.
Figure 1.14. Mutations in *fra* and *robo* affect axon guidance at the *Drosophila* embryonic CNS midline. (A) In wild-type embryos, ~90% of interneurons cross the CNS midline once before projecting longitudinally and never recrossing the midline. Note that FasII-positive bundles never cross the CNS midline (right panel). The midline is an important boundary for all axons because midline glial cells secrete attractive (Netrin) and repulsive (Slit) ligands (cartoon, left). The anterior commissural tract (AC) and posterior commissural tract (PC) are labeled in the cartoon, left panel. (B) In *fra* mutants, some commissures are thin or missing (left panel), since axons’ ability to perceive the midline as attractive is compromised. FasII-positive pathways are largely normal (right panel) with the occasional exception of thinning of the lateral fascicle. (C) In *robo1* mutants, axons cross and re-cross the midline boundary inappropriately (left panel) since their ability to sense Slit is compromised. FasII-positive axon bundles, especially the medial fascicles, collapse onto the CNS midline (right panel).
Figure 1.14. Continued.
CHAPTER 2

IDENTIFICATION OF TRIO AS A GENETIC ENHANCER OF MUTATIONS IN THE ABELSON TYROSINE KINASE GENE

2.1. INTRODUCTION

The Abelson non-receptor tyrosine kinase (Abl) is an important regulator of actin cytoskeletal dynamics. In this chapter, the identification of trio, a guanosine exchange factor (GEF) that regulates Rho-family small GTPases, as a genetic enhancer of mutations in the Drosophila Abl gene is described. First, the original discovery of tyrosine kinases as oncogenes is briefly recounted, followed by a more detailed consideration of Abl’s role in the modulation of cytoskeletal dynamics in both vertebrates and flies. A description of the molecular genetic isolation of trio follows, along with data implicating trio in Abl signaling pathways during embryonic axonogenesis. Finally, the implications of these data and potential future investigative directions shall be discussed.

2.1.1. Tyrosine kinases as oncogenes

Protein tyrosine kinases are enzymes that catalyze the transfer of a phosphate group from adenosine triphosphate to a tyrosine amino acid residue in another protein
This covalent modification (reversible by another enzyme, the protein tyrosine phosphatase) alters the substrate protein’s activity, ability to interact with other proteins, and/or localization (Cohen, 2000; Hubbard and Till, 2000). Since their initial discovery in the late 1970’s and early 1980’s, over 90 tyrosine kinases have been implicated in nearly all cellular signal transduction pathways, and are known to regulate diverse cellular functions including metabolism, growth, proliferation, differentiation, survival, and cytoskeletal motility (Manning et al., 2002b; Robinson et al., 2000). Tyrosine kinases have been identified in most metazoan phyla, and function during normal developmental processes, homeostasis, and nervous and immune system activity (Manning et al., 2002a; Manning et al., 2002b; Robinson et al., 2000).

Additionally, tyrosine kinases or their mutant or oncogenic forms have been implicated in a large number of congenital disorders, diseases, and cancer (Blume-Jensen and Hunter, 2001; Manning et al., 2002b; Robertson et al., 2000). Understanding of tyrosine kinases and the cellular signaling pathways in which they function continues to be an intense area of biological investigation.

Tyrosine kinases can be categorized into two broad classes: receptor or transmembrane and non-receptor or cytoplasmic (Hunter, 1987; Robinson et al., 2000). The first tyrosine kinase to be purified, pp60\(^{v-Src}\), was a cytoplasmic tyrosine kinase (Erikson et al., 1979; Hunter, 1987; Hunter and Sefton, 1980). v-Src was identified as the transforming protein encoded by the genome of Rous Sarcoma Virus, which had been isolated and described in 1911 by Peyton Rous as a “filterable agent” which could induce tumor formation in chickens (Martin, 2004; Rous, 1911). \(v-ssrc\) had been identified as the first retroviral oncogene, and subsequently, Harold Varmus and Michael Bishop
discovered that sequences homologous to \textit{v-src} were encoded by the vertebrate genome (Martin, 2001; Stehelin et al., 1976). Thus, \textit{c-src} (the “cellular” or non-transforming homolog) was also the first proto-oncogene to be discovered (Martin, 2004). Soon after, point mutations in \textit{v-src} were discovered that led to activation of Src kinase activity, suggesting that \textit{v-Src}’s ability to transform cells could be due to increased activation of biochemical pathways normally regulated by c-Src (Martin, 2001). Indeed, in the last 25 years c-Src has been implicated in signaling pathways controlling cell adhesion, motility, proliferation, survival, and cell cycle progression, among others, and the growth factor-independence, decreased adhesion, and increased invasiveness of RSV-transformed cells is caused by inappropriate activation of these pathways (Martin, 2001).

Also in the early 1980’s, another proto-oncogene, the Abelson non-receptor tyrosine kinase or \textit{c-Abl}, was identified as the homolog of the murine leukemia retroviral oncogene, \textit{v-Abl} (Goff et al., 1980; Wang et al., 1984; Witte et al., 1979a; Witte et al., 1979b; Wong and Witte, 2004). The Abelson murine leukemia virus (a variant of Moloney murine leukemia virus found to cause lymphosarcoma) was found to express a chimeric fusion protein, Gag-Abl, composed of N-terminal viral \textit{Gag} sequences and C-terminal \textit{Abl} sequences of cellular origin (reviewed in Wong and Witte, 2004). Transformation-defective variants of Gag-Abl had reduced kinase activity, again (as in the case of Src) suggesting that \textit{v-Abl} inappropriately upregulated signaling pathways normally controlled by c-Abl (Witte et al., 1980).

Following these initial discoveries in mice, others showed that the human \textit{c-Abl} gene localized to the distal tip of the long arm of chromosome 9, which translocates to the long arm of chromosome 22 in 95% of patients diagnosed with chronic myelogenous
leukemia (CML) (Bartram et al., 1983; de Klein et al., 1982; Heisterkamp et al., 1982; Wong and Witte, 2004). In patients carrying this chromosomal aberration, called the Philadelphia chromosome, c-Abl is fused to the breakpoint cluster region (BCR) gene; the resulting Bcr-Abl protein, composed of N-terminal BCR sequences and most of the Abl open reading frame (ORF), is oncogenic (reviewed in Saglio and Cilloni, 2004). Bcr-Abl has elevated tyrosine kinase activity, a consequence of both the deletion of an auto-inhibitory N-terminal myristoylation sequence in Abl, as well as oligomerization sequences in Bcr which promote activating “transphosphorylation” by Abl (Saglio and Cilloni, 2004; Wong and Witte, 2004). The mechanism of transformation by Bcr-Abl is not completely understood, but signaling pathways regulating proliferation, DNA repair, cell adhesion, and cytoskeletal dynamics are all known to be regulated by c-Abl (Saglio and Cilloni, 2004; Woodring et al., 2003). A recently-developed drug, Imatinib mesylate/Gleevec/STI571, selectively inhibits a small number of tyrosine kinases (four) including Abl and is effective in the treatment of CML; however, some CML patients are or eventually become resistant to Imatinib treatment (Wong and Witte, 2004). Thus, the cellular signaling pathways normally regulated by c-Abl continue to be of interest, as components of these pathways may become important drug targets in CML patients. In addition, Abl’s role in the regulation of cell shape and movement has garnered increasing attention in recent years, as cytoskeletal dynamics are modulated during a variety of developmental and physiological processes, including nervous system development, wound healing, immune cell homing, and cancer metastasis (Hernandez et al., 2004a; Moresco and Koleske, 2003; Woodring et al., 2003).
2.1.2. *Abl-family kinases regulate nervous system development and cytoskeletal dynamics*

In the mouse, c-Abl is not absolutely essential for development. Approximately 75% of mice homozygous for a targeted mutation in *c-Abl* are born, but survivors are runted and develop lymphopenia, osteoporosis, and thymic and splenic atrophy, and die within 1-2 weeks after birth (Li et al., 2000; Schwartzberg et al., 1991; Tybulewicz et al., 1991). Mice homozygous for mutations in the closely related *Abl-related gene, arg*, have no gross developmental defects, but do display several behavioral defects, suggesting a role in CNS morphogenesis or synapse function (Koleske et al., 1998). Both *c-Abl* and *arg* are widely expressed in the mouse embryo, including the brain, but defects in brain morphology have not been detected in *Abl* or *arg* mutants or even in animals carrying only one functional copy of either *Abl* or *arg* (Koleske et al., 1998). *Abl, arg* double mutant mice, however, die during embryogenesis (possibly due to pericardial or peritoneal hemorrhage) and have severe defects in neurulation and in F-actin organization in the neuroepithelium (Koleske et al., 1998). Immunohistochemical analysis of the neuroepithelium reveals that Abl and Arg colocalize with each other and apical F-actin in the cytoplasm (Koleske et al., 1998). Since the neuroepithelial defects occur early, before significant neurogenesis has occurred, the precise *in vivo* functions of Abl and Arg in nervous system development (especially at later time points) will have to be evaluated using conditional alleles of these genes that can be deleted in subsets of neurons during specific phases of neuronal specification, axon guidance, and synaptogenesis.
(Moresco and Koleske, 2003). However, the severity of defects in Abl, arg double mutants as compared to individual Abl and arg single mutants suggests that these genes have at least partially redundant functions.

While in vivo data on c-Abl’s function during neurodevelopment and regulation of cytoskeletal dynamics are not abundant, experiments in cultured cells have revealed more about how Abl regulates these processes. Abl localizes to neurite extensions and growth cones of embryonic mouse cortical neurons in primary culture (Zukerberg et al., 2000). Neurite extension and branching is promoted by overexpression of truncated, active forms of c-Abl in cultured cortical neurons, and inhibited in neurons derived from Abl-/- embryos or by pharmacological inhibition of c-Abl (Woodring et al., 2002; Zukerberg et al., 2000). Arg promotes neuritogenesis by phosphorylating and activating p190RhoGAP, and phosphorylation of p190RhoGAP is reduced in postnatal arg mutant brains (Hernandez et al., 2004b). Abl regulates dendritic outgrowth and branching in cultured hippocampal neurons through a pathway that likely inhibits the RhoA GTPase, although it is not known whether p190RhoGAP might also be regulated by Abl (Jones et al., 2004). c-Abl interacts biochemically with the nerve growth factor (NGF) receptor TrkA and the EphB2 receptor, both of which regulate axon outgrowth and guidance (Koch et al., 2000; Yano et al., 2000; Yu et al., 2001). Abl may negatively regulate N-cadherin/β-catenin-mediated adhesion in response to signaling by the repulsive Roundabout receptor (Rhee et al., 2002). Cables, an adaptor protein that is phosphorylated by both c-Abl and Cdk5, interacts physically with Abl in neurons, colocalizes with Abl in axons and growth cones, and promotes neurite extension.
(Zukerberg et al., 2000). Similarly, Abi (Abl-interactor) proteins, Abl substrates that regulate cytoskeletal dynamics, are expressed in developing neurons and localize to neuronal processes in culture (Courtney et al., 2000; Stradal et al., 2001).

Abl’s ability to regulate cytoskeletal dynamics has also been studied in vitro and in non-neuronal cells. The Abl C-terminal F-actin binding domain bundles actin in vitro, and Abl associates with actin filaments in fibroblasts (Van Etten et al., 1994). In cultured fibroblasts, c-Abl promotes filopodia extension, leading edge membrane ruffling, and spreading, and regulates migration rate (reviewed in Woodring et al., 2003). Abl is activated upon engagement of integrins, as well as PDGF (platelet-derived growth factor) and EGF (epidermal growth factor) receptor activation; miniruffling in response to PDGF is impaired in Abl-/- cells (Lewis et al., 1996; Plattner et al., 1999; Woodring et al., 2003). Additionally, Abl binds and/or directly or indirectly regulates the phosphorylation and activity of a growing list of cytoskeletal effectors involved in cell migration, including the focal adhesion protein Paxillin (whose phosphorylation is correlated with increased cell migration), the Dok-1 adaptor protein (to promote filopodia extension), the Son-of-sevenless GEF (to promote Rac activation, lamellipodia extension, and ruffling), and Ena/VASP proteins (which localize to the tips of extending filopodia and focal adhesions and also regulate lamellipodial stability) (Escalante et al., 2000; Howe et al., 2002; Lambrechts et al., 2000; Lewis and Schwartz, 1998; Sini et al., 2004; Tani et al., 2003; Woodring et al., 2003; Woodring et al., 2004). Finally, c-Abl is activated by Src and Fyn kinases in cells and in vitro, suggesting that multiple signaling pathways may intersect to regulate Abl and its effectors (Plattner et al., 1999).
2.1.3. The Drosophila Abl homolog regulates axon outgrowth and guidance and functions in signaling networks that regulate cytoskeletal dynamics

In the 1980’s, F. Michael Hoffmann’s group and others isolated Drosophila Abl in DNA cross-hybridization experiments designed to identify fly homologs of c-Abl and c-Src kinases (Henkemeyer et al., 1988; Henkemeyer et al., 1987; Hoffman-Falk et al., 1983; Hoffmann et al., 1983; Lev et al., 1984; Telford et al., 1985). These researchers hypothesized that isolation of proto-oncogene homologs in the fly might aid in the elucidation of these gene products’ normal cellular functions (Henkemeyer et al., 1988; Hoffman-Falk et al., 1983; Shilo and Weinberg, 1981).

Like its vertebrate homologs, Drosophila Abl encodes a protein tyrosine kinase domain preceded N-terminally by a myristoylation sequence, an SH3 (Src homology) domain, an SH2 domain, and a region of homology to the F-actin binding domain identified in murine Abl (Figure 2.2) (Lanier and Gertler, 2000; Van Etten et al., 1994; Woodring et al., 2003). In the coding region between the tyrosine kinase domain and the F-actin binding domain, D-Abl encodes two PxxPxK/R and one K/RxxPxxP consensus/putative SH3-domain binding sites. D-Abl also contains six other PxxP motifs in this region, although the functional importance of these PxxP domains in D-Abl has not been elucidated experimentally (DJF, not shown, and Woodring et al., 2003; Zarrinpar et al., 2003). In addition, D-Abl contains two predicted EVH1-binding domains (F/L-PPPP) absent in other Abl family members (DJF, data not shown, and Lanier and Gertler, 2000; Woodring et al., 2003). Conversely, nuclear export and localization
sequences, DNA binding domains, and G-actin binding sequences found in c-Abl have not been described in *Drosophila* Abl (DJF, not shown, and Moresco and Koleske, 2003; Woodring et al., 2003).

Investigators working in the Hoffmann lab initially found that mutations in *D-Abl* caused semilethality: only a percentage of homozygous *Abl* mutants hatched from their pupae and survived to adulthood (Henkemeyer et al., 1987). In addition, adult *Abl* mutants were short-lived, semi-fertile, and had a “rough eye” phenotype in which the organization of retinal cells within ommatidia was disrupted (Henkemeyer et al., 1987). The first immunohistochemical analysis of D-Abl protein in the embryo indicated that it localized primarily to the axons of the CNS and PNS, suggesting a role for Abl in the development or maintenance of embryonic axonal architecture (Gertler et al., 1989). Somewhat surprisingly, though, no defects in the organization of the CNS neurons or their projections were detected in *Abl* zygotic mutants (Gertler et al., 1989). However, Frank Gertler and others in the Hoffman lab had observed that when *Abl* alleles were heterozygous with chromosomal deficiencies that removed *Abl* as well as other nearby genes on the third chromosome, the number of animals surviving to pupation and adulthood dramatically decreased, and these animals died as embryos or larvae (Gertler et al., 1989). Thus, one or more genes deleted on the deficiency chromosomes displayed “haploinsufficiency dependent on *Abl*”, or the “HDA effect” (Gertler et al., 1989). Gertler and colleagues capitalized on this observation, and screened EMS-mutagenized *Abl* mutant third chromosomes for genes that displayed the HDA effect. Mutations in the first of the genes identified in this manner, *disabled (dab)*, exerted dominant effects on CNS axon organization in *Abl* mutants, and in *Abl,dab* double mutants, CNS architecture was
severely disrupted (Gertler et al., 1989). This early report demonstrated a role for Abl during CNS axonogenesis, and suggested that Abl was functionally redundant with one or more other molecules.

In a twist of science, Liebl and colleagues discovered in a subsequent investigation that these original “HDA” mutations were incorrectly ascribed to the dab gene (Liebl et al., 2003). Direct sequencing (which was not feasible at the time of the original Abl screen) of the large transcriptional units on these chromosomes revealed that an adjacent gene, neurotactin (nrt), not dab, was the mutant gene responsible for enhancement of Abl mutant phenotypes (Gertler et al., 1993; Liebl et al., 2003). Furthermore, in recent experiments in the Seeger laboratory, Chad Campbell, an Ohio State Molecular Genetics graduate student, found that co-injection of dab dsRNA with Abl dsRNA did not significantly enhance commissural axon pathfinding defects in embryos vs. injection of Abl alone (in contrast to coinjection of nrt and Abl dsRNA, which does have a dramatic effect, data not shown). Thus, although Dab colocalizes with Abl in embryonic axons, and is tyrosine phosphorylated in S2 cells that endogenously express Abl (Gertler et al., 1993), it seems unlikely that these two genes interact during embryonic nervous system development.

The fact that mutations in nrt enhance Abl semilethality and CNS phenotypes is intriguing, as nrt encodes a receptor that mediates heterophilic cell:cell adhesion of cultured Drosophila cells in the presence of Nrt’s ligand, Amalgam (Ama) (Barthalay et al., 1990; Darboux et al., 1996; Fremion et al., 2000). A novel mutation, ama^{M109}, which was isolated in a similar screen for enhancers of the Abl semilethality phenotype, also enhances axonal organization defects in Abl mutant embryos, and double mutant Abl, ama
embryos have defects in CNS organization (disrupted commissures and longitudinal connectives) approaching the severity seen in Abl,nrt mutants (Liebl et al., 2003). Both Nrt and Ama accumulate on neuronal cell bodies (specifically at points of contact between Nrt-expressing cells) and axons in the embryonic CNS, and nrt also interacts genetically with several other cell-adhesion molecules leading to disruption of axon outgrowth and fasciculation (Barthalay et al., 1990; de la Escalera et al., 1990; Hortsch et al., 1990; Seeger et al., 1988; Speicher et al., 1998). While an in vivo role in adhesion for Ama and Nrt has not been demonstrated, one interpretation of the genetic interactions between Abl, nrt, and ama is that Abl functions as a cytoplasmic effector of Ama-Nrt signaling during cell or axon adhesion in the CNS (Liebl et al., 2003). Future work must refine our understanding of the in vivo role of Ama and Nrt, and explore whether Abl modulates signaling by these molecules.

Since the initial description of dab, mutations in several other genes have been reported that interact synergistically with mutations in Abl, although as with nrt and ama, the functional significance of these interactions is not understood. For example, embryos doubly mutant for fasciclin I (which encodes a GPI-linked homophilic cell adhesion molecule) and Abl display an increased occurrence of defects in commissure formation (Elkins et al., 1990). Similarly, mutations in midline fasciclin (a gene related to fasciclin I whose expression is restricted to CNS midline cells) and failed-axon-connections (fax, which encodes a membrane-targeted protein with sequence similarity to neurofilaments) also enhance axon outgrowth defects in Abl mutants (Bedian and Jungklaus, 1987; Hill et al., 1995; Hu et al., 1998). Mutations in prospero, a transcription factor that regulates asymmetric divisions in neuroblasts, and neuronal and glial cell proliferation, enhance
semilethality in Abl mutants, but not CNS defects; the functional basis, if any, of this interaction is not known (Gertler et al., 1993; Griffiths and Hidalgo, 2004; Jan and Jan, 2001; Li and Vaessin, 2000).

In other cases, Abl interacts with genes encoding receptors known to play more specific roles during axon guidance (Notch, Dlar, and robo), or genes whose homologs in other systems are known to regulate cytoskeletal dynamics (enabled (ena), chickadee/profilin, capping protein, capulet, orbit/MAST/CLASP, armadillo/β-catenin, and shotgun/E-cadherin). For example, the transmembrane receptor Notch, better known for its role in embryonic neurogenesis, also modulates axon outgrowth in the CNS and defasciculation at a specific choice point for segmental nerve b (SNb) motoneurons in the ventral muscle field; these functions in guidance are genetically separable from Notch’s role in regulating cell fate (Crowner et al., 2003; Giniger, 1998). In the CNS, mutations in Abl and Notch interact synergistically, enhancing axon outgrowth defects (Giniger, 1998). In the PNS, however, the Notch temperature-sensitive loss-of-function phenotype, in which SNb axons fail to defasciculate from the intersegmental nerve (ISN) and bypass their muscle targets, is suppressed by mutations in Abl and other genes that interact with Abl, nrt and trio (Crowner et al., 2003).

Mutations in Drosophila lar, a receptor protein tyrosine phosphatase (RPTP), affect the same motoneuron choice point as mutations in Notch (Wills et al., 1999a). In Dlar mutants, SNb motoneurons fail to defasciculate at the ventral choicepoint, instead following other neurons in the ISN to more distal targets, a phenotype called “bypass,” since neurons bypass their correct branch point and in so doing, their muscle targets. Interestingly, mutations in ena lead to an identical phenotype (Wills et al., 1999a). ena
was originally isolated as a dominant genetic suppressor of semilethality in *Abl* homozygous mutants, and Ena was subsequently shown to bind and be phosphorylated *in vitro* by Abl (Comer et al., 1998; Gertler et al., 1995; Gertler et al., 1990). Mutations in *Abl* potently suppress the *Dlar* bypass phenotype, while overexpression of *Abl* phenocopies the bypass phenotype (Wills et al., 1999a). Abl and Ena both bind *Dlar*’s cytoplasmic domain, Abl tyrosine phosphorylates *Dlar* (in addition to Ena) *in vitro*, and *Dlar* dephosphorylates both Abl and Ena (Wills et al., 1999a). Taken together, these data suggest that *Dlar* and Ena (and possibly dephosphorylation of Abl, Ena, or other substrates) are required for SNb axons to defasciculate at the ventral choice point, while Abl (and possibly phosphorylation of Ena, *Dlar*, or other substrates) promotes adhesion and axon extension past this choice point (Wills et al., 1999a).

The antagonistic relationship between Ena and Abl may not be due to Abl’s ability to tyrosine phosphorylate Ena. A transgene encoding a non-phosphorylatable mutant Ena protein is capable of partially rescuing semilethality in *ena* mutants (Comer et al., 1998), and also rescues the *ena* bypass phenotype (cited in Krause et al., 2003). Ena’s murine homolog Mena, which also regulates neuronal migration and axon guidance, is phosphorylated by c-Abl, although the functional significance of this (as with Ena) is unknown (Lanier et al., 1999; Tani et al., 2003). Ena tyrosine residues targeted by D-Abl are not conserved in Mena, and Tyr-296, c-Abl’s substrate in Mena, is not conserved in Ena (Krause et al., 2003). However, these residues in both Ena and Mena are located in or near the proline-rich region, and thus may function similarly by regulating Ena ligand-binding; for example, D-Abl binds only unphosphorylated Ena via Abl’s SH3 domain (Comer et al., 1998; Krause et al., 2003). D-Abl also encodes two
predicted EVH1-binding domains in its C-terminus (Krause et al., 2003; Woodring et al., 2003). Thus, Abl’s physical interaction with Ena may be more functionally significant than Abl’s phosphorylation of Ena. D-Abl binding of Ena may regulate Ena’s localization, as Ena is mislocalized in epithelial cells during cellularization and dorsal closure in Abl mutants (Grevengoed et al., 2003). D-Abl binding may also regulate Ena’s interaction with other ligand(s); vertebrate Profilin II, for example, competes with n-Src’s (a neuronal Src splice variant) SH3 domain for binding to EVL (Lambrechts et al., 2000).

In cultured Drosophila and vertebrate cells Ena/VASP proteins localize to the leading edge of lamellipodia, the tips of filopodia and neurite-like cellular extensions, focal adhesions, and F-actin fibers themselves (Bear et al., 2002; Biyasheva et al., 2004; Krause et al., 2003; Rogers et al., 2003; Rothenberg et al., 2003). In vertebrate cell culture, Ena/VASP proteins promote the rate of actin polymerization by antagonizing capping protein at the barbed end of actin filaments, leading to filopodia extension in neurons (Bear et al., 2002; Lebrand et al., 2004). Interestingly, mutations in capping protein subunit β enhance axon pathfinding defects in D-Abl mutants, including commissure formation, suggesting that an antagonistic relationship between Ena and capping protein might be regulated by Abl in Drosophila (Grevengoed et al., 2003). Structure-function analysis of both Abl and Ena in cell culture, during nervous system development and other events during embryogenesis will be useful in clarifying the functional importance of physical interactions between Abl and Ena.

After SNb neurons have defasciculated, they extend dorsolaterally, ultimately synapsing on muscles 6,7,12, and 13 in the ventrolateral body wall. Abl appears to promote neurite extension towards these targets, since in Abl mutants, neurons that
innervate the distal-most muscle 12 often stall before reaching their final target (Wills et al., 1999b). Abl’s kinase domain is required for SNb axon extension, because Abl transgenes encoding a mutant kinase domain fail to rescue the SNb stalling phenotype (Wills et al., 1999b). Intriguingly, mutations in chickadee (chic), which encodes the Drosophila homolog of Profilin, have an identical SNb arrest phenotype, and mutations in Abl and chic interact genetically, leading to severe defects in axon outgrowth in the CNS (Wills et al., 1999b). Profilin is an actin-monomer binding protein that promotes ADP:ATP exchange by G-actin (Witke, 2004). Experimental observations support a role for Profilin in both inhibiting and promoting actin polymerization, suggesting that it may either sequester or supply actin monomers to barbed actin filament ends, depending on cellular context (Witke, 2004). Profilin is also believed to nucleate signaling complexes during actin polymerization (Witke, 2004). Biochemical interactions between Abl and Profilin have not been reported, but Drosophila Ena and its orthologs Mena, VASP, and EVL all bind Profilin in vitro, and Mena and profilin interact genetically in mice leading to perinatal lethality and neural tube defects (Krause et al., 2003; Lanier et al., 1999). The functional consequences for Profilin of Ena binding (or of binding by any of Profilin’s 50+ known ligands in various organisms) are not understood (Witke, 2004). Ena/VASP proteins recruit Profilin to promote actin polymerization and motility of the intracellular pathogen Listeria monocytogenes, but Profilin-binding is not required for Ena/VASP proteins during normal (random) fibroblast motility, and the necessity for Ena/VASP proteins’ proline-rich domains during axon guidance or filopodia generation has not been explored (Krause et al., 2003; Kwiatkowski et al., 2003). As previously mentioned, D-AblSH3 may compete with Profilin for binding to proline-rich regions in Ena, but the
molecular events that lead to specific motor axon phenotypes in Abl, ena, and chic mutants remain obscure. In any case, existing genetic data suggest that Abl and Profilin promote similar processes during motoneuron outgrowth; the nature of their cooperation, if any, will likely require careful structure-function analysis of Abl, Profilin, and Ena.

Both Abl and Ena also interact with the Roundabout repulsive guidance receptor at the Drosophila CNS midline. Overexpression of Abl promotes inappropriate crossing of the midline by longitudinally-projecting neurons in a kinase-dependent manner, suggesting that Abl might inhibit repulsive signaling (Bashaw et al., 2000). Loss-of-function mutations in ena interact genetically with mutations in slit and robo, also leading to inappropriate crossing of the midline by axons, consistent with a role for Ena during Robo-mediated repulsive signaling (Bashaw et al., 2000). Human Robo1 is tyrosine phosphorylated in cells and in vitro by Abl (Bashaw et al., 2000). Mutation of the corresponding tyrosine residue in dRobo1 (Tyr-1040) results in an activated receptor whose expression upregulates repulsion and causes thin or missing commissures, suggesting that Abl-mediated phosphorylation of Robo at this site normally inhibits Robo function (Bashaw et al., 2000). Mutations in ena suppress the phenotype of this activated receptor, consistent with a role for Ena as an effector of Slit-Robo signaling (Bashaw et al., 2000). The mechanism by which Abl regulates Robo is not known, but most likely involves modulation of the Robo cytoplasmic domain’s protein:protein interactions. It is also not clear if Abl constitutively inhibits Robo, or must be instructed to do so.

Similarly, Ena’s molecular role during Robo signaling has not been elucidated. However, in fibroblasts, Ena/VASP proteins inhibit overall migration rate by promoting excessive
actin polymerization and unstable lamellae formation at the leading edge, so Robo may be able to harness or localize Ena’s anti-capping activity for this purpose during Slit signaling (Bashaw et al., 2000; Bear et al., 2000; Bear et al., 2002).

Perplexingly, Abl also may promote repulsion, as mutations in Abl interact with robo and slit, also leading to inappropriate midline crossing (Hsouna et al., 2003; Wills et al., 2002). Interestingly, an intact kinase domain is required for Abl constructs to rescue inappropriate midline crossing in Abl mutants, suggesting that Abl’s kinase domain may function to both inhibit as well as promote repulsion (Hsouna et al., 2003; Wills et al., 2002). Robo and Abl may signal repulsion through Capulet (Capt—Drosophila adenyl cyclase-associated protein, whose homologs in other organisms suppress actin polymerization and whose human homolog binds c-Abl) and through Orbit/MAST/CLASP (a plus-end microtubule tracking protein) (Lee et al., 2004; Wills et al., 2002). Mutations in capt and orbit interact with Abl, robo, and slit, leading to inappropriate axon crossing at the CNS midline (Lee et al., 2004; Wills et al., 2002). In addition, Capt coimmunoprecipitates with Abl from Drosophila cell lysates, and orbit behaves genetically as an effector of Abl signaling (mutations in orbit suppress the Abl gain-of-function bypass phenotype in SNb) (Lee et al., 2004; Wills et al., 2002).

Finally, Abl also plays a role in the organization of the actin cytoskeleton at cell:cell adherens junctions. While most zygotic Abl mutants survive to pupation or adulthood, researchers in Mark Peifer’s laboratory found that animals mutant for both the zygotic and maternal contributions of Abl die at the end of embryogenesis (Grevengoed et al., 2001). In these maternal/zygotic mutants, CNS axon architecture is more dramatically disrupted than in Abl zygotic mutants (Grevengoed et al., 2001). In addition,
multiple developmental events requiring cell shape changes and/or cell migration are affected in \textit{Abl} maternal/zygotic \((Abl^{MZ})\) mutants, including head involution, germ band retraction, and dorsal closure (Grevengoed et al., 2001). During early embryogenesis, \textit{Abl} is also required for proper syncytial nuclear divisions and cellularization, when epithelial adherens junctions are first established (Grevengoed et al., 2003). In \(Abl^{MZ}\) mutants, the adherens junction components Armadillo (Arm)/\(\beta\)-catenin and \(\alpha\)-catenin, as well as F-actin, Ena, the formin Diaphanous, and the Arp2/3 complex all mislocalize during cellularization and/or dorsal closure (Grevengoed et al., 2003; Grevengoed et al., 2001). Furthermore, mutations in \textit{shotgun/E-cadherin, armadillo/\(\beta\)-catenin, diaphanous, and capping protein \(\beta\)} interact genetically with \(Abl\), enhancing defects in epithelial morphogenesis (Grevengoed et al., 2003; Grevengoed et al., 2001). Since \textit{Drosophila} \(N\)-cadherin (neuronal cadherin) and Arm/\(\beta\)-catenin function during CNS development, and \textit{arm/\(\beta\)-catenin} mutations interact with \(Abl\) leading to CNS axon outgrowth phenotypes, cadherin-catenin mediated adhesion probably regulates Abl function (or vice versa) in the nervous system as well as in epithelial cells (Iwai et al., 1997; Loureiro and Peifer, 1998). As with most of the gene products D-Abl interacts with, the exact molecular sequence of events in which Abl functions to regulate adherens junction organization remains to be characterized.

### 2.1.4. \textit{Drosophila trio} also interacts genetically with \(Abl\) to regulate axon pathfinding

Abl interacts biochemically and genetically with a plethora of molecules that regulate axon guidance and cytoskeletal dynamics. It should perhaps not be surprising that more Abl signaling partners remain to be discovered. In this Chapter, the
identification of trio as a novel genetic enhancer of Abl mutant phenotypes is described. trio, like nrt, fax, ama<sup>M109</sup>, and ena, was identified in a genetic screen for mutations that modify the Abl semilethality phenotype. trio encodes a cytoplasmic guanine nucleotide exchange factor highly similar to <i>C. elegans</i>, mouse, and human orthologs that regulate actin cytoskeletal dynamics through Rho-family small GTPases. trio’s orthologs UNC-73 in <i>C. elegans</i> and Trio in mouse and human also regulate axon pathfinding and outgrowth and neuronal positioning (Bateman and Van Vactor, 2001). Phenotypes in trio and trio,Abl mutants suggest that trio, like Abl and other “Abl network” genes, regulates axon outgrowth and/or guidance, and that tyrosine kinase activity may be functionally linked to GEF and GTPase activity in the growth cone.

[Much of the data presented in this Chapter was published in Liebl et al. (2000).]

2.2. MATERIALS AND METHODS

2.2.1. Fly Maintenance and Stocks

All genetic crosses were carried out using standard cornmeal, yeast, sucrose, agar medium in humidified 25° incubators. Stocks were maintained at room temperature (25°). Chromosomes used included Abl<sup>1</sup>; Abl<sup>4</sup>; trio<sup>M89</sup>,Abl<sup>1</sup>; trio<sup>M89</sup>,Abl<sup>4</sup>; Df(3L)FpaI; Df(3L)FpaI,Abl<sup>4</sup>; trio<sup>P0368/10</sup>, Tn[Abl<sup>+</sup>] or P[Abl<sup>+</sup>]A2.8; endothelium<sup>G10</sup>, fax<sup>M7</sup>,Abl<sup>1</sup>; Abl<sup>1</sup>,dab<sup>M221</sup>; In(3L)std11; trio<sup>P0368/10</sup>,fax<sup>M7</sup>; pUAST[trio<sup>+</sup>]; and scabrous-GAL4 (Mlodzik et al., 1990). The trio<sup>M89</sup> allele was generated in the Hoffmann laboratory by mutagenizing the Abl<sup>1-254C</sup> chromosome with EMS and screening for genetic enhancement of the Abl semilethality phenotype (the HDA effect), as previously
described (Gertler et al., 1989; Hill et al., 1995). Fly strains and stocks not generated by
the Liebl or Seeger laboratories were obtained from the Bloomington *Drosophila* Stock
Center (Indiana University, Bloomington, IN), Dr. F. M. Hoffmann (University of
Wisconsin, Madison, WI), Dr. F. Laski (University of California at Los Angeles, Los
Angeles, CA), Dr. A. Simcox (The Ohio State University, Columbus, OH), Dr. A.
Spradling (Howard Hughes Medical Institute, Carnegie Institute of Washington,
Baltimore, MD), Dr. J. Szidonya (University of Szeged, Szeged, Hungary), and other
members of the *Drosophila* community.

2.2.2. Dosage-Sensitive Genetic Tests

Stocks were balanced over either *TM6,B, Hu, e, Tb, ca* or *T(2;3), Cy, pr, Roi, cn; Hu, e, Tb, ca* balancer chromosomes. Crosses were conducted with 5 males and 5 females
of the appropriate genotype in a single vial, and transferred to fresh food every two days
three times for a total of four vials. For each genotype, at least 1300 progeny were
counted. To calculate the percent of expected *Tb*+ pupae from a cross, the number of *Tb*+ 
pupae was divided by one half the number of *Tb* pupae. Percent of expected *Hu*+ adults
were calculated in the same way. Values were normalized by carrying out a control cross
with one relevant chromosome to *Df(3L)emc-E12* (a neutral deficiency which
complements mutations in all genes in this study) and dividing experimental percent of
expected progeny by percent of expected progeny in the control cross.
2.2.3. Isolation and Sequencing of the trio Genomic DNA and cDNA

Plasmid rescue was used to isolate flanking genomic DNA sequences from the trio\textsuperscript{P0368/10} chromosome. Non-TnDoc sequences were used to probe a \textit{Drosophila} 0-18 hour embryonic \(\lambda\)gt10 cDNA library (Clontech, Palo Alto, CA). 5’ and 3’ RACE reactions were carried out using embryonic 0-18 hour poly(A)\(^{+}\) mRNA (Clontech, Palo Alto, CA) and the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). trio ESTs were obtained from Research Genetics (Birmingham, AL). The conceptual translation of the trio cDNA was aligned with the amino acid sequence of trio orthologs using Clustal-X.

Colony lifts of an array of P1 clones from the 61E;61F region of chromosome III (T. Kaufman, Indiana University, Bloomington, IN) were probed with DIG-labeled trio cDNA sequences. Positive clones (DSO2448 and DSO7840) were sequenced directly and compared to full-length trio cDNA sequence to determine the intron/exon boundaries of the trio gene. Small introns (<200bp) were sequenced directly. To estimate the size of larger introns, southern blots of the products of EcoRI and HindIII restriction digests of P1 clones were probed with various trio cDNA sequences.Comparison with completed genomic sequences (\url{http://flybase.bio.indiana.edu}) validated the genomic organization determined experimentally.

To determine the nature of the trio\textsuperscript{M89} allele, genomic DNA was obtained from \(ena^{GC10/+; trio^{M89}/Df(3L)FpaI}\) and from \(ena^{GC10/+; Abl1/Df(3L)FpaI}\) animals. Exons from the trio\textsuperscript{M89} allele and the parental, wild-type trio gene were amplified by PCR and sequenced directly. Differences between the EMS-induced trio allele and the parental allele were verified at least twice in independent amplification and sequencing reactions.
The L1412F mutation in the trio<sup>M89</sup> allele was modeled with the Quant/CHARMm software package (Molecular Simulations, San Diego, CA). Analogous wild-type residues in hTrio and hSos1 were imaged by DJF using Swiss-PdbViewer v3.7 (Guex and Peitsch, 1997) (http://www.expasy.org/spdbv/). Drawing in Figure 2.8 was generated using hTrio DH/PH1 coordinates in entry (residues 1225-1535) “1NTY” in the RCSB protein data bank (www.rcsb.org) (Skowronek et al., 2003; Skowronek et al., 2004).

### 2.2.4. Rescue of trio Mutant Phenotypes

A full-length trio ORF was constructed by ligating sequences from three ESTs into pBSK (Stratagene): an EcoRI(224)-MluI(2427) fragment from LD28463, an MluI(2427)-NheI(5217) fragment from LD34378, and an NheI(5217)-EcoRI(7152) fragment from LD19830. The full-length construct was fully sequenced, then subcloned into pUAST (Brand and Perrimon, 1993), and transgenic flies carrying pUAST[trio<sup>+</sup>] on the second chromosome were generated using standard methods (Spradling, 1986). For rescue, pUAST[trio<sup>+</sup>];trio<sup>M89</sup>,A<sup>d</sup> flies were crossed to scabrous-GAL4;Df(3L)Fpa1,A<sup>d</sup> flies and progeny were collected and processes for immunohistochemistry as described below.

