Frazzled And Abelson Interact To Regulate The Actin Cytoskeleton In Drosophila

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FRAZZLED AND ABELSON INTERACT TO REGULATE
THE ACTIN CYTOSKELETON IN DROSOPHILA

by

BRIDGET ELSA VARUGHESE

DISSERTATION

Submitted to the Graduate School
of Wayne State University,
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________________________________________
Advisor Date
DEDICATION

I would like to dedicate this work to my parents for their love, encouragement, and understanding throughout my life in both personal and professional aspects. They have been my support and stronghold through my life.
ACKNOWLEDGMENTS

Foremost, I would like to extend my gratitude to my mentor, Dr. Mark VanBerkum for his immense patience and guidance through the years. He taught me what I know of science - research, how to read papers and scientific writing, among other things and not limited to science. I would also like to thank all my committee members – Dr. Russ Finley, Dr. Markus Friedrich and Dr. Miriam Greenberg for their invaluable input and critique during various meetings. I am especially indebted to Dr. Russ Finley for opening his lab to me to work after my lab got burned down, for allowing me to participate in lab meetings and for always having a different perspective on the project.

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Chapter 1

Introduction: Background and Significance

The growth cone at the leading edge of an axon (Ramon Cajal, 1889) acts as a molecular sensor to explore the extracellular environment in search of diffusible and membrane bound guidance cues (Garbe and Bashaw, 2004; Letourneau et al., 1994; Van Vactor, 1999). The growth cone expresses membrane receptors that recognize these attractive or repulsive cues and initiates a cascade of intracellular signaling pathways that converge on the cytoskeleton (Stein and Tessier-Lavigne, 2001; Yu and Bargmann, 2001). Two main classes of receptors namely, attractive Netrin and repulsive Slit receptors coordinate the cytoskeletal dynamics underlying axon guidance. Netrin initiates signaling through the Frazzled (Fra)/Deleted in Colorectal Cancer (DCC)/Unc40 family of receptors to mediate growth cone movement (Evans and Bashaw, 2010). Upon activation, these receptors either recruit cytoplasmic proteins such as Abelson Tyrosine kinase (Abl) or simply activate downstream effectors (e.g. GTPases) to orchestrate the rearrangement of the cytoskeleton elements required to achieve the directed steering of an outgrowing axon (Dorsten et al., 2007; Dorsten et al., 2010; Forsthoefel et al., 2005; Gitai et al., 2003). Experiments in vertebrate primary neurons and cultured cells demonstrate that attractive receptor signaling leads to the formation of F-actin filaments that push the plasma membrane forward by forming long protrusions called filopodia and thereby aid the cell to move forward (Shekarabi and Kennedy, 2002).
This thesis specifically studies the mechanism of Fra signaling and the role of Abl downstream of Fra during midline guidance in *Drosophila melanogaster*. To better understand how Fra signaling cross talks to actin, a *Drosophila* S2 cell culture assay system, analogous to the vertebrate model system (Shekarabi and Kennedy, 2002) has been established here. Quantification of the distribution of cell morphologies as determined by phalloidin (F-actin) staining has been used as readout for the rearrangement of the actin cytoskeleton. Loss and gain of function assays in the presence and absence of ectopic Fra allowed us to determine the role of Abl in this process in S2 cells.

*Drosophila as a model system*

The fruit fly *Drosophila melanogaster* is an excellent system to study gene-gene, protein-protein interactions and signal transduction processes during axon pathway formation. It is endowed with multiple advantages including a short life cycle, conservation of gene function, straight-forward genetic manipulations, and availability of nervous system specific antibodies, tissue specific promoters and immortalized cell lines.

*Drosophila* has only four chromosomes which allows relatively easy manipulation of the levels of ligands (e.g. Netrin), guidance receptors (e.g., Fra) and downstream effectors (e.g. Abl). A major strength of fruit fly genetics lies in the UAS-Gal4 system that allows straight forward over-expression or knockdown of the genes of interest in a tissue specific manner (Brand and Dormand, 1995). A tissue specific promoter from
Drosophila and a Gal4 transcription factor from yeast are used in the UAS-Gal4 system. The presence of both the promoter and the upstream activating sequence (UAS) in the same fly will allow Gal4 to bind to the upstream activating sequence, which in turn drives expression of the adjacent gene. For example, Embryonic Lethal Abnormal Vision (Elav) is ubiquitously transcribed in all developing neurons both in the central nervous system and the peripheral nervous system, thus qualifying as a pan neural gene. A mating between flies that have ElavGal4 transgene in one fly and a UAS Fra transgene in another will allow pan neural expression of Frazzled in the fraction of embryos that inherit both Elav Gal4 and UAS Frazzled transgenes (Fig. 1.1A).