### 2.2.5. In Situ Analysis and Antibody Staining of Embryos

Digoxigenin-11-UTP-labeled, single-stranded RNA probes sense and anti-sense probes were synthesized in vitro using the LD19830 EST clone (3’ trio sequences including the Trio GEF2 domain and 3’UTR) as a template. Hybridizations were carried out on wild-type Canton-S embryos as described (O’Neill and Bier, 1994). Antibody
staining of 0-24 hour embryos was conducted essentially as described (Patel et al., 1987). mAb BP102 (used at 1:20) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). mAb 1D4 (used at 1:20) was a gift from Dr. C. Goodman (University of California, Berkeley, CA). mAb anti-β-galactosidase (Promega) was used at 1:500 to detect lacZ expression from balancer chromosomes to determine embryo genotypes. Horseradish-peroxidase conjugated secondary antibodies (Jackson) were used to detect primary antibodies.

2.3. RESULTS

2.3.1. The M89 HDA mutation maps to the trio gene

As in other screens for genetic enhancers and suppressors of the Abl mutant phenotype, an ethylmethanesulfonate(EMS)-induced allele, originally identified as M89, displayed haploinsufficiency dependent on Abl mutations, or the HDA effect (Table 2.1) (Hill et al., 1995; Hoffmann, 1991). In the Liebl laboratory, the analysis of meiotic recombination products between the M89, AblI chromosome and the multiply-marked ru, h, th, st, cu, sr, e, ca (rucuca) chromosome revealed that 100% (n=110) of ru, AblI chromosomes had lost the ability to enhance Abl semilethality, and therefore the M89 mutation. This indicated that M89 mapped near roughoid (61F8), distal to hairy (66D10) on the left arm of chromosome III. Complementation analysis with chromosomal deficiencies narrowed M89’s location to 61D3;61F2, based on the fact that Df(3L)emc-E12 (61A;61D3) (data not shown), but not Df(3L)Fpal (61D1-2;61F1-2) (Table 2.1) complemented the M89 mutation.
Next, the Liebl laboratory screened available P-element insertion lines in 61D3;61F2 for their ability to complement the M89 mutation. The Szeged P[w+] insertion line l(3)\(^{0368/10}\) (Deak et al., 1997) failed to complement the M89, Abl\(^l\) chromosome (Table 2.1) but complemented the parental Abl\(^l\) chromosome (data not shown). Mobilization of P[w+] in l(3)\(^{0368/10}\) was conducted to generate “excision lines” which had lost P[w+]; these were also tested for their ability to complement M89, Abl\(^l\). 95% (103 of 108) of these chromosomes complemented, suggesting that l(3)\(^{0368/10}\) is an allele of the gene containing the M89 mutation.

Genomic DNA flanking l(3)\(^{0368/10}\) was isolated by plasmid rescue and sequenced, revealing that the P[w+] was inserted into a partial Tn Doc transposable element (Bender et al., 1983). To isolate full-length cDNA, non-Tn Doc flanking sequences were used to screen a 0-18 hour embryonic cDNA library, and 5’ and 3’ RACE reactions were conducted using embryonic mRNA. Ultimately, we assembled a full-length cDNA from sequences identified through the Berkeley Drosophila Genome Project. Because of extensive similarity to orthologs in human and C. elegans, the gene encoding this cDNA was named Drosophila trio.

2.3.2. trio is highly similar to the N-terminal 80% of human trio

The full-length cDNA encodes an open reading frame of 2263 amino acids, a protein with a predicted molecular weight of 257 kDa (Figure 2.3). Database searches with this sequence revealed highest similarity to human Trio and Kalirin, and C. elegans...
UNC-73A (Alam et al., 1997; Debant et al., 1996; Steven et al., 1998). These molecules have been implicated in the regulation of cytoskeletal dynamics in fibroblasts and neurons, and activate Rho-family small GTPases \textit{in vitro}.

An alignment of \textit{Drosophila} Trio, human Trio, and \textit{C. elegans} UNC-73A amino acid sequences reveals multiple regions of high similarity (Figures 2.3 and 2.4). \textit{Drosophila trio} encodes a protein including a conserved N-terminal domain with similarity to yeast Sec14p, a phosphatidylinositol transfer protein involved in transporting secretory proteins from the Golgi complex; this domain is found in a number of metazoan GEFs, and may regulate localization or intramolecular interactions (Kostenko et al., 2005; Ueda et al., 2004). Immediately C-terminal to this region are 4-6 spectrin-like repeats. The function of these domains in other GEFs has also not been explored extensively, although they may play a role in oligomerization or Trio molecules, or localization or assembly of Trio signaling complexes (Djinovic-Carugo et al., 2002). Trio encodes two Dbl homology (DH) and two pleckstrin homology (PH) domains (Figures 2.3 and 2.4). In human Trio, the N-terminal DH/PH domain promotes GDP-GTP exchange by the Rac1 and RhoG GTPases, while in \textit{C. elegans} UNC-73A, the N-terminal DH/PH domain promotes GDP-GTP exchange by Rac1; the hTrio C-terminal DH/PH domain promotes GDP-GTP exchange by the RhoA GTPase (Blangy et al., 2000; Debant et al., 1996; Steven et al., 1998). While the DH domain actually catalyzes GDP-GTP exchange by GTPases, the adjacent PH domain binds phospholipids and regulates exchange activity and/or molecular interactions (Bellanger et al., 2000; Rossman et al.,
Between the DH/PH domains, an SH3 domain likely mediates interactions with signaling partners, although the only binding partner identified so far is focal adhesion kinase (FAK), which interacts with hTrio (Medley et al., 2003).

C-terminal to DH/PH2, hTrio encodes a second SH3 domain, an immunoglobulin (Ig)-like domain, and a serine-threonine kinase domain, while UNC-73A encodes an Ig-like domain and a fibronectin type III (FnIII) domain (Figure 2.4). These domains are not likely encoded by Drosophila trio, because in the Liebl laboratory, 3’RACE experiments with embryonic cDNA did not identify alternative ends (data not shown). Furthermore, searches of the Drosophila genome using C-terminal hTrio and UNC-73 amino acid sequences did not identify similar sequences in or near the Drosophila trio ORF (DJF, not shown). Finally, three other groups cloned Drosophila trio simultaneously, and none of these reported additional protein-coding sequences 3’ to DH/PH2 (Awasaki et al., 2000; Bateman et al., 2000; Newsome et al., 2000).

Northern blot analysis of Drosophila embryonic RNA with full-length and 3’UTR probes against the trio coding region revealed two prominent transcripts, an ~8.0 Kb and a slightly larger, but minor ~8.9-9.2 Kb message (Figure 2.5). The 8.0 Kb transcript corresponds to the size of our full-length trio cDNA (8033bp, Genbank Accession AF216663), which is sufficient for rescue of the trio,Abl double mutant phenotype (see below). The identity of the longer message is not clear, but Awasaki et al. (2000) independently reported a larger, 9238bp full-length cDNA (Genbank Accession AB035419), whose ORF is identical to our sequence (with the exception of single nucleotide polymorphisms) but that encodes a 3’UTR that is 1.2 Kb longer than our cDNA. Given that probes generated from both full-length trio or just the 3’ UTR
hybridized to both messages in our northern blot analyses, it seems likely that this larger message is a less-widely or less-robustly expressed trio RNA species with a longer 3’UTR of unknown function.

2.3.3. Molecular basis of trio alleles

The trio gene spans ~40 Kb, and is encoded by 16 exons (Figure 2.6). Genomic DNA from flies carrying the trio<sup>M89</sup> chromosome was isolated, and all exons and splice sites were sequenced and compared to sequence from the parental trio<sup>+</sup> chromosome. This revealed a C to T transition resulting in a missense mutation in codon 1412, converting a leucine residue to phenylalanine (L1412F) (Figures 2.6 and 2.7) in trio’s first (N-terminal) DH domain. This residue (L1412) is conserved at this position in both DH domains in human and Drosophila trio, and is highly conserved in the N-terminal DH domains of Trio orthologs in human, mosquito, worm, and Drosophila pseudoobscura, in addition to other DH-containing human proteins, including Sos1, Dbl, Vav, and Tiam1 (Figure 2.7) (Soisson et al., 1998). L1412 is analogous to L343 in the DH domain of human Sos1 and L1363 in hTrio; in both hTrio and hSos1, this residue’s side chain projects into a hydrophobic pocket at the confluence of helices that form the DH catalytic domain (Figure 2.8) (Liu et al., 1998; Skowronek et al., 2003; Skowronek et al., 2004; Soisson et al., 1998). Molecular modeling conducted by Dr. Liebl and collaborators at Denison University suggests that conversion of leucine 1412 to the bulkier phenylalanine may alter the packing between F1412 and F1331 in this hydrophobic pocket (not shown). Drosophila trio F1331 is analogous to F246 in hSos1 and F1282 in hTrio, an invariant residue within DH domains (Figure 2.7) (Soisson et al., 1998). Although these residues
(hSos1 L343 and F246) are not directly involved in DH catalytic activity, alteration of side chain packing in this hydrophobic pocket may disrupt the position of DH1 alpha helices, which in turn could compromise or completely disrupt DH1’s catalytic activity or protein:protein interactions; this remains to be explored experimentally (Soisson et al., 1998).

The P[w\+] insertion site in line 0368/10 has been mapped to the intron between exons 7 and 8; the splice sites of this intron span a glycine codon (G1756) between the SH3 domain and the second DH domain (Figure 2.6). Aberrantly-sized transcripts in embryos carrying trio\textsuperscript{P0368/10} have not been detected (Figure 2.5), but in situ hybridization of embryos which were the progeny of heterozygous trio\textsuperscript{P0368/10} parents with a 3’UTR probe stained only ~75% of embryos, suggesting that P[w\+] may disrupt transcription 3’ to the insertion site (Table 2.2). Additionally, Awasaki et al. (2000) were unable to detect either full-length Trio protein or a smaller, 130 kDa protein in lysates generated from homozygous trio\textsuperscript{P0368/10} animals (both of these Trio isoforms are detected in lysates from wild-type flies). Since the antibody used in these experiments was generated against an epitope encoded by trio sequence 3’ to the P[w\+] insertion site, these authors could not rule out that a truncated, but undetectable, Trio protein was produced in trio\textsuperscript{P0368/10} homozygotes. However, their results clearly demonstrate that this insertion line affects production of full-length Trio protein (Awasaki et al., 2000).

### 2.3.4. trio is expressed in the developing CNS

The temporal and spatial pattern of trio expression was examined in embryos by conducting in situ hybridization of wild-type embryos with antisense riboprobes. trio
mRNA was detected in early cleavage stage embryos, indicating that trio is maternally contributed (Figure 2.9A). By cellular blastoderm stage, the level of trio mRNA was reduced compared to earlier stages (not shown). As germ band extension proceeded, mRNA accumulation was detected in the invaginating mesodermal layer (not shown). trio mRNA was detected in the developing nervous system by stage 10, and persisted through stage 15 (Figure 2.9B, C, and D). Most, if not all, neurons appeared to express trio (Figure 2.9F). In addition, patches of epidermal expression appeared at stages 13-15, corresponding to muscle attachment sites for the somatic musculature (Bate, 1993; Frommer et al., 1996). During the process of dorsal closure, increased levels of trio mRNA accumulation were observed in leading edge cells that were enclosing the yolk sack along the dorsal surface (Figure 2.9G). By stage 16, the trio message was restricted to cells surrounding the developing gut (Figure 2.9H).

2.3.5. Reducing the gene dose of trio exacerbates the Abl mutant phenotype

The EMS-induced trio<sup>M89</sup> allele was isolated based on its ability to enhance the Abl semilethality phenotype in a dosage-sensitive manner, shifting the lethal phase to prepupal stages (Table 2.1, Table 2.3). This “HDA effect” is similar to that observed for mutations in nrt and fax (Gertler et al., 1989; Hill et al., 1995; Hoffmann, 1991). Dr. Liebl found that trio<sup>M89</sup> is not haploinsufficient itself, since trio<sup>M89</sup>,Abl<sup>+/Abl</sup> animals carrying a wild-type Abl minigene survived to adulthood (Table 2.3) (Henkemeyer et al., 1990). A chromosomal deficiency that completely removes the trio gene, Df(3L)FpaI, also showed the HDA effect, indicating that the effect on Abl semilethality is not a unique property of the trio<sup>M89</sup> allele (Table 2.3).
As both *Abl* and *trio* are expressed in a number of tissues (see *in situ* analysis above and Awasaki et al., 2000; Bateman et al., 2000; Bennett and Hoffmann, 1992; Liebl et al., 2000; Newsome et al., 2000) and likely function in a variety of developmental processes (see Chapter 2 Introduction and Chapter 4 of this Dissertation), the exact cause of lethality in *Abl* and *trio* mutant animals is not known.

**2.3.6. Mutations in Abl reciprocally enhance semilethality in trio hypomorphic mutants**

Investigators in the Liebl laboratory also discovered that although the majority of *trio*-M89/Df(3L)FpaI animals died prior to pupation, the *trio*-P0368/10 allele proved to be a hypomorphic allele, and the majority of *trio*-P0368/10/trio-M89 animals survived to adulthood (Table 2.4). In this adult, viable *trio* background, reducing the gene dose of *Abl* using two different alleles of *Abl* resulted in genetic enhancement of the *trio* semilethality phenotype, shifting lethality to pupal and prepupal phases, similar to *trio*’s ability to enhance the *Abl* semilethality phenotype (Tables 2.3 and 2.4). An allele of *fax*, originally identified as an enhancer of *Abl* mutant semilethality, also reduced the viability of *trio* hypomorph mutants (Table 2.4). A chromosome that carries a deletion of *Abl, dab*, and *nrt, In(3L)std11*, also enhanced the *trio* mutant phenotype in animals that also carried a wild-type *Abl* transgene, suggesting that *dab, nrt*, or some other gene removed by this deficiency also interacts genetically with *trio*. Interestingly, Dr. Liebl also found that 23% of *trio*-P0368/10/trio-M89 adults had an uninflated, blistered wing phenotype, and that mutations in *Abl* and *fax* enhanced this defect (100% for *Abl*1, 89% for *Abl*4, and 72% for *fax*M7), suggesting that *trio* may also function during wing morphogenesis.
2.3.7. trio also interacts genetically with ena

Mutations in ena were originally isolated as genetic suppressors of the Abl semilethality phenotype (Gertler et al., 1995; Gertler et al., 1990). In agreement with these previous findings, Dr. Liebl and colleagues found that an allele of ena suppressed semilethality in Abl mutants (Table 2.5). Heterozygosity for ena also suppressed the enhancement of Abl semilethality by trio, as well as the enhancement of the Abl mutant phenotype by nrt or fax (Table 2.5). Heterozygosity for ena also increased the percentage of Df(3L)Fpal/trioM89 animals surviving to pupariation, although these animals never survived to adulthood (Table 2.5).

2.3.8. Mutations in Abl and trio interact synergistically to disrupt the developing CNS

Abl interacts genetically with both fax and nrt leading to severe disruptions of axonal architecture in the embryonic CNS (Gertler et al., 1989; Gertler et al., 1993; Hill et al., 1995). Because trio was isolated in the same manner as these genes, we asked whether mutations in trio and Abl would also interact genetically to cause disruptions in the developing CNS.

Immunohistochemistry with mAb BP102 reveals the ladder-like scaffold of axons in the CNS of wild-type embryos (Figure 2.10A). Axons are bundled into two commissures per segment (an anterior and posterior commissure, the “rungs” of the ladder), and two longitudinal tracts that run the length of the entire embryo. In embryos homozygous mutant for Abl (Abl<sup>l</sup>/Abl<sup>l</sup>), the organization of the CNS is relatively unaffected, although longitudinal connectives between segments are often thinner than in
wild-type (Figure 2.10B). Reducing the dose of trio by half in Abl mutants resulted in more frequent thinning of longitudinal tracts and obvious disruptions in the formation of commissures (Figures 2.10C and D). For example, in trio heterozygous, Abl homozygous (trioM89.Abl1/Abl4) embryos, 31% (n=210) of segments scored had clear defects in commissures, compared to only 8% (n=240) of segments in Abl1/Abl4 embryos (compare panel C vs. B in Figure 2.10). Similarly, the trio deficiency also enhanced commissure disruptions in Abl mutants; in Abl1/Df(3L)FpaI, Abl4 embryos, 42% (n=230) segments had disrupted commissures (Figure 2.10D). This enhancement was dependent on Abl mutations, as trio heterozygous, Abl homozygous embryos also carrying an Abl minigene, P[Abl+], exhibited commissure defects in 0% of segments (n=90).

In trio homozygous mutant embryos stained with mAb BP102, as in Abl mutants, CNS defects were mild and infrequent; no commissure disruptions were observed, and occasionally longitudinal connectives were thin, as in Abl mutants (Figure 2.10E). Reciprocal dosage-sensitive interactions between trio and Abl affecting commissure formation were not observed. For example, in trioM89.Abl1/Df(3L)FpaI embryos, only 1.5% of segments (n=210) had disrupted commissures, vs. 0% (n=200) in trioM89/Df(3L)FpaI embryos (Figure 2.10E and F). However, longitudinal connectives seemed to be thinner more frequently in trio homozygous, Abl heterozygous embryos (Figure 2.10F and data not shown), a phenotype we quantified more carefully using a different axonal marker (below).

In embryos homozygous for both trio and Abl, disruptions in the axon scaffolding were severe (Figure 2.10G). In these embryos, 100% (n=200) of commissures were disrupted, and very few axons successfully crossed the CNS midline boundary compared
to animals in which one copy of either *trio* or *Abl* was wild-type (Figure 2.10C, D, and F). Expression of a full-length *trio* cDNA under the control of the UAS promoter using the panneural *scabrous*-GAL4 driver line dramatically rescued the synergistic *trio*,*Abl* double mutant CNS phenotype (Figure 2.10H) (Brand and Perrimon, 1993). In these embryos, only 28% of segments (n=86) had disrupted commissures. These embryos still displayed thin longitudinal connectives, due to the fact that they are still homozygous mutant for *Abl* (Figure 2.10H). The ability of our full-length *trio* cDNA to rescue the commissure phenotype indicates that this cDNA encodes a functional Trio protein, and verifies that the *trio*<sup>M89</sup> and *Df(3L)FpaI* chromosomes’ ability to enhance *Abl* phenotypes is due primarily to mutation or deletion of the *trio* gene.

During our analysis of enhancement of CNS phenotypes in homozygous *trio* embryos by mutations in *Abl* with mAb BP102, we noted that often longitudinal connectives between segments were thin or, in more severe cases, missing (Figure 2.10E and F and data not shown). We explored the dosage-sensitive genetic interactions between *trio* and *Abl* further by analyzing a subset of longitudinally-projecting axons with mAb 1D4, which recognizes the FasII protein. This reagent marks axons in three distinct fascicles that project on either side of the CNS midline (Figure 2.10I). In *trio* mutants (*trio*<sup>M89</sup>/*Df(3L)FpaI*), there were clear breaks in the lateral-most FasII-positive fascicle in 58% of hemisegments assayed (n=100) (Figure 2.10M). Reducing the gene dose of *Abl* in this background dramatically increased the severity of this phenotype, as 98% of hemisegments (n=80) had breaks in the lateral fascicle. Additionally, there were occasional disruptions in more medial fascicles (Figure 2.10M and N). In the hypomorphic *trio* homozygotes (*trio*<sup>P0368/10</sup>/*trio*<sup>M89</sup>), 14% (n=104) of hemisegments had
breaks in the lateral FasII-positive fascicle; in \textit{trio}^{P0368/10}/\textit{trio}^{M89}\textit{Abl}^{1} embryos, 47% (n=106) of hemisegments were disrupted (Figure 2.10O and P). We also found that mutations in \textit{trio} enhanced lateral fascicle defects in \textit{Abl} mutants, and in \textit{trio},\textit{Abl} double mutants, there were severe disruptions in all three FasII-positive fascicles on either side of the midline (Figure 2.10I, K, and L). In \textit{trio},\textit{Abl} double mutants, three distinct fascicles were often not distinguishable, as pathways appeared to fuse or collapse onto each other; additionally, in some hemisegments, only one fascicle appeared to extend between segments (Figure 2.10L). Despite these severe defects, motoneurons (which are also recognized by mAb 1D4) still exit the CNS in \textit{trio},\textit{Abl} mutants (not shown). Taken together, our data indicate that both \textit{Abl} and \textit{trio} function during axon guidance and/or outgrowth in both longitudinal and commissural pathways in the \textit{Drosophila} embryonic CNS.

2.4. DISCUSSION

In this Chapter, I have reviewed the identification of \textit{Drosophila trio} as a genetic enhancer of \textit{Abl} mutant phenotypes. \textit{trio} interacts in a dosage-sensitive, reciprocal manner with \textit{Abl}, enhancing both semilethality and axon pathfinding phenotypes. Furthermore, \textit{trio} also interacts genetically with the \textit{ena} and \textit{fax}, suggesting that these molecules may function in common signaling networks during axon outgrowth and other developmental processes. The original HDA mutation, \textit{M89}, was verified to be an allele of \textit{trio} since (a) sequencing of the \textit{trio} gene on the \textit{M89} mutant chromosome revealed a C to T transition resulting in a missense mutation (L1412F) in the first DH domain, (b) the
P[w+ ] insertion in trio\textsuperscript{P0368/10} maps to an intron in the trio gene and precise excision of this P-element reverts this trio allele to wild-type, and (c) CNS defects in trio, Abl double mutants are rescued by panneural expression of a full-length trio cDNA. The trio gene is a large, multi-exonic transcriptional unit spanning \~40 Kb. trio encodes a large, multi-domain protein of predicted \~257 kDa mass. Trio is highly similar to orthologs in human, mouse, and \textit{C. elegans}, encoding a conserved N-terminal domain, spectrin-like repeats, two DH/PH domains or GEFs, and an SH3 domain. The simultaneous demonstration by another group that DTrio, like its orthologs, regulates cytoskeletal reorganization in cultured cells and activation of Rac GTPases \textit{in vitro} suggests that the Abl tyrosine kinase may function with Trio in axons to regulate cytoskeletal dynamics with Rho-family GTPases (Newsome et al., 2000).

2.4.1. Trio’s orthologs in other systems regulate cell migration, axon guidance, and Rho GTPase-mediated cytoskeletal dynamics

Human Trio (so named because it encodes three catalytic domains) was first identified as a binding partner for the LAR (leukocyte antigen receptor) transmembrane protein tyrosine phosphatase in a yeast two hybrid assay (Debant et al., 1996). Trio is a member of the large family of Dbl homology (DH) or GEF (guanosine exchange factor) proteins that promote GDP:GTP exchange on the Rho-family of small GTPases (Rossman et al., 2005). In their GTP-bound form, GTPases are activated and interact with downstream effectors to regulate cytoskeletal dynamics, cell cycle progression, and transcription (Etienne-Manneville and Hall, 2002). hTrio’s N-terminal GEF domain (GEF1) specifically activates Rac1 GTPase \textit{in vitro}, and in cultured fibroblasts, promotes
lamellipodia extension and miniruffling, cell spreading, migration, and anchorage-independent growth (Bellanger et al., 1998; Debant et al., 1996; Seipel et al., 1999). hTrio’s C-terminal domain specifically activates RhoA in vitro, and promotes the formation of stress fibers in cells (Bellanger et al., 1998; Debant et al., 1996; Seipel et al., 1999). Subsequently, it was demonstrated that in vivo, Trio primarily activates RhoG, a GTPase that activates both Cdc42 and Rac1, since expression of dominant-negative RhoG as well as expression of a peptide that specifically inhibits RhoG’s interactions with effectors abolished Trio GEF1’s ability to promote miniruffling and cell spreading (Blangy et al., 2000). In human tissues, Trio is expressed widely, including in the brain (Debant et al., 1996). trio mutant mice die between E15.5 and birth, and display defects in skeletal muscle formation and cellular organization in the hippocampus and olfactory bulb (O'Brien et al., 2000). In cultured neurons, Trio functions during nerve growth factor (NGF)-induced neurite outgrowth, but Trio has not yet been linked to signaling by any growth cone receptor (Estrach et al., 2002). Trio interacts biochemically with focal adhesion kinase (FAK) and the actin-crosslinking protein filamin, in addition to LAR and the GTPases (Bellanger et al., 2000; Medley et al., 2003).

In *C. elegans*, the Trio ortholog UNC-73 functions during cell migrations, axon guidance, vulval morphogenesis, and phagocytosis (deBakker et al., 2004; Hedgecock et al., 1987; Kishore and Sundaram, 2002; Lundquist et al., 2001; Siddiqui, 1990; Spencer et al., 2001; Steven et al., 1998; Struckhoff and Lundquist, 2003; Wu et al., 2002 and references therein). UNC-73 activates the Rac-related GTPases CED-10/Rac-1 and MIG-2/RhoG in vitro, and genetic analyses indicate a role for these GTPases, as well as Rac-2/3, downstream of UNC-73 in vivo (Kishore and Sundaram, 2002; Lundquist et al.,
2001; Steven et al., 1998; Struckhoff and Lundquist, 2003; Wu et al., 2002) The signaling pathways in which UNC-73 functions have not been extensively characterized, but unc-73 interacts genetically with the Semaphorin receptor plexin-1 during vulval cell migration and male sensory ray positioning, and with unc-115/AbLIM (an actin-binding protein) and ced-5/DOCK180 (a Rac activator) during neuronal cell migration, P cell migration, and axon guidance (Dalpe et al., 2005; Dalpe et al., 2004; Struckhoff and Lundquist, 2003; Wu et al., 2002).

*Drosophila trio* was isolated simultaneously by three other groups in addition to the Liebl and Seeger laboratories (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). Awasaki et al. (2000) reported defects in trio mutants in longitudinal axon pathfinding in the CNS of embryos (similar to our observations) and larvae, stalling of embryonic ISNb motoneurons, and malformations of the mushroom body and defects in neurite outgrowth within this structure in the larval brain. Consistent with these observations, a Trio mAb immunolabeled axons in the embryonic and larval CNS (Awasaki et al., 2000). Bateman et al. (2000) observed similar defects in embryonic CNS and motoneuron pathfinding, and also found that trio interacted genetically with Dlar, enhancing the ISNb “bypass” phenotype observed in Dlar mutants (Bateman et al., 2000). Additionally, these investigators noted that the gaps in the longitudinal CNS fascicles in trio mutants resembled similar disruptions caused by expression of DN Rac1, and that reducing the gene dose of trio genetically suppressed disruption in the adult retina caused by overexpression of wild-type Rac1 (Bateman et al., 2000). Newsome et al. (2000) found that mutations in trio affected photoreceptor axon pathfinding in larval and adult brains, and that trio interacted genetically with dreadlocks (dock)/Nck1, an
SH2-SH3 adaptor protein, and Pak (p21-activated kinase), a Rac effector. These authors found that DTrio’s N-terminal GEF domain activated Rac1, Rac2, and Mtl (mig-two like, named after its most similar ortholog in C. elegans) in vitro, and expression of GEF1 in rat embryonic fibroblasts (REF-52 cells) promoted actin polymerization, miniruffling, and lamellipodial formation (Newsome et al., 2000). Trio GEF2 did not activate RhoA or RhoL in vitro, however, expression of GEF2 in REF-52 cells did promote the formation of F-actin stress fibers (Newsome et al., 2000). In a later publication, investigators in the Dickson and Luo labs reported that mutations in Rac1 and Rac2, but not Mtl, suppressed defects in photoreceptor axon guidance caused by overexpression of TrioGEF1, suggesting that Rac1 and Rac2 are TrioGEF1’s preferred substrates in vivo (Hakeda-Suzuki et al., 2002).

2.4.2. GTPases, trio, and Abl function during axon guidance in the Drosophila embryonic CNS

Taken together, our data along with the observations of others in Drosophila, C. elegans, and mammalian systems strongly support a role for Drosophila trio in the regulation of Rho GTPases during axon outgrowth and pathfinding. GTPases have been extensively implicated in axon outgrowth and guidance (Dickson, 2001; Govek et al., 2005; Luo, 2000). Although there are an increasing number of exceptions (see Chapter 4 Introduction), in general Cdc42 and Rac-related GTPases promote axon outgrowth and function during attractive growth cone signaling, while Rho-related GTPases promote axon retraction and/or growth cone collapse, and function during repulsive growth cone signaling (Govek et al., 2005; Guan and Rao, 2003; Huber et al., 2003). The GTPases
Cdc42, Rac1, and RhoA have been implicated in axon pathfinding at the *Drosophila* embryonic CNS midline, as neuronal expression of CA and/or DN GTPases, and loss-of-function mutations in *Rac1, Rac2*, and *Mtl*, lead to disruptions in axon guidance at this important choice point (Fan et al., 2003; Fritz and VanBerkum, 2002; Hakeda-Suzuki et al., 2002; Hu et al., 2005; Matsuura et al., 2004). In addition, many of the GTPases’ downstream effectors, including *Myosin Light Chain Kinase, spaghetti squash* (the myosin light chain gene), *Pak, dock, SCAR, Wasp*, and *Arp2/3* complex components, have been implicated in axon guidance and/or outgrowth at the midline choice point (Fan et al., 2003; Kim et al., 2002; Zallen et al., 2002). Upstream of the GTPases, GEF64C, a RhoGEF, promotes axon crossing at the midline, while CrossGAP (CrGAP), a putative RacGAP, may function with Robo to regulate repulsion (although inappropriate axon crossing at the midline is observed whether CrGAP levels are increased or decreased) (Bashaw et al., 2001; Hu et al., 2005). The fact that *trio* contains both a Rac-GEF and a putative Rho-GEF domain is intriguing, as it suggests that Trio may be integrated into signaling pathways that both promote axon extension/attraction as well as axon retraction/repulsion. Because *trio* interacts genetically with *Abl*, and *Abl* has been implicated in repulsive Robo signaling (Hsouna et al., 2003; Wills et al., 2002), it is tempting to speculate that *trio* might also function during repulsion. However, *trio,Abl* double mutants display a dramatic loss of commissural axons, suggesting a role in growth cone attraction, and furthermore, in these mutants, few axons cross the midline inappropriately, as would be expected if loss of *trio* compromised repulsive signaling (see Figure 2.10, and data not shown). The clarification of Trio’s role with respect to guidance receptor signaling remains a major current goal (see Chapters 3 and 4).
The reciprocal, dosage-sensitive genetic interactions between \textit{Abl} and \textit{trio}, and the synergistic CNS phenotypes in \textit{trio,Abl} double mutants suggest that these two molecules might function together to regulate cytoskeletal dynamics. One possibility is that Abl might regulate Trio by tyrosine phosphorylation. hTrio is tyrosine phosphorylated by FAK, and 19 tyrosine residues are conserved in UNC-73, DTrio, and hTrio, supporting the idea that hTrio orthologs might be regulated by tyrosine phosphorylation (DJF, data not shown, and Medley et al., 2003). Tyrosine phosphorylation of GEFs generally regulates their activity. For example, c-Abl regulates the phosphorylation and Rac-GEF activity of hSos1, while VavGEF activity is increased upon phosphorylation by Hck, Lck, Fyn, and Syk kinases (Tani et al., 2003; Turner and Billadeau, 2002). In neurons, EphA4-mediated tyrosine phosphorylation of Ephexin1 specifically enhances its RhoA-GEF activity, and Ephexin1 phosphorylation is required for growth cone collapse (Sahin et al., 2005). Of course, Abl and Trio might function together in some other manner. In \textit{Drosophila}, alleles of \textit{Son-of-sevenless (Sos)}, a Ras-GEF, were originally isolated as dosage-sensitive suppressors of the \textit{sevenless (sev)} receptor protein tyrosine kinase (Rogge et al., 1991; Simon et al., 1991). Although Sos is not a Sev substrate, it is recruited and indirectly activated during Sev signaling (Raabe, 2000). Abl and Trio may also function in parallel pathways, exclusively of or in addition to a cooperative biochemical interaction. As both \textit{Abl} and \textit{trio} mRNAs are maternally contributed, interpretation of the loss-of-function genetic interactions presented here is difficult (Bennett and Hoffmann, 1992; Liebl et al., 2000; Wadsworth et al., 1985). Ultimately, biochemical and cell biological assays will be required to address this issue (see Chapter 3).
As with genetic interactions between *Abl* and *trio*, the significance of the genetic interaction between *ena* and *trio* is also unclear, although the fact that *ena* suppresses semilethality in *trio* mutants hints that their biochemical relationship may be antagonistic. Ena encodes an ortholog of the Ena/VASP family of proteins that regulate F-actin capping, efficient lamellipodial extension, and filopodial extension in mammalian cells (Bear et al., 2000; Bear et al., 2002; Krause et al., 2003; Lebrand et al., 2004). Interestingly, Mena may function as part of a signaling complex formed during Cdc42-induced filopodia formation (Krugmann et al., 2001). Cdc42 has been observed to inhibit the formation of stress fibers (Kozma et al., 1995), while Rho inhibition leads to more rapid formation of Cdc42-induced filopodia (Nobes and Hall, 1995), suggesting that Cdc42 and Rho GTPases may antagonize each others’ function (Giniger, 2002; Lim et al., 1996; Nobes and Hall, 1995). This concept has not been explored in *Drosophila*, however, and thus the basis for *trio*’s interaction with *ena* awaits further characterization of these molecules’ roles in cytoskeletal dynamics during axon guidance. Similarly, the role of *fax* with respect to growth cone dynamics is unknown, therefore the significance of *fax*’s genetic interactions with *Abl* and *trio* awaits analysis.

The *Drosophila* lar receptor, which interacts with both *Abl* and *trio* during motor axon outgrowth, also regulates synapse morphogenesis at the neuromuscular junction (Bateman et al., 2000; Kaufmann et al., 2002; Wills et al., 1999a). Interestingly, both c-Abl and Arg are enriched in axonal pre-synaptic terminals and post-synaptic dendritic spines in the mouse hippocampus (Moresco et al., 2003); it will be interesting to determine whether *Abl* or *trio* functions during synapse formation or maintenance in *Drosophila*. As mentioned in the Introduction to this Chapter, *Abl* regulates cytoskeletal
dynamics in a number of tissues undergoing cell shape changes and/or migrations. Since both \textit{Abl} and \textit{trio} are widely expressed, \textit{trio} may function with \textit{Abl} outside the CNS, for example during cellularization or in epithelial tissues. For example, Dr. Liebl noted that mutations in \textit{Abl} enhanced the blistered wing phenotype in \textit{trio} hypomorphs, potentially linking Trio and Abl to regulation of actin dynamics by Rho-family GTPases during wing morphogenesis (Eaton et al., 1995; Eaton et al., 1996). Unlike \textit{Abl}, though, removing both maternal and zygotic \textit{trio} does not seem to affect dorsal closure, despite \textit{trio} expression in the leading edge cells and the requirement for GTPases during this process; perhaps \textit{trio} plays a minor or redundant role (Hakeda-Suzuki et al., 2002; Jacinto et al., 2002). Bateman et al. (2000) noted that reducing the dose of \textit{trio} in the eye modified a rough-eye phenotype caused by \textit{Rac} overexpression, suggesting a role for \textit{trio} in this tissue as well. \textit{trio}’s functions outside of the CNS has enabled additional strategies for dissecting the function of this important molecule (see Chapter 4).

In summary, the \textit{Drosophila trio} GEF has been isolated as a genetic enhancer of the \textit{Abl} semilethality phenotype. Efforts to understand the mechanisms by which Trio regulates cytoskeletal dynamics and axon guidance in \textit{Drosophila} are ongoing.
Figure 2.1. Protein Tyrosine Kinases and Phosphatases. Tyrosine kinases catalyze the transfer of a phosphate group from adenosine triphosphate (“ATP”) to a tyrosine residue on the target protein (“substrate”). Tyrosine phosphatases can reverse this reaction by catalyzing the release of the phosphate, reverting the substrate to its unphosphorylated form.
Figure 2.2. Structure of normal and mutant Abl and Abl-related gene (Arg) kinases. The Abl protein in mammals, Drosophila, and C. elegans consists of the tyrosine kinase domain, preceded N-terminally by an SH2 (Src homology 2, phosphotyrosine-binding) domain, an SH3 (proline-binding) domain, and a filamentous (F) actin-binding domain at the C-terminus. Consensus SH3 binding sites (PxxPxK/R and K/RxxPxxP) are encoded by Abl and arg genes, but their position and number vary by organism; additional PxxP motifs are found in all Abl orthologs/homologs (not shown). In human and mouse, alternative splicing leads to the inclusion of one of two N-terminal exons, one of which encodes a myristoylation sequence. In Drosophila and C. elegans, there is no evidence of alternative splicing of the N-terminus, but a myristoylation sequence is normally encoded by the Abl ORF. Vertebrate Abl and Arg encode nuclear localization and export sequences and DNA binding domains, while D-Abl encodes two Ena/VASP homology (EVH1) binding domains that are not contained in orthologs. Adapted from (Hernandez et al., 2004a; Woodring et al., 2003).
<table>
<thead>
<tr>
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<th></th>
<th></th>
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<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
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<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>trio&lt;sup&gt;P0368/10&lt;/sup&gt; / trio&lt;sup&gt;M89&lt;/sup&gt;, Abt&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>0.5</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2.1.** “M89” is a dosage-sensitive modifier of the Abt mutant phenotype and encodes a mutation in trio. The “M89” allele dominantly enhances the semilethality phenotype in Abt mutants, similar to previously identified enhancers, fax and nrt. In another test during the mapping of “M89,” Df(3L)Fpa<i>i</i> failed to complement “M89,” in contrast to an adjacent deficiency (not shown, see Results). Similarly, trio<sup>P0368/10</sup> failed to complement the trio<sup>M89</sup>, Abt<sup>i</sup> chromosome, but did complement the parental Abt<sup>i</sup> chromosome (not shown, see Results). Taken together, these data indicate “M89” is an allele of trio.

* “Wild-type dose” refers to the number of intact, wild-type alleles of each gene shown.

** The percent of the expected progeny class of both pupae and viable adults of the indicated genotype is shown. A minimum of 1,300 animals was counted in each cross, and values are normalized to an appropriate control cross.

<sup>a</sup> The trio<sup>P0368/10</sup> allele is indicated as 0.5 of a wild-type gene dose to reflect its hypomorphic character.
Figure 2.3. Alignment of human and *Drosophila* Trio proteins. Predicted 2261 amino acid sequence of *Drosophila* Trio (Genbank Accession AF216663) aligned with the 2861 amino acid human Trio protein (Genbank Accession U42390). Residue numbers are indicated at right. Identical residues are highlighted in reverse text. The conserved N-terminal domains are indicated below the sequence by plus signs. The region of spectrin repeats is indicated by solid underlining. DH domains are indicated by single dashed underlining, while PH domains are indicated by double dashed underlining. The SH3 domain is indicated by asterisks.
Figure 2.4. Domain arrangement of *Drosophila* Trio and homology to orthologs *hTrio* and *C. elegans* UNC-73A. Spatial arrangement of domains in the three proteins is shown. Percent amino acid identity is indicated by numbers placed in “flags” between domains. These values were generated by alignments with Clustal-X.
Figure 2.5. Northern blot of total RNA reveals two *Drosophila trio* transcripts. ~30 µg of total RNA from a 0-24h embryo collection was electrophoresed, transferred to nitrocellulose, and incubated with either a full-length *trio* radiolabeled DNA probe (A) or a probe synthesized from only the *trio* 3’ UTR (B). Lane (1), RNA from wild-type embryos; lane (2), RNA was obtained from embryos that were the progeny of heterozygous *trio*P0368/10 parents; lane (3), RNA from embryos that were the progeny of *trio*M89 heterozygous parents. The major *trio* transcript (~8.0 Kb) and the minor transcript (~8.9-9.2 Kb) are indicated on each blot. Aberrantly-sized transcripts were not detected in total RNA from *trio* mutants (lanes 2 and 3) using either the full-length probe or the smaller 3’ UTR probe. In (A), a loading control probe (RP49) runs at ~300 bp, near the bottom of the gel.
Figure 2.6. Trio genomic organization. trio spans ~40 Kb of genomic DNA. The sixteen coding exons are shown as shaded boxes, while 5’ and 3’ UTR sequences are indicated with open boxes; the trio transcription start site has not been determined. Introns are indicated by lines drawn between exons. AUG and UGA indicate start and stop codons, respectively. Above the exon-intron cartoon, a restriction map indicating positions of EcoRI (E) and HindIII (H) sites that were initially determined by Southern mapping and later verified by analysis of sequence from the Drosophila genome project. Below the exon-intron cartoon, the domains encoded by the exons are listed. The open reading frame is organized as follows: exon 1, amino acids M1-S60; exon 2, D61-Q241; exon 3, A242-Q943; exon 4, I944-Q1570; exon 5, I1571-K1638; exon 6, L1639-S1737; exon 7, N1738-G1756; exon 8, R1757-R1787; exon 9, T1758-M1873; exon 10, A1874-T1895; exon 11, E1896-E1927; exon 12, S1928-G1966; exon 13, Y1967-D2005; exon 14, F2006-D2068; exon 15, L2069-Q2195; exon 16, L2196-K2263. All introns are shorter than 100 base pairs, except between exons 1 and 2, 2 and 3, 5 and 6, 7 and 8, and 9 and 10. P[w²] (~11Kb total length, not drawn to scale) in line 0368/10 is inserted into intron 7, shown above the restriction map. The position of the C to T transition in trioₚ₧ (which produces a missense mutation, L1412F) is indicated with an asterisk in coding exon 4.
Figure 2.7. Alignment of DH domains from Trio orthologs and other DH domain-containing proteins. L1412 (indicated in red, which is mutated to phenylalanine in the trio<sup>M89</sup> allele) is highly conserved in DH domains from <i>C. elegans</i> to human. In this alignment, only DH2 of <i>Drosophila melanogaster</i> (Dm) and <i>Drosophila pseudoobscura</i> (Dp) contain isoleucine instead of leucine at this position, a highly similar residue. F1331 (in orange), whose packing is predicted to be affected by the L1412F mutation, is invariant in these and other DH domains (Soisson et al., 1998). “AgTrio” is the mosquito <i>Anopheles gambiae</i> Trio ortholog. UNC-73 is the UNC-73A isoform from <i>C. elegans</i>. “Mm Trio” is the <i>Mus musculus</i> Trio ortholog and “GgTrio” is the <i>Gallus gallus</i> Trio ortholog, which were identified in database searches based on homology to hTrio. In “DpTrioDH2” two stop codons were found in the genome sequence (probably genome sequencing errors); these were converted to “X” before alignment. In Trio orthologs “DH1” is the N-terminal DH domain, while “DH2” is the C-terminal DH domain. Residue numbering is based on isolated DH domain sequence, not DH domains in the context of their full-length protein.

Continued on next page
Figure 2.7. Continued.

L1412 (F1412 in trio^{M89})
Figure 2.8. Ribbon drawing of human Trio GEF1. Arrows identify hTrio L1363 (analagous to L1412 in DTrio, which is mutated to phenylalanine in trio<sup>M89</sup>), and hTrio F1282 (analagous to F1331 in DTrio). These residues’ side chains are positioned internally in a hydrophobic pocket between alpha helices in the DH domain, colored in green, orange, red, and blue. The DH domain of GEF1 is on top, composed of alpha helices, while the PH domain is below, composed primarily of β-sheets.
<table>
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<th>trio mRNA+lacZ</th>
<th>lacZ only</th>
<th>neither</th>
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<td>$trio^{IMP159.4}$ (126)</td>
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<td>73</td>
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<td>23</td>
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</table>

Table 2.2. **An antisense 3’ UTR trio riboprobe does not detect trio expression in homozygous trio$^{P0368/10}$ embryos.** Embryos were collected for 24 hours from adults carrying the indicated chromosome over a balancer chromosome that expresses β-galactosidase in embryos (TM3[Ubx::lacZ]). After in situ hybridization with a riboprobe recognizing only the trio 3’ UTR, embryos were then stained with an anti-β-galactosidase antibody, allowing embryos homozygous for the indicated trio allele (approximately 25%) to be distinguished from the other classes of progeny (either heterozygous or homozygous for the balancer). Embryos between stages 8 and 14, when trio is most widely expressed (see Figure 2.9), were scored for trio and β-galactosidase expression. $Df(3L)Fpal$ completely removes the trio gene; 22% of embryos (expected 25%) in this class do not express lacZ, nor is trio mRNA detected. Similarly, trio expression was not detected in ~25% of embryos homozygous for the hypomorphic trio$^{P0368/10}$ allele, or for an imprecise excision line, trio$^{IMP159.4}$, that was generated by mobilizing the P element in trio$^{P0368/10}$. In contrast, embryos homozygous for the precise excision allele trio$^{PRECI59.2}$ did not express β-galactosidase, but did express trio. Perplexingly, embryos homozygous for the precise excision allele trio$^{PRECI59.1}$ did not express trio, or at least the 3’ UTR. Because precise and imprecise excisions were isolated in the Liebl laboratory based on their ability or failure to complement the trio$^{M89,Abl^d}$ chromosome (not shown), this result suggests that a truncated or less stable trio message might still be sufficient to rescue the trio semilethality phenotype. Molecular characterization of the lesion(s) caused by excision of P[w$^+$] in these lines will be necessary before the basis for these observations can be understood.
Figure 2.9. Expression of trio mRNA in Drosophila embryos. (A) An early cleavage stage embryo (stage 2) showing maternal loading of trio mRNA. (B) During germ band extension (stage 10), the beginning of segmentally repeated neuroblast staining is evident (arrowheads); mesodermal expression is still detectable at this stage. (C and D). Strong CNS expression is observed through germ band retraction. (C) is a stage 12 embryo, while (D) is stage 14. (E and F). Segmentally repeated expression of trio in the lateral epidermis, corresponding to muscle attachment sites (arrowheads), is evident at stage 13. (G) Dorsal view showing trio expression in leading edge cells during dorsal closure (arrowheads). (H) Stage 16 embryo. trio is expressed in cells surrounding the gut.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>trio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Abl</th>
<th>fax</th>
<th>dab</th>
<th>ena</th>
<th>pupae**</th>
<th>adults**</th>
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**Table 2.3. Dosage-sensitive modification of the \(Abl\) mutant phenotype.**
* “Wild-type dose” refers to the number of intact, wild-type alleles of each gene shown.
** The percent of the expected progeny class of both pupae and viable adults of the indicated genotype is shown. A minimum of 1,300 animals was counted in each cross, and values are normalized to an appropriate control cross.
<table>
<thead>
<tr>
<th>Genotype</th>
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<th>adults**</th>
</tr>
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<td>0.5</td>
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Table 2.4. Dosage-sensitive modification of the trio mutant phenotype.
* “Wild-type dose” refers to the number of intact, wild-type alleles of each gene shown.
** The percent of the expected progeny class of both pupae and viable adults of the indicated genotype is shown. A minimum of 1,300 animals was counted in each cross, and values are normalized to an appropriate control cross.
<sup>a</sup> The trio<sup>P0368/10</sup> allele is indicated as 0.5 of a wild-type gene dose to reflect its hypomorphic character.
<table>
<thead>
<tr>
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<th>ena</th>
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<th>adults**</th>
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Table 2.5. *ena* modifies the enhancement of the *Abl* phenotype by *trio*, and suppresses the *trio* semilethality phenotype.

*“Wild-type dose” refers to the number of intact, wild-type alleles of each gene shown.

**The percent of the expected progeny class of both pupae and viable adults of the indicated genotype is shown. A minimum of 1,300 animals was counted in each cross, and values are normalized to an appropriate control cross.
Figure 2.10. Mutations in Abl and trio affect embryonic CNS development.
Embryos of different genotypes were stained with either mAb BP102 to visualize the ladder-like organization of all CNS axons (A-H), or with mAb 1D4 to visualize a subset of axons that project in three longitudinal fascicles on either side of the midline (I-P). In trio, Abl mutant embryos, both commissural bundles (C, D, and G) are often disrupted (arrowheads in C and D). Expression of a full-length trio cDNA using the UAS-GAL4 binary expression system rescues commissure defects in trio, Abl double mutants (H). Similarly, trio and Abl mutants often display defects in the organization of longitudinal pathways (J-P), especially gaps in the lateral-most fascicle. Arrowheads in (C) and (D) indicate examples of segments scored with disrupted commissures, while arrowheads in (J), (K), and (M) through (P) indicate segments scored as having disruptions in FasII-positive fascicles.

Continued on next page
Figure 2.10. Continued.
CHAPTER 3

THE ABELSON TYROSINE KINASE, THE TRIO GEF, AND ENABLED INTERACT WITH THE NETRIN RECEPTOR FRAZZLED IN DROSOPHILA

3.1 INTRODUCTION

The signaling molecules Abelson tyrosine kinase (Abl), the guanine nucleotide exchange factor Trio, and the Abl substrate Enabled (Ena) all regulate axon pathfinding at the Drosophila embryonic CNS midline (see Chapters 1 and 2). In this Chapter, I describe the detection of genetic and/or physical interactions between the attractive Netrin receptor Frazzled (Fra) and these effector molecules that suggest that they act in concert to guide axons across the midline. Mutations in Abl and trio dominantly enhance fra and Netrin mutant CNS phenotypes, and fra;Abl and fra;trio double mutants display a dramatic loss of axons in a majority of commissures. Conversely, heterozygosity for ena reduces the severity of the CNS phenotype in fra, Netrin, and trio,Abl mutants. Consistent with an in vivo role for these molecules as effectors of Fra signaling, heterozygosity for Abl, trio, or ena reduces the number of axons which inappropriately cross the midline in embryos expressing the chimeric Robo-Fra receptor. Fra interacts physically with Abl and Trio in GST pulldown assays and co-immunoprecipitation
experiments. In addition, tyrosine phosphorylation of Trio and Fra is elevated in S2 cells when Abl levels are increased. Together, these data suggest that Abl, Trio, Ena and Fra are integrated into a complex signaling network that regulates axon guidance at the CNS midline.

3.1.1. *Frazzled and its homologs regulate axon attraction*

Extracellular guidance cues, their receptors on the growth cone surface, and intracellular effectors function together to regulate directional axon extension (Dent and Gertler, 2003; Guan and Rao, 2003; Huber et al., 2003; Lee and Van Vactor, 2003; Luo, 2002). Genetic screens for mutants defective in axon pathfinding at the midline in the *Drosophila* embryo have identified many of these evolutionarily conserved molecules, and suggest that growth cones respond to a balance of extracellular matrix chemoattractants (e.g., Netrins) and chemorepellents (e.g., Slit) expressed by midline cells (Araujo and Tear, 2003). *frazzled (fra)* encodes an attractive Netrin receptor related to mammalian Deleted in Colorectal Cancer (DCC) and *C. elegans* UNC-40 (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). Mutations that remove both of the closely related *Netrin* genes or mutations in *fra* result in thin or missing commissural axon bundles, reflecting a decrease in growth cone attraction to the central nervous system (CNS) midline (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Conversely, members of the Roundabout (Robo) family of Slit receptors mediate growth cone repulsion, and mutations in *slit* or in *robo* receptors cause CNS axons to cross the
Netrins and their receptors also mediate growth cone guidance at the midline in other model systems. The first Netrin homolog to be discovered was the *C. elegans* gene *unc-6*, and was identified in screens for worms with uncoordinated movement (Hedgecock et al., 1990; Hedgecock et al., 1987; Ishii et al., 1992; Wadsworth et al., 1996 and references therein). UNC-6 is expressed by ventral neurons and epidermal cells, and guides the ventral circumferential projections of lateral neurons by attraction through the UNC-40 receptor, which is required cell autonomously in neurons (Chan et al., 1996; Hedgecock et al., 1990; Ishii et al., 1992; Wadsworth et al., 1996). Conversely, ventral axons are repelled by UNC-6 and extend dorsally, because they express the Netrin co-receptor UNC-5 that functions with UNC-40 to mediate growth cone migration away from UNC-6 (Hamelin et al., 1993; Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992).

Two vertebrate UNC-6 homologs (Netrin-1 and Netrin-2, named after the Sanskrit word meaning “to guide”) were first isolated biochemically from chick brain as factors that promoted the outgrowth of commissural axons from rat dorsal spinal cord explants (Serafini et al., 1994). Subsequently, the genes encoding these proteins were cloned, and it was demonstrated that COS cells transfected with Netrin expression vectors secreted these proteins and attracted cultured dorsal commissural axons at a distance (Kennedy et al., 1994). Consistent with these observations in culture, in the spinal cord of mouse, chick, and *Xenopus*, Netrin-1 is expressed in ventral floor plate
cells, while in mouse and chick Netrin-2 is expressed in the ventral two thirds of the
total cord (Kennedy et al., 1994; MacLennan et al., 1997; Serafini et al., 1996). In
Netrin-1 knockout mice, dorsal commissural axons are no longer attracted to the floor
plate, and commissural tracts are absent in the spinal cord and several regions of the
forebrain (Serafini et al., 1996). Like their invertebrate homologs, vertebrate Netrins are
laminin B2-related proteins with three epidermal growth factor repeats and a positively-
charged C-terminal domain (Harris et al., 1996; Ishii et al., 1992; Serafini et al., 1994).

The Netrin receptor DCC, the vertebrate homolog of frazzled and unc-40, was
originally identified as a candidate tumor suppressor gene that was deleted in colorectal
cancer (Fearon et al., 1990). Doubts about DCC’s role in cancer arose, however, as DCC
heterozygous mice did not display a predisposition to cancer, and other tumor
suppressors in the same chromosomal region as DCC (18q21) were found (Arakawa,
2004; Fazeli et al., 1997; Mehlen and Fearon, 2004). Recently, though, interest in the role
of the Netrins and their receptors DCC and UNC5H in cancer has been revived, as a
number of studies have reported reduced expression or mutation of the Netrin and
UNC5H genes, in addition to DCC, in a number of cancers (Arakawa, 2004). Transgenic
overexpression of Netrin in the mouse colon results in decreased apoptosis in intestinal
epithelial cells, and increased rates of hyperplasia and adenoma (Mazelin et al., 2004).
Additionally, DCC and UNC5H have been shown to function as dependence receptors,
promoting apoptosis in the absence of ligand (Llambi et al., 2001; Mehlen and Fearon,
2004; Meheen et al., 1998).

DCC, like fra and unc-40, encodes a type I, transmembrane immunoglobulin (Ig)
family member with 4 Ig C2 type domains and 6 fibronectin type III repeats in its
extracellular domain (Culotti and Merz, 1998). In the cytoplasmic domain, there are 3 short regions of conservation (the “P” domains) between fra, DCC, and a related vertebrate molecule, neogenin, although in unc-40, the amino acid conservation in these regions is low (DJF, not shown) and (Kolodziej et al., 1996). DCC is expressed in commissural axons and growth cones, and in DCC knockout mice (as in Netrin-1 knockouts), commissural axon tracts are disrupted in the spinal cord and brain (Fazeli et al., 1997; Keino-Masu et al., 1996). DCC-expressing cells bind Netrin, and DCC also binds Netrin in vitro (Keino-Masu et al., 1996; Stein et al., 2001). Pre-treatment of spinal cord explants with an anti-DCC antibody blocks commissural axon outgrowth in response to Netrin-1 (Keino-Masu et al., 1996). In addition to promoting outgrowth, Netrins also induce growth cone turning in cultured Xenopus retinal ganglion cell and spinal axons, a response that is also blocked by anti-DCC antibody (de la Torre et al., 1997; Ming et al., 1997). Taken together, data from invertebrate and vertebrate organisms support the idea that Netrins/UNC-6 and DCC/Fra/UNC-40 function together as a conserved ligand-receptor system, regulating chemoattraction by DCC-/Fra-/UNC-40-expressing axons towards cells that express the Netrins/UNC-6.

3.1.2. **DCC and UNC-40 signaling regulates F-actin dynamics and tyrosine phosphorylation**

An emerging theme in axon guidance is that growth cone receptors recruit cytoplasmic effectors to modulate reorganization of the actin cytoskeleton (Huber et al., 2003; Patel and Van Vactor, 2002). Recently, a number of studies have begun to illuminate how Frazzled’s homologs DCC and UNC-40 signal to the cytoskeleton during
axon guidance and outgrowth. A screen in *C. elegans* for genetic suppressors of an UNC-40 gain-of-function phenotype identified molecules which may function with UNC-40 and Netrin/UNC-6 to regulate actin dynamics (Gitai et al., 2003). These include the actin-binding protein AbLIM/UNC-115, Enabled (Ena)/UNC-34, and the Rho-family guanosine triphosphatase (GTPase) Rac/CED-10. AbLIM/UNC-115 behaves genetically as an effector of signaling by the Rac-2 GTPase (Struckhoff and Lundquist, 2003). Ena/UNC-34’s vertebrate orthologs Mena, vasodilator-stimulated protein (VASP), and Ena/VASP-like (EVL) antagonize F-actin capping and allow F-actin filament elongation (Bear et al., 2002; Gitai et al., 2003). Netrin stimulation of cultured mouse neurons results in Ena/VASP-dependent filopodia formation and Mena phosphorylation at a protein kinase A regulatory site (Lebrand et al., 2004). In cultured vertebrate cells, the adaptor Nck1 and the GTPases Cdc42 and Rac1 affect Netrin- and DCC-dependent neurite outgrowth, growth cone expansion, cell spreading, substrate adhesion, and filopodia extension (Li et al., 2002a; Li et al., 2002b; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Pharmacological inhibition of Cdc42, Rac, and Rho GTPases and the Rho effector Rock affects axon outgrowth and orientation towards Netrin1 in explanted precerebellar neurons (Causeret et al., 2004). Nck1 binds DCC in vitro, and can regulate actin nucleation in concert with Rac through WAVE1, an Arp2/3 complex activator; however, it is not known if the WAVE complex is activated in response to Netrin-DCC signaling (Eden et al., 2002; Li et al., 2002a). Similarly, although Netrin stimulation of DCC-expressing non-neuronal cells leads to activation of Cdc42 and Rac1, the mechanisms by which DCC regulates small GTPase activity have not been elucidated (Li et al., 2002b; Shekarabi and Kennedy, 2002). In cultured commissural neurons,
Netrin stimulation activates Cdc42, Rac1, and the Rac effector Pak1, and promotes the formation of a protein complex that includes Cdc42, Rac1, Pak1, DCC, and N-WASP; but again, the mechanism of DCC-mediated GTPase activation is unknown (Shekarabi et al., 2005).

Other pathways from DCC to the F-actin cytoskeleton likely involve cytoplasmic tyrosine kinases. DCC interacts with focal adhesion kinase (FAK), Src, and Fyn, and DCC is tyrosine phosphorylated in cells expressing increased levels of these kinases or upon Netrin stimulation; furthermore, phosphorylation of DCC is required for attractive axon turning in cultured neurons and Rac1 activation in non-neuronal cells (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Tyrosine phosphorylation of UNC-40 has also been observed, and genetic interactions indicate that UNC-40 signaling is regulated by the receptor protein tyrosine phosphatase (RPTP) CLR-1 (Chang et al., 2004; Tong et al., 2001).

### 3.1.3. Drosophila Fra signaling is poorly understood

In *Drosophila*, by contrast, much less is known about signaling by Fra and the Netrins. Genetic interactions with *fra* suggest that *GEF64C, wenerig, ARF6-GEF/Schizo, Myosin Light Chain Kinase*, and the G-protein *Gαq* promote commissure formation (Bashaw et al., 2001; Hummel et al., 1999a; Hummel et al., 1999b; Kim et al., 2002; Onel et al., 2004; Ratnaparkhi et al., 2002). However, none of the molecules encoded by these genes nor any others have been linked biochemically to Fra signaling.

The *Drosophila* Abelson cytoplasmic tyrosine kinase (Abl), the Trio Rac/Rho guanosine exchange factor (GEF), and Ena are expressed in the nervous system and
interact genetically and/or biochemically with receptors known to regulate nervous system development (Chapters 1 and 2) and (Awasaki et al., 2000; Bashaw et al., 2000; Bateman et al., 2000; Crowner et al., 2003; Gertler et al., 1989; Gertler et al., 1995; Liebl et al., 2003; Wills et al., 1999a). These molecules and their homologs in other organisms regulate cytoskeletal dynamics during diverse developmental processes (Bateman and Van Vactor, 2001; Hakeda-Suzuki et al., 2002; Kwiatkowski et al., 2003; Lanier and Gertler, 2000; Moresco and Koleske, 2003; Van Etten, 1999; Woodring et al., 2003). In cultured cells, these molecules regulate cell migration, neurite extension, and leading edge actin dynamics (Bateman and Van Vactor, 2001; Estrach et al., 2002; Kwiatkowski et al., 2003; Moresco and Koleske, 2003).

In this study, we expand the understanding of signaling networks in which Abl, Trio, and Ena function by uncovering genetic and biochemical interactions between these molecules and the Netrin receptor Fra. Our results indicate that Abl, Trio, and Ena likely function as effectors of Fra signaling in commissural axons, in addition to roles downstream of other growth cone receptors. Furthermore, our observations suggest potential mechanisms by which Fra and other receptors might coordinate actin cytoskeletal dynamics through these molecules.

[Many of the results presented here were published in (Forsthoefel et al., 2005).]
3.2. MATERIALS AND METHODS

3.2.1. Genetics and Immunohistochemistry

A complete list of alleles, transgenic chromosomes, and driver lines with references can be found in Table 3.1. The following alleles/chromosomes were used:

fra\(^4\); Df(2R)vg135, nomp\(^A\)\(^{vg135}\) (Df(2R)vg135 is a chromosomal deficiency that removes fra); Df(1)NP5 (removes NetA and NetB); ena\(^{GC10}\); ena\(^{GC5}\); ena\(^{210}\); ena\(^{23}\); Abl\(^1\); Abl\(^4\); Df(3L)st-j7 (removes Abl); trio\(^{M89}\); Df(3L)FpaI (removes trio); trio\(^{P0368/10}\); trio\(^{IMP159.4}\) (an imprecise excision allele generated by mobilizing the P-element in trio\(^{P0368/10}\)); trio\(^{M89}\), Abl\(^1\); Df(3L)FpaI, Abl\(^4\); trio\(^{IMP159.4}\), Abl\(^1\); elav\(^{C155}\)-GAL4; 1407-GAL4; UAS-fra-HA; UAS-fra\(^{ACYTO}\)-HA; UAS-fra-myc; and UAS-Robo-Fra-myc (kindly provided by Greg Bashaw). fra\(^4\), ena\(^{GC10}\) recombinant chromosomes were generated by meiotic recombination and isolated based on their failure to complement both ena\(^{GC8}\) and Df(2R)vg135.

All flies were maintained in standard cornmeal-yeast medium at room temperature. Embryos were fixed in 4% paraformaldehyde/1X PBS, and the CNS was visualized using mAb BP102 (1:20, Developmental Studies Hybridoma Bank, Univ. of Iowa), mAb 1D4/anti-Fasciclin II (1:10, Developmental Studies Hyrbridoma Bank, Univ. of Iowa), anti-β-galactosidase (1:500, Promega), goat anti-mouse-HRP (1:500, Jackson), and standard immunohistochemical procedures (Patel et al., 1987). All alleles were maintained over lacZ-expressing balancers to distinguish the genotype of embryos. Stage 14-16 embryos were filleted and scored at 400X magnification.
3.2.2. Constructs

pMET Abl-myc, pMET Trio-myc, pMET Trio^{ASPR}-myc, pMET Fra-myc, pMET Fra-HA, pMET Fra^{ACYTO}-HA (deleted for amino acids P1123-C1375 (GenBank Accession U71001)), pBSK Abl-myc, pBKS Trio-myc, pBKS Trio^{ASPR}-myc (deleted for aa’s L285-D1199 (GenBank Accession AF216663)), pBSK Ena-myc, pGEX2T-Fra^{ACYTO} (aa’s C1098-C1375 (GenBank Accession U71001)), pGEX2T-AblSH3 (aa's E202-K268 (GenBank Accession AH001049)), and pGEX2T-TrioSH3 (aa's E1177-L1840, deleted for GEF1 (A1281-P1596) (GenBank Accession AF216663)) were all constructed using standard molecular techniques; details are available upon request. pPAC Ena was provided by A. Comer (Comer et al., 1998). pMET Fra constructs were generated using the short isoform which rescues fra mutant phenotypes (Kolodziej et al., 1996). Myc and HA tags were added C-terminally. Poonam Bheda subcloned Fra-HA, Fra^{ACYTO}-HA, and Fra-myc constructs from pMET into pUAST (Brand and Perrimon, 1993), and generated transgenic Drosophila lines via germline transformation using standard techniques (Spradling, 1986).

3.2.3. Protein-Protein Interactions and Phosphorylation Assays

GST and GST-Fra_{cyto} were generated in E. coli (BL21) as described in Amersham Pharmacia's Gene Fusion System Guide. GST pulldowns of in vitro-translated proteins were performed essentially as described in (Bashaw et al., 2000), except that non-radiolabeled, epitope-tagged proteins were generated in vitro using Promega's TnT T7 Coupled rabbit reticulocyte lysate system and Abl, Trio, and Ena constructs cloned into pBluescript (Stratagene). 15-25 µl of each reaction was added to ~10 µg fusion protein
bound to beads suspended in 200 µl binding buffer. Binding was overnight, and after washing ~20% of total protein was separated by SDS-PAGE. For GST pulldowns from S2 cell extracts, 2x10⁷ S2 cells were transiently transfected with pMET Abl-myc, pMET Trio-myc, or pPAC Ena with CellFectin Reagent (Invitrogen). 24 hours after induction, cells were lysed in 1 ml IP buffer (Comer et al., 1998), and lysates were precleared with 100 µl of Glutathione Sepharose4B beads prior to GST pulldowns.

For coimmunoprecipitations, 2x10⁷ S2 cells were transiently transfected with the relevant constructs, and 24 hours after induction, cells were rinsed once in 1X PBS and lysed in 50 mM Tris pH 8, 100 mM NaCl, 1 mM MgCl₂, 1% NP-40, 10 mM NaF, 2 mM Na₃VO₄, and 5 µg/ml each Aprotinin and Leupeptin (Roche). Cell extracts were cleared by centrifugation, lysates were pre-cleared with 40 µl Protein G Sepharose beads (Sigma) for 30 minutes, and protein complexes were immunoprecipitated with 1 µg rabbit anti-myc (Santa Cruz), anti-Ena (5G2, Developmental Studies Hybridoma Bank (Bashaw et al., 2000)) diluted 1:20, or anti-HA (HA.11, Covance) diluted 1:150, for 60 minutes. Immune complexes were recovered on 40 µl Protein G beads for 60 minutes, washed 3-4X in lysis buffer containing 1 µg/ml Aprotinin and Leupeptin, and boiled in 6X Sample Buffer.

For Trio and Fra phosphorylation experiments, 2x10⁷ S2 cells were transiently transfected with pMET Trio-myc, pMET Fra-myc, pMET Abl, and/or empty pMET vector (to control for transfection efficiency in experiments receiving less than 5 µg pMET Abl). For pervanadate treatment, cells (in Schneider's Media (BRL/Invitrogen), plus 10% FBS) were treated with 2 mM Na₃VO₄ and 3 mM H₂O₂ for 30 minutes at room temperature. In all experiments, cells were rinsed once in PBS and then lysed in 50 mM
Tris pH 8, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 2 mM Na$_3$VO$_4$, and 5 µg/ml each Aprotinin and Leupeptin. After IP, beads were washed 4-6X in lysis buffer containing 1 µg/ml Aprotinin and Leupeptin, and boiled in 2X Sample Buffer.

Phosphotyrosine was detected using 4G10 mouse anti-phosphotyrosine (Upstate) at 1:10,000 and goat anti-mouse-HRP (Jackson) at 1:10,000 in low-salt TBST (25 mM NaCl)/5% BSA. Mouse anti-myc (Roche), rabbit anti-myc (Santa Cruz), mouse anti-HA, and rabbit anti-Abl (kindly provided by A. Comer) were used at 1:2000 in 5% milk/TBST. Mouse anti-Ena 5G2 was used at 1:200 in 5% milk/TBST. Proteins were visualized using ECL (for phosphotyrosine detection), ECL PLUS (Amersham Pharmacia, for protein-protein interactions), or NBT/BCIP detection (for loading controls in co-IP and phosphorylation experiments). Prior to re-probe of co-IP and phosphotyrosine westerns, blots were stripped in 50 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol overnight at 65°C.

3.3. RESULTS

3.3.1. Mutations in Abelson and trio dominantly enhance the fra and Netrin CNS phenotypes

Noting that the trio,Abl double mutant CNS phenotype is qualitatively similar, but much more severe, than the phenotype of fra mutant embryos, and that Fra, Trio, and Abl localize to CNS axons, we hypothesized that these molecules collaboratively regulate commissure formation (Fig. 3.1A-F, Tables 3.2, 3.3, and 3.8) (Awasaki et al., 2000; Gertler et al., 1989; Kolodziej et al., 1996; Liebl et al., 2000). Since dosage-sensitive
genetic interactions often indicate that gene products function in the same biological process, we asked whether mutations in *Abl* or *trio* dominantly modify the *fra* CNS phenotype.

In homozygous *fra* (*fra^4/Df(2R)vg135*) mutant embryos, 21% of segments had defective commissures. As in a previous study (Kolodziej et al., 1996), we found that the majority of defective segments in *fra* embryos (13% in this case) had thin or missing commissures, and that the posterior commissure was most often affected (Fig. 3.1F and Table 3.2). In 8% of segments, an approximately wild-type number of axons crossed the midline, but commissures were disorganized (examples of errors are indicated in Fig. 3.1 and described in the Table 3.2 legend).

In homozygous *fra*, heterozygous *Abl* (*fra^4/Df(2R)vg135;Abl^1/+*) embryos, the percentage of segments with defective commissures increased to 30%, and this increase was due solely to an increase in the number of thin or missing commissures (24%, Fig. 3.1G, Table 3.2). The independently-generated *Abl^1* allele also dominantly enhanced the *fra* phenotype, demonstrating that lesions in *Abl* were responsible for the genetic interaction with *fra*, and not accessory mutations on any of the chromosomes tested (Table 3.2).

Heterozygosity for *trio* also enhanced the CNS phenotype in *fra* mutant embryos. For example, in *fra^4/Df(2R)vg135;trio^{IMP159.4}/+* embryos, 53% of segments had defective commissures (Fig. 3.1H, Table 3.3). 42% of segments in *fra^4/Df(2R)vg135;trio^{IMP159.4}/+* embryos had thin or missing commissures (vs. 13% in *fra^4/Df(2R)vg135* animals) and 11% of segments had commissural pathfinding errors (vs. 8% in *fra^4/Df(2R)vg135* embryos) (Fig. 3.1H, Table 3.3). Milder dominant enhancement of the *fra* phenotype was
observed for a number of other trio alleles, including the deficiency Df(3L)Fpal, the hypomorphic P-element insertion allele trio\(^{P0368/10}\), and the trio\(^{M89}\) allele (which encodes a point mutation in Trio’s GEF1 domain) originally identified as a dominant enhancer of the Abl semilethality phenotype (Liebl et al., 2000) (Table 3.3). It is not clear why trio\(^{IMP159.4}\) (an imprecise excision allele generated by mobilizing the P element on the trio\(^{P0368/10}\) chromosome) enhances the fra phenotype so strongly. Since the deficiency Df(3L)Fpal (which completely removes the trio gene) behaves similarly to the other trio alleles we tested, it is likely that trio\(^{IMP159.4}\) is not a null or hypomorphic allele, but rather encodes a Trio protein with unusual properties. It is also not clear why fra\(^d\) homozygotes have more disrupted commissures than fra\(^d\)/Df(2R)vg135 animals–this fra allele has not been characterized, although fra\(^d\) homozygous animals are not immunoreactive with the polyclonal anti-fra serum generated by Kolodziej et al. (1996).

While in most fra mutant combinations tested the posterior commissure (PC) was affected more often than the anterior commissure (AC), heterozygosity for Abl or trio increased the frequency of defects in both commissures. For example, in fra\(^d\)/fra\(^d\) embryos 20% (n=212) of PCs scored were thin or missing, vs. 31% (n=215) in fra\(^d\)/fra\(^d\);Abl\(^1\)/+ mutants and 39% (n=262) in fra\(^d\)/fra\(^d\);trio\(^{M89}\)/+ embryos. Similarly, 5% (n=212) of ACs scored were thin or missing in fra\(^d\)/fra\(^d\) embryos, vs. 14% (n=215) in the fra\(^d\)/fra\(^d\);Abl\(^1\)/+ background and 18% (n=262) in fra\(^d\)/fra\(^d\);trio\(^{M89}\)/+ embryos. Thus, although mutations in fra seem to affect posterior commissural axons preferentially (Kolodziej et al., 1996), fra's genetic interactions with Abl and trio are consistent with a significant role for all of these genes in anterior commissure formation as well.
We next asked whether heterozygosity for \textit{Abl} or \textit{trio} modifies the CNS phenotype in embryos mutant for the genes encoding Frazzled's ligands, \textit{Netrin A} and \textit{Netrin B}. \textit{NetA} and \textit{NetB} are both removed by a deficiency on the X chromosome, \textit{Df(1)NP5} (Mitchell et al., 1996). Like \textit{fra} mutant embryos, \textit{Netrin} mutant embryos have fewer commissural axons, with those in the posterior commissure being most affected (Mitchell et al., 1996). As observed for \textit{fra}, both \textit{Abl} and \textit{trio} dominantly enhanced the \textit{Netrin} deficiency phenotypes and increased the frequency of defects in both commissures (Fig. 3.1P, Q, and R and data not shown). In \textit{Df(1)NP5/Y;Abl}^+/+ embryos, 33% of segments had thin or missing commissures, vs. only 24% in \textit{Df(1)NP5/Y} hemizygotes; in \textit{Df(1)NP5/Y;trio}^{IMP159.4/+} embryos, 56% of segments had thin or missing commissures (Tables 3.2 and 3.3, Fig. 3.1P, Q, and R). \textit{Abl}'s and \textit{trio}'s genetic interactions with the \textit{Netrins} further support the idea that \textit{Abl} and \textit{trio} are required to attract growth cones to the CNS midline.

Although mutations in \textit{Abl} and \textit{trio} dominantly enhanced the loss-of-commissure phenotype in \textit{fra} and \textit{Netrin} mutants, we did not observe other dose-sensitive interactions between \textit{fra}, \textit{Abl}, and \textit{trio}. For example, mutations in \textit{fra} did not reciprocally enhance the loss-of-commissure phenotype in \textit{Abl} or \textit{trio} mutants, although the percentage of segments with axon pathfinding errors increased when one dose of \textit{fra} was removed in the \textit{Abl} mutant background (Tables 3.2 and 3.3, Fig. 3.1I, J). We also did not observe transheterozygous interactions between \textit{Abl}, \textit{trio}, and \textit{fra} in single, double, and triple transheterozygous mutant combinations. In all of these cases, no more than 5% of segments had disrupted commissures (Table 3.4, Fig. 3.1K). Furthermore, even in embryos that were homozygous mutant for one of these three genes, heterozygosity for
the two remaining genes did not lead to additive or synergistic increases in commissure defects (Table 3.4). Since in other experiments, Abl and trio behaved genetically as fra effectors (see below), the inability of fra to dominantly enhance Abl or trio loss-of-commissure defects and the lack of transheterozygous interactions between fra, Abl, and trio may be due to the presence of maternally-contributed Abl and trio in the embryo (Bennett and Hoffmann, 1992; Liebl et al., 2000; Wadsworth et al., 1985). Another possibility is that heterozygosity for fra simply does not reduce the effective dose enough to enhance Abl or trio loss-of-commissure phenotypes, and/or the presence of another attractive receptor compensates for reduction in Fra levels in Abl and trio backgrounds.

Interestingly, heterozygosity for fra does enhance the Abl semilethality phenotype, provided that females do not carry the Abl allele, which encodes a mislocalized, truncated Abl protein that likely includes the kinase domain but not C-terminal sequences (Table 3.5) (Henkemeyer et al., 1990). Reducing the dose of fra does not modify the trio semilethality phenotype, however (Table 3.5). Additionally, although female embryos that are heterozygous for the Netrins, fra, Abl, and trio do not display commissure defects as visualized with mAb BP102 immunolabeling (not shown), the percent of these “quintuple transheterozygotes” surviving to adulthood is reduced (Table 3.6). Thus, although for the most part we have not observed transheterozygous genetic interactions between loss-of-function mutations in Netrin, fra, trio, and Abl that affect CNS axon pathfinding, evidence from semilethality assays is consistent with the idea that these genes function together during development.
3.3.2. Commissure formation is severely disrupted in double mutant fra;Abl and fra;trio embryos

As in trio,Abl double mutant embryos, fra;Abl and fra;trio double mutant embryos had severe CNS phenotypes in which the majority of segments had thin or missing commissures. In fra\textsuperscript{4}/Df(2R)vg135;Abl\textsuperscript{f}/Abl\textsuperscript{f} embryos, 85% of segments had thin or missing commissures (Fig. 3.1L, M, and Table 3.2). In fra\textsuperscript{4}/Df(2R)vg135;trio\textsuperscript{IMP159.4}/trio\textsuperscript{M89} embryos, 66% of segments had thin/missing commissures (Fig. 3.1N, O, and Table 3.3). In these animals, as in homozygous fra, heterozygous Abl or trio mutants, the posterior commissure was affected more often than the anterior commissure. For example, in fra\textsuperscript{4}/Df(2R)vg135;Abl\textsuperscript{f}/Abl\textsuperscript{f} embryos, 79% of PCs were thin/missing, vs. only 49% of ACs (n=166 segments); in fra\textsuperscript{4}/Df(2R)vg135;trio\textsuperscript{IMP159.4}/trio\textsuperscript{M89} embryos, 62% of PCs were thin/missing, vs. only 33% of ACs (n=172 segments).

Additionally, while there were occasional breaks (>75% of axons missing) in longitudinal connectives in fra homozygotes (10% (n=519) in fra\textsuperscript{4}/Df(2R)vg135 embryos), in fra;Abl and fra;trio double mutants, this type of defect did not increase considerably. For example, only 11% (n=387) of connectives had breaks in fra\textsuperscript{4}/Df(2R)vg135;Abl\textsuperscript{f}/Abl\textsuperscript{f} embryos, and 16% (n=368) in fra\textsuperscript{4}/Df(2R)vg135;trio\textsuperscript{IMP159.4}/trio\textsuperscript{M89} embryos. However, analysis of subsets of longitudinally-projecting axons in stage 17 embryos using the monoclonal antibody 1D4 (anti-Fasciclin II) revealed significant disorganization of these pathways, especially in fra;Abl double mutants (Fig. 3.2 and Table 3.7). In fra;Abl and fra;trio double mutants, FasII-positive longitudinal pathways wandered medially or laterally, often seeming to
intertwine so that individual bundles were indistinguishable. In addition, while breaks in all three longitudinal bundles between segments were rare, often 1-2 (usually lateral) 1D4-positive bundles were discontinuous between segments. Similar defects were observed at a lower frequency in individual fra, Netrin, Abl, and trio mutants (Fig. 3.2 and Table 3.7). Thus, although BP102 immunohistochemistry did not reveal consistent defects in longitudinal pathways, the disorganization of FasII-positive axon bundles suggests that Fra, Abl, and Trio function during axon pathfinding in longitudinal pathways in addition to their roles during commissure formation.

3.3.3. Mutations in enabled suppress frazzled, Netrin, and trio,Abl CNS phenotypes

In the CNS, ena interacts genetically with the repulsive receptor robo, leading to inappropriate crossing of the midline by longitudinal axons (Bashaw et al., 2000). In Abl, trio, and fra mutant combinations numerous axons fail to cross the midline (Liebl et al., 2000) (Tables 3.2 and 3.3). We explored this apparently antagonistic relationship further by analyzing genetic interactions among trio, Abl, fra, the Netrins, and ena in the CNS.

Mutations in ena dominantly reduced the severity of the CNS phenotype in trio,Abl mutants. For example, in ena heterozygous, trio,Abl homozygous (ena\(^{GC10/+; trio^{M89}Abl^1/Df(3L)FpaI,Abl^4}\) embryos, only 78% of segments had defective commissures, vs. 100% in the trio,Abl (trio\(^{M89}Abl^1/Df(3L)FpaI,Abl^4\) double mutant (Table 3.8, and compare Figs. 3.1D, E to Fig. 3.1T). Overall, there was a 20% reduction in the number of segments with thin or missing commissures (Table 3.8). However, analysis of individual commissures revealed a dramatic increase in the number of axons which crossed the midline compared to the trio,Abl double mutant, especially in the AC.
For example, in *ena* heterozygous, *trio,Abl* homozygous

\((ena^{GC10}+/;trio^{M89},Abl^{1}/Df(3L)FpaI,Abl^{1})\) embryos only 24% of ACs and 59% of PCs were thin or missing (n=206 segments), compared to 64% of ACs and 84% of PCs in *trio,Abl* embryos (n=160 segments) (Fig. 3.1D, E, and T). In another mutant combination, the increase in the number of axons crossing the midline was even more striking. In *Df(3L)FpaI,Abl^{4}/trio^{IMP159.4},Abl^{1}\) embryos, 65% of segments had thin or missing commissures, while in *ena^{GC5}/+;Df(3L)FpaI,Abl^{4}/trio^{IMP159.4},Abl^{1}\) embryos only 9% of segments' commissures were thin or missing (Table 3.8). Removing one dose of *ena* in the *trio,Abl* homozygous mutant background also decreased the number of breaks in longitudinal connectives (Fig. 3.1D,E,T and Table 3.7).

Mutations in *ena* also dominantly suppressed CNS defects in *fra* and *Netrin* mutants. For example, in *Df(1)NP5/Y;ena^{GC10}+/\) embryos, only 7% of segments had thin/missing commissures, vs. 24% in *Df(1)NP5/Y* embryos (Fig. 3.1P, S, and Table 3.8). In *fra^4,ena^{GC10}/Df(2R)vg135* embryos, 10% of segments had thin or missing commissures, vs. 13% in *fra^4/Df(2R)vg135* animals (Table 3.8). Heterozygosity for *ena* also suppressed enhancement of the *fra* CNS phenotype by *Abl* or *trio*. For example, in *fra^4,ena^{GC10}/fra^4;Abl^{1}/+\) embryos 14% of segments had thin/missing commissures, vs. 33% in *fra^4/fra^4;Abl^{1}/+\) mutants, and 23% in *fra^4/fra^4* embryos. Similarly, in *fra^4/fra^4;trio^{IMP159.4}/+\) embryos 80% of segments had thin or missing commissures, compared to only 15% in *fra^4,ena^{GC10}/fra^4;trio^{IMP159.4}/+\) embryos (Table 3.8).
3.3.4. *Heterozygosity for Abl, trio, and ena suppresses inappropriate midline crossing by axons expressing the chimeric Robo-Fra receptor*

The genetic interactions described support positive roles for Abl and Trio, and an antagonistic role for Ena, during commissure formation. However, the types of interactions we observed between *fra, Abl,* and *trio* loss-of-function mutations (dominant enhancement and synergistic double mutant genetic interactions) are usually interpreted to mean that gene products function in parallel pathways. Especially since *fra* is not maternally contributed, it is likely, for example, that Abl and Trio are instructed by at least one other receptor that positively regulates commissure formation. Similarly, although Fra or Abl might inhibit Ena function *in vivo,* it is equally likely that Ena simply functions in a pathway whose ultimate output is antagonistic to Fra, Abl, and/or Trio function (e.g., repulsion). To test genetically whether Abl, Trio, and/or Ena might function as Fra effector(s) *in vivo,* we took advantage of the fact that neuronal expression of a chimeric receptor composed of the extracellular and transmembrane domains of the repulsive Roundabout receptor and the intracellular domain of Fra causes CNS axons to inappropriately sense the midline-secreted repellent Slit as an attractant and cross the midline boundary inappropriately (Bashaw and Goodman, 1999). If Abl, Trio, and/or Ena function as effectors of Fra signaling in axons that cross the midline, then reducing the dose of these molecules genetically would be expected to reduce the severity of the chimeric Robo-Fra receptor phenotype. We chose this strategy since, in the *Drosophila* embryo, over-expressing full-length Fra in CNS neurons does not cause robust ectopic midline crossing by CNS axons (D.J. Forsthoefel and P.A. Kolodziej, data not shown) and (Kim et al., 2002).
In wild-type stage 17 embryos, FasII-positive axons project longitudinally in three bundles on either side of the midline, but these axons never cross the midline boundary (Fig. 3.3A). In embryos expressing UAS-Robo-Fra in all neurons, numerous FasII-positive axon bundles crossed the midline inappropriately (Table 3.9, Fig. 3.3B). Reducing the gene dose of Abl in embryos expressing the chimeric receptor led to a moderate reduction in the number of these ectopic crossovers (Table 3.9). Similarly, in the case of 3 out of 4 alleles tested, heterozygosity for trio also reduced the severity of the Robo-Fra phenotype (Table 3.9). In these experiments, the deficiency Df(3L)FpaI suppressed the Robo-Fra receptor phenotype most strongly, and not the imprecise excision allele trioIMP159.4, which acted as the strongest dominant enhancer of the fra loss-of-function phenotype (Table 3.3). It is possible that other, unidentified genes removed by the FpaI deficiency also function as Fra effectors, that the other trio alleles encode Trio proteins that retain partial function downstream of Fra signaling, or that trioIMP159.4 disrupts signaling by other receptor(s) that mediate commissure formation more strongly than this allele interferes with Fra signaling. We also discovered that if the Df(3L)FpaI chromosome was contributed to progeny by the male parent, rather than the female, genetic suppression of the Robo-Fra phenotype was less severe (Table 3.9), suggesting that maternal contribution of trio (or another gene removed by this deficiency) plays a role.

Interestingly, although heterozygosity for Abl4 (the Abl point mutant allele thought to be protein-null or nearly null (Bennett and Hoffmann, 1992)) led to a negligible reduction in ectopic crossovers on its own (0.84 crossovers/segment), heterozygosity for this allele of Abl and three different trio mutations led to a moderately
synergistic reduction in the number of inappropriate crossovers. For example, in ELAV-GAL4; UAS-Robo-Fra; +, Abl4/trio\textsuperscript{P0368/10}, + embryos, only 0.47 axon bundles per segment crossed the midline inappropriately, compared to 0.61 crossovers/segment in ELAV-GAL4; UAS-Robo-Fra; trio\textsuperscript{P0368/10}/+ embryos (Table 3.9, Fig. 3.3C). These data are consistent with positive roles for both Abl and Trio as effectors of Fra signaling in axons which cross the CNS midline.

Heterozygosity for three different alleles of ena also reduced the severity of the Robo-Fra phenotype (Table 3.9). Furthermore, reducing the gene dose of both Abl and ena also led to a synergistic reduction in the number of ectopic midline crossovers by FasII-positive axons, similar to the genetic interaction between Abl and trio in embryos expressing Robo-Fra. For example, in ELAV-GAL4; UAS-Robo-Fra/ena\textsuperscript{23}; Abl4/+ embryos, there were only 0.23 crossovers per segment, compared to 0.47 crossovers/segment in Robo-Fra-expressing embryos heterozygous for ena\textsuperscript{23} only (Table 3.9, Fig. 3.3D). While these data were initially surprising since heterozygosity for ena led to an increase in the number of axons that crossed the midline in fra, Netrin, Abl, and trio loss-of-function mutants (Table 3.8), they are consistent with the idea that Ena may be functioning positively as an effector of signaling by the Fra cytoplasmic domain, similar to Ena’s orthologs in other organisms (see Discussion and (Gitai et al., 2003; Lebrand et al., 2004)).

3.3.5. Physical interactions between Fra, Abl, and Trio

To test whether Fra could physically interact with Abl, Trio, or Ena, we first asked whether glutathione-S-transferase (GST) fusions with the cytoplasmic domain of
Fra (generated in *E. coli*) could bind *in vitro*-translated Abl, Trio, or Ena. In these experiments, Abl and Trio, but not Ena, specifically bound GST-Fracyto, but not GST or glutathione beads alone (Fig. 3.4A and data not shown), indicating that the cytoplasmic domain of Fra can interact directly with Abl and Trio. In parallel experiments, we also found that GST-Fra could interact with Abl-myc and Trio-myc in extracts from *Drosophila* Schneider-2 (S2) cells that had been engineered to express each protein (not shown).

In addition, *in vitro*-translated Abl-myc bound specifically to GST-TrioSH3 (Fig. 3.4B), and Trio-myc bound specifically to GST-AblSH3 (Fig. 3.4C), indicating that Abl and Trio can interact directly as well via their SH3 domains. GST-TrioSH3 includes linker sequences on either side of the SH3 domain. Deleting most of the SH3 domain and C-terminal linker sequences in GST-TrioSH3 (L1624-L1840) abolishes Abl-myc binding (not shown), suggesting that the SH3 domain mediates the interaction with Abl. In all of the GST-pulldown experiments, the Trio constructs used were deleted for the spectrin-like repeats in order to maximize expression level and stability (full-length, wild-type Trio’s molecular mass is greater than 225 kDa). Thus, at least in these *in vitro* assays, the spectrin-like repeats are not required for Trio’s interaction with either the cytoplasmic domain of Fra or the SH3 domain of Abl.

Next, we asked whether full-length Fra could interact with full-length Abl, Trio, and/or Ena in S2 cells. Hemagglutinin (HA)-tagged Fra (which rescues the loss-of-commissure phenotype in *fra* mutant embryos, Fig. 3.5A,B,D and Table 3.10) was transiently co-expressed with either Abl-myc, Trio-myc, or Ena, and extracts were subjected to immunoprecipitation. Fra-HA specifically co-immunoprecipitated in the
presence of Abl-myc or Trio-myc, but not in their absence (Fig. 3.4D and E, lanes 1 and 2). This interaction is mediated by Fra's cytoplasmic domain (which is required for Fra function in vivo, Fig. 3.5E), since a Fra protein deleted for this domain did not co-IP with either Abl or Trio (Fig. 3.4D and E, lane 3). We did not observe a similar association between Ena and Fra-HA in S2 cells, using either anti-Ena or anti-HA antibodies for co-IP (data not shown).

3.3.6. Fra and Trio are tyrosine phosphorylated in S2 cells

The genetic and physical interactions that we observed among Fra, Trio, and Abl raised the possibility that Fra and Trio might be substrates for the Abl tyrosine kinase. To determine whether or not phosphotyrosine could be detected on Fra or Trio, we transiently expressed full-length, epitope-tagged versions of Trio and Fra in S2 cells, and treated the cells with pervanadate, a potent phosphotyrosine phosphatase inhibitor that has been used previously to sustain tyrosine phosphorylation of proteins in Drosophila cells (Fashena and Zinn, 1997; Muda et al., 2002). (Fra-myc and Trio-myc rescue CNS defects in vivo; see Fig. 3.5F, Table 3.10, and Chapter 4). In control S2 cells, Trio tyrosine phosphorylation was not detected, while Fra was moderately tyrosine-phosphorylated (Fig. 3.6A and B, lane 1). After a 30 minute pervanadate treatment, both molecules were robustly tyrosine phosphorylated, indicating that Trio and Fra are substrates of tyrosine kinases and phosphatases expressed endogenously in S2 cells (Fig. 3.6A and B, lane 2).

Next, we asked whether elevating Abl levels in S2 cells would increase tyrosine phosphorylation of Trio or Fra. Phosphotyrosine on Trio increased dramatically when
Abl was co-expressed (Fig. 3.6C, lane 2). Fra was also tyrosine phosphorylated at a higher level when Abl was co-expressed, although not quite as robustly as Trio (Fig. 3.6D, lanes 2 and 3). We observed similar elevation of tyrosine phosphorylation of Ena, a known Abl substrate, in S2 cells treated with pervanadate or cotransfected with Abl (not shown). These results indicate that Abl either phosphorylates Fra and Trio directly, or indirectly regulates Fra and Trio tyrosine phosphorylation.

3.4. DISCUSSION

Recent investigations into the molecular mechanisms of axon guidance have focused on identifying which cytoplasmic molecules cooperate with which growth cone receptors to regulate actin cytoskeletal dynamics and growth cone motility (Huber et al., 2003). In this study, we present evidence that Abl, Trio, and Ena function together with the Netrin receptor Fra to regulate chemoattraction to the *Drosophila* embryonic CNS midline.

We found that mutations in *Abl* and *trio* dominantly enhance the CNS phenotype in *fra* and Netrin mutant embryos, and that *fra;Abl* and *fra;trio* double mutants have a severe CNS phenotype in which a majority of commissures are thin or missing, similar to the *trio;Abl* double mutant phenotype. Mutations in *Abl* and *trio* reduce the number of axons that cross the midline inappropriately in embryos expressing the chimeric Robo-Fra receptor. Abl and Trio interact physically with Fra’s cytoplasmic domain, and increasing Abl kinase expression in cells increases tyrosine phosphorylation of Fra and Trio. Interpreting these data together, we conclude (1) that Abl, Trio, and Fra function
together during commissure formation, (2) that the severe double mutant phenotypes reflect the disruption of multiple signaling pathways or networks in the growth cones of commissural axons (i.e., Abl and Trio function downstream of at least one other receptor that positively regulates commissure formation), and (3) that the lack of other dose-sensitive interactions between \textit{fra}, \textit{Abl}, and \textit{trio} is a result of redundancy (other receptors/effectors mediating commissure formation) or the presence of maternally contributed proteins.

Abl’s interactions with Fra are intriguing, as they suggest that in \textit{Drosophila} as in other organisms, this evolutionarily conserved guidance receptor is regulated by tyrosine phosphorylation, and also that Fra may regulate Abl substrates (Fig. 3.7A). Recently, others have demonstrated Netrin-dependent tyrosine phosphorylation of DCC, Netrin/DCC-dependent activation of the tyrosine kinases FAK, Src, and Fyn, and the requirement of DCC tyrosine phosphorylation for Netrin-dependent Rac1 activation and growth cone turning (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Interestingly, the tyrosine residue in DCC identified as the principal target of Fyn/Src kinases is not conserved in \textit{Drosophila} Fra or \textit{C. elegans} UNC-40, suggesting that the precise mechanisms by which Fra/DCC/UNC-40 signaling is regulated by tyrosine kinases may differ between organisms (Li et al., 2004; Meriane et al., 2004). Tyrosine phosphorylation of UNC-40 has also been observed, and although the kinase(s) responsible have not been identified, genetic interactions suggest that UNC-40 signaling is regulated by the RPTP CLR-1, supporting the idea that regulation of tyrosine phosphorylation is a consequence of UNC-6/Netrin signaling in \textit{C. elegans} as well (Chang et al., 2004; Tong et al., 2001). In this study, we observed more robust tyrosine
phosphorylation of Fra in cells with pervanadate stimulation than with Abl overexpression alone, raising the possibility that additional kinase(s) may function during Fra signaling. Further investigation will be needed to address this issue and to determine how Abl-mediated phosphorylation of Fra modulates commissural growth cone guidance.

Abl is thought to control actin dynamics in part through its ability to regulate other proteins through tyrosine phosphorylation (Lanier and Gertler, 2000; Woodring et al., 2003). Thus, in addition to potential regulation of Fra, Fra may recruit Abl to regulate other Abl substrates (Fig. 3.7A). Abl interacts genetically with trio (Liebl et al., 2000), and in this study, we have found that Trio physically interacts with Abl in vitro and that Trio tyrosine phosphorylation increases dramatically with coexpression of Abl.

Phosphorylation of Trio may affect its activity, as observed for other GEFs. For example, c-Abl regulates phosphorylation and Rac-GEF activity of Sos-1, and Lck, Fyn, Hck, and Syk kinases tyrosine phosphorylate Vav GEF and stimulate its activity (Sini et al., 2004; Turner and Billadeau, 2002). In neurons, phosphorylation of the GEF Ephexin1 by Src family kinases and/or the EphA4 receptor promotes RhoA-dependent growth cone collapse in response to Ephrin stimulation (Sahin et al., 2005). As with Fra, Abl’s ability to modulate Trio phosphorylation may involve other kinases and/or phosphatases. Future experiments must determine whether Abl phosphorylates Trio directly, and the functional consequences of Trio phosphorylation.

Trio physically interacts with Fra in vitro and in S2 cells, suggesting that Fra can recruit Trio directly. In addition, heterozygosity for trio dominantly modifies the Robo-Fra chimeric receptor phenotype, consistent with a positive role for Trio as a downstream effector of Fra signaling in vivo. As a Rac/Rho GEF, Trio may link Netrin-Fra signaling...
to regulation of Rho-family GTPases in commissural axons (Fig. 3.7A). Rho-family GTPases have been rigorously studied with regard to their role in the regulation of cytoskeletal dynamics and axon guidance, outgrowth, and branching (Dickson, 2001; Govek et al., 2005; Luo, 2000). While positive roles for GTPases in commissure formation in the *Drosophila* embryo have not been directly demonstrated, *trio* (in this study), and *GEF64C*, a Rho GEF (Bashaw et al., 2001) interact genetically with *fra* leading to dramatic disruption of commissures. Additionally, expression of constitutively active or dominantly negative isoforms of both Rac and Rho, as well as constitutively active Cdc42 causes axons to cross the CNS midline inappropriately (Fan et al., 2003; Fritz and VanBerkum, 2002; Matsuura et al., 2004). Recent studies have implicated Cdc42 and Rac1/CED-10 as effectors of DCC and UNC-40 signaling, but the biochemical mechanisms by which GTPases are regulated have been elusive (Gitai et al., 2003; Li et al., 2002a; Li et al., 2002b; Shekarabi and Kennedy, 2002). Future experiments must determine whether Netrin-Fra signaling modulates Trio’s GEF activity, and how this occurs.

In this study, we found that reducing the genetic dose of *ena* causes either more or fewer axons to cross the CNS midline, depending on the genetic background, suggesting that Ena’s role in the growth cone is complex. Heterozygosity for *ena* in embryos expressing the Robo-Fra chimeric receptor reduces the number of axon bundles which inappropriately cross the CNS midline, consistent with a role for Ena as a positive effector of Fra signaling. Ena/UNC-34 has been identified genetically as an effector of DCC/UNC-40 in *C. elegans* (Gitai et al., 2003). In cultured mouse neurons, Ena/VASP proteins are required for Netrin-DCC-dependent filopodia formation, and Mena is
phosphorylated at a PKA regulatory site in response to Netrin stimulation (Lebrand et al., 2004). In migrating fibroblasts, increasing Ena/VASP proteins at the leading edge leads to unstable lamellae and decreased motility; in contrast, increasing Ena/VASP levels at the leading edge in growth cones causes filopodia formation, possibly due to differences in the distribution of actin bundling or branching proteins (Bear et al., 2000; Bear et al., 2002; Lebrand et al., 2004). Although *Drosophila* Ena’s role in actin reorganization has not been rigorously studied, Ena localizes to filopodia tips in cultured *Drosophila* cells, suggesting that Ena’s role in filopodia formation may be conserved (Biyasheva et al., 2004).

We have not observed a direct biochemical interaction between Fra and Ena. However, Abl binds and phosphorylates Ena, and heterozygosity for both *Abl* and *ena* further suppresses the Robo-Fra phenotype, suggesting that Fra may recruit Abl to regulate filopodial extension through Ena (Comer et al., 1998; Gertler et al., 1995). Alternatively, Fra may regulate Ena through other molecule(s), and the synergistic suppression of the Robo-Fra phenotype by *Abl* and *ena* is a result of the compromise of parallel pathway(s) regulated by Fra (Fig. 3.7A). It is important to note that the functional consequences of biochemical interactions between Abl and Ena are not understood (Comer et al., 1998; Grevengoed et al., 2003; Krause et al., 2003). Therefore it will be of particular interest to determine whether Ena is tyrosine phosphorylated in response to Netrin-Fra signaling, and if Ena phosphorylation regulates its activity during filopodial extension. Additionally, as Ena binds Profilin and may also negatively regulate F-actin branching, the full significance of Ena’s interactions with Fra and Abl remains to be elucidated (Fig. 3.7A) (Krause et al., 2003).
In addition to suppressing the Robo-Fra chimeric receptor phenotype, mutations in \textit{ena} also suppress the loss-of-commissure phenotype in \textit{fra}, Netrin, trio, and Abl mutant combinations. In \textit{Drosophila} (as well as \textit{C. elegans}), Ena interacts genetically and biochemically with the repulsive receptor Robo, indicating that Ena may restrict axon crossing at the midline (Fig. 3.7B) (Bashaw et al., 2000; Yu et al., 2002). Thus, the fact that mutations in \textit{ena} dominantly suppress \textit{fra}, Netrin, trio and Abl CNS phenotypes could simply reflect the compromise of a parallel, opposing signaling pathway. Consistent with this idea, some axons which cross the midline in \textit{ena} heterozygous, trio, Abl homozygous embryos are FasII-positive (see Table 3.7), indicating a partial reduction in repulsive signaling. However, \textit{ena} also dominantly suppresses \textit{fra} and Netrin commissural pathfinding defects, without causing longitudinal FasII-positive axons to cross the midline (see Table 3.7). Reductions in Robo signaling therefore may not fully explain \textit{ena}’s ability to suppress defects in \textit{fra}, Netrin, Abl, and trio mutants.

Based on the fact that mutations in \textit{ena} suppress a number of Abl mutant phenotypes, it has been proposed that Abl antagonizes Ena function (Grevengoed et al., 2003; Grevengoed et al., 2001; Lanier and Gertler, 2000). In Abl mutant embryos Ena and actin mislocalize during dorsal closure and cellularization, and apical microvilli are abnormally elongated, indicating that Abl regulates Ena’s localization (Grevengoed et al., 2003; Grevengoed et al., 2001). In migrating fibroblasts, increasing Ena/VASP levels at the leading edge results in long, unbranched actin filaments, unstable lamellae, and decreased motility due to increased antagonism of capping protein (Bear et al., 2000; Bear et al., 2002). Interestingly, mutations in the gene encoding \textit{Drosophila} capping protein \(\beta\) enhance CNS axon pathfinding defects in \textit{Abl} mutants, including commissure
formation (Grevengoed et al., 2003). Therefore, if Fra and/or Abl regulate Ena localization in commissural axons, then in fra, Netrin, or Abl mutants, Ena may be mislocalized in the growth cone, leading to inappropriate inhibition of capping protein and excessive F-actin filament elongation. Additionally, reducing regulation of Ena by Fra or Abl may also allow greater Ena regulation by Slit-Robo signaling. In either case, reducing the gene dose of ena in fra, Netrin, and trio, Abl mutant embryos would partially relieve these effects, allowing axons to respond more efficiently to other cues and cross the midline, as we observed (Fig. 3.7B). Consistent with this idea, Lebrand et al. (2004) found that either increasing or decreasing Ena/VASP proteins at the leading edge impaired the elaboration of growth cone filopodia in response to Netrin-DCC signaling, suggesting that Ena/VASP levels must be tightly regulated in order for the growth cone to respond optimally to extracellular signals.

The role of Abl in the growth cone is also likely to be complex. Our observations implicate Abl as an effector of attractive Fra signaling. In addition, tyrosine phosphorylation of Robo by Abl is thought to negatively regulate repulsive signaling by Robo (Bashaw et al., 2000). Paradoxically, though, loss-of-function mutations in Abl, robo, and slit interact genetically, resulting in inappropriate axon crossing at the midline, and indicating that Abl may also promote repulsion in longitudinally migrating growth cones (Hsouna et al., 2003; Wills et al., 2002). Additionally, Abl interacts genetically and/or biochemically with a number of additional substrates or putative effectors (see Chapter 2 Introduction), including the RPTP Dlar, the plus-end microtubule tracking protein Orbit/MAST/CLASP, and the adenylyl cyclase associated protein Capulet (Fig. 3.7A) (Lee et al., 2004; Wills et al., 1999a; Wills et al., 2002; Wills et al., 1999b).
Obviously, much remains to be understood about the molecular basis for *Abl’s* genetic interactions, particularly how Abl and its various substrates cooperate with different growth cone receptors to yield specific cytoskeletal outputs.

In summary, we have observed genetic and biochemical interactions indicating that Abl, Trio, and Ena are integrated into a complex signaling network with Fra and the Netrins during commissure formation. These observations identify another receptor that acts through these effectors, and provide a framework for further investigation of signaling by this key, evolutionarily conserved guidance receptor.
<table>
<thead>
<tr>
<th>Allele/Chromosome</th>
<th>Nature of allele/description</th>
<th>Reference(s)</th>
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<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;</td>
<td>EMS-induced allele, nature unknown, but homozygotes not immunoreactive with Fra polyclonal antisera against cytoplasmic domain</td>
<td>(Kolodziej et al., 1996)</td>
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<tr>
<td>fra&lt;sup&gt;5&lt;/sup&gt;</td>
<td>EMS-induced stop codon in extracellular domain</td>
<td>(Kolodziej et al., 1996)</td>
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<td>Df(2R)vg135</td>
<td>γ-ray induced chromosomal deficiency, fails to complement fra alleles, completely deletes fra based on cytology (49A;49E1-2) (fra at 49B2-3), also removes G-protein subunit Gαq49B</td>
<td>(Kolodziej et al., 1996; Lasko and Pardue, 1988; Ratnaparkhi et al., 2002)</td>
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<tr>
<td>Abl&lt;sup&gt;1&lt;/sup&gt;</td>
<td>EMS-induced, encodes mislocalized Abl protein that is truncated C-terminal to kinase domain, hypomorphic</td>
<td>(Bennett and Hoffmann, 1992; Henkemeyer et al., 1990; Henkemeyer et al., 1987)</td>
</tr>
<tr>
<td>Abl&lt;sup&gt;2&lt;/sup&gt;</td>
<td>EMS-induced, exact nature unknown, but may encode truncated protein, hypomorphic</td>
<td>(Bennett and Hoffmann, 1992; Henkemeyer et al., 1987)</td>
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<tr>
<td>Abl&lt;sup&gt;4&lt;/sup&gt;</td>
<td>EMS-induced, exact nature unknown, but likely protein null</td>
<td>(Bennett and Hoffmann, 1992)</td>
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<tr>
<td>Df(3L)st-j7</td>
<td>73A1-2;73B1-2, protein null</td>
<td>(Bennett and Hoffmann, 1992; Henkemeyer et al., 1987; McKeown et al., 1987)</td>
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<td>trio&lt;sup&gt;MB9&lt;/sup&gt;</td>
<td>EMS-induced missense (L1412F) mutation in DH1 domain, hypomorphic</td>
<td>(Liebl et al., 2000)</td>
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<td>trio&lt;sup&gt;P0368/10&lt;/sup&gt;</td>
<td>P-element insertion in large intron between SH3 and DH2, hypomorphic, C-terminal Ab fails to detect Trio protein</td>
<td>(Awasaki et al., 2000; Deak et al., 1997; Liebl et al., 2000)</td>
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<tr>
<td>trio&lt;sup&gt;IMP159.4&lt;/sup&gt;</td>
<td>imprecise excision of trio&lt;sup&gt;P0368/10&lt;/sup&gt;, nature unknown but transcription disrupted 3’ of P-element insertion site (see Chapter 2)</td>
<td>(Forsthoefel et al., 2005)</td>
</tr>
<tr>
<td>Df(3L)FpaI</td>
<td>chromosomal deficiency, fails to complement trio alleles, completely removes trio gene based on cytology (61D1-2;61F1-2) (trio at 61E1-2)</td>
<td>(Awasaki et al., 2000; Liebl et al., 2000)</td>
</tr>
</tbody>
</table>

Table 3.1. List of alleles, transgenes, and driver lines used in this study. All alleles are recessive.

Continued on next page
Table 3.1. Continued.

<table>
<thead>
<tr>
<th>Allele/Chromosome</th>
<th>Nature of allele:description</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Df(1)NP5)</td>
<td>completely removes (NetA) and (NetB)</td>
<td>(Mitchell et al., 1996)</td>
</tr>
<tr>
<td>(ena^{GC10})</td>
<td>(\gamma)-ray induced deletion, fails to complement (ena) alleles; exact breakpoints unknown, but one breakpoint within (ena) gene</td>
<td>(Gertler et al., 1995)</td>
</tr>
<tr>
<td>(ena^{GC5})</td>
<td>(\gamma)-ray induced inversion, fails to complement (ena) alleles; breakpoint within (ena) gene, exact rearrangement unknown</td>
<td>(Gertler et al., 1995)</td>
</tr>
<tr>
<td>(ena^{23})</td>
<td>ENU-induced missense (N379F) mutation in proline-rich region, nonsense (K636stop) mutation that deletes EVH2 domain</td>
<td>(Ahern-Djamali et al., 1998)</td>
</tr>
<tr>
<td>(ena^{210})</td>
<td>EMS-induced missense (A97V) mutation in EVH1 domain</td>
<td>(Ahern-Djamali et al., 1998; Gertler et al., 1990)</td>
</tr>
<tr>
<td>UAS-Robo-Fra-myc</td>
<td>transgene, encodes extracellular and transmembrane domains of Robo, cytoplasmic domain of Fra, Myc-tagged</td>
<td>(Bashaw and Goodman, 1999)</td>
</tr>
<tr>
<td>UAS-Fra-HA</td>
<td>transgene, Fra tagged at C-terminus with 1X HA tag</td>
<td>this study</td>
</tr>
<tr>
<td>UAS-Fra&lt;sup&gt;CYTO&lt;/sup&gt;-HA</td>
<td>transgene, Fra deleted for cytoplasmic sequences including and C-terminal to P1 domain, tagged with 1X HA</td>
<td>this study</td>
</tr>
<tr>
<td>UAS-Fra-myc</td>
<td>transgene, Fra tagged at C-terminus with 6X myc tag</td>
<td>this study</td>
</tr>
<tr>
<td>(ELAV^{155})-GAL4</td>
<td>enhancer-trap line, expresses GAL4 in all neurons post-mitotically (stage 12-13+)</td>
<td>(Lin et al., 1994)</td>
</tr>
<tr>
<td>(1407)-GAL4</td>
<td>enhancer-trap line, expresses GAL4 in all neurons pre-mitotically (stage 8-9+)</td>
<td>(Luo et al., 1994; Sweeney et al., 1995)</td>
</tr>
</tbody>
</table>
Figure 3.1. *Abl, trio, and ena* interact genetically with *fra* and the *Netrins*. CNS axons were labeled with mAb BP102, and stage 14-16 embryos were dissected and scored for defects. In wild-type embryos (A), CNS axons are organized into two commissures per segment (AC and PC, panel A), and two longitudinal tracts which run the length of the nerve cord on either side of the midline. In mutant embryos (genotypes are indicated in the figure), commissure formation is defective. Examples of segments with thin or missing commissures are indicated with arrows in panels F, G, H, P, and T. Examples of segments with errors in commissural axon pathfinding are indicated with arrowheads in panels C, H, I, R, and S. Examples of breaks in longitudinal pathways are indicated with asterisks in panels F, H, L, N, and O. Panels D, L, and N are examples of moderate *trio,Abl, fra;Abl*, and *fra;trio* phenotypes, respectively, while panels E, M, and O are severe. *Df(2R)vg135* and *Df(3L)FpaI* are deficiencies for *fra* and *trio*, respectively. *Df(1)NP5* removes both *NetA* and *NetB*. Anterior is at the top of each image. Scale bar (A) spans ~25 μm.
Figure 3.1. Continued.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures</th>
<th>% segments with pathfinding errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>fra^d/Df(2R)vg135</td>
<td>21 (1279)</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>fra^d/Df(2R)vg135:Abl^+/+</td>
<td>30 (511)</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>fra^d/Df(2R)vg135:Abl^+/+</td>
<td>47 (186)</td>
<td>39</td>
<td>8</td>
</tr>
<tr>
<td>fra^d/Df(2R)vg135:Abl^+/Abl^d</td>
<td>95 (166)</td>
<td>85</td>
<td>10</td>
</tr>
<tr>
<td>fra^d/fra^d</td>
<td>36 (212)</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>fra^d/fra^d:Abl^+/+</td>
<td>45 (458)</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>fra^d/fra^d:Abl^+/+</td>
<td>54 (215)</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td>fra^d/fra^d:Abl^+/Abl^d</td>
<td>100 (158)</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>Df(1)NP5/Y</td>
<td>34 (277)</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Df(1)NP5/Y;Abl^+/+</td>
<td>41 (312)</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>Abl^+/Abl^d</td>
<td>6.6 (257)</td>
<td>1.2</td>
<td>5.4</td>
</tr>
<tr>
<td>fra^d/+;Abl^+/Abl^d</td>
<td>11.8 (286)</td>
<td>1.0</td>
<td>10.8</td>
</tr>
<tr>
<td>Df(2R)vg135/+;Abl^+/Abl^d</td>
<td>19 (234)</td>
<td>2</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 3.2. *Abl* enhances comissure defects in *fra* and *Netrin* mutant embryos.
Number of segments scored (n) are indicated in parentheses in column 2. Segments were scored as having "Thin/Missing" commissures if >75% of BP102-staining axons were absent in either commissure. "Errors" category includes ectopic wandering or defasciculation of axons between commissures, fused commissures, and commissures that split into smaller bundles along their length, often at the junction of the commissure with the longitudinal axon tract. Percentages were not rounded when overall defects were less than 15%.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures</th>
<th>% segments with pathfinding errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/Df(2R)vg135</td>
<td>21 (1279)</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/Df(2R)vg135;Df(3L)FpaI/+</td>
<td>33 (497)</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/Df(2R)vg135;trio&lt;sup&gt;M89&lt;/sup&gt;/+</td>
<td>25 (185)</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/Df(2R)vg135;trio&lt;sup&gt;IMP159.4&lt;/sup&gt;/+</td>
<td>53 (208)</td>
<td>42</td>
<td>11</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/Df(2R)vg135;trio&lt;sup&gt;IMP159.4&lt;/sup&gt;/trio&lt;sup&gt;M89&lt;/sup&gt;</td>
<td>79 (172)</td>
<td>66</td>
<td>13</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;</td>
<td>36 (212)</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;;trio&lt;sup&gt;M89&lt;/sup&gt;/+</td>
<td>64 (262)</td>
<td>44</td>
<td>20</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;;trio&lt;sup&gt;P0368/10&lt;/sup&gt;/+</td>
<td>69 (162)</td>
<td>47</td>
<td>22</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;;trio&lt;sup&gt;IMP159.4&lt;/sup&gt;/+</td>
<td>90 (213)</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;;Df(3L)FpaI/+</td>
<td>62 (152)</td>
<td>47</td>
<td>15</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;;trio&lt;sup&gt;IMP159.4&lt;/sup&gt;/trio&lt;sup&gt;M89&lt;/sup&gt;</td>
<td>96 (118)</td>
<td>92</td>
<td>4</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;;trio&lt;sup&gt;M89&lt;/sup&gt;/trio&lt;sup&gt;P0368/10&lt;/sup&gt;</td>
<td>85 (189)</td>
<td>75</td>
<td>10</td>
</tr>
</tbody>
</table>

| Df(1)NP5/Y                                   | 34 (277)                         | 24                                     | 10                               |
| Df(1)NP5/Y;trio<sup>M89</sup>/+             | 43 (214)                         | 29                                     | 14                               |
| Df(1)NP5/Y;trio<sup>IMP159.4</sup>/+        | 71 (143)                         | 56                                     | 15                               |

| trio<sup>IMP159.4</sup>/Df(3L)FpaI           | 6.4 (421)                        | 1.9                                     | 4.5                              |
| fra<sup>4</sup>+/ trio<sup>IMP159.4</sup>/Df(3L)FpaI | 3.3 (216)                        | 0.5                                     | 2.8                              |
| Df(2R)vg135/+; trio<sup>IMP159.4</sup>/Df(3L)FpaI | 6.1 (195)                        | 1.0                                     | 5.1                              |
| trio<sup>IMP159.4</sup>/trio<sup>M89</sup>   | 9.9 (354)                        | 4.8                                     | 5.1                              |
| fra<sup>4</sup>+/ trio<sup>IMP159.4</sup>/trio<sup>M89</sup> | 5.9 (153)                        | 1.3                                     | 4.6                              |

**Table 3.3.** trio enhances commissure defects in fra and Netrin mutant embryos.

Percentages were not rounded when overall defects were less than 15%.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures</th>
<th>% segments with pathfinding errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>crosses at [25°C]:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fra(^4)/+</td>
<td>1.9 (209)</td>
<td>0</td>
<td>1.9</td>
</tr>
<tr>
<td>Abl(^1)/+</td>
<td>1.8 (217)</td>
<td>0</td>
<td>1.8</td>
</tr>
<tr>
<td>trio(^{IMP159.4})/+</td>
<td>0.9 (218)</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>fra(^4)/+ ; Abl(^1)/+</td>
<td>1.5 (205)</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>fra(^4)/+ ; trio(^{IMP159.4})/+</td>
<td>4.3 (208)</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td>trio(^{IMP159.4}), +/-, Abl(^1)</td>
<td>1.4 (211)</td>
<td>0</td>
<td>1.4</td>
</tr>
<tr>
<td>Df(2R)vg135; Df(3L)Fpal, Abl(^4)/+, +</td>
<td>0.5 (191)</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>fra(^4)/+ ; trio(^{IMP159.4}), +/-, Abl(^1)</td>
<td>3.3 (212)</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>fra(^4)/fra(^4)</td>
<td>36 (212)</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>fra(^4)/fra(^4), Abl(^1)/+</td>
<td>45 (458)</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>fra(^4)/fra(^4), trio(^{P036810})/+</td>
<td>69 (162)</td>
<td>47</td>
<td>22</td>
</tr>
<tr>
<td>fra(^4)/fra(^4), trio(^{IMP159.4})/+</td>
<td>90 (213)</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>fra(^4)/fra(^4), trio(^{P036810}), +/-, Abl(^4)</td>
<td>66 (196)</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>fra(^4)/fra(^4), trio(^{IMP159.4}), +/-, Abl(^4)</td>
<td>88 (140)</td>
<td>76</td>
<td>12</td>
</tr>
<tr>
<td>Abl(^1)/Abl(^1)</td>
<td>6.6 (257)</td>
<td>1.2</td>
<td>5.4</td>
</tr>
<tr>
<td>fra(^4)/ + ; Abl(^1)/Abl(^1)</td>
<td>11.8 (286)</td>
<td>1.0</td>
<td>10.8</td>
</tr>
<tr>
<td>Df(3L)Fpal, Abl(^1)/+, Abl(^1)</td>
<td>31 (227)</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>fra(^4)/ + ; Df(3L)Fpal, Abl(^1)/+, Abl(^1)</td>
<td>28 (235)</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>trio(^{IMP159.4})/Df(3L)Fpal</td>
<td>6.4 (421)</td>
<td>1.9</td>
<td>4.5</td>
</tr>
<tr>
<td>fra(^4)/ + ; trio(^{IMP159.4})/Df(3L)Fpal</td>
<td>3.3 (216)</td>
<td>0.5</td>
<td>2.8</td>
</tr>
<tr>
<td>trio(^{IMP159.4}), +/Df(3L)Fpal, Abl(^4)</td>
<td>22 (212)</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>fra(^4)/ + ; trio(^{IMP159.4}), +/Df(3L)Fpal, Abl(^4)</td>
<td>16 (220)</td>
<td>3</td>
<td>13</td>
</tr>
</tbody>
</table>

**Table 3.4.** *fra, Abl, and trio do not interact transheterozygously.* For single transheterozygous combinations, crosses were performed at 25°C (top panel). All other crosses were conducted at room temperature (22°C). Percentages were not rounded when overall defects were less than 15%.
Table 3.5. Mutations in *fra* enhance semilethality in *Abl*, but not *trio* mutants.
Semilethality was assayed as in Chapter 2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of expected:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pupae (n)</td>
<td>Adults (n)</td>
<td></td>
</tr>
<tr>
<td><em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; male / <em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; female</td>
<td>51 (605)</td>
<td>17 (614)</td>
<td></td>
</tr>
<tr>
<td><em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; male / <em>fra</em>&lt;sup&gt;4&lt;/sup&gt;;<em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; female</td>
<td>43 (513)</td>
<td>25 (488)</td>
<td></td>
</tr>
<tr>
<td><em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; male / <em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; female</td>
<td>97 (171)</td>
<td>42 (150)</td>
<td></td>
</tr>
<tr>
<td><em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; male / <em>fra</em>&lt;sup&gt;4&lt;/sup&gt;;<em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; female</td>
<td>61 (184)</td>
<td>28 (178)</td>
<td></td>
</tr>
<tr>
<td><em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; male / <em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; female</td>
<td>97 (205)</td>
<td>41 (117)</td>
<td></td>
</tr>
<tr>
<td><em>fra</em>&lt;sup&gt;4&lt;/sup&gt;;<em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; male / <em>Abl</em>&lt;sup&gt;l&lt;/sup&gt; female</td>
<td>76 (222)</td>
<td>6 (161)</td>
<td></td>
</tr>
<tr>
<td><em>trio</em>&lt;sup&gt;P0368/10&lt;/sup&gt; male / <em>trio</em>&lt;sup&gt;M89&lt;/sup&gt; female</td>
<td>63 (400)</td>
<td>60 (356)</td>
<td></td>
</tr>
<tr>
<td><em>fra</em>&lt;sup&gt;4&lt;/sup&gt;;<em>trio</em>&lt;sup&gt;P0368/10&lt;/sup&gt; male / <em>trio</em>&lt;sup&gt;M89&lt;/sup&gt; female</td>
<td>71 (602)</td>
<td>62 (589)</td>
<td></td>
</tr>
<tr>
<td><em>trio</em>&lt;sup&gt;M89&lt;/sup&gt; male / <em>trio</em>&lt;sup&gt;P0368/10&lt;/sup&gt; female</td>
<td>67 (327)</td>
<td>53 (321)</td>
<td></td>
</tr>
<tr>
<td><em>trio</em>&lt;sup&gt;M89&lt;/sup&gt; male / <em>fra</em>&lt;sup&gt;4&lt;/sup&gt;;<em>trio</em>&lt;sup&gt;P0368/10&lt;/sup&gt; female</td>
<td>77 (561)</td>
<td>58 (598)</td>
<td></td>
</tr>
<tr>
<td><em>trio</em>&lt;sup&gt;M89&lt;/sup&gt; male / <em>trio</em>&lt;sup&gt;P0368/10&lt;/sup&gt; female</td>
<td>58 (197)</td>
<td>52 (188)</td>
<td></td>
</tr>
<tr>
<td><em>fra</em>&lt;sup&gt;4&lt;/sup&gt;;<em>trio</em>&lt;sup&gt;M89&lt;/sup&gt; male / <em>trio</em>&lt;sup&gt;P0368/10&lt;/sup&gt; female</td>
<td>52 (164)</td>
<td>53 (168)</td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>% of expected adults eclosing (n):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; fra^4/+ ; +/+)</td>
<td>121 (220)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; +/+ ; Abl^4/+)</td>
<td>130 (202)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; +/+ ; trio^{p0368/10}/+)</td>
<td>98 (150)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; +/+ ; Df(3L)FpaI/+)</td>
<td>103 (235)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; +/+ ; Df(3L)FpaI,Abl^4/+)</td>
<td>123 (309)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; fra^4/+ ; Abl^4/+)</td>
<td>102 (212)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; fra^4/+ ; trio^{p0368/10}/+)</td>
<td>86 (228)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; fra^4/+ ; Df(3L)FpaI/+)</td>
<td>140 (343)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Df(1)NP5/+ ; fra^4/+ ; Df(3L)FpaI,Abl^4/+)</td>
<td>67 (388)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.6. Adults transheterozygous for the Netrins, fra, Abl, and trio are semiviable.** Males with alleles balanced over 2\(^{nd}\) and 3\(^{rd}\) chromosome balancers were crossed to \(Df(1)NP5/FM7c\) females. Semilethality was assayed as in Chapter 2, with the exception that in this assay up to three balancers were involved, depending on how many genes were transheterozygous. Progeny carrying two or three balancers were often less viable than other classes of balancer-carrying progeny, resulting in percents of expected mutant progeny that were greater than 100% in some cases.
Figure 3.2 Mutations in fra, Abl, trio, ena, and the Netrins affect the organization of longitudinal axon pathways. A subset of longitudinally projecting axons in stage 17 embryos was labeled with mAb 1D4 (anti-FasII). Genotypes are indicated. In wild-type embryos (A), axons course in three distinct bundles on either side of the CNS midline. In mutant embryos, two distinct phenotypes were observed, breaks in longitudinal pathways in which longitudinal fascicles were discontinuous between segments, and fascicle fusions in which two or more fascicles merged. Small arrows in B, D, and H indicate mild breaks in which only the lateral pathway is discontinuous. Large arrows in K and N indicate severe breaks in which none of the three longitudinal pathways projects into the next segment. Small arrowheads in G, I, and L indicate mild fusions in which two fascicles have collapsed onto each other. Large arrowheads in J and N indicate severe fusions in which it appears that most longitudinal axons are present, but individual fascicles are indistinguishable. Severe breaks in all three fascicles were rarely observed, even in trio,Abl mutants. Severe fusions were observed most frequently in fra:Abl, fra;trio, and trio,Abl double mutants.

Continued on next page
Figure 3.2. Continued.
Table 3.7. Organization of longitudinally projecting axons is affected by mutations in *Abl*, *trio*, *fra*, *ena*, and the *Netrins*. Stage 17 embryos were stained with mAb 1D4 and dissected. Fascicle breaks were scored if one or more longitudinally projecting fascicles within the longitudinal connective were discontinuous between hemisegments. Fascicle “fusions” were scored when two or three fascicles appeared to fasciculate with each other within a segment or the longitudinal connective posterior to a segment. In more severe cases, distinct fascicles could not even be distinguished, although the width of the resulting bundle or intensity of staining suggested that most axons were present, but simply had collapsed into one larger longitudinal bundle.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% segments with acopic midline crossing (n)</th>
<th>% hemisegments with fascicle breaks (n)</th>
<th>% hemisegments with fascicle fusions in total hemisegments scored</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mild (1-2)</td>
<td>severe/complete (3)</td>
<td>total (n)</td>
</tr>
<tr>
<td>WT</td>
<td>0.9 (109)</td>
<td>0.9</td>
<td>0.9 (220)</td>
</tr>
<tr>
<td>fra^+/fra^+</td>
<td>10.2 (88)</td>
<td>19.3</td>
<td>0</td>
</tr>
<tr>
<td>fra^+/Df(2R)vg135</td>
<td>1.0 (96)</td>
<td>5.6</td>
<td>0</td>
</tr>
<tr>
<td>Df(1)NP5Y</td>
<td>0.8 (118)</td>
<td>12.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Abl^+/Abi^+</td>
<td>27.1 (96)</td>
<td>15.7</td>
<td>0</td>
</tr>
<tr>
<td>fra^+/Abi^+/Abi^+/</td>
<td>13.3 (128)</td>
<td>8.6</td>
<td>0</td>
</tr>
<tr>
<td>Df(3L)Fpal/trio^MP159.4</td>
<td>14.7 (109)</td>
<td>69.7</td>
<td>0</td>
</tr>
<tr>
<td>fra^+/Df(3L)Fpal/trio^MP159.4</td>
<td>28.4 (88)</td>
<td>58.8</td>
<td>0.6</td>
</tr>
<tr>
<td>fra^+/fra^+/Abi^+/+</td>
<td>2.1 (97)</td>
<td>28.1</td>
<td>0.5</td>
</tr>
<tr>
<td>fra^+/Df(3L)Fpal/+</td>
<td>24.8 (109)</td>
<td>28.6</td>
<td>0</td>
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<tr>
<td>fra^+/fra^+/Abi^+/Abi^+/</td>
<td>36.5 (85)</td>
<td>63.1</td>
<td>3.1</td>
</tr>
<tr>
<td>fra^+/Df(3L)Fpal/trio^MR9</td>
<td>26.4 (110)</td>
<td>34.0</td>
<td>0</td>
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<tr>
<td>Df(3L)Fpal,Abi^+/trio^MP159.4,Abi^+</td>
<td>22.8 (101)</td>
<td>42.5</td>
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<tr>
<td>fra^+,ena^Gcs10,Df(2R)vg135</td>
<td>0 (66)</td>
<td>13.7</td>
<td>0</td>
</tr>
<tr>
<td>Df(1)NP5Y,ena^Gcs10</td>
<td>0 (65)</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>ena^Gcs10,tri^MP159.4,Abi^+/Df(3L)Fpal,Abi^+</td>
<td>77.1 (109)</td>
<td>22.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Genotype</td>
<td>% defective segments, overall (n)</td>
<td>% segments with thin/missing commissures</td>
<td>% segments with pathfinding errors</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------</td>
<td>----------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>trio&lt;sup&gt;M89&lt;/sup&gt;, Abl&lt;sup&gt;l&lt;/sup&gt;/Df(3L)FpaI,Abl&lt;sup&gt;l&lt;/sup&gt;</td>
<td>100 (160)</td>
<td>86</td>
<td>14</td>
</tr>
<tr>
<td>ena&lt;sup&gt;GC10&lt;/sup&gt;/+: trio&lt;sup&gt;M89&lt;/sup&gt;, Abl&lt;sup&gt;l&lt;/sup&gt;/ Df(3L)FpaI,Abl&lt;sup&gt;l&lt;/sup&gt;</td>
<td>78 (206)</td>
<td>65</td>
<td>13</td>
</tr>
<tr>
<td>Df(3L)FpaI,Abl&lt;sup&gt;l&lt;/sup&gt;/trio&lt;sup&gt;IMP159.4&lt;/sup&gt;, Abl&lt;sup&gt;l&lt;/sup&gt;</td>
<td>85 (196)</td>
<td>65</td>
<td>20</td>
</tr>
<tr>
<td>ena&lt;sup&gt;GC5&lt;/sup&gt;/+: Df(3L)FpaI,Abl&lt;sup&gt;l&lt;/sup&gt;/ trio&lt;sup&gt;IMP159.4&lt;/sup&gt;, Abl&lt;sup&gt;l&lt;/sup&gt;</td>
<td>29 (208)</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/Df(2R)vg135</td>
<td>21 (1279)</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;, ena&lt;sup&gt;GC10&lt;/sup&gt;/Df(2R)vg135</td>
<td>17 (799)</td>
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<td>7</td>
</tr>
<tr>
<td>Df(1)NP5/Y</td>
<td>34 (277)</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Df(1)NP5/Y; ena&lt;sup&gt;GC10&lt;/sup&gt;/+</td>
<td>17 (308)</td>
<td>7</td>
<td>10</td>
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<tr>
<td>Df(1)NP5/Y; ena&lt;sup&gt;210&lt;/sup&gt;/+</td>
<td>26 (222)</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Df(1)NP5/Y; ena&lt;sup&gt;23&lt;/sup&gt;/+</td>
<td>20 (212)</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;</td>
<td>36 (212)</td>
<td>23</td>
<td>13</td>
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<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;; Abl&lt;sup&gt;l&lt;/sup&gt;/+</td>
<td>45 (458)</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;, ena&lt;sup&gt;GC10&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;; Abl&lt;sup&gt;l&lt;/sup&gt;/+</td>
<td>26 (221)</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;; trio&lt;sup&gt;IMP159.4&lt;/sup&gt;/+</td>
<td>90 (213)</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;, ena&lt;sup&gt;GC10&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;; trio&lt;sup&gt;IMP159.4&lt;/sup&gt;/+</td>
<td>25 (197)</td>
<td>15</td>
<td>10</td>
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</table>

Table 3.8. Heterozygosity for ena dominantly suppresses commissure defects in fra, Netrin, and trio,Abl mutant embryos. Percentages were not rounded when overall defects were less than 15%.
Figure 3.3. Heterozygosity for *Abl*, *trio*, and *ena* reduces the severity of inappropriate midline crossing by axons expressing the chimeric Robo-Fra receptor. A subset of longitudinally-projecting axons in stage 17 embryos were labeled with mAb 1D4 (anti-FasII). Examples of FasII-positive bundles scored as ectopic crossovers are indicated with arrows. (A) In wild-type embryos, FasII-positive axons project in three distinct longitudinal bundles on either side of the CNS midline, but never cross the midline. (B) In embryos expressing UAS-Robo-Fra under the control of the ELAV-GAL4 postmitotic neuronal driver, numerous FasII-positive axon bundles cross the midline, reflecting inappropriate attraction towards the midline repellent Slit. (C) In embryos heterozygous for *Abl* and *trio*, fewer axon bundles cross the midline. (D) Heterozygosity for *Abl* and *ena* also reduces the severity of the Robo-Fra phenotype.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Crossovers/Segment (n)</th>
<th>Penetrance (n)</th>
<th>Expressivity (defects/embryo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELAV-G4; UAS-Robo-Fra</td>
<td>0.86 (261)</td>
<td>100% (24)</td>
<td>9.3</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; Abt^{t/+}</td>
<td>0.84 (242)</td>
<td>100% (22)</td>
<td>9.2</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; Abt^{t/+}</td>
<td>0.78 (183)</td>
<td>100% (17)</td>
<td>8.5</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; Df(3L)st-j7^{+/+}</td>
<td>0.74 (247)</td>
<td>100% (21)</td>
<td>8.8</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; trio^{M89/+}</td>
<td>0.88 (217)</td>
<td>100% (19)</td>
<td>10.1</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; trio^{P0358/10/+}</td>
<td>0.61 (241)</td>
<td>100% (22)</td>
<td>6.8</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; trio^{BMP159.4/+}</td>
<td>0.65 (242)</td>
<td>96% (22)</td>
<td>7.5</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; Df(3L)FpaI^{+/a}</td>
<td>0.39 (229)</td>
<td>57% (21)</td>
<td>7.5</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; Df(3L)FpaI(MALE)/^{+/b}</td>
<td>0.62 (95)</td>
<td>100% (8)</td>
<td>7.4</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; +,Abt^{t}/trio^{M89,+}</td>
<td>0.78 (302)</td>
<td>100% (26)</td>
<td>9.0</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; +,Abt^{t}/trio^{P0358/10,+}</td>
<td>0.47 (176)</td>
<td>100% (15)</td>
<td>5.5</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra; Df(3L)FpaI,Abt^{t/+},+</td>
<td>0.28 (318)</td>
<td>79% (29)</td>
<td>3.9</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra/ena^{GC10}</td>
<td>0.65 (246)</td>
<td>100% (23)</td>
<td>6.9</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra/ena^{23}</td>
<td>0.47 (201)</td>
<td>88% (17)</td>
<td>6.3</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra/ena^{210}</td>
<td>0.59 (257)</td>
<td>96% (22)</td>
<td>7.2</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra/ena^{GC10}; Abt^{t/+}</td>
<td>0.35 (172)</td>
<td>81% (16)</td>
<td>4.6</td>
</tr>
<tr>
<td>ELAV-G4; UAS-Robo-Fra/ena^{23}; Abt^{t/+}</td>
<td>0.23 (223)</td>
<td>74% (19)</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 3.9. Heterozygosity for *Abt*, *trio*, and *ena* genetically suppresses ectopic midline crossing by axons expressing the Robo-Fra chimeric receptor. Stage 17 embryos were stained with mAb 1D4 and dissected. At least 9 segments/embryo were scored for FasII-positive axon bundles which crossed the midline inappropriately. Since some segments had two crossovers, the "crossovers/segment" score is the total number of crossovers divided by total number of segments (n) scored for that genotype. Penetrance is the # of embryos with defects divided by the total number (n) of embryos scored. Expressivity is the total number of ectopic crossovers divided by the number of affected embryos. The same UAS-Robo-Fra transgenic chromosome ("3.1") was used in each cross. *a*In these embryos, the Df(3L)FpaI chromosome was contributed by the female parent, while in *b*, the male parent contributed this chromosome.
**Figure 3.4. Physical interactions between Fra, Abl, and Trio.** (A) The cytoplasmic domain of Fra interacts directly with Abl and Trio. Beads only (lane 1), GST bound to beads (lane 2), or GST-FraCYTO bound to beads (lane 3) were incubated with *in vitro*-translated, epitope-tagged Abl or Trio. ~20% of each pulldown (~2 µg of fusion protein) and bound target proteins were resolved by SDS-PAGE. Bound proteins (top 2 blots) were visualized by anti-myc immunoblotting, and fusion proteins (bottom gel) were visualized by Coomassie staining. "In" lane represents 2% of total input protein incubated with beads, GST, or GST-FraCYTO. Abl and Trio specifically interact with GST-FraCYTO (lane 3), but not GST or beads (lanes 1 and 2). TrioΔSPR-myc is deleted for the spectrin-like repeats to optimize expression *in vitro*. (B and C) Trio and Abl interact directly via their SH3 domains. (B) GST-TrioSH3 (lane 2), but not GST (lane 1) specifically pulls down *in vitro*-translated Abl-myc. (C) GST-AblSH3 (lane 2), but not GST (lane 1) specifically pulls down *in vitro*-translated TrioΔSPR-myc. "In" represents ~2.5% of input incubated with GST or fusion protein. Target proteins were visualized by anti-myc staining, while fusion proteins (bottom gel) were visualized by anti-GST staining. Panels B and C were assembled from different lanes on the same gel. (D and E) Fra complexes via its cytoplasmic domain with Abl and Trio in S2 cells. (D) HA-tagged Fra (arrow, lanes 1 and 2, bottom gel) or FraΔCYTO, a Fra molecule lacking the intracellular domain, (arrowhead, lane 3, bottom gel) were co-expressed in S2 cells with Abl-myc (lanes 2 and 3, middle gel) and complexes were immunoprecipitated with anti-myc antibody. Fra-HA (arrow, top gel) coimmunoprecipitates only in the presence of Abl-myc (compare lanes 1 and 2). FraΔCYTO-HA (arrowhead indicates FraΔCYTO-HA's absence, top gel) does not coimmunoprecipitate with Abl-myc (compare lanes 2 and 3). When expressed in S2 cells, Abl-myc runs as a ~180-190kD doublet, similar to untagged Abl (D, compare to Fig. 4C, D). Additional low mobility bands in panel D, top gel, lanes 2 and 3 are background staining of Abl-myc, which migrates at nearly the same size as Fra-HA. (E) Fra-HA (lane 2) but not FraΔCYTO-HA (lane 3) coimmunoprecipitates with Trio-myc.

Continued on next page
Figure 3.4. Continued.
Figure 3.5. HA- and Myc-tagged fra transgenes rescue the loss-of-commissure phenotype in fra mutant embryos. UAS-fra transgenes were expressed in embryos under the control of the 1407-GAL4 driver, which expresses in all CNS neurons from stage 8-9 through the end of embryogenesis. (A-F), mAb BP102-stained embryos. (G,H), anti-HA stained embryos, (I), anti-myc stained embryo. (A,B) In fra mutant embryos, commissures are occasionally thin or missing (arrows), and the presence of the 1407-GAL4 driver does not significantly affect this phenotype (see also Table 3.10). (C) Expression of two copies of UAS-fraΔCYTO-HA by two copies of the 1407-GAL4 driver does not dominantly cause thin or missing commissures in wild-type embryos. Although this may be due to low expression level of this particular transgene, its expression level is equal to or slightly higher than a UAS-fra-HA transgene that rescues the fra phenotype (see D, E, G, and H, below). (D-F) Fra-HA (D) or Fra-myc (F) expression rescues the loss-of-commissure phenotype in fra mutant embryos. However, the breaks in longitudinal connectives observed in fra mutants are incompletely rescued by Fra-HA or Fra-myc (asterisks in A, D, and E). (E) Expression of UAS-fraΔCYTO-HA does not rescue commissure defects or longitudinal connective defects in fra mutants. (G-H) Epitope-tagged Fra proteins localize to commissural axons in vivo. Anti-HA signal in (G) and (H) is weaker than anti-Myc signal in (I) for two reasons: first, because six Myc epitopes are encoded at the C-terminus of Fra-myc, while only one HA epitope is encoded by UAS-fra-HA and UAS-fraΔCYTO-HA, and second, the driver used in (G) and (H) (ELAV-GAL4) expresses less robustly than the 1407-GAL4 driver (I) at similar stages of development. In all three cases, Fra protein appears to localize slightly less well to posterior commissures (PC) than anterior commissures (AC) (G-I). Full genotypes of (A) and (B) are indicated in the Figure. Full genotypes of embryos in other panels are as follows: (C) 1407-GAL4/1407-GAL4; UAS-fraΔCYTO-HA/ΔUAS-fraΔCYTO-HA, (D) fra4/1407-GAL4,fra3; UAS-fra-HA/+ (E) fra4/1407-GAL4,fra3; UAS-fraΔCYTO-HA/+ (F) fra4/1407-GAL4,fra3; UAS-fra-myc/+ (G) 1407-GAL4/+; UAS-fra-HA/+ (H) 1407-GAL4/+; UAS-fraΔCYTO-HA/+ (I) 1407-GAL4/+; UAS-fra-myc/+.

Continued on next page
Figure 3.5. Continued.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures</th>
<th>% segments with pathfinding errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>fra&lt;sup&gt;1&lt;/sup&gt;/fra&lt;sup&gt;4&lt;/sup&gt;</td>
<td>31 (213)</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/1407-G4,fra&lt;sup&gt;3&lt;/sup&gt;</td>
<td>38 (230)</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/1407-G4,fra&lt;sup&gt;3&lt;/sup&gt;; UAS-fra-HA</td>
<td>7.6 (236)</td>
<td>0</td>
<td>7.6</td>
</tr>
<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/1407-G4,fra&lt;sup&gt;3&lt;/sup&gt;; UAS-fra&lt;sup&gt;ΔCTTO&lt;/sup&gt;-HA</td>
<td>44 (144)</td>
<td>22</td>
<td>22</td>
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<tr>
<td>fra&lt;sup&gt;4&lt;/sup&gt;/1407-G4,fra&lt;sup&gt;3&lt;/sup&gt;; UAS-fra-myc</td>
<td>19 (127)</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>1407-G4/1407-G4; UAS-fra&lt;sup&gt;ΔCTTO&lt;/sup&gt;-HA/UAS-fra&lt;sup&gt;ΔCTTO&lt;/sup&gt;-HA</td>
<td>4.8 (124)</td>
<td>0</td>
<td>4.8</td>
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Table 3.10. Myc- and HA-tagged Fra transgenes rescue the thin/missing commissure phenotype in fra mutant embryos. HA- and Myc-tagged UAS-fra transgenes were expressed in CNS neurons using the panneural driver 1407-GAL4, which expresses in all neurons beginning at stage 8-9 and continuing through stage 17. HA-tagged fra also partially rescues pathfinding errors in the CNS. The UAS-fra<sup>ΔCTTO</sup>-HA transgene includes the transmembrane domain and 25 residues C-terminal to the transmembrane domain, but not the conserved P1 domain or sequences C-terminal to this domain. Although fra’s cytoplasmic domain is required for rescue, overexpressing two copies of UAS-fra<sup>ΔCTTO</sup>-HA in wild-type embryos with two copies of the 1407-GAL4 driver does not cause thinning or loss of commissural axons. Values were not rounded if total defect number was less than 15%.
Figure 3.6. Trio and Fra are tyrosine phosphorylated in S2 cells. (A and B)
Pervanadate treatment results in robust elevation of phosphotyrosine levels in both Trio and Fra proteins. S2 cells transiently expressing Trio-myc (A) or Fra-myc (B) were mock-treated with PBS (lane 1) or treated with pervanadate (lane 2) for 30 minutes. Target proteins were immunoprecipitated, and equivalent aliquots of immune complexes were resolved by SDS-PAGE. Blots were probed with either anti-phosphotyrosine (top gel) or anti-myc (bottom gel). (C and D) Tyrosine phosphorylation of both Trio (C, lane 2) and Fra (D, lanes 2 and 3) is elevated in the presence of increased Abl levels (top gel). S2 cells were co-transfected with 5 µg of pMET Trio-myc (C) or pMET Fra-myc (D) and 0, 2, or 5 µg of pMET Abl, as indicated. 24 hours after induction, target proteins were immunoprecipitated and resolved via SDS-PAGE. Blots were probed with anti-phosphotyrosine (top gel), then stripped and re-probed with anti-myc to verify equivalent loading of samples (middle gel). ~2% of total lysates used in each IP were resolved separately and elevated Abl level was verified with anti-Abl (bottom gel).
Figure 3.7. Potential mechanisms by which Abl, Trio, and Ena may function with Fra and other receptors during growth cone signaling. (A) Trio interacts directly with Fra, suggesting that Netrin-Fra signaling may recruit or modulate GTPase activity through Trio’s GEF domains. Abl also interacts directly with Fra, and may regulate Fra tyrosine phosphorylation directly or indirectly. Abl interacts biochemically with Trio, and thus may regulate Trio during Fra signaling. Ena does not interact directly with Fra, but genetic interactions suggest it may be a Fra effector, similar to orthologs in *C. elegans* and mouse. Abl, PKA, or some other molecule may link Fra to Ena function. Ena’s best understood activity is its ability to promote actin polymerization by inhibiting capping protein; in cultured mouse neurons, this results in filopodia extension, but Ena may also regulate Profilin, actin nucleation, and/or actin filament branching. Other *Drosophila* Abl substrates or signaling partners (Dlar, Orbit, Capulet, etc.) may also be regulated by Fra. Finally, loss-of-function genetic interactions suggest that Abl and Trio likely function downstream of at least one additional attractive receptor, whose identity is unknown. (B) Ena also interacts genetically and biochemically with the repulsive Robo receptor in *Drosophila*. It is thought that in this context (by analogy to observations in fibroblasts), Ena-induced actin polymerization is unproductive and leads to unstable lamellae, possibly due to the absence of bundling or branching proteins. In *fra* or *Abl* mutant backgrounds, reducing the dose of Ena may therefore compromise repulsive signaling, balancing the loss of attractive signaling by Fra. Alternatively, in the absence of Fra and/or Abl, Ena is simply mislocalized in the growth cone, as in other tissues in *Drosophila* (see text), leading to excessive actin polymerization at inappropriate subcellular locations. In this scenario, reducing Ena levels relieves these effects. Ultimately, each receptor’s complete repertoire of binding partners and their effects on cytoskeletal geometry will need to be identified before the genetic interactions between *fra, Abl, ena,* and *trio* can be fully understood.

Continued on next page
Figure 3.7. Continued.
CHAPTER 4

MOLECULAR GENETIC ANALYSIS OF THE REQUIREMENT FOR PROTEIN DOMAINS IN THE GUANOSINE EXCHANGE FACTOR TRIO DURING AXONOGENESIS IN THE DROSOPHILA EMBRYO

4.1. INTRODUCTION

In Drosophila, the guanosine exchange factor (GEF) Trio is a large, multidomain protein composed of an N-terminal Sec14p-like domain, a region of spectrin-like repeats, two GEF domains, and an SH3 domain positioned between the GEF domains. In Trio and its orthologs in worms, rats, mice, and humans, the GEF domains regulate cytoskeletal dynamics in migrating cells and extending axons by activating Rho-family GTPases, but the functions of Trio’s other domains are not as well understood. Since Trio and other GEFs likely play a crucial role in transducing signals from guidance receptors to the GTPases, a precise understanding of GEF function will contribute to a more complete understanding of the specificity of GTPase function in growth cones. In the Drosophila embryo, very few axons successfully cross the CNS midline in trio,Abl and fra, trio double mutants (Chapters 2&3), suggesting that trio plays an important role at this choice point. However, which of Trio’s individual domains are required for axons to cross the
midline is not known. In this Chapter, I present an analysis of the ability of a series of transgenic trio deletion constructs to rescue commissure formation in trio,Abl double mutant embryos. Interpreted together with phenotypes caused by overexpression of Trio deletion mutants in eye and wing imaginal discs and in cultured cells, the results of these experiments suggest that activation of Rac1, Rac2, and/or Mtl GTPases by TrioGEF1 is necessary for axons to cross the embryonic CNS midline, while the spectrin-like repeats and SH3 domain appear to negatively regulate GEF activity. The N-terminal and GEF2 domains are dispensible in some assays, but deletion of these domains affects cell spreading and signaling by the Robo-Fra chimeric receptor.

4.1.1. Regulators of GTPases function with axon guidance receptors

Axons are guided toward their targets by attractive, repulsive, and adhesive molecules that are either secreted into the extracellular environment or presented on the surface of cells along an axon’s trajectory (Tessier-Lavigne and Goodman, 1996). At the tip of a growing axon, a dynamic structure called the growth cone senses these cues through a repertoire of membrane-associated molecules (Dickson, 2002; Huber et al., 2003). For example, the vertebrate receptor Deleted-in-Colorectal-Cancer (DCC) and its homologs Frazzled (Fra) in Drosophila and UNC-40 in C. elegans mediate attraction toward sources of the secreted Netrin/UNC-6 family of ligands, while Roundabout (Robo)-family receptors in vertebrates, flies, and worms generally mediate growth cone repulsion away from cells that secrete its ligand, Slit (Guan and Rao, 2003). Cell adhesion molecules (CAMs) of the immunoglobulin and cadherin families and integrin adhesion receptors also modulate a growth cone’s trajectory by regulating axon-axon,
axon-cell, and axon-extracellular matrix adhesion (Nakamoto et al., 2004; Rougon and Hobert, 2003). Additionally, many recent reports have revealed cross-talk between attractive, repulsive, and adhesive signaling mechanisms, both at the receptor level and at points of convergence in downstream signaling cascades, suggesting that diverse extracellular signals must be integrated for proper axon pathfinding to occur (Huber et al., 2003; Nakamoto et al., 2004; Rougon and Hobert, 2003).

The detection of extracellular cues at the surface of the growth cone ultimately leads to changes in the dynamics and geometry of the actin- and microtubule-based cytoskeletons (Dent and Gertler, 2003). One extensively studied family of molecules that act as a point of convergence for multiple signaling pathways are the Rho GTPases, including Cdc42, Rac, and Rho. GTPases are molecular switches that are active when bound to GTP, but inactive when bound to GDP. This GTP- or GDP-bound status is regulated by three classes of molecules, GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), and GDIs (guanosine dissociation inhibitors) (Chapter 1 and Rossman et al., 2005). When active, GTPases modulate specific changes in the dynamics of the actin and microtubule cytoskeletons, such as polymerization and depolymerization rate, the geometry of polymerization (for example whether actin filaments are organized into parallel, bundled filaments or lattice-like meshworks), and polymer stability (Giniger, 2002; Meyer and Feldman, 2002; Raftopoulou and Hall, 2004). Generally, Cdc42 and Rac1 have been implicated in axon extension and growth cone attraction, while Rho is thought to function during axon retraction, collapse, and growth cone repulsion. However, mounting evidence suggests that the role of GTPases during axon guidance is considerably more complex than this oversimplified model (see
Chapter 1 and Giniger, 2002; Govek et al., 2005). Since GTPases are regulated by diverse upstream signals, it is necessary to understand the mechanisms by which individual GTPases are regulated during specific guidance decisions, and how activation of a GTPase can lead to different cytoskeletal outcomes by different receptors.

For example, at the *Drosophila* embryonic CNS midline, both dominant negative (DN) and constitutively active (CA) Rac1 and RhoA promote inappropriate axon crossing (Fan et al., 2003; Fritz and VanBerkm, 2002; Matsuura et al., 2004). Mutations in *rac1, rac2, and mil* interact genetically with *slit*, causing axons to inappropriately cross the midline, and genetic interactions between a constitutively active Robo receptor and DN Rac also support a positive role for Rac1 downstream of Robo signaling (Fan et al., 2003; Matsuura et al., 2004). In fact, activation of Robo in cell culture leads to Rac activation in a Dreadlocks/Nck1 and Pak (p21-activated kinase)-dependent manner (Fan et al., 2003). However, increasing or decreasing levels of *crossGAP*, a Rac-specific GAP, also enhances the *slit/robo* phenotype (Hu et al., 2005), confounding an interpretation of Rac’s role during Robo signaling at the midline. Heterozygosity for *robo1* in embryos expressing either DN or CA RhoA also enhances inappropriate axonal midline crossing (Fritz and VanBerkm, 2002). However, mutations in *gef64C*, a RhoA-specific GEF, interact genetically with mutations in the attractive Netrin receptor *fra*, leading to a loss of commissural pathways and implicating Rho GTPases in attraction at the midline (Bashaw et al., 2001). Similarly, mutations in *trio*, a Rac/Rho GEF, interact genetically with mutations in the *Abelson tyrosine kinase (Abl)* and *fra* genes, leading to a dramatic loss of commissural pathways and implicating Rac and/or Rho as positive regulators of
axon guidance across the midline boundary (Forsthoefel et al., 2005; Liebl et al., 2000). Taken together, these data support a role for Rac and Rho GTPases during both attraction and repulsion at the CNS midline.

The identification of signaling pathways from specific guidance receptors to GAPs, GEFs, and/or GDIs will undoubtedly increase our understanding of GTPase regulation. However, the specification of attraction or repulsion is likely to be determined not just by the collection of molecules recruited by a specific receptor, but also by the collaborative functions of multiple catalytic and regulatory domains within each effector molecule. For example, in the case of large, multidomain GEFs such as Trio, the selective activation of Trio’s N-terminal or C-terminal GEF domain may allow Trio to intricately control temporal and spatial changes in growth cone cytoskeletal dynamics. This possibility necessitates a more rigorous analysis of the contribution of individual domains in these molecules with respect to specific guidance decisions.

4.1.2. Trio is a multidomain GEF that links Rac and Rho activity

*Drosophila trio* encodes a molecule with an N-terminal Sec14p-like domain, a region of spectrin-like repeats, two GEF domains composed of paired DH (dbl homology) and PH (pleckstrin homology) domains, and an SH3 domain positioned between the GEF domains (Chapter 2, Fig. 4.9 and Liebl et al., 2000). In addition to commissure formation, dTrio regulates longitudinal and peripheral axon guidance in the embryo, as well as larval CNS organization and photoreceptor and mushroom body axon guidance in the larval brain (Awasaki et al., 2000; Bateman et al., 2000; Hakeda-Suzuki et al., 2002; Newsome et al., 2000).
C-terminally, vertebrate Trio encodes an additional catalytic domain, a serine-threonine kinase, preceded by a second SH3 domain and an immunoglobulin-like domain (Chapter 2 and Bateman and Van Vactor, 2001). In the mouse, mutation of trio results in disrupted skeletal muscle development and subtle defects in brain organization (O'Brien et al., 2000). Trio also functions during nerve growth factor (NGF)-stimulated neurite outgrowth in cultured PC12 cells (Estrach et al., 2002).

unc-73, dTrio’s C. elegans ortholog, encodes an Ig-like domain and a fibronectin-type repeat C-terminally, but N-terminally, UNC-73’s domain organization is identical to dTrio and hTrio (Bateman and Van Vactor, 2001). unc-73 functions during axon guidance by touch receptor neurons, motoneurons, and others, and during various cell migrations (reviewed in Steven et al., 1998 and see also Chapter 2 Discussion).

Similar to Kalirin, a related GEF, alternative neuronal-specific isoforms that encode N-terminally or C-terminally truncated versions of vertebrate trio have been detected in developing rat brains and embryonic and adult human and mouse brains, suggesting that trio is alternatively spliced for specialized functions in neurons (McPherson et al., 2005; Portales-Casamar et al., 2005). unc-73 is also alternatively spliced, but the exact distribution or functional significance of unc-73 isoforms is not well understood (Steven et al., 1998). Alternative splice variants of Drosophila trio have not been reported, and in northern blots of total RNA from embryos with full length or 3’UTR probes, the only prominent transcripts detected were ~8.0 Kb or larger (Fig. 2.5 and Bateman et al., 2000). However, several truncated trio isoforms are predicted in the latest Drosophila genome annotation (Release 4.2, www.flybase.org, Annotation ID CG18214, Gene ID FBgn0024277), so perhaps alternative trio isoforms are present at
later stages of development, or more sensitive methods of detection such as RACE or RT-PCR will be needed to detect these variants, as with vertebrate trio isoforms (McPherson et al., 2005; Portales-Casamar et al., 2005). A second prominent but truncated Trio protein was detected in western analysis of lysates from adult flies, and has been hypothesized to be an alternative splice variant, but further analysis of Drosophila trio alternative splicing has not been reported (Awasaki et al., 2000).

In vitro, vertebrate TrioGEF1 activates both Rac1 and RhoG; in vivo, GEF1 likely activates RhoG only, which in turn activates both Cdc42 and Rac1 (Bellanger et al., 1998; Blangy et al., 2000; Debant et al., 1996). In cultured fibroblasts, expression of GEF1 promotes cell spreading as well as miniruffling and lamellipodial extension at the leading edge, similar to expression of activated Rac1 and RhoG (Bellanger et al., 1998; Blangy et al., 2000; Seipel et al., 1999). Vertebrate TrioGEF2 activates RhoA in vitro, and in fibroblasts, promotes the formation of F-actin stress fibers (Bellanger et al., 1998; Debant et al., 1996; Seipel et al., 1999). In PC12 cells and neuroblastoma cells, expression of GEF1 alone promotes cell spreading, while GEF2 promotes retraction (Estrach et al., 2002). However, expression of full-length hTrio promotes the formation of long neurites in many cells, suggesting that the GEF domains likely cooperate with other domains in Trio to specifically yield neurite outgrowth in this cell type (Estrach et al., 2002). In the context of the full-length molecule, the spectrin-like repeats, the SH3 domain, and GEF1, but not GEF2, are required to promote neurite extension in PC12 cells (Estrach et al., 2002; Portales-Casamar et al., 2005). Trio also mediates neurite outgrowth promoted by NGF treatment, as expression of mutant Trio isoforms with point mutations in the GEF1 or SH3 domains, or expression of only the N-terminal and
spectrin repeat domains reduces the number of cells that elaborate NGF-induced neurites (Estrach et al., 2002). Deletion of Trio’s N-terminal domain, GEF2, second SH3 domain, Ig repeat, or the serine-threonine kinase domain does not interfere with NGF-induced neurite outgrowth (Estrach et al., 2002).

hTrio interacts with the actin filament-crosslinking protein filamin through its first PH domain, an interaction that appears to be necessary for TrioGEF1 to induce membrane ruffling (Bellanger et al., 2000). Trio’s C-terminal SH3 and kinase domains bind focal adhesion kinase (FAK), stimulating its autophosphorylation, and Trio’s kinase domain is phosphorylated by FAK, suggesting a bidirectional signaling complex that may be active in focal adhesions during cell spreading (Medley et al., 2003). Trio GEF1 also interacts with Tara, an F-actin binding molecule that is proposed to stabilize actin filaments during Trio activation and cell spreading (Seipel et al., 2001). Trio was originally isolated in a yeast-two hybrid screen for molecules that interact with the leukocyte antigen-related protein (LAR) receptor protein tyrosine phosphatase (RPTP) (Debant et al., 1996). LAR has since been implicated in neurite outgrowth in cultured cells and sciatic nerve regeneration in mice (Xie et al., 2001; Yang et al., 2003).

UNC-73 GEF1 also activates CED-10/Rac1 and MIG-2 in vitro, and when injected into Rat2 fibroblasts, promotes actin enrichment at the leading edge (Steven et al., 1998; Wu et al., 2002). Additionally, several rigorous genetic studies suggest that unc-73 functions collaboratively with ced-10/rac1, rac2/3, and mig-2 to regulate axon pathfinding as well as a number of cell migrations (Kishore and Sundaram, 2002; Lundquist et al., 2001; Struckhoff and Lundquist, 2003; Wu et al., 2002). In vitro experiments with UNC-73 GEF2 were unable to be conducted due to technical
difficulties in purifying this domain from bacteria, but this domain is similar to GEF2 in both vertebrate and *Drosophila trio* orthologs (~40% amino acid identity with *Drosophila trio* DH1, see Fig. 2.4), suggesting a conserved function in regulation of RhoA (Steven et al., 1998). Much less is known about the signaling pathways in which UNC-73 functions in *C. elegans*, but unc-73 interacts genetically with *unc-115/abLIM*, an actin-binding protein, and *ced-5/DOCK180*, a Rac activator, during neuronal cell migrations and axon guidance (Struckhoff and Lundquist, 2003; Wu et al., 2002).

*Drosophila* TrioGEF1 activates Rac1, Rac2, and Mig-two like (Mtl) *in vitro*, and promotes leading edge actin polymerization, miniruffling, and lamellipodia formation in rat fibroblasts (Newsome et al., 2000). RhoA activation was unable to be demonstrated for dTrioGEF2, but when expressed in rat fibroblasts, this domain induces stress fiber formation, consistent with observations with hTrioGEF2 (Bellanger et al., 1998; Newsome et al., 2000). Mutations in *trio* suppress the rough-eye phenotype caused by expression of *rac1*, and reciprocally, the rough eye phenotype generated by overexpressing the isolated GEF1 domain is suppressed by mutations in *rac1*, *rac2*, or *mtl* (Bateman et al., 2000; Hakeda-Suzuki et al., 2002). Similarly, expression of GEF1 in photoreceptors causes a dominant projection defect in which fewer axons enter the optic lobe of the larval brain from the eye imaginal disc, and those which do project abnormally (Newsome et al., 2000). This phenotype is exacerbated by coexpression of *rac1* and *mtl*, but not *rhoA* or *cdc42*, and is suppressed by mutations in *rac1* and *rac2* (Hakeda-Suzuki et al., 2002; Newsome et al., 2000). A similar phenotype was observed with a *trio* isoform deleted for both the N-terminal and spectrin domains, suggesting that GEF1 is constitutively active in the absence of these domains (Newsome et al., 2000).
GEF2 overexpression does not generate a similar photoreceptor projection defect, nor do mutations in trio suppress the rough eye phenotype caused by rhoA overexpression, a phenotype that has raised questions about the activity of GEF2, at least in photoreceptor axons and eye tissue (Bateman et al., 2000; Newsome et al., 2000). Similarly, in the developing brain, genetic interactions suggest that TrioGEF1, but not GEF2, may act through a Rac/Pak/LIM kinase pathway to negatively regulate Cofilin/Twinstar and inhibit axon growth in the mushroom body (Ng and Luo, 2004). trio interacts genetically with pak and dreadlocks/nck1 (an SH2-SH3 adaptor molecule) in photoreceptor axons, and with the Dlar RPTP in embryonic motoneurons (Bateman et al., 2000; Newsome et al., 2000). Functions of the SH3 domain in Drosophila Trio have not been reported.

Summarizing the data above, Drosophila TrioGEF1, like its counterparts in vertebrates and C. elegans, regulates Rac-like GTPases to promote actin polymerization at the leading edge and lamellipodia formation. While the spectrin-like repeats and SH3 domain are required for Trio-mediated, NGF-induced neurite outgrowth in PC12 cells, the N-terminal and spectrin repeats in Drosophila trio appear to be inhibitory, leading to constitutive activation of GEF1 when deleted. These observations may not be contradictory, since GEF1 hyperactivation leads to severe photoreceptor axon projection defects in Drosophila that are worse than loss of trio function (Newsome et al., 2000). In PC12 cells, expression of hTrioGEF1 only in PC12 cells caused spreading, at the expense of neurite formation (Estrach et al., 2002; Newsome et al., 2000). The authors did not report whether deletion of spectrin and SH3 domains in hTrio caused better spreading, but it would certainly be consistent with regulatory roles for these domains (Estrach et al.,
Drosophila GEF2 (like hTrioGEF2) promotes stress fiber formation in fibroblasts, but in vivo, experiments in the eye and brain have not revealed a role for this domain, nor has a role for GEF2 been identified in PC12/NGF experiments.

We have previously identified a role for trio during axon guidance at the CNS midline (Forsthoefel et al., 2005; Liebl et al., 2000). Our previous genetic and biochemical observations suggested that Trio functions with the Abl kinase and the attractive Fra receptor to guide axons across the midline. However, given the complex organization of the Trio molecule, we wished to further address how Trio’s different domains contribute to guidance at the midline. Since we were interested in how these domains function in the context of the entire molecule, we generated a collection of epitope-tagged isoforms that were deleted for each domain individually. We also deleted GEF domains in combination with the SH3 domain, since this domain’s function has not been well studied in any system. We expressed these isoforms in vivo in the CNS, in eye and wing tissue, and in cultured cells. Our results suggest that GEF1 is the most essential domain for commissure formation, and that GEF2 is indeed functional, promoting spreading in cultured cells, but inhibiting axon crossing at the midline downstream of Fra signaling. Additionally, our results suggest that TrioSH3 is inhibitory at the CNS midline, and that this domain regulates the function of GEF1 in the eye, and both GEF domains in cultured cells.
4.2. MATERIALS AND METHODS

4.2.1. Trio deletion constructs

For wild-type, Myc-tagged trio, C-terminal sequences in trio were amplified by PCR using a 3’ primer that encoded a BamHI site after the codon encoding L2258, and subcloned into pBluescript SK (Stratagene). A BamHI-NotI fragment of pCS2+MT (Rupp et al., 1994; Turner and Weintraub, 1994) encoding six Myc epitopes, stop codons, and an SV40 late polyadenylation site was then subcloned into this construct at trio’s 3’ end at the engineered BamHI site. Finally, a SalI (5951)-NotI fragment from this construct was then subcloned into pBluescript trio cDNA to generate the full-length, trio-myc ORF. This construct was used to make other trio deletion mutants with the exception of isoforms deleted for GEF2. For the GEF2 deletion, the BamHI site from pCS2+MT upstream of Myc sequences was filled in by Klenow polymerase and ligated to the filled-in NcoI site in trio (6098, ~100bp 5’ of the beginning of the GEF2 domain), such that the GEF2 deletion is simply missing all sequences 3’ of the NcoI site (GEF2 and ~100bp of linker sequence).

For the GEF1 deletion, sequences flanking the DH1/PH1 domains were amplified by PCR and subcloned into pBluescript, and subsequently subcloned into a full-length trio-myc construct. For the spectrin deletion, sequences 5’ of the first spectrin repeat were amplified by PCR and ligated to the EcoRV site (3942) that coincides with the 3’ end of the last spectrin repeat in full-length trio. Part of this construct was then cloned into trio-myc to generate spectrin-deleted trio-myc. For N-terminal and and SH3 deletions, short regions of trio were subcloned into pBluescript, and then PCR using primers flanking the
appropriate domain was initiated to amplify the entire plasmid with the exception of the N-terminal or SH3 domain; this PCR product was then religated to generate a plasmid containing the trio region deleted for each domain. Part of this region was then subcloned into trio-myc. For trioM89, sequences encoding the L1412F mutation were amplified using genomic DNA from trioM89/Df(3L)FpaI escaper pupae as a template, and then subcloned into full length trio-myc. trio isoforms containing multiple deletions were generated by subcloning relevant fragments from constructs containing individual domain deletions. All deletion junctions and amplified regions were sequenced to verify in-frame ligation and PCR fidelity. In most cases, constructs were first partially or completely made in pBluescript and/or pMET (pRmHa-3) (Bunch et al., 1988), and subsequently cloned into pUAST (Brand and Perrimon, 1993). In some cases, pMET and pUAST constructs were generated simultaneously, depending on the restriction sites utilized. Amino acid sequences deleted in each construct are as follows: N-terminal domain, Q4-A279; spectrin repeats, L285-D1199; GEF1, R1282-P1596; SH3, G1630-P1708; GEF2, G1919-K2263. Sequences numbered as in GenBank Accession AF21663. Further details on plasmid construction are available upon request.

4.2.2. Generation of transgenic lines, genetics, and immunohistochemistry

pUAST constructs were injected into Drosophila embryos with helper plasmid to generate germline transformants, and transgenic chromosomes were isolated using standard methods (Spradling, 1986). Other chromosomes used in this study include ELAVC155-GAL4 (Lin and Goodman, 1994); 1407-GAL4 (Luo et al., 1994); sca-GAL4
(Mlodzik et al., 1990); \textit{GMR-GAL4} (Sawado et al., 1998); \textit{71B-GAL4} and \textit{69B-GAL4} (Brand and Perrimon, 1993); \textit{Df(3L)Fpal,Abl} \textsuperscript{d} (Liebl et al., 2000); \textit{trio} \textsuperscript{IMP159.4,Abl} \textsuperscript{d} (Forsthoefel et al., 2005); \textit{UAS-robo-fra-myc} (Bashaw and Goodman, 1999).

Stocks were maintained on standard corn meal, sucrose, yeast, agar medium. All embryo collections were conducted at 25°C on standard apple juice plates supplemented with yeast paste. Chromosomes were maintained over \textit{lacZ} expressing balancers to allow immunohistochemical genotyping of embryos. 0-24 hour embryo collection, fixation, and antibody staining was conducted essentially as described (Patel et al., 1987). mAb BP102 (used at 1:20) and mAb 1D4/anti-FasII (used at 1:10) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Other antibodies used included mAb anti-c-myc (clone 9E10, Roche), 1:500; mAb anti-β-galactosidase (Promega), 1:500; and goat anti-mouse-HRP (Jackson), 1:500.

\textit{Drosophila} wings were dehydrated in isopropanol, and mounted in Permount (Fisher).

\textbf{4.2.3. Cell culture}

pMET (pRmHa-3) constructs encoding C-terminally Myc-tagged \textit{trio} deletion transgenes were transfected into 1-2 x 10\textsuperscript{7} \textit{Drosophila} S2 cells in serum-free Schneider’s \textit{Drosophila} media (Invitrogen/GibcoBRL) using CellFectin (Invitrogen) essentially according to the manufacturer’s protocol. Fetal bovine serum was added to 10% 24 hours after transfection, and expression was induced with 0.7mM copper sulfate at 48 hours. 16-20 hours later, 10\textsuperscript{6} cells were plated onto nitric acid-washed 22mm glass coverslips (in 6-well tissue culture dishes) that had been coated with with 1 mg/ml poly-D-lysine.
(Sigma) and ~7 µg/ml laminin (Becton Dickinson). After 4 hours, coverslips were gently rinsed once in 1XPBS to remove excess media and unattached cells, then fixed in 4%
paraformaldehyde/1X PBS for 20 minutes at room temperature. After 3 1XPBS washes, cells were permeabilized and blocked in S2 blocking buffer (0.1% BSA (Roche)/0.1%
Triton X-100/1X PBS) for 15 minutes. Cells were stained with TRITC-phalloidin
(Sigma) at 0.5 µg/ml, DAPI (Sigma) at 0.5 µg/ml, and mAb anti-c-myc 9E10 (Roche) at
1:500 (~1 µg/ml) in S2 blocking buffer for 1 hour at room temperature, followed by three
5 minute 1XPBS washes. Cells were then incubated with FITC donkey anti-mouse
(Jackson) at 1:200 (~8 µg/ml) in S2 blocking buffer for 30 minutes at room temperature.
Following 3 5 minute 1XPBS washes, coverslips were mounted on slides in Vectashield
(Vector Laboratories). Cell surface areas were quantified using Metavue software
(Universal Imaging).

4.3. RESULTS

4.3.1. Early expression of trio rescues the trio,Abl commissure phenotype

We have previously reported that expression of UAS-trio by the pre-mitotic,
panneural/panectodermal driver scabrous-GAL4 significantly rescued the
missing/defective commissure phenotype in trio,Abl double mutant embryos (Liebl et al.,
2000; Mlodzik et al., 1990). As a prerequisite to the current study, we wished to
determine which of a number of CNS GAL4 driver lines were best suited for driving trio
expression to rescue the trio,Abl CNS phenotype, and whether the C-terminal Myc tag
would interfere with Trio function in vivo.
In these experiments, we found that *sca-GAL4*-driven expression of *UAS-trio-myc* significantly rescued the thin/missing commissure phenotype in *trio,Abl* embryos, as in our previous study using an untagged *UAS-trio* transgene (Table 4.1 and Figs. 4.1-4.3) (Liebl et al., 2000). By contrast, *1407-GAL4*-driven expression of the same *UAS-trio-myc* transgene rescued the *trio,Abl* commissure phenotype to a much lesser degree (Figs. 4.1-4.3 and Table 4.1). In these embryos, there was a moderate rescue of the thin/missing commissure phenotype in individual anterior and posterior commissures (Table 4.1 and Fig. 4.3). However, since most segments had at least one thin or missing commissure or had other errors in midline guidance, the overall number of segments with defective commissures was not appreciably different compared with control crosses (Table 4.1). Finally, with the exception of a slight reduction in the number of thin/missing anterior commissures, *ELAVC155-GAL4*-driven expression of *UAS-trio-myc* was completely unable to rescue the CNS phenotype in *trio,Abl* mutants (Table 4.1 and Figs. 4.1-4.3).

The inability of other drivers to rescue the *trio,Abl* midline phenotype was initially surprising, as *1407-GAL4* has been used to rescue the commissure phenotype in *fra* mutant embryos, and an *ELAV-GAL4* promoter fusion construct is able to rescue motoneuron and longitudinal pathway defects in *trio* mutants (Bateman et al., 2000; Kolodziej et al., 1996). However, the *ELAV-GAL4* promoter fusion likely drives expression of higher protein levels than both the *ELAVC155-GAL4* and *1407-GAL4* enhancer trap drivers, since the *ELAV-GAL4* promoter fusion generates the strongest *Abl* gain-of-function phenotype in the ISNb motoneuron (Wills et al., 1999a). Furthermore, our analysis of transgenic Trio protein expression driven by the different enhancer trap
lines tested suggests that only $sca$-$GAL4$ turns on early enough to allow sufficient Trio accumulation by stage 12, when the earliest axons begin to extend across the CNS midline.

In $UAS$-$trio$-$myc$/$ELAV^{c155}$-$GAL4$ embryos, Trio-myc accumulation was detectable in a few neuronal cell bodies at stage 12, but not robustly in axons until stage 15 (Fig. 4.5 and 4.6). In $UAS$-$trio$-$myc$/$1407$-$GAL4$ embryos, Trio-myc expression was moderate in the CNS at stage 11, and axonal accumulation was high from stage 13 onward, consistent with $1407$-$GAL4$’s ability to drive Trio-myc expression sufficiently to slightly decrease the number of thin/missing commissures in trio,$Abl$ mutants (Fig. 4.5, Fig. 4.7, and Table 4.1). Early $sca$-$GAL4$-driven expression of $UAS$-$trio$-$myc$ was significantly stronger than the other two drivers. In whole-mount embryos, Trio-myc expression was high by stage 11 (the end of neuroblast formation, but prior to axon extension), and gradually decreased after stage 13 (Fig. 4.5). In dissected embryos, Trio-myc expression was so high at early stages that we were unable to distinguish axon-specific staining until stage 14 (Fig. 4.8). By stage 16, Trio-myc was only faintly visible in neuronal cell bodies, but axonal Trio-myc accumulation was still readily detectable, indicating significant perdurance of the transgene-derived protein (Fig. 4.8).

Thus, all three CNS GAL4 lines we tested drove $UAS$-$trio$-$myc$ expression sufficiently for it to accumulate in axons at later stages of CNS development, but at earlier stages, $sca$-$GAL4$-driven expression was the highest. Others have found that the drivers we used turn on slightly earlier than we have reported here. For example, robust β-gal accumulation driven by $1407$-$GAL4$ has been observed at stage 11 in neuroblasts (Luo et al., 1994), and $sca$-$GAL4$ has been reported to drive lacZ expression in
neuroepithelium at stage 8 (Mlodzik et al., 1990). With another transgene, \textit{UAS-fra-myc}, we observe significantly more protein accumulation than in \textit{UAS-trio-myc} embryos at early stage 12 in \textit{ELAV}^{C155} \textit{GAL4} embryos and at stage 11 in \textit{1407-GAL4} embryos (not shown). It seems likely, therefore, that the longer Trio ORF (~7.5 Kb, with Myc sequences) results in a delay in protein accumulation, which may necessitate strong, early expression for effective rescue of commissure defects.

In wild-type embryos, \textit{trio} mRNA begins accumulating in the developing CNS by stage 10; mRNA levels are high by stage 11, in germ-band extended embryos, and do not begin to decrease until around stage 15 or 16 (Fig. 2.9 and Bateman et al., 2000; Liebl et al., 2000). Trio protein accumulation in axons is quite high and readily detectable by late stage 12/early stage 13, when commissural axons first begin to extend, through stage 16, when the majority of commissures and longitudinal pathways have formed (Awasaki et al., 2000). The early requirement for Trio protein might indicate a role in neurogenesis; however the axonal localization of Trio in wild-type embryos argues against this (Awasaki et al., 2000). Additionally, in an initial experiment, there were no apparent differences in the number or relative position of Even-skipped positive neurons in \textit{trio,Abl} mutants (Fig. 4.4). We cannot rule out the possibility that \textit{sca-GAL4} drives expression in non-neuronal tissues that are required for commissure formation, for example midline glial cells. However, given that neither \textit{trio} mRNA nor Trio protein has been detected in midline cells, it does not seem likely that Trio expression in these cells would contribute significantly to rescue (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000). \textit{sca-GAL4} also drives significant expression in epidermal cells at stages 13-16 (Fig. 4.5). However, as epidermal cells have no known role during CNS
axon guidance, it is doubtful that this domain of *sca-GAL4*-driven expression contributes to rescue of the *trio,Abl* commissure phenotype either. Therefore, we conclude that *sca-GAL4* is most effective at rescue of commissure formation because it most closely recapitulates the endogenous pattern of Trio expression, particularly at early stages of CNS development.

In the course of our initial experiments, we also found that an untagged *UAS-trio* transgene, *UAS-trio*\textsuperscript{D29-1}, rescued commissure formation nearly completely in the *Df(3L)Fpal,Abl\textsuperscript{4}/trio\textsuperscript{IMP159.4},Abl\textsuperscript{4}* background, much better than Myc-tagged *trio* transgenes (Table 4.1, Figs. 4.1-4.3). However, when expression was driven by *1407-GAL4* rather than *sca-GAL4*, many axons crossed the midline, but in a disorganized fashion, supporting the idea that *sca-GAL4* is more effective at driving levels of *trio* expression appropriate for rescue (Table 4.1, Figs. 4.2 and 4.3). It is possible that the presence of a C-terminal Myc tag interferes with Trio’s localization, function, or accumulation. This may be a general effect, and not specific to any particular domain in Trio, since most Myc-tagged Trio deletion isoforms rescued the *trio,Abl* mutant phenotype as well as or better than wild-type Trio-myc (see below). Alternatively, the C-terminal position of the Myc tag may interfere specifically with GEF2 function. However, others have found that N-terminally Myc tagged mammalian Trio and Kalirin GEF1 and GEF2 domains were functional in cultured cells, and C-terminally Myc-tagged *Drosophila trio* transgenes rescue photoreceptor axon guidance in *trio* mutants as well as untagged transgenes (Blangy et al., 2000; Newsome et al., 2000; Penzes et al., 2001). Additionally, deletion of GEF2 has phenotypic consequences in cell culture (see Fig. 4.18), and deletion of GEF2 exacerbates an SH3-deletion phenotype in *Drosophila* eyes.
(preliminary results, see Discussion), suggesting that this domain is at least partially functional in Myc-tagged constructs. It is also possible that \textit{UAS-trio}^{D29-1} simply expresses at higher levels than our \textit{trio-myc} transgenes, but we were unable to verify this \textit{in vivo} since the publicly available monoclonal anti-Trio mAB 9.4A (Awasaki et al., 2000) does not recognize Trio in fixed embryos (data not shown). We are currently testing other untagged full-length \textit{trio} transgenes to determine whether they rescue as well as \textit{UAS-trio}^{D29-1}.

4.3.2. TrioGEF1 promotes commissure formation, while TrioSH3 is inhibitory

In order to further understand Trio’s role during axon guidance in the embryo, we constructed a series of transgenes deleted for each of Trio’s conserved domains (Fig. 4.9). These constructs were C-terminally Myc-tagged so that accumulation of the full-length proteins could be monitored \textit{in vivo}. Each construct was subcloned into pMET (pRmHa-3), permitting experiments in cultured cells, and pUAST, allowing the generation of transgenic \textit{Drosophila} lines (Brand and Perrimon, 1993; Bunch et al., 1988). In S2 cells, the stability of each Trio deletion isoform was verified (not shown). Subsequently, the ability of each Trio isoform to accumulate in embryonic CNS axons was confirmed by crossing independently-generated transgenic lines to \textit{ELAV^{Cl55}-GAL4}-carrying flies (Fig. 4.10). Interestingly, no single protein domain appears to be required for axonal localization of Trio (Fig. 4.10). In fact, deletion of all three C-terminal domains (GEF1, SH3, and GEF2) does not interfere with localization to axons (not shown). Therefore, both the N-terminal and spectrin-like repeats may collaboratively mediate Trio’s transport along axons; alternatively, linker regions in Trio might regulate axonal
localization. *In vivo,* expression levels of independently-generated Trio transgenes varied, likely due to position effects. Transgenes were arbitrarily grouped into zero, low, medium, or high categories based on intensity of anti-Myc staining in Stage 16 embryos (not shown). Unless otherwise noted, trio transgenes expressing at medium or high levels were used in the rescue and overexpression experiments in this study.

In trio homozygous mutants, commissure formation is unaffected (Chapter 2, Chapter 3, and Forsthoefel et al., 2005; Liebl et al., 2000). However, trio plays a crucial role in the guidance of axons across the midline choice point, because in trio,Abl double mutants, very few axons cross this important boundary (Fig. 4.1) (Liebl et al., 2000). Therefore, to analyze the contribution of trio’s individual conserved domains to commissure formation, we expressed trio deletion mutant transgenes in the trio,Abl background. For each deletion construct, we tested two independently-generated Myc-tagged transgenes in the trio,Abl (Df(3L)FpaI,Abl1/trioIMP159.4,Abl1) double mutant background using the sca-GAL4 driver (Table 4.2). As described above, wild-type trio rescued the CNS phenotype, increasing the percentage of intact anterior commissures by about 50%, and the percentage of intact posterior commissures between 40-60% (Table 4.2, Figs. 4.3, 4.11A&B, 4.13). In addition, in about 20% of segments, an approximately wild-type number of axons crossed the midline, but in a disorganized fashion (categorized as “fusions” or “errors”, see Table 4.1 legend), suggesting that Trio levels were sufficiently restored to allow growth cone attraction to the midline, but that signaling pathway(s) regulating more precise commissural axon organization (for example, bundling, sorting, fasciculation, or perhaps commissure choice) were disrupted (Table 4.2, Fig. 4.12).
The conserved N-terminal domain and the GEF2 domain were not required for axons to cross the CNS midline (Table 4.2, Figs. 4.11C, D, K, and L, and 4.12). Compared to the best wild-type transgene ($UAS\text{-}\text{trio}^{\text{R2-1b}}\text{-myc}$), the overall percentage of thin/missing commissures was at most 7% higher in embryos expressing either N-terminally- or GEF2-deleted trio (Table 4.2). In fact, deletion of the N-terminal domain led to a moderate (~10% of segments) improvement in anterior commissure rescue compared to wild-type Trio, possibly indicating a regulatory role for this domain, at least in the anterior commissure (Table 4.2). In the case of one N-terminally-deleted ($UAS\text{-}\text{trio}^{\Delta\text{NTM}}\text{-myc}^{J38}$) and one GEF2-deleted ($UAS\text{-}\text{trio}^{\Delta\text{GEF2}}\text{-myc}^{R1-4B}$) transgene, the percentage of disorganized commissures (fusions and errors) was slightly higher compared to both wild-type transgenes (Table 4.2 and Figure 4.12), suggesting that these domains might have subtle, but relatively dispensable roles during commissural axon organization.

$\text{trio}$ transgenes deleted for the spectrin-like repeats rescued anterior commissure formation as well as or better than wild-type $\text{trio}$ transgenes (Table 4.2, Fig. 4.11E&F, Fig. 4.13). The ability to rescue posterior commissure formation was intermediate between the two wild-type trio transgenes, making interpretation of this result difficult (Table 4.2, Fig. 4.13). In any case, if the spectrin-like repeats function to guide posterior axons across the midline, this role is a minor one. Like the N-terminally- and GEF2-deleted trio transgenes, a high percentage of segments (40-48%) had disorganized commissural pathways; in particular, the percentage of segments with pathfinding errors was higher for embryos expressing $UAS\text{-}\text{trio}^{\Delta\text{SPR}}\text{-myc}$ than for any other transgene (Table 4.2). Qualitatively, this disorganization seemed more severe than in embryos.
expressing other transgenes. Commissures were more likely to be loosely bundled, frequently small gaps could be seen within the commissures, and fewer axons (although not less than 25%, our criteria for scoring a commissure as thin) seemed to be present generally at the midline (Fig. 4.11E&F). Additionally, in some embryos expressing UAS-trioΔSPR-myc, the bundles of longitudinal axons within segments were more disorganized than in trio,Abl mutants and embryos expressing other transgenes (Fig. 4.11E&F). There were frequently “holes” in these longitudinal bundles, as axons appeared to be fasciculating around individual neuronal cell bodies, and a greater number of axons often seemed to extend laterally, away from the midline (Fig. 4.11E&F). Overall, greater than 70% of segments were defective in some way in embryos expressing UAS-trioΔSPR-myc, making this transgene the least efficient at overall commissure rescue with the exception of UAS-trioΔGEF1-myc (below). We should note here that sca-GAL4 driven expression of spectrin-deleted trio transgenes caused gross morphological defects in a percentage of wild-type and trio,Abl mutant embryos, likely due in part to defects in epidermal integrity (see Fig. 4.15). While we were careful to exclude embryos with any such detectable abnormalities from our analysis of commissure formation, we cannot rule out the possibility that some axon guidance defects observed may be secondary to defects in other cellular processes disrupted by spectrin-deleted Trio.

The presence of Trio’s GEF1 domain was most critical for commissure formation. One GEF1-deleted transgene, UAS-trioΔGEF1-mycR1-3, was completely unable to rescue axon crossing at the midline in either commissure (Table 4.2, Figs. 4.11G&H, Fig. 4.12, Fig. 4.13). Surprisingly, the other transgene, UAS-trioΔGEF1-mycR1-8, partially rescued
both anterior and posterior commissure formation, although not nearly as well as transgenes deleted for other domains (Table 4.2, Figs. 4.11-4.13). In these embryos, 50% of ACs and 76% of PCs were still thin or missing. Additionally, often only one or the other commissure in each segment was rescued, therefore overall either the AC or the PC was thin or missing in 84% of segments (Table 4.2). These results indicate that the GEF1 domain (and therefore activation of Rac1/Rac2/Mtl by Trio) is very important, but not absolutely required, for trio transgenes to rescue midline guidance in trio,Abl mutants.

By contrast to the other domains in trio, deletion of the SH3 domain resulted in a more efficient rescue of commissure formation compared to wild-type transgenes (Table 4.2, Figs. 4.11I&J, Figs. 4.12 & 4.13). Very few (5% or less) of segments had thin or missing commissures, compared to 25% of segments in embryos expressing the wild-type UAS-trio-mycR2-1b transgene (Table 4.2). As with N-terminal, spectrin, and GEF2 deletion mutants, though, a greater percentage of segments had disorganized commissures compared to rescue with wild-type transgenes (Table 4.2, Fig. 4.13).

Thus, the preliminary picture that emerges from our rescue experiments is as follows. GEF1 directly promotes axon crossing at the midline, since deletion of this domain severely impairs trio’s ability to rescue the thin/missing commissure phenotype. Trio’s other domains contribute more subtly to the organization (fasciculation, bundling, etc.) of axons within commissural bundles, since deletion of these domains does not significantly affect the ability to reduce the number of thin or missing commissures, but does lead to a modest increase in the percentage of segments with disorganized commissural pathways compared with wild-type transgenes. Additionally, Trio’s SH3
domain may somehow negatively regulate Trio function, since deletion of this domain leads to a greater reduction in thin or missing commissures than all other trio transgenes.

4.3.3. Misexpression of trio isoforms in the embryonic CNS causes infrequent defects in longitudinal axon pathfinding

Next, we asked whether expressing high levels of the trio deletion mutants in the CNS of wild-type embryos would lead to defects in axon pathfinding at the midline. In these experiments, we drove trio transgene expression with sca-GAL4, since it was most effective at rescue of defects in trio,Abl double mutants. [Laura A. Carver, an OSU Molecular Genetics graduate student, and Amer Jameel, an OSU Molecular Genetics undergraduate, conducted most of the genetic crosses listed in Table 4.3, and processed embryos for immunohistochemical visualization of axon tracts. Laura Carver also conducted the initial analysis of phenotypes. DJF dissected the majority of embryos and quantified specific defects.] In BP102-stained embryos, no trio deletion mutant causes thin or missing commissures, although overexpression of GEF1-deleted Trio causes slightly more disorganized commissures overall than other Trio isoforms (Fig. 4.14, Table 4.3). Thus, although commissures are severely disrupted in trio,Abl double mutants and deletion of the GEF1 domain severely impairs trio transgenes’ ability to rescue commissure formation (Table 4.2), commissural axons are insensitive to overexpression of GEF1-deleted trio (and all other deletions) in a wild-type background.

In these experiments, we also discovered that overexpression of spectrin-deleted trio with sca-GAL4 (but not 1407-GAL4 or ELAV$^{C155}$-GAL4, not shown) caused more general morphological defects in 40-60% of embryos, including protrusion of the gut
through the epidermis, and gaps, twisting, and constrictions of ventral cord tissue (Fig. 4.15). We excluded embryos with any such abnormalities from our analysis of CNS axon pathfinding defects. Deletion of trio’s N-terminal and spectrin repeats was previously hypothesized to cause constitutive activation of the GEF1 domain, since expression of Trio deleted for its N-terminal and spectrin repeats or expression of only the isolated GEF1 domain caused similar dominant phenotypes during photoreceptor axon guidance (Newsome et al., 2000). Therefore, since sca-GAL4 drives expression in ectodermal tissues (see Fig. 4.5 and Mlodzik et al., 1990), the morphological defects we observed in sca-GAL4 embryos could be caused by hyperactivation of Rac1, Rac2, or Mtl (GEF1 targets) in epidermal/epithelial tissues.

Overexpression of trio deletion mutants caused minor disruptions in longitudinal axon pathfinding (Fig. 4.14, Table 4.4). Expression of UAS-trioΔSH3-mycR1-7, which expressed at higher levels than any other SH3-deleted trio transgenes (not shown), with the sca-GAL4 driver causing the greatest increase in ectopic midline crossovers (5.4%, Fig. 4.14, Table 4.4). Expressing this same transgene with 1407-GAL4 did not cause midline crossing (Table 4.4). Simultaneously expressing two independently-generated SH3-deleted transgenes (that express at intermediate levels compared with UAS-trioΔSH3-mycR1-7, not shown) using sca-GAL4 caused only infrequent midline crossovers (Table 4.4). Expression of spectrin-deleted Trio with sca-GAL4 did not cause significant midline crossovers (Table 4.4), but we noticed that some axons appeared to cross the CNS midline in embryos that were excluded due to the presence of gross morphological defects (Fig. 4.14B). Therefore, we also expressed spectrin-deleted Trio with the 1407-GAL4 driver, which rescues commissure defects less effectively than sca-GAL4 (see
Table 4.1). In these embryos, a slightly higher percentage of segments (up to 3.9%) had midline crossovers, indicating that deletion of the spectrin repeats (similar to the SH3 domain) can also cause mild midline crossovers, albeit infrequently. Deletion of the N-terminal and GEF2 domains also led to midline crossovers, although at even lower frequency than in embryos expressing spectrin- or SH3-deleted trio. Overexpression of both GEF1- and spectrin-deleted trio also caused occasional breaks in the lateral longitudinal fascicle and fusions between longitudinal fascicles (Table 4.4). Previously, breaks in the lateral longitudinal fascicle have been observed in trio mutants or in embryos expressing dominant negative Rac1 (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000). The fact that overexpression of spectrin-deleted trio causes a similar phenotype, albeit infrequently, suggests that axons in the lateral fascicle may be exquisitely sensitive to either over- or under-activation of Rac1/Rac2/Mtl GTPases.

4.3.4. Expression of Trio isoforms disrupts eye and wing morphology

Rho GTPases function during development in many tissues, regulating cell adhesion, polarity, migration, and cell shape changes (Johndrow et al., 2004). In Drosophila, Rac and Rho GTPases have been shown to play a key role during wing and eye development (Bateman et al., 2000; Eaton et al., 1995; Eaton et al., 1996; Fanto et al., 2000; Hakeda-Suzuki et al., 2002; Hariharan et al., 1995; Hu et al., 2005). Mutations in trio cause wing posture and blistering phenotypes in homozygous adult escapers, suggesting a role for trio in wing formation (Bateman et al., 2000; Liebl et al., 2000). Similarly, heterozygosity for trio suppresses the rough eye phenotype caused by Rac1 overexpression, and homozygosity for rac1, rac2, or mtl GTPases suppresses the rough
eye phenotype caused by overexpression of the isolated TrioGEF1 domain (Bateman et al., 2000; Hakeda-Suzuki et al., 2002). We therefore overexpressed our collection of trio deletion mutants in the eye and wing in an attempt to glean further clues about the role of Trio’s various domains.

The wild-type Drosophila adult eye is organized into orthogonal arrays of ommatidia, inside of which are precisely arranged photoreceptor and support cells. This organization is disrupted by expression of Rac or Rho GTPases using the eye-specific promoter GMR, leading to a “rough” phenotype in which ommatidia are no longer organized into highly ordered rows (Bateman et al., 2000). Expression of SH3-deleted trio using GMR-GAL4 (Sawado et al., 1998) caused a slightly milder but completely penetrant rough-eye phenotype, in which all ommatidia seemed to be present, but ommatidial sizes varied slightly and the orthogonal rows of ommatidia were disrupted (Fig. 4.16). This phenotype was similar whether low-, medium-, or high-expressing trio transgenes were expressed (not shown). Expression of spectrin-deleted trio caused an even more severe phenotype. Firstly, many flies expressing spectrin-deleted trio failed to hatch; in fact, when highly-expressing transgenes were used, complete lethality was observed (not shown). For spectrin-deleted transgenes that expressed at lower levels, often very few or no ommatidia formed; only a flat, unpigmented membrane was visible where the eye should be, and the few ommatidia that did form were highly disorganized (Fig. 4.16). Occasionally, wild-type, N-terminally-deleted, GEF1-deleted, or GEF2-deleted trio caused a mild roughening of the eye, but only a portion of the eye was disorganized in these flies, and only some flies in each cross displayed a phenotype (not
shown). Additionally only highly-expressing transgenes yielded a phenotype, suggesting that these phenotypes were less specific than the SH3- and spectrin-deletion phenotypes.

The severe spectrin-deletion phenotype we observed in the eye is similar to that caused by expression of the isolated GEF1 domain (Hakeda-Suzuki et al., 2002), suggesting that at least in the eye, the spectrin repeats may negatively regulate GEF1 function. The fact that expression of the SH3 deletion mutant also causes rough eyes is a novel observation, and might indicate a GEF regulatory role for this domain as well. We are currently pursuing this idea further by expressing trio transgenes deleted for both the SH3 domain and individual GEF domains. In fact, in preliminary experiments, deletion of GEF1 and SH3 together almost completely abolishes the rough eye phenotype, while deletion of SH3 and GEF2 together seems to exacerbate the phenotype in a percentage of flies (data not shown). While we must test more lines, these results may suggest that not only does SH3 regulate GEF1, but there may be a “balance” of activity between GEF1 and GEF2 activity in eye tissue.

We also expressed the trio deletion mutants in the wing imaginal disc using the driver 71B-GAL4 (Brand and Perrimon, 1993), and the driver 69B-GAL4, which expresses in the wing, eye-antennal, and leg imaginal discs, as well as in embryonic ectoderm (Brand and Perrimon, 1993; Staehling-Hampton et al., 1994; Wilder and Perrimon, 1995). [Laura A. Carver conducted a majority of the crosses to wing drivers, mounted wings for microscopy, and captured many of the images in Fig. 4.17.] Expression of wild-type (untagged) or Myc-tagged trio caused minor defects in wing morphogenesis, including thin or missing cross veins, or occasional blistering or failure of the wings to unfold after hatching (Fig. 4.17A, B, J-L). In general, these defects were
more penetrant with the $69B$-$GAL4$ driver, or with transgenes that expressed at high levels (not shown). Wing phenotypes caused by expression of GEF2-deleted trio were not noticeably different from those in flies expressing wild-type trio (Fig. 4.17I, Q, R). Phenotypes in flies expressing N-terminally deleted trio with $71B$-$GAL4$ were also similar to wild-type and GEF2-deletion phenotypes (Fig. 4.17C). However, $69B$-$GAL4$-driven expression of N-terminally deleted trio led to the appearance of dark “spots” on the wings of some flies, in addition to the other phenotypes just mentioned (Fig. 4.17M). Expression of GEF1-deleted trio generated a very specific phenotype with both drivers that was not observed with any other trio deletion: a “Y-shaped” second cross vein in which the anterior (or occasionally, the posterior) portion of the cross vein extended a second “branch” (Fig. 4.17D, E, N). This phenotype varied in penetrance, but was generally more frequent with highly-expressing transgenes and with $69B$-$GAL4$. It also varied in expressivity, since in some flies the “Y” branch was incomplete, and did not extend all the way to longitudinal vein IV. Interestingly, flies expressing $UAS$-$trio^{M89}$-myc, the trio allele originally identified as a dominant enhancer of Abl semilethality, displayed a similar phenotype, although it was generally less penetrant and expressive than in flies expressing $UAS$-$trio^{ΔGEF1}$-myc (Fig. 4.17O, P and data not shown). Since the M89 allele encodes a missense mutation in the GEF1 domain, the specificity of the GEF1-deletion and M89 wing phenotypes suggests that deletion of GEF1 specifically disrupts the activation of Rac-like GTPases, and does not disrupt the function of Trio’s other domains. Additionally, the lower frequency and expressivity of wing phenotypes caused by expression of trio$^{M89}$ suggests that this allele only partially inactivates GEF1.
Expression of spectrin-deleted trio was lethal when expressed with either driver, and even with transgenes that expressed at low levels. Expression of SH3-deleted trio was also lethal with the 69B-GAL4 driver (multiple transgenes were tested, not shown). With 71B-GAL4, SH3-deletion phenotypes were similar to phenotypes observed in embryos expressing wild-type, N-terminally deleted, or GEF2-deleted trio (Fig. 4.17F, G, H). However, phenotypes were more penetrant and expressive, since cross veins were more likely to be completely missing than partially missing, and longitudinal vein thickening, wing blisters, and failure to unfold were observed more frequently than for any other genotype. The reason for the lethality caused by 69B-GAL4-driven expression of SH3- and spectrin-deleted trio isoforms is likely due in part to the cumulative effects of expression of these transgenes in multiple tissues (Staehling-Hampton et al., 1994; Wilder and Perrimon, 1995). Indeed, with some spectrin-deleted transgenes, not even larval activity was observed in crosses with homozygous stocks (not shown). As in the eye, the phenotypes in the wing suggest that deletion of Trio’s SH3 domain may also lead to activation of GEF domain(s). We are currently testing this idea by expressing transgenes deleted for both SH3 and either GEF1 or GEF2 in the wing. If the phenotypes in the wing are due to elevation of GEF1 activity, for example, and not the activity of some other domain in Trio (such as the N-terminal domain or spectrin repeats) the “Y-shaped” posterior cross vein phenotype observed in flies expressing GEF1-deleted trio would be expected to be epistatic to the phenotypes in flies expressing other trio isoforms.
4.3.5. Trio’s SH3 domain may regulate both GEF1 and GEF2 during lamellipodial extension and cell spreading

In mammalian fibroblasts, expression of the N-terminal GEF domain of hTrio promotes cell spreading and the extension of lamellipodia, while expression of the C-terminal GEF domain causes the formation of stress fibers (Bellanger et al., 1998; Seipel et al., 1999). Similarly, expression of dTrio GEF1 in rat fibroblasts promotes actin polymerization at the membrane, inducing the formation of lamellae and miniruffles, while dTrio GEF2 promotes stress fiber formation (Newsome et al., 2000). In PC12 cells expressing full-length hTrio, GEF2 and other C-terminal sequences are not required for neurite formation, but deletion or mutation of the spectrin repeats, SH3 domain, or GEF1 domain interferes with outgrowth (Estrach et al., 2002). Overexpression of *Drosophila trio* deleted for the N-terminal domain and spectrin repeats is believed to generate a constitutively active Trio molecule, since defects in photoreceptor axon guidance caused by expression of this isoform can be phenocopied by expression of the isolated GEF1 (but not GEF2) domain (Newsome et al., 2000). However, the contribution of N-terminal, spectrin, as well as SH3 domains in the context of full-length dTrio have not been reported in cultured cells. Accordingly, we expressed our collection of Trio isoforms in cultured *Drosophila* cells to complement our *in vivo* studies.

Untransfected S2 cells spread well on polylysine/laminin-coated coverslips. After 24h, nearly all cells spread broad, actin-rich lamellae (not shown) similar to previous reports using other substrates such as serum or concanavalin-A (Kunda et al., 2003; Rogers et al., 2003). We wished to analyze whether Trio isoforms would promote or inhibit spreading, but at 24h, we detected little difference between cells expressing
different Trio isoforms (not shown). We therefore explored the effect of expressing Trio deletion mutants at earlier timepoints. At 1h, cells had only just begun to adhere to the substrate and spread; few cells had lamellae, and many displayed filopodia-like extensions (not shown). However, there were only subtle differences between cells expressing different Trio mutants (not shown). At 4h, differences were much more pronounced and detectable by even a cursory perusal of the cells (Fig. 4.18).

Surprisingly, expression of wild-type Trio inhibited spreading. Not all untransfected cells extended lamellae, but Trio-myc expression seemed to interfere with leading edge protrusion in a percentage of cells (Fig. 4.18A). In others, lamellipodial extension occurred normally (Fig. 4.18B). In these cells, Trio-myc localized at intermediate levels to lamellipodia, as well as the F-actin “ribs” in these lamellae (Fig. 4.18B). Overall, average surface area was 28% lower for cells expressing Trio than untransfected cells (253±8 µm² vs. 198±11 µm², Fig. 4.18F).

Consistent with in vivo observations made by others (Newsome et al., 2000), deletion of trio’s N-terminal domain, and especially its spectrin repeats, led to increases in surface area compared to cells expressing wild-type Trio (Fig. 4.18C and data not shown). In these cells, a higher percentage of cells extended lamellae, and in cells expressing TrioΔSPR-myc, these lamellae were generally wider with respect to the radius of the cell than in cells expressing any other Trio isoform (Fig. 4.18C). The average surface area for cells expressing N-terminally deleted Trio was similar to untransfected cells, but 35% higher (268±15 µm²) than cells expressing wild-type Trio (Fig. 4.18F).
Expression of spectrin-deleted Trio caused an even greater 57% increase in surface area (311±17 µm²) compared to cells expressing wild-type Trio; many cells spread 500 µm² or more.

We also found that deletion of Trio’s SH3 domain allowed more efficient spreading than in cells expressing wild-type Trio (Fig. 4.18D,F). TrioΔSH3-myc-expressing cells frequently extended broad lamellipodia (Fig. 4.18D), but even cells that did not were often similarly or slightly better spread than untransfected cells. Overall, deletion of TrioSH3 led to a 32% increase in average surface area (263±14 µm²) compared to wild-type Trio-expressing cells.

Deletion of TrioGEF1 or TrioGEF2 alone did not appear to dramatically affect surface area (Fig. 4.18F). Interestingly, though, deletion of either of these domains together with the SH3 domain almost completely abolished the efficient spreading observed when only the SH3 domain was deleted (Fig. 4.18F). This suggests that both GEF1 and GEF2 function during leading edge protrusion, and additionally, that the SH3 domain may regulate the activity of both GEF domains. Expression of TrioΔGEF1+ΔSH3-myc or TrioΔSH3+ΔGEF2-myc did not completely eliminate lamellipodial extension, but did reduce its frequency (Fig. 4.18E and data not shown). Interestingly, TrioΔGEF1+ΔSH3-myc was the only Trio isoform we analyzed that seemed to mislocalize in cells relative to full-length Trio (Fig. 4.18E, compare to Figs. 4.18A and B). In many cells, TrioΔGEF1+ΔSH3-myc levels appeared to be reduced in lamellipodia, and enriched in a “ring” either at the central region of the lamellipodia, or cortically at the membrane, in regions above the plane of lamellae that do not directly contact the substrate (Fig. 4.18E). This phenotype may suggest that both SH3 and GEF1
domains collaborate to localize Trio in lamellae. Alternatively, Trio may be relocalized away from lamellae as a secondary consequence of changes in cytoskeletal dynamics caused by overactivation of GEF2 in the absence of GEF1 co-activation and SH3 regulation. We did not find any differences in TrioΔSH3+ΔGEF2-myc localization compared with other Trio isoforms (not shown).

In parallel experiments, we transfected a number of other Drosophila cell lines including S2R+ (Yanagawa et al., 1998), as well as embryonic cell lines 4875-8, UC13-1, 5697-8, and UC88-8, which displayed various filopodial and/or lamellipodial morphologies (not shown) (Simcox et al., 1985). Of these lines, Amer Jameel (an OSU Molecular Genetics undergraduate) and Laura Carver (an OSU Molecular Genetics graduate student) found that 4875-8 cells were less efficiently transfected than S2 cells, but behaved similarly to S2 cells, in that SH3 and spectrin-deleted Trio promoted cell spreading and lamellipodia extension after 4-24 hours (not shown). However, in some 4875-8 cells expressing spectrin-deleted Trio, vacuole-like spaces were evident in the cytoplasm, and nuclear morphology was complex, indicating potential defects in cytokinesis (not shown). 4875-8 cells spread equally well (but more slowly than S2 cells) on plain glass as on polylysine/laminin or serum-coated coverslips (not shown). UC88-8 and S2R+ cells were efficiently transfected (respectively), but when expressing spectrin-deleted trio, these cells did not extend obvious lamellae, although they did seem to increase slightly in surface area (not shown). Untransfected S2R+ cells on polylysine/laminin were also more variable in their ability to spread as a population; many cells did not extend lamellae, even after 24 hours (not shown). We also noticed an increase in the percentage of cells with complex nuclear morphology in UC88-8 and
S2R+ cells, suggesting that their increased size might be due partially to defects in cytokinesis (not shown). UC13-1 and 5697-8 displayed extremely low transfection efficiencies (<0.1%), making them unsuitable for analysis (not shown).

4.3.6. Expression of trio deletion isoforms modifies the Robo-Frazzled chimeric receptor phenotype

Previously, we demonstrated that Trio interacts biochemically with the attractive Netrin receptor Frazzled/DCC, and that mutations in trio suppress the inappropriate midline crossing phenotype in axons expressing the chimeric Robo-Frazzled receptor (Chapter 3 and Forsthoefel et al., 2005). Our results in this study suggest that TrioGEF1 may promote midline crossing by axons, while TrioSH3 may negatively regulate Trio-mediated midline crossing. One hypothesis is that attractive signaling by the Fra cytoplasmic domain may recruit or activate TrioGEF1 in order to modulate Rac1, Rac2, or Mtl activity in growth cones. If this is the case, then coexpression of trio deletion transgenes would be expected to genetically modify the Robo-Fra chimeric receptor phenotype.

To test this idea, we used the sca-GAL4 driver that most effectively rescued the commissure phenotype in trio,Abl mutants (Fig. 4.1). In embryos expressing only the Robo-Fra receptor, bundles of FasII-positive axons crossed the midline; these axons never cross the midline in wild-type embryos (Fig 4.19A-B). We scored crossovers by generating a “crossovers per segment” score, and also by calculating penetrance and expressivity (Table 4.5). In embryos expressing Robo-Fra alone, there were 0.28 crossovers per segment, and 58% of embryos were affected, with an average of 5.5
crossovers per affected embryo. This phenotype was milder than the Robo-Fra phenotype we reported in an earlier study (Chapter 3 and Forsthoefel et al., 2005). Here we chose to use an independently-generated \textit{UAS-robo-fra} transgene (Bashaw and Goodman, 1999) that expressed at significantly lower levels so that we could monitor increases as well as decreases in the severity of the phenotype with the strong, early \textit{sca-GAL4} driver. With \textit{ELAVC155-GAL4} (the neuronal driver used in Chapter 3 with the chimeric receptor), the transgenic \textit{UAS-robo-fra} chromosome used in this study (“3.3”) causes a negligible number of crossovers (not shown).

In embryos coexpressing wild-type \textit{trio-myc}, the crossovers per segment score increased only slightly to 0.36 (Table 4.5). 10% fewer embryos were affected, but crossovers were far more frequent and severe in these embryos (8.8 crossovers/embryo, Figure 4.19, C-E and Table 4.5). In fact, in 12% (n=25) of embryos analyzed (25% of affected embryos), axons completely collapsed onto the midline, a phenotype that was never observed in embryos expressing Robo-Fra alone. This phenotype was scored as one crossover in each segment that it occurred.

In embryos expressing SH3-deleted \textit{trio}, the Robo-Fra phenotype was even more dramatically enhanced (Figure 4.19, I-K and Table 4.5). A higher percentage of embryos had crossover phenotypes, and in these embryos, more crossovers were present (10.7 crossovers/embryo, see Table 4.5). Overall, there were 0.56 crossovers/segment. Additionally, phenotypes were qualitatively more severe, since 21% (n=29) of embryos (33% of affected embryos) displayed the “collapse” phenotype.

By contrast to the enhancement observed with coexpression of wild-type or SH3-deleted \textit{trio}, coexpression of GEF1-deleted \textit{trio} caused a mild reduction in the number of
crossovers at the midline compared with embryos expressing only Robo-Fra (Table 4.5, Fig. 4.19H). More illuminating is a comparison of the effect of coexpressing GEF1-deleted trio with the phenotype in embryos coexpressing wild-type trio. Although coexpression of GEF1-deleted trio affected 10% more embryos than wild-type trio, there were almost half as many crossovers per embryo (Table 4.5). Furthermore, only one of 24 embryos scored (4%) displayed the “collapse” phenotype. GEF1 is therefore required in order for trio coexpression to enhance the Robo-Fra chimeric receptor phenotype, an observation that is consistent with a role for TrioGEF1 regulation downstream of signaling by the Fra cytoplasmic domain.

Coexpression of N-terminally-deleted or GEF2-deleted Trio resulted in unexpected phenotypes, as compared to these isoforms’ ability to rescue commissure defects in trio,Abl mutants (Table 4.2). N-terminally-deleted Trio neither enhanced nor suppressed the Robo-Fra phenotype (Fig. 4.19F, Table 4.5), and the collapse phenotype was observed in only 8% of embryos (12% of affected embryos). This suggests that although this domain is not required for commissure formation (at least in the trio,Abl mutant background, Table 4.2), the N-terminal domain may nonetheless be important for Trio-mediated attraction downstream of Fra. By contrast to wild-type Trio, deletion of GEF2 enhanced penetrance (in addition to expressivity) of midline crossovers when coexpressed with Robo-Fra (Fig. 4.19L, Table 4.5). In addition, the GEF2 deletion mutant caused the greatest increase in frequency of the collapse phenotype, which was observed in 23% of scored embryos (35% of affected embryos). Thus, although deletion
of GEF2 did not affect Trio’s ability to rescue commissure formation (Table 4.2), GEF2 activity appears to inhibit midline crossing as significantly as the SH3 domain in embryos coexpressing Robo-Fra.

We also coexpressed spectrin-deleted trio with Robo-Fra, and after discarding embryos with morphological defects, we found that while the penetrance of the crossover phenotype was similar to other genotypes tested, there were only 1.5 crossovers per embryo (Table 4.5 and Fig. 4.19G). It is possible that the presence of spectrin-deleted trio might suppress the Robo-Fra phenotype by inappropriately (subcellularly and/or temporally) overactivating Rac1, Rac2, and/or Mtl, analagous to the defect in axon extension caused by expression of activated Rac1 in peripheral neurons (Kim et al., 2003; Luo et al., 1994). However, we suspect that the number of defects scored for spectrin-deleted Trio are artificially low, since some embryos that were excluded from analysis due to morphological defects appeared to display midline crossovers (although in some cases the ventral nerve cord was very misshapen, not shown). We attempted to address this issue further by coexpressing the same UAS-robo-fra and trio deletion transgenes with the 1407-GAL4 driver, which generates a midline crossing phenotype similar to embryos expressing Robo-Fra only with sca-GAL4 (not shown). Surprisingly, in these embryos, coexpression of wild-type Trio did not enhance or suppress the Robo-Fra phenotype (not shown), and furthermore, spectrin-deleted, SH3-deleted, and GEF1-deleted trio all enhanced the number of midline crossovers by about 50% compared to embryos expressing Robo-Fra only (~0.3 crossovers vs. ~0.45 crossovers per segment, Table 4.5). However, the “collapse” phenotype observed in sca-GAL4 embryos with coexpression of wild-type or SH3-deleted trio was never observed, again supporting the
idea that for genetic analysis of trio function, 1407-GAL4 is a less suitable driver. The fact that no differences were observed between these different Trio deletion isoforms using 1407-GAL4 is also perplexing. One explanation is that multiple domains in trio collaborate with Fra to drive axons across the midline, but if this were the case, then we would expect wild-type trio to enhance the Robo-Fra phenotype as well as or better than the deletion mutants with 1407-GAL4. An alternative explanation is that since 1407-GAL4 did not rescue trio,Abl commissure defects nearly as efficiently as sca-GAL4 (Fig. 4.1), the Robo-Fra coexpression results with 1407-GAL4 may reflect non-specific cytoskeletal disruption caused by expressing trio isoforms at inappropriate times or levels during CNS development. In any case, we are unable to further address the role of the spectrin repeats in the context of signaling by the Robo-Fra chimeric receptor, since embryos with gross morphological defects had to be excluded from our analysis.

In the case of the other trio deletions, however, the robo-fra/sca-GAL4 coexpression results are consistent with the idea that Fra may recruit Trio to regulate Rac1, Rac2, and/or Mtl, and that the SH3 domain somehow regulates TrioGEF1 activity at the CNS midline. If this is the case, then deletion of GEF1 would also be expected to suppress the dramatic enhancement of the Robo-Fra phenotype by SH3-deleted Trio, similar to the results we observed in cells. In addition, GEF1 deletion might also be expected to suppress enhancement by TrioΔGEF2, which would support the idea that GEF2 activity may somehow “balance” GEF1 function, at least downstream of signaling by Fra. Experiments to test these ideas are underway using trio transgenes deleted for multiple domains.
4.4. DISCUSSION

A major goal of ongoing investigations in the axon guidance field is to identify the signaling pathways used by the many guidance receptors to regulate cytoskeletal dynamics, and ultimately to understand how these changes lead to directional axon extension. Previously, we identified *Drosophila trio* as a genetic enhancer of mutations in the *Abelson tyrosine kinase* gene, implicating trio in development of central nervous system axonal architecture (Chapter 2 and Liebl et al., 2000). Subsequently, we discovered that Trio, currently the only Rac-specific GEF known to function during *Drosophila* axon guidance, interacted genetically and biochemically with the Netrin receptor Frazzled, reinforcing the idea that Trio functions at the important CNS midline choice point (Chapter 3 and Forsthoefel et al., 2005). In the current study, we have begun a molecular dissection of the contribution of Trio’s multiple domains during embryonic axon guidance and leading edge extension in cultured cells (Table 4.6). Our results have lead to some novel and intriguing insights into how Trio’s domains collaboratively regulate axon pathfinding at the CNS midline.

4.4.1. *Drosophila Trio: Two catalytic domains and three regulatory domains*

We found that the Rac-specific GEF1 domain was most critical for commissure formation in *trio,Abl* mutants. GEF1-deleted *trio*, unlike wild-type *trio*, is also unable to enhance the inappropriate midline crossover phenotype in embryos coexpressing the chimeric Robo-Fra receptor. Furthermore, co-deletion of GEF1 inhibits the efficient spreading in cultured cells promoted by deletion of the SH3 domain, and in preliminary
experiments (not shown), co-deletion of GEF1 also completely suppresses the rough-eye phenotype generated by overexpression of SH3-deletion mutants. We must still test double deletion mutants’ ability to rescue commissure formation or suppress the midline crossing phenotype in embryos coexpressing Robo-Fra and TrioΔSH3. But up to this point, our results suggest that GEF1 is the domain responsible for promoting axon guidance across the CNS midline, and that this domain may be recruited or activated in response to signaling by the Fra receptor.

TrioGEF1 has been shown to activate Rac1, Rac2, and Mtl in vitro, and mutations in the genes encoding these GTPases suppress the rough eye phenotype in flies expressing the isolated GEF1 domain (Hakeda-Suzuki et al., 2002; Newsome et al., 2000). Thus, our results imply a role for Rac-like GTPases in the promotion of axon crossing at the CNS midline. While constitutively active Rac1 has been shown to promote inappropriate midline crossing, so has dominant negative Rac1 (Fan et al., 2003; Fritz and VanBerkum, 2002; Matsuura et al., 2004). Furthermore, mutations in rac enhance slit/robo mutant phenotypes, and Rac has been shown to be activated downstream of Robo, Pak, and Dock in response to Slit signaling (Fan et al., 2003). Our results suggest a mechanism by which Fra/DCC might activate Rac GTPases, but future experiments must clarify how these GTPases function downstream of receptors that signal opposite outputs, attraction and repulsion.

Furthermore, our results potentially indicate differences between signaling by Drosophila Fra and its homologs in C. elegans and vertebrate species. Mutations in ced-10/rac1, but not unc-73/trio suppress an unc-40 gain-of-function phenotype in the nervous system of the worm; currently, no GEF has been identified as an UNC-40
signaling partner (Gitai et al., 2003). In vertebrates, Rac1 is activated by Netrin-DCC signaling, and this activation is required for growth cone turning to occur in response to Netrin signaling (Li et al., 2002a; Li et al., 2002b; Meriane et al., 2004; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005) Recent evidence suggests that Rac1 may be regulated by Cdc42 downstream of DCC signaling, since expression of DN Cdc42 inhibits Rac1 activation, but not vice versa (Shekarabi et al., 2005). However, it is important to mention that no vertebrate GEFs, GAPs, or GDIs that function during DCC signaling have been identified. In a similar vein, it is interesting to note that the tyrosine residue in DCC targeted by Src/Fyn kinases is not conserved in worms or flies (Li et al., 2004; Meriane et al., 2004). Differences in signaling between Fra, Unc-40, and DCC will be fascinating to unravel and will provide valuable insights into the evolution of guidance signaling pathways and the various mechanisms that can be used to achieve similar growth cone behaviors.

GEF2, Trio’s other catalytic domain, has not been shown to activate Rho GTPases in vitro, but this domain can induce the formation of stress fibers in rat fibroblasts, suggesting that GEF2’s role in dTrio is similar to that in hTrio (Newsome et al., 2000). In previous studies, GEF2 was not required during photoreceptor axon guidance, and mutations in trio suppress the rough eye phenotype caused by overexpression of rac1, but not rhoA (Bateman et al., 2000; Newsome et al., 2000). These results suggested that at least in these tissues, GEF2 is either not functional or not regulated by upstream signaling pathways. However, in preliminary results (not shown), we have found that deletion of TrioGEF2 in combination with the SH3 domain exacerbates the rough eye phenotype observed in flies expressing SH3-deleted Trio. This indicates that this domain is indeed
active in the eye, and its activity may be required to somehow “balance” that of GEF1, since deletion of GEF1, SH3, and GEF2 together completely abolishes the rough eye phenotype (preliminary results, not shown).

Additionally, we also found that although GEF2 was not required for rescue of commissure formation, compared to wild-type Trio, GEF2-deleted Trio enhanced the midline collapse phenotype in embryos expressing the Robo-Fra chimeric receptor, indicating that GEF2 activity may oppose GEF1 function, at least downstream of Fra signaling. However, in spreading cells expressing SH3-deleted Trio, codeletion of GEF2 dramatically reduced average cell surface area, implying that GEF2 promotes leading edge protrusion in the context of the full-length Trio molecule. We have not observed stress fibers in S2 cells, but Rho also regulates the formation and dynamics of focal adhesions (which do appear to form in S2 cells on polylysine/laminin, not shown), which are crucial for attachment of newly extended membrane to the substrate, and thus for stable lamellae (Raftopoulou and Hall, 2004). Rho also regulates myosin motors in cells and growth cones, and both Rho and myosins are likely involved in axon extension at the Drosophila CNS midline (Bashaw et al., 2001; Brown and Bridgman, 2003; Brown and Bridgman, 2004; Kim et al., 2002). Thus, in the context of cell spreading, GEF2 appears to be functioning differently compared to its role in vivo downstream of Fra signaling. Intriguingly, although our results suggest that Trio-mediated Rho activation inhibits midline crossing downstream of Fra, another RhoA-specific GEF, GEF64C, likely functions with Fra to promote midline crossing (Bashaw et al., 2001). Clearly, Rho activation must be tightly regulated, since excessive adhesion and/or Rho/myosin-mediated contractility could be as detrimental to growth cone migration as too little
Our results suggest that GEF2-mediated Rho activation constrains the outcome of GEF1-mediated Rac activation downstream of Fra signaling, but it remains formally possible that TrioGEF2 also functions negatively downstream of another, unidentified receptor. Future experiments must address more directly whether one or both of Trio’s GEF domains are activated in response to signaling by Fra and/or other receptor(s), and how cytoskeletal organization is affected when both domains are active concurrently.

In large, multidomain GEFs, a variety of N-terminal sequences have been implicated in the regulation of C-terminal GEF activity; deletion of these sequences leads to constitutive GEF activation (Katzav et al., 1991; Miki et al., 1993; Sone et al., 1997). In Dbs, Trio orthologs, and Kalirin, a Sec14p-like domain is positioned at the N-terminus, followed by a region of spectrin repeats (Kostenko et al., 2005; Liebl et al., 2000; Penzes et al., 2001). In Dbs, the Sec14p domain mediates intramolecular interactions with the pleckstrin homology domain; deletion of this domain in Dbs also leads to relocalization of the molecule away from the perinuclear region and to the plasma membrane (Kostenko et al., 2005). Deletion of the Sec14p and spectrin repeats in dTrio is believed to generate a constitutively active molecule, since the phenotype caused by expression of this deletion mutant in photoreceptor cells is similar to the phenotype caused by expression of GEF1 only (Newsome et al., 2000). In our experiments, we did not notice a change in localization of N-terminally deleted Trio in Drosophila cells, but nonetheless, deletion of this domain did lead to more efficient spreading compared to expression of wild-type Trio. We did not observe a phenotype when N-terminally deleted Trio was expressed in the eye, however, when expressed in wings with the stronger 69B-
*GAL4* driver, the phenotype caused by this deletion isoform did seem slightly more severe than expression of full-length Trio. In the CNS, N-terminally deleted Trio rescued anterior commissure formation slightly better than wild-type Trio, but there were a few more errors in pathfinding when N-terminally deleted Trio was expressed. Interestingly, deletion of the N-terminal domain essentially abolished Trio’s ability to enhance the Robo-Fra receptor phenotype. This suggests that this domain may be important during Fra-mediated attraction, but that it is less important during other signaling pathways that regulate commissure formation. In any case, this domain does seem to be regulatory based on results in wings, cells, and anterior commissure formation. However, this role is likely to be less significant than that played by the spectrin repeats and the SH3 domains.

Expression of spectrin-deleted *trio* in wings caused lethality, and when expressed in the eye, caused semilethality and the most severe phenotype of all the deletion isoforms, consistent with Rac hyperactivation. Similarly, in cells, expression of Trio∆SPR caused the greatest increase in spreading efficiency, again consistent with Rac activation. However, given that deletion of GEF2 affects efficient spreading in cells expressing the SH3 deletion mutant, we cannot formally rule out that the spectrin repeats also regulate GEF2 function. The spectrin repeats were not required for axons to cross the midline in our commissure rescue experiments; however, the CNS was generally more disorganized in embryos expressing this isoform than any other with the exception of the GEF1 deletion. Trio∆SPR overexpression caused infrequent defects in CNS axon pathfinding in wild-type embryos. Trio∆SPR also strongly suppressed the Robo-Fra chimeric receptor phenotype. However, expression of the spectrin-deleted mutant with *sca-GAL4* also caused highly penetrant epidermal and other gross morphological defects,
confounding our analysis of the spectrin repeat function during axon guidance in the CNS. Since the SH3 domain likely also regulates GEF1 function (see below) and rescues commissure formation more efficiently than wild-type or spectrin-deleted Trio, it seems that Rac activation, at intermediate levels, promotes axon guidance across the CNS midline. However, based on our results with the spectrin deletion mutant, and keeping in mind the gross morphological defects observed in embryos, we hesitantly conclude that too much Rac activation is as detrimental as not enough for faithful axon pathfinding, similar to effects observed when activated Rac is expressed in peripheral neurons, or when N-terminally truncated Trio is expressed in photoreceptor axons (Kim et al., 2003; Luo et al., 1994; Newsome et al., 2000). These results would also be consistent with the fact that deletion of the spectrin repeats in Trio inhibits NGF-induced neurite extension in PC12 cells, although it has not been formally verified that deletion of spectrin repeats in hTrio leads to constitutive GEF1 activation (Estrach et al., 2002).

Our experiments also revealed a novel regulatory role for Trio’s SH3 domain. In the CNS, the SH3 deletion mutant rescued commissure formation better than full-length trio, and caused subtle, infrequent midline crossovers when overexpressed in wild-type embryos. When coexpressed with the chimeric Robo-Fra receptor, the SH3 deletion mutant caused the strongest enhancement of inappropriate axon crossing at the midline. Although we must verify that SH3-deleted Trio can bind the Fra cytoplasmic domain, these results are consistent with the idea that TrioGEF(s) function downstream of Netrin-Fra signaling. Interestingly, we have been unable to generate a midline crossing phenotype by overexpressing both wild-type Fra and wild-type Trio. However, our results with the SH3 deletion mutant in the robo-fra background have prompted us to
revisit this experiment using SH3-deleted trio transgenes; this work is in progress. In the eye, TrioΔSH3 was the only other deletion mutant that caused a rough eye phenotype. This phenotype was abolished by codeletion of GEF1 (preliminary results, not shown), consistent with a GEF1-regulatory role for the SH3 domain. Additionally, deletion of either GEF1 or GEF2 also inhibited the efficient spreading observed in cells expressing SH3-deleted Trio, indicating that SH3 may also regulate GEF2 activity, at least in this context. We are currently conducting rescue experiments and Robo-Fra coexpression experiments with Trio molecules deleted for GEF1, SH3, and/or GEF2 in combination to further analyze the collaborative interactions between these domains at the CNS midline. Interestingly, a point mutation in hTrioSH3 dramatically inhibited NGF-induced neurite outgrowth in PC12 cells (Estrach et al., 2002). Either neurite extension in PC12 cells is more sensitive to increased GEF activity than Drosophila commissural axons or spreading S2 cells, or the hTrioSH3 domain, at least in PC12 cells, functions differently than dTrioSH3.

4.4.2. The bigger picture: what is the functional basis for regulation of GEF activity by trio’s regulatory domains?

The immediate question raised by our results is “how do Trio’s Sec14p, spectrin, and SH3 domains regulate GEF function?” The Sec14p domain may regulate Trio’s subcellular localization or intramolecular interactions, as in Dbs. Although we currently have no evidence that N-terminally-deleted Trio is mislocalized, this Trio isoform is unable to enhance the Robo-Fra chimeric receptor phenotype, implying that this domain is indeed functional in some signaling pathways. Trio’s spectrin repeats, by analogy to
other spectrin-containing proteins, may serve as a structural platform for the assembly of
duprotein signaling complexes (Djinovic-Carugo et al., 2002). Their presence might
serve as a docking site for proteins that inhibit TrioGEF function, or alternatively, for
proteins that mold or harness the cytoskeletal changes induced by Rac and Rho GTPase
activation. In their absence, cultured cells may spread well, but in vivo, the lack of a
structural motif combined with overactivated GTPases may have detrimental effects in
the context of signaling by diverse growth cone receptors. There is also no reason at this
time to rule out an intramolecular interaction mediated by the spectrin repeats. It will be
important to understand the precise mechanism by which deletion of the spectrin repeats
leads to GEF activation.

Trio’s SH3 domain may also mediate intramolecular interactions, or,
alternatively, may bind an inhibitory molecule that is only released when appropriate
upstream signals are relayed to Trio. We have previously observed that Trio’s SH3
domain binds the Abl kinase in vitro, that increasing Abl levels modulates Trio tyrosine
phosphorylation in cells, and that very few axons cross the midline in trio,Abl double
mutants (Forsthoefel et al., 2005; Liebl et al., 2000). It is intriguing to speculate that Abl
may be involved in relieving intramolecular interactions in Trio, analogous to activation
of N-WASP by interaction with Cdc42, PIP2, or SH3-domain-containing proteins, or
activation of Pak (p21-activated kinase) when bound to active Rac or Cdc42 (Bokoch,
2003; Bompard and Caron, 2004).

Clearly, upstream signals must regulate Trio. We are struck by the fact that in
cultured cells, wild-type Trio inhibits spreading, while in embryos expressing the Robo-
Fra receptor, coexpression of wild-type Trio dramatically enhances the midline crossover

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phenotype. It should theoretically be possible to recapitulate signaling pathways downstream of Fra in cultured *Drosophila* cells to gain greater understanding of how *Drosophila* Fra/DCC regulates cytoskeletal dynamics. This avenue of research is being intensively pursued by the Seeger laboratory.

4.4.3. Summary

*Drosophila* Trio is an important molecule that regulates cytoskeletal dynamics during axon guidance in the developing nervous system (Awasaki et al., 2000; Bateman et al., 2000; Forsthoefel et al., 2005; Liebl et al., 2000; Newsome et al., 2000; Ng and Luo, 2004). Previous investigations have highlighted important functions for Trio’s GEF1, N-terminal, and spectrin repeats during photoreceptor axon guidance (Newsome et al., 2000), but the function of Trio’s domains during embryonic axon guidance, and the collaborative nature of these domains in the context of the full-length molecule has not been as fully explored in *Drosophila* as it has in mammalian cell culture.

Here, we have presented evidence that Trio’s GEF1 domain promotes axon guidance across the CNS midline, implicating Rac1, Rac2, and/or Mtl at this important choice point. We also find that both GEF1 and GEF2 are important for leading edge extension in cultured cells, but that *in vivo* in the eye and downstream of Fra signaling in the embryo, GEF2 activity may oppose GEF1 activity. The results from our experiments with spectrin-deleted Trio concur with the results of others, that this domain likely negatively regulates GEF activity. In cells, the N-terminal domain also appears to inhibit GEF activity, but *in vivo*, this domain appears to be required during Fra signaling.
Additionally, our experiments have also revealed a novel regulatory role for Trio’s SH3 domain. The mechanisms by which Trio’s N-terminal, spectrin, and SH3 domains regulate GEF activity remain to be investigated.
Figure 4.1. **trio-myc** expression driven by **sca-GAL4** partially rescues commissure formation in **trio,Abl** mutants. Stage 14-16 embryos were stained with mAb BP102 and filleted. The full genotype of the **trio,Abl** double mutant background is *Df(3L)Fpal,Abl^{IMP159.4},Abl^{1}*. (A) In **trio,Abl** double mutants, commissure formation is severely disrupted, and very few axons cross the midline. (B) **ELAV^{C155}-GAL4**-driven expression of **UAS-trio-myc** is not sufficient for axons to cross the CNS midline. (C) **1407-GAL4** is capable of driving levels of **trio-myc** expression sufficient for axons to cross the midline in some commissures. (D) **sca-GAL4**-driven **trio-myc** expression most successfully rescues commissure formation in **trio,Abl** mutants, although not in all commissures. (E) In the absence of a driver, **UAS-trio-myc** does not rescue commissure defects. (F) An untagged **trio** transgene driven by **sca-GAL4** rescues commissure formation almost completely. In (B-E), the actual **trio** transgene used was **UAS-trio-myc^{B2-1b}**, in (F), the transgene used was **UAS-trio^{D29.1}**. Arrows in (C) and (D) indicate examples of segments with thin or missing commissures. Scale bar in (A), ~25 µm.
<table>
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<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures, total &amp; (AC/PC)</th>
<th>% segments with fused or collapsed commissures</th>
<th>% segments with pathfinding errors</th>
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<td>93 (80/87)</td>
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<td>88 (68/82)</td>
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<td>UAS-trio-myc&lt;sup&gt;R2-1b&lt;/sup&gt;/1407-G4; trio.Abl</td>
<td>92 (203)</td>
<td>74 (58/65)</td>
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<td>5</td>
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<td>UAS-trio&lt;sup&gt;D29-1&lt;/sup&gt;/1407-G4; trio.Abl</td>
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<td>19 (12/14)</td>
<td>40</td>
<td>9</td>
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<tr>
<td>+/sca-G4; trio.Abl</td>
<td>100 (149)</td>
<td>96 (85/95)</td>
<td>1</td>
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<tr>
<td>UAS-trio-myc&lt;sup&gt;R2-1b&lt;/sup&gt;/sca-G4; trio.Abl</td>
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<td>25 (21/22)</td>
<td>15</td>
<td>5</td>
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<td>47 (24/43)</td>
<td>15</td>
<td>7</td>
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<tr>
<td>UAS-trio&lt;sup&gt;D29-1&lt;/sup&gt;/sca-G4; trio.Abl</td>
<td>33 (252)</td>
<td>4 (0/4)</td>
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<td>10</td>
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**Table 4.1. Transgenic expression of trio-myc by sca-GAL4 rescues the trio.Abl mutant commissure phenotype.** The full genotype of the trio.Abl double mutant background is Df(3L)FpaI,Abl<sup>IMP159.4</sup>,Abl<sup>1</sup>. Number of segments scored (n) are indicated in parentheses in column 2. Segments were scored as having "Thin/Missing" commissures if >75% of BP102-staining axons were absent in either commissure. Listed are total % of segments with thin/missing commissures, followed by the % of thin/missing anterior (AC) vs. posterior (PC) commissures in parentheses. In segments scored as “Fused” or “collapsed,” an approximately wild-type or greater number of axons crossed the midline, but individual commissures were not distinguishable. "Errors" category includes ectopic wandering or defasciculation of axons between commissures, and commissures that split into smaller bundles along their length, often at the junction of the commissure with the longitudinal axon tract. Percentages were not rounded when overall defects were less than 15%.
Figure 4.2. The *sca-GAL4* driver is most effective at driving sufficient *trio* expression levels to rescue commissure defects in *trio, Abl* mutants. In addition to transgenes and drivers indicated, all embryos are *Df(3L)Fpa1, Abl1*/*trioIMP158.4, Abl1*. Thin/missing commissures are shown in blue, fusions are shown in orange, and errors are shown in yellow. Phenotypic classes are described in the Table 4.1 legend.
Figure 4.3. Transgenic trio and trio-myc expression rescues anterior and posterior commissure formation. In addition to transgenes and drivers indicated, all embryos are $Df(3L)Fpal,Abl^{1}/trio^{IMP159.4},Abl^{1}$. Commissures were scored as “thin/missing” if >75% of BP102-positive axons were missing. Anterior commissure is the top bar (light blue) for each genotype, and the posterior commissure value is the bottom bar (dark blue). Actual values are listed Table 4.1.
Figure 4.4. Neurogenesis defects are not detected in trio,Abl mutants. Stage 13 embryos were stained with anti-Even-skipped and filleted. (A,B) Wild-type. (C,D) Df(3L)Fpa1,Abl1/trioIMP159.4,Abl1. (A) and (C) are dorsal (superficial) focal planes. The aCC/pCC/CQ cluster is indicated with a large arrow, and the RP2 neuron is indicated with a small arrow. Note that in the trio,Abl mutant, the number and position of neurons is similar to wild-type, with the exception that RP2 is often laterally displaced in some segments relative to the midline. (B) and (D) are ventral (deep) focal planes. The EL cluster is indicated with a large open arrow. There are a similar number of neurons in this cluster in trio,Abl mutants as in wild-type, although again, these neurons are laterally displaced in some segments. All images were taken at the same magnification. Scale bar in (A), ~25µm.
Figure 4.5. The pattern of Trio-myc expression generated by three different GAL4 driver lines in Drosophila embryos. UAS-trio-mycR2-2 was crossed to either ELAVC155-GAL4 (A-G), 1407-GAL4 (H-N), or sca-GAL4 (M-U). Embryos were labeled immunohistochemically with anti-myc antibody. Embryo stages are shown in the bottom right hand corner of each panel. In ELAVC155-GAL4 embryos, Trio-myc is not detectable in the CNS until stage 13 (C). ELAVC155-GAL4 sustains Trio-myc expression through stage 17 (D-G). In 1407-GAL4 embryos, Trio-myc is weakly detectable at stage 11 (H), but not robustly until stage 12 (I). 1407-GAL4 drives very high Trio-myc expression levels in the CNS until stage 16 (M); at stage 17, Trio-myc expression decreases, but only slightly. Trio-myc expression can also be seen at stages 13 and 14 (J,K) in lateral peripheral neurons. At early stages, sca-GAL4 drives high levels of Trio-myc expression in the ventral neuroectoderm, as well as in developing neurons (O,P). At stage 14, sca-GAL4-driven Trio-myc expression begins to decrease, but is detectable through stage 17 (R-U). Peripheral neuron staining can be seen at stages 13-15 (Q-S), and broad epidermal staining is also visible at stages 13-16 (Q-T). Arrows in G, N, and U indicate Trio-myc enrichment in the axons of the neuropil, which lies dorsal to the majority of neurons in the nerve cord.

Continued on next page
Figure 4.5. Continued.
Figure 4.6. *ELAV\textsuperscript{C155}-GAL4* driven expression of Trio-myc. Stages are indicated at the bottom of each panel. Note that Trio-myc expression is only barely detectable at stage 12 (B), and that high levels of axonal Trio-myc are not detectable until stage 15 (E), when many axons have already crossed the midline, and commissures have formed. In (B) and (C), the future position of the anterior (AC) and posterior (PC) commissures are indicated, but at stages 12 and early 13, the axons have not yet sorted into two distinct commissures.
**Figure 4.7. 1407-GAL4 driven expression of Trio-myc.** Trio-myc is weakly detectable in neurons at stage 11 (A). (B) By stage 12, Trio-myc expression is strong, but very little staining is seen at the midline, even though a few axons have begun to cross the midline at this point in development. By early stage 13 (C), commissural axon staining is present; the axons have not sorted into individual commissures at this early stage. At stages 14-16 (D-F), Trio-myc levels are very high in both axons and neuronal cell bodies.
Figure 4.8. *sca-GAL4*-driven expression of Trio-myc. In (A), Trio-myc expression is already high in neurons by stage 11. These high levels are maintained through stages 12-14 (B-D). Neuronal staining is very high, making it difficult to distinguish axonal Trio-myc at stages 12-13, but by stage 14 (D), Trio-myc can clearly be detected in both longitudinal and commissural axons. By stage 16 (F), Trio-myc expression has all but disappeared in neuronal cell bodies, but persists in both longitudinal and commissural axons. In (B) and (C), the future position of the anterior (AC) and posterior (PC) commissures are indicated as landmarks, but at stages 12 and early 13, the axons have not yet sorted into two distinct commissures.
Figure 4.9. Cartoon of Trio deletion constructs generated for this study. “NTM” is the conserved, N-terminal Sec14p-like domain found in Trio’s orthologs in C. elegans, mouse, and humans, as well as in Kalirin, a closely-related GEF. Protein alignments suggest that there are likely seven spectrin-like repeats in Drosophila trio (McPherson et al., 2002). “M” represents the 6X-myc epitope engineered at the C-terminus of each trio construct. The trioM89 construct was created by amplifying the sequence encoding the L1412F missense mutation from Drosophila genomic DNA and subcloning into full-length trio.
Figure 4.10. Wild-type and mutant Trio proteins localize to axons in the embryonic CNS. Flies bearing \textit{UAS-trio} transgenes were crossed to flies carrying \textit{ELAV}\textsubscript{C155}-\textit{GAL4}, and embryos were stained with anti-myc. Stage 16 embryos were selected for dissection. Transgenes are indicated. Trio deleted for any two or all three C-terminal domains (GEF1, SH3, and GEF2, shown in Fig. 4.9) in combination also efficiently localize to commissural and longitudinal axons (not shown).
Figure 4.11. TrioGEF1 promotes commissure formation. CNS axons were labeled immunohistochemically using mAb BP102, and stage 14-16 embryos were dissected and scored for defects in axon pathfinding. All embryos shown are homozygous mutant for trio and Abl (Df(3L)Fpal,Abl\textsuperscript{trioIMP159.4;/},Abl\textsuperscript{1}), and carry one copy of the sca-GAL4 driver. The specific UAS-trio-myc isoform is listed below each panel. Thin/missing commissures are indicated with arrows, while disorganized commissures (fusions and errors) are indicated with arrowheads. (A) and (B) are representatative of embryos expressing wild-type UAS-trio-myc. Commissure formation is rescued in most segments, although in UAS-trio-myc\textsuperscript{R2-2} embryos (B), axons do not cross in posterior commissure as often as in the anterior commissure (see also Table 4.2). (C) and (D), phenotypes in embryos expressing N-terminally-deleted Trio. (C) is an example of a mild phenotype in which most axons cross the midline, but in one segment (arrowhead) distinct commissures are not discernible. (D) is an example of a more severely-affected embryo expressing UAS-trio\textsuperscript{ΔNTM\textsuperscript{44}-myc}, in which fewer axons cross the midline, and two segments are more severely disorganized. (E) and (F) Expression of spectrin-deleted Trio caused axons to cross the midline, but in a more disorganized manner in some segments. In (E), commissures converge on one side of the midline, joining the longitudinal bundle to form a sideways “V” shape rather than the normal ladder-like architecture (top arrowhead). In another segment (bottom arrowhead), many axons cross the midline, but commissures appear irregularly bundled and closer together than normal. In (E), axons appear to be more loosely bundled than in embryos expressing other Trio isoforms. Additionally, axons seem to fasciculate around nearby neuronal cell bodies more often compared to other genotypes. In (F), commissures are thin or missing in two segments (arrows), while in the bottom segment, many axons cross the midline but the anterior and posterior commissures have fused together. (G) and (H) Examples of embryos expressing GEF1-deleted Trio. (G) is representative of embryos expressing UAS-trio\textsuperscript{ΔGEF1\textsuperscript{R1-3}-myc}; very few axons cross the midline, and this transgene is unable to rescue the trio,Abl commissure phenotype. (H) is a moderate example of embryos expressing UAS-trio\textsuperscript{ΔGEF1\textsuperscript{R1-8}-myc}. All segments shown in this panel are defective, but in 4 of 5 segments only the posterior commissure was absent. (I) and (J), examples of embryos expressing SH3-deleted Trio. A wild-type number of axons crosses the midline in nearly all segments in these embryos, although occasionally commissures are disorganized in some fashion. (K) and (L), embryos expressing GEF2-deleted Trio. This transgene rescues as well as wild-type and N-terminally-deleted trio. Examples of thin/missing commissures (arrows) and disorganized commissures (arrowheads) are indicated.
Figure 4.11. Continued.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures, total &amp; (AC/PC)</th>
<th>% segments with fused or collapsed commissures</th>
<th>% segments with pathfinding errors</th>
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<tbody>
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<td>trio.Abl</td>
<td>99 (179)</td>
<td>93 (80/87)</td>
<td>4</td>
<td>2</td>
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<tr>
<td>+/sca-G4; trio.Abl</td>
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<td>96 (85/95)</td>
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<td>3</td>
</tr>
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<td>95 (77/84)</td>
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<td>3</td>
</tr>
<tr>
<td>UAS-trio-mycR2-1b/sca-G4; trio.Abl</td>
<td>45 (254)</td>
<td>25 (21/22)</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>UAS-trio-mycR2-2/sca-G4; trio.Abl</td>
<td>69 (364)</td>
<td>47 (24/43)</td>
<td>15</td>
<td>7</td>
</tr>
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<td>15</td>
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<td>12</td>
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<td>UAS-trioαGEF2-mycR1-4B/sca-G4; trio.Abl</td>
<td>65 (232)</td>
<td>28 (18/26)</td>
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Table 4.2. TrioGEF1 is required for transgenic rescue of commissure formation in *trio,Abl* mutants. Defects were scored as described in the legend to Table 4.1. The full genotype of the *trio,Abl* double mutant background is *Df(3L)FpaI,Abl^IMP159.4,Abl^1*.
Figure 4.12. TrioGEF1 promotes commissure formation. In addition to transgenes and drivers indicated, all embryos are $Df(3L)Fpa1, Abl^{1}/trio^{IMP159.4}, Abl^{1}$. Thin/missing commissures are shown in blue, fusions are shown in orange, and errors are shown in yellow. Actual values are listed in Table 4.2. Phenotypic classes are described in the Table 4.1 legend.
Figure 4.13. Trio deletion isoforms rescue anterior and posterior commissure formation. Note that UAS-trio-mycR2-2 rescues anterior, but not posterior commissure formation as well as UAS-trio-mycR2-1b. The N-terminal deletions rescue anterior commissure formation slightly better than posterior commissure formation; these values are also lower than wild-type or GEF2-deleted trio. GEF1-deleted trio does not rescue nearly as well as all other transgenes. SH3-deleted Trio is more effective than any other transgene, including wild-type. In addition to transgenes and drivers indicated, all embryos are Df(3L)FpaI,Abl1/trioIMP159.4,Abl1. Commissures were scored as “thin/missing” if >75% of BP102-positive axons were missing. Anterior commissure is the top bar (light blue) for each genotype, and the posterior commissure value is the bottom bar (dark blue). Actual values are listed Table 4.2.
Figure 4.14. Overexpression of Trio deletion mutants does not cause CNS axon guidance defects. Trio mutant isoforms were expressed under the control of \textit{sca-GAL4} (A-G), or \textit{1407-GAL4} (H). Specific transgenes are indicated in the figure. In (A-D), all axons were visualized using mAb BP102 in stage 15/16 embryos. In (E-H), a subset of ipsilaterally-projecting longitudinal axons were visualized in stage 17 embryos using mAb 1D4. In (B-D), note that overall CNS architecture is normal. In (F), an embryo expressing wild-type \textit{trio-myc} displays no disruptions in axon guidance. In (G), an example of a bundle of axons ectopically crossing the CNS midline in an embryo expressing SH3-deleted \textit{trio} is indicated with an arrow. In (H), an example of a gap in the lateral FasII-positive fascicle is indicated with an arrow in an embryo expressing spectrin-deleted \textit{trio}. 
<table>
<thead>
<tr>
<th>Genotype</th>
<th>% defective segments, overall (n)</th>
<th>% segments with thin/missing commissures, total</th>
<th>% segments with fused or collapsed commissures</th>
<th>% segments with pathfinding errors</th>
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<td>0</td>
<td>1.7</td>
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<td>UAS-trio&lt;sup&gt;∆&lt;/sup&gt;SPR-myc&lt;sup&gt;R1-8&lt;/sup&gt;/sca-G4</td>
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<td>0</td>
<td>1.7</td>
<td>2.5</td>
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<td>UAS-trio&lt;sup&gt;∆&lt;/sup&gt;GEF1-myc&lt;sup&gt;R1-8&lt;/sup&gt;/sca-G4</td>
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<td>4.8 (1050)</td>
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Table 4.3. Overexpression of trio isoforms causes very few defects in CNS axon guidance. Axons in stage 14-16 embryos were labeled with mAb BP102, and embryos were dissected and scored for defects. Number of segments scored (n) are indicated in parentheses in column 2. Segments were scored as having "Thin/Missing" commissures if >75% of BP102-staining axons were absent in either commissure. Description of criteria for fused and errors categories are listed in the Table 4.1 legend. Percentages were not rounded when overall defects were less than 15%.
<table>
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<tr>
<th>Genotype</th>
<th>% segments with ectopic midline crossovers (n)</th>
<th>% hemisegments with fascicle breaks (n)</th>
<th>% hemisegments with fascicle fusions (n)</th>
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<td>0 (256)</td>
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<td>0.4 (250)</td>
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<td>UAS-trio(\Delta)SPR-myc(^{R1,6})/sca-G4</td>
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<td>0 (224)</td>
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<td>1.3 (240)</td>
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<td>0 (289)</td>
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<td>UAS-trio(\Delta)SH3-myc(^{R1,4}); UAS-trio(\Delta)SH3-myc(^{R1,5})/sca-G4</td>
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<td>0 (289)</td>
<td>0 (289)</td>
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<td>0 (118)</td>
<td>2.0 (196)</td>
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**Table 4.4.** Overexpression of trio isoforms causes very few defects in longitudinal axon guidance. A subset of longitudinally-projecting axons in stage 17 embryos were visualized immunohistochemically using mAb 1D4. Number of segments or hemisegments scored (n) are indicated in parentheses. Segments were scored as having ectopic midline crossovers if obvious 1D4-positive bundles crossed the CNS midline. Hemisegments were scored as having fascicle breaks if any of the 3 1D4-positive fascicles were discontinuous between segments. Only the lateral fascicle was affected by overexpression of Trio deletion mutants. Hemisegments were scored as having fascicle fusions if two or three fascicles merged together so that they were indistinguishable within a segment. Percentages were rounded when overall defects were less than 15%.
Figure 4.15. Expression of *UAS-trioΔSPR-myc* under the control of *sca-GAL4* causes gross morphological disruptions in embryos. (A, C, E, and G) wild-type embryos. (B, D, F, and H) *UAS-trioΔSPR-myc/* *sca-GAL4*. (A) and (B) are ventral views of stage 17 embryos stained with mAb 1D4. In (B), epidermal integrity has been compromised, and portions of the gut protrude laterally and posteriorly (arrows). Note also the highly aberrant motoneuron trajectories, as well as areas of CNS constriction posteriorly. In several segments, axons appear to cross the CNS midline. (C) and (D) are lateral views of stage 16 embryos stained with mAb 1D4. In (D), there is a severe gap in the ventral tissues of the animal, accompanied by a displacement and thinning of the CNS in this region (arrow). (E) and (F), stage 14 embryos stained with mAb BP102. Note severe gaps and constrictions in the CNS in (F). (G) and (H), stage 15 embryos tained with mAb BP102. Severely disrupted ventral cord morphology is evident in (H).

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Figure 4.15. Continued.
Figure 4.16. Expression of SH3- and spectrin-deleted trio disrupts eye development. (A) In a wild-type fly or in UAS-trio-myc/GMR-GAL4 flies, ommatidia are arranged precisely into orthogonal rows. In flies expressing trio isoforms deleted for the N-terminal (B), GEF1 (D), or GEF2 (F) domains, ommatidial organization is largely unaffected. In flies expressing spectrin-deleted trio (C), eye morphology is severely disrupted. Only a few ommatidia form, and the rest of the region where the eye should be (dotted line) is simply covered by a flat, unpigmented membrane. (E) In flies expressing SH3-deleted trio, the orthogonal array of ommatidia is disorganized, leading to a “rough” eye appearance.
Figure 4.17. Expression of trio isoforms in the wing disc leads to distinct morphological effects. (A-I) Expression trio transgenes was driven by 71B-GAL4. (J-R) Expression was driven by 69B-GAL4. The specific transgene is listed in each panel. Phenotypes were generally more severe when expression was driven by 69B-GAL4 than by 71B-GAL4. In (A), (C), (D) and (I), examples of unaffected wings are shown. In (B) (arrow) and (L), note the shortened posterior cross vein in flies expressing wild-type trio-myc. This phenotype was also observed in flies expressing N-terminally deleted trio (not shown) and GEF2-deleted trio (Q). When wild-type, N-terminally deleted, and GEF2-deleted trio were expressed with 69B-GAL4, the frequency of more severely affected wings increased. For example, note the partially folded edges and “mottled” appearance of wings in (J), (K), and (R), and the dark “spots” on the posterior portion of the wing in (M). In flies expressing GEF1-deleted trio or trio^{M89}, the posterior cross vein became “Y”-shaped (E, N). In some cases the second branch of the “Y” failed to form completely (O). (D) is an example of an unaffected wing from a fly expressing GEF1-deleted trio. In flies expressing SH3-deleted trio, phenotypes ranged from thin or missing crossveins (F), to a partial blistering and thickening of longitudinal veins (usually longitudinal vein III) (G), to a complete blistering of the entire wing (H).
Figure 4.17. Continued.
Figure 4.18. Expression of SH3- and spectrin-deleted Trio in Drosophila S2 cells promotes cell spreading. S2 cells were transfected with pMET-trio-myc constructs. After induction of Trio expression, cells were plated onto acid-washed, polylysine and laminin-coated glass coverslips for 4 hours, and then fixed and stained. In (A-E), F-actin is labeled with TRITC-phalloidin (red), Trio-myc is visualized with an FITC-conjugated secondary antibody (green), and DNA is visualized with DAPI (blue). Wherever Trio and F-actin colocalize, signal is orange or yellow. All images were taken with the 40X objective, and were processed identically in Photoshop. Scale bar in (A), ~10 µm. (A) A representative example of S2 cells expressing wild-type Trio-myc. One cell (arrow) is as well spread as adjacent untransfected cells, but most cells expressing Trio-myc are less well spread. Note that although F-actin is enriched peripherally, cells extend only narrow, ruffle-like lamellae, and few, if any, filopodia. (B) An example of an infrequently occurring well-spread cell expressing wild-type Trio-myc; note the more pronounced lamella surrounding the cell that is filled with numerous F-actin “ribs.” Trio is cytoplasmic, and is found at significant levels in lamellae and the actin “ribs.” (C) S2 cells expressing spectrin-deleted Trio frequently extend very broad lamellae, consistent with a regulatory role for this domain. Note the significant difference in surface area between this cell and adjacent untransfected cells, and also to the cell expressing wild-type Trio-myc in panel (B). Also, a third cell expressing spectrin-deleted Trio-myc does not spread well (arrow), suggesting some variation in expression of Trio effectors in the S2 cell population. (D) Cells expressing SH3-deleted Trio-myc spread better than cells expressing wild-type Trio-myc, but not as well as those expressing Trio∆SPR-myc. Although many cells extend lamellae (arrow), a significant number do not (arrowhead), although surface area is not reduced compared to untransfected cells. (E) Trio deleted for both GEF1 and SH3 domains often mislocalizes, being dramatically reduced in lamellipodia and enriched more centrally, especially in the cortical/membrane region that does not make contact with the substrate (arrow). A similar localization is observed in cells expressing lower levels of this isoform (arrowhead). (F) Histogram shows the average surface area of cells (µm²) expressing each Trio isoform. For each genotype, cells were imaged from from at least 5 randomly selected fields. Surface area was calculated for at least 30 cells of each genotype, and data were averaged. Isoforms that spread better than wild-type Trio are shown in yellow. Error bars indicate standard error of the mean (SEM) for each sample. For the “untransfected” category, non-expressing cells on coverslips plated with wild-type (n=28) and spectrin-deleted Trio-myc (n=27) were averaged together. For WT Trio-myc, (n=63). TrioANTM-myc, (n=39). Trio∆SPR-myc, (n=56). Trio∆GEF1-myc, (n=40). Trio∆SH3-myc, (n=66). Trio∆GEF2-myc, (n=39). Trio∆GEF1+∆SH3-myc, (n=32). Trio∆SH3+∆GEF2-myc, (n=38).

Continued on next page
Figure 4.18. Continued.
Figure 4.19. *sca-GAL4* driven coexpression of *trio* deletion mutants modifies the chimeric Robo-Frazzled receptor phenotype. (A) In wild-type stage 17 embryos, three 1D4-positive fascicles project longitudinally on either side of the midline, but never cross this boundary. (B) In embryos expressing the chimeric Robo-Fra receptor, axons cross the midline boundary inappropriately (arrows) because they are attracted to Slit. (C-E) Examples of the range of phenotypes caused by coexpression of wild-type Trio. In most affected embryos, distinct bundles of axons cross the midline as in (C) and (D). In a few cases, medial 1D4-positive fascicles collapse completely onto the midline (E). (The “collapse” phenotype was scored as such if, in >75% of segments, the two medial fascicles were indistinguishable.) (F) Deletion of the N-terminal domain leads to a milder phenotype compared to expression of full-length Trio. (G) Coexpression of spectrin-deleted Trio suppresses the Robo-Fra phenotype in scorable embryos, but interpretation of this phenotype is difficult, since some embryos with other gross morphological defects were excluded from analysis. (H) Coexpression of GEF1-deleted Trio leads to fewer axon crossovers at the midline relative to coexpression of wild-type Trio; the “collapse” phenotype was only observed in one of 24 embryos scored. (I-K) Coexpression of SH3-deleted Trio causes an increase in the number of axons crossing the midline, and the “collapse” phenotype is more frequent. In (J), most axons of the medial fascicle have collapsed onto the midline, while in (K), complete collapse of medial fascicles has occurred. (L) Coexpression of GEF2-deleted Trio causes the “collapse” phenotype more often than wild-type Trio, similar to coexpression of SH3-deleted Trio.
### Table 4.5

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<th>Genotype</th>
<th>Crossovers/segment (n)</th>
<th>Penetrance (n)</th>
<th>Expressivity</th>
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<tr>
<td>UAS-robo-fra / sca-G4</td>
<td>0.28 (274)</td>
<td>58 (24)</td>
<td>5.5</td>
</tr>
<tr>
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<td>UAS-robo-fra / sca-G4; UAS-trio\textsuperscript{ΔSH3-myc}\textsuperscript{R1,5}</td>
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<tr>
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<td>96 (23)</td>
<td>5.3</td>
</tr>
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**Table 4.5** *scabrous-GAL4* driven coexpression of SH3-deleted Trio with the chimeric Robo-Fra receptor increases the severity of inappropriate axon crossing at the midline. Crossovers per segment score equals the total number of ectopic crossovers by 1D4-positive axons divided by the total number (n) of segments scored. Penetrance is the number of affected embryos divided by the total number (n) of embryos analyzed. Expressivity is the number of crossovers per affected embryo. The same *UAS-robo-fra* chromosome (“3.3”) was used in all crosses.
Table 4.6 Summary of results from expression of Trio deletion mutants. The phenotypic consequences of deleting Trio’s individual domains in different assays are listed, along with an overall conclusion about the function of each domain (bottom row). GEF1 is the only domain required for commissure formation and attraction downstream of Fra, while the spectrin repeats and SH3 domain appear to regulate GEF activity. The N-terminal domain is not required for commissure formation, but is required to enhance the Robo-Fra chimeric receptor phenotype. Deletion of GEF2 enhances the Robo-Fra phenotype, but only partially rescues the trio,Abl phenotype.
CHAPTER 5

DISCUSSION

Great strides have been made in recent years in the identification of molecules that function during axon guidance, and more generally, during cell migration and motility. The modern field of axon guidance was born in the 1940’s, due to the revival of the “chemoaffinity” hypothesis by Roger Sperry. In the 1970’s and 1980’s, Corey Goodman, Marc Tessier-Levigne, and others began the quest for the surface molecules which allowed axons to recognize and extend along specific pathways during embryonic development (Goodman et al., 1984; Tessier-Lavigne and Goodman, 1996).

Concurrently, another branch of axon guidance focused on the cytoskeletal “motor,” the many molecules which interact directly with actin and microtubules, or indirectly with actin- and microtubule-binding proteins to regulate the rate and geometry of their assembly and disassembly in cultured neurons (Gallo and Letourneau, 2004).

Investigators’ work in this field overlapped with that of others studying cell motility more generally (Pollard and Borisy, 2003). In recent years, many researchers have identified molecules that link receptors to their ultimate cytoskeletal effectors, including adaptors, kinases, phosphatases, GEFs, GAPs, GDIs, and others, an effort that has begun to bear fruit in both vertebrates and invertebrates (Guan and Rao, 2003; Huber et al., 2003).
some cases, specific cytoskeletal changes such as filopodial protrusion, lamellipodial extension, focal adhesion formation and the development of traction forces have been implicated downstream of specific guidance signals (Gallo and Letourneau, 2004; Govek et al., 2005).

It is into this last category, the problem of how surface receptors are linked to changes in cytoskeletal modulation, that the results presented in this Dissertation fall. In Chapter 2, the isolation of *Drosophila trio* as a genetic enhancer of *Abelson tyrosine kinase* mutant phenotypes was described. In Chapter 3, genetic and biochemical evidence that Abl, Trio, and Enabled function as effectors of the Netrin receptor Frazzled were presented. Finally, in Chapter 4, a structural analysis of the Trio molecule at the CNS midline was instigated. Together, these studies parallel contemporary discoveries by others concerning homologous receptors and effectors in other organisms.

A number of recent discoveries (all cited in earlier Chapters) have begun to delineate the signaling pathways downstream of the attractive Netrin/UNC-6 receptor Fra/DCC/UNC-40 (Fig. 5.1). Two molecules, Ena/VASP/UNC-34 and the Rac1/CED-10 GTPase, have been implicated in signaling by DCC homologs in worms, flies, and vertebrates (assuming Trio GEF1 activates Rac1 in commissural axons). More generally, it seems that tyrosine phosphorylation of DCC and/or its effectors is a regulatory step shared across species as well, since UNC-40 interacts genetically with a phosphatase, and phosphorylation of Fra and DCC have been demonstrated biochemically. In the case of DCC, the pathways from the receptor to actin modulation is perhaps best understood, but questions remain, for example whether DCC recruits a GEF(s) in order to regulate Cdc42 and/or Rac1. In *Drosophila*, signaling partners have been identified genetically, but the
molecular sequence of events in which these molecules are involved needs to be investigated. For example, does Abl phosphorylate Fra, Trio, or Ena in response to Netrin engagement of Fra? If so, what are the consequences of phosphorylation? Are other kinases involved? Is Trio constitutively bound to Fra, or recruited upon Fra activation? Does Fra activate Trio GEF domain(s)? In worms, similar questions exist, for example the identity of kinases or GEFs which may function in collaboration with UNC-40.

We have made significant inroads into the mechanisms by which this phylogenetically conserved receptor signals attraction, but the identification of the remaining signaling partners and the biochemical sequence of events during Fra/DCC/UNC-40 signaling is required. With this information in hand, a comparison of the complete signaling pathways for each molecule will be possible. As DCC, Fra, and UNC-40 were among the first attractive guidance receptors to be identified, they are also on track to be among the first for which we will have an answer to a very basic, fascinating, and difficult question: how do different species accomplish the same net output, growth cone attraction, on a molecular level? Such information will be invaluable in building a platform of knowledge from which to understand cytoskeletal signaling in a variety of contexts, from axon guidance, to angiogenesis, wound healing, and cancer metastasis.

The discoveries in this Dissertation also parallel those in other organisms in another respect: they reveal deficiencies in our knowledge and in the current approaches to studying this important problem. A catalog of the molecules involved in a particular signaling pathway is important, but not sufficient. To illustrate this point, recent discoveries have found that many cytoskeletal molecules can function downstream of
multiple receptors, yielding opposite outputs: attraction and repulsion. For example, in *Drosophila*, the Rho GTPases, the Abelson tyrosine kinase, and Enabled are likely regulated by both attractive and repulsive guidance receptors (see Chapters 3 and 4) (Bashaw et al., 2000; Fan et al., 2003; Forsthoefel et al., 2005; Fritz and VanBerkum, 2002; Hsouna et al., 2003; Matsuura et al., 2004; Wills et al., 2002). In vertebrate cells, Ena/VASP proteins promote unproductive lamellar protrusion or filopodial extension, depending on cell type and cytoskeletal context (Bear et al., 2000; Bear et al., 2002; Lebrand et al., 2004; Mejillano et al., 2004). The adaptor Nck1/Dock and and the Rac effector Pak have been implicated as repulsive Robo effectors in flies, but they function downstream of the attractive DCC receptor in mammalian neurons (Fan et al., 2003; Shekarabi et al., 2005). If identical molecules can be used for seemingly opposite purposes, how is specificity of response achieved? As another specific example, how can Rac be involved in endocytosis during growth cone collapse, but lamellipodial protrusion during DCC signaling (Jurney et al., 2002; Shekarabi et al., 2005)?

The answer in part may be that the total collection of molecules recruited by an activated receptor determines its output. As a hypothetical example, the spectrin repeats of Trio may predetermine the localized, subcellular effect of Rac activation downstream of Fra by constraining the cytoskeleton in certain ways. Downstream of Robo (which also activates Rac), a different GEF or the absence of a GEF might contribute to a different cytoskeletal response. This is a very strong argument for not only identifying a receptor’s effectors, but also for a structure-function analysis of these effectors, which we have begun for Trio (Chapter 4). Interestingly, there are at least 9 GEFs and 9 GAPs expressed
in the *Drosophila* embryonic nervous system (Hu et al., 2005). A molecular understanding of the cytoskeletal outputs of different GEFs that activate the same GTPase would be informative.

It also must be considered that the output of the response is biased by the status of the cytoskeleton prior to the signal. Recent studies suggest that the growth cone cytoskeleton may be intrinsically different than the fibroblast cytoskeleton. For example, elevation of the ratio of Ena/VASP:capping protein in fibroblasts leads to a decrease in motility and unproductive lamellipodial extension, but in mouse melanoma cells or hippocampal neurons, it results in filopodial extension (Bear et al., 2000; Bear et al., 2002; Lebrand et al., 2004; Mejillano et al., 2004). The authors of these studies propose that the difference is due to the levels or distribution of other cytoskeletal proteins, such as fascin, an actin bundling protein. Another interesting example is the Arp2/3 complex, which nucleates dense dendritic arrays of branched actin filaments and promotes migration rate in fibroblasts. In the growth cone, looser networks of branched filaments exist between parallel bundles, not the dense dendritic arrays seen at the leading edge of fibroblasts, suggesting that growth cones may have a more “filopodial” mode of migration (Strasser et al., 2004). Furthermore, Arp2/3 localizes more centrally in the growth cone, and appears to negatively regulate growth cone translocation, and be dispensible for actin polymerization and filopodial extension, at least in the absence of extracellular guidance cues (Shekarabi et al., 2005; Strasser et al., 2004). If such global differences are possible between growth cones and fibroblasts due to differences in expression level of a relatively small number of proteins, then it formally is possible that differences may exist between, for example, the growth cones of longitudinal vs.
commissural axons. It has already been mentioned in the Introduction that Commissureless is tightly regulated in order to, in turn, tightly regulate Robo levels on the axon’s surface. Although currently no examples have been reported, similar regulation of expression or localization may exist for one or more cytoskeletal effectors.

Finally, studies of axon guidance at the *Drosophila* midline have lagged behind other systems (particularly zebrafish) with respect to live growth cone imaging (for example, see Hutson and Chien, 2002). Observation of missing or extra axon pathways in fixed embryos is informative as to a gene’s general function or interaction with other genes, but it does not allow a mechanistic understanding of the basis for the phenotype. For example, most axons do not cross the CNS midline in *fra;Abl* mutants, but why not? Is there a defect in filopodial or lamellipodial extension? Is there a defect in traction forces or adhesive contacts? The power of *Drosophila* genetics theoretically makes possible the generation of the GAL4-drivers which would enable the expression of fluorescent markers in the growth cones of individual commissural neurons. Currently, there is one potentially useful reagent, *eagle-GAL4*, that drives in a subsets of 5-10 commissural neurons per hemisegment, although even this may be too many neurons to discern individual growth cones (Dittrich et al., 1997). Single-neuron labeling *in vivo* via injection of lipophilic dye has been done successfully, but only rarely, suggesting that significant technical base must be developed in order to proceed in this fashion (Murray et al., 1998). *In vivo* imaging of commissural growth cones would be technically challenging, but invaluable for a more precise understanding the function of Fra, Abl, Trio, Ena, and other molecules in specific neurons before, during, and after midline crossing.
In summary, we have identified *Drosophila trio* as a genetic enhancer of mutations in the *Abelson* tyrosine kinase gene, and have implicated Abl, Trio, and Ena as downstream effectors of the attractive Netrin receptor. In addition, genetic experiments indicate that Trio promotes commissure formation through its Rac-specific GEF1 domain. Future goals include a more complete mechanistic explanation of Fra signaling, and the identification of additional ligands, receptors, and effectors that mediate commissure formation.
Figure 5.1. Comparison of known signaling pathways downstream of *Drosophila* Fra, *C. elegans* UNC-40, and vertebrate DCC. (A) For *Drosophila* Fra, the only molecules for which there is biochemical evidence of an interaction are Abl and Trio (Forsthoefer et al., 2005). Molecules studied in this dissertation are indicated in red. Question marks indicate cases where genetic interactions in other studies or in other systems suggest a potential mechanism by which these molecules might be linked. For example, PKA regulates Mena downstream of DCC in mouse neurons, and MLCK may regulate traction forces based on studies in mammalian cells. (B) It is not known whether *C. elegans* CLR-1 inhibits UNC-40, or other effectors, only that signaling by CLR-1 seems to be antagonistic to UNC-40 function. (C) The identity of a GEF downstream of DCC signaling is hypothetical at this time. The hierarchy of interactions downstream of Nek1 and Pak were recently proposed in (Shekarabi et al., 2005).
Figure 5.1. Continued.
LIST OF REFERENCES