Second, a disruption in the axon scaffold due to genetic interactions is easy to visualize and quantify with the availability of antibodies such as 1D4 (α-FasII) that detects Fascilin II expressed in the longitudinal tracts (Garcia-Alonso et al., 1995) and BP102 that recognizes a glycoprotein ubiquitously expressed in the CNS allowing visualization of longitudinal and commissural axons. The combination of such antibodies and a wide range of tissue specific driver lines make the Drosophila embryonic nerve cord an ideal system to study signal transduction during axon guidance.

While gross axon guidance defects can be observed in a whole animal, very little information can be retrieved about the effect or distribution of the proteins at the cellular level. Therefore, the availability of immortalized cell lines (e.g. S2 and S2R+) serves as an important tool to investigate the molecular and cellular mechanisms underlying nerve cord phenotypes.
In this study, semi-adherent S2 cells of macrophage – like lineage are used to study the effect of Fra and Abl interaction on actin cytoskeleton. The S2 cell lines derived from 20-24 hours old Drosophila embryos are karyotyped as female, and exhibit 60-80% tetraploidy (Schneider, 1972). The ease of maintaining and manipulating these cells make it researcher-friendly and has various applications including biochemical analysis of genetic interactions, large scale identification of known and novel binding partners, effects on cell proliferation and cell cycle (Bjorklund et al., 2006; Chang et al., 2008; Rogers et al., 2003). Simple gene delivery using recombinant vectors allows...
heterologous protein expression and subsequent analysis. In this thesis, pMT vector containing the promoter region of the *Drosophila* metallothionein (Mtn) gene is used. Mtn gene expression is driven by a transcription factor MTF-1 loaded with heavy metals such as copper and cadmium (Selvaraj et al., 2005). Thus, in cultured cells, adding copper facilitates the expression of the protein of interest in a controlled manner [Fig. 1.1B; (Bunch et al., 1988)]. S2 cells treatment with doublestranded RNA (dsRNA) allows the functional role of genes to be assayed by knocking down the endogenous mRNA via RNAi.

Normal S2 cells are round and small (10µm) in culture. However, when these cells are plated on the substrate, concanavalin A (Con A), it spreads to form a flat lamellar structure that is approximately 20µm in diameter (Rogers et al., 2003). These cells can then be fixed, and immunostained against proteins of interest to address questions such as sub cellular localization, morphological changes and alterations in cytoskeletal elements - actin, myosin and microtubules. The major caveat to such an approach lies in the heterogeneous nature of the cells, where some cells express very few copy numbers while others express many copy numbers of transgene. However, optimizing the transfection efficiency such that most cells express approximately the same amount of protein will aid in quantifiying and analysing of the data.

The goal of my thesis project is to elucidate the cellular and molecular mechanism behind Frazzled and Abelson Tyrosine Kinase interaction in the regulation of the actin dependent cell morphological changes in S2 cells. Thus, the following introduction is divided into three sections: First, keeping in view that Frazzled is a major axon guidance receptor, an overview of *Drosophila* nerve cord
development and role of the attractive and repulsive receptors in axon guidance particularly focusing on Frazzled will be discussed. In the second part, the growth cone movement that involves the concerted effort of actin, myosin and microtubules of the cytoskeleton is discussed. Finally, the third part will cover the role of tyrosine phosphorylation in the growth cone, particularly shedding light on the structure and function of Abelson and its interactions with different actin modifiers.

**Drosophila Nerve Cord Development**

The central nervous system of *Drosophila* is divided into the brain and the ventral nerve cord (VNC). During early embryogenesis, patterning genes act in a gradient along the anterior-posterior and dorso-ventral axis; this in turn leads to expression of segment polarity and columnar genes in each segment and the neuroectoderm respectively (Skeath and Thor, 2003). Here, each hemi segment attains a unique combination of expressed genes giving rise to an equivalence group. The expression of the ac/sc genes namely, *achaete* (ac), *scute* (sc) and *lethal of scute* (l'sc) in an equivalence group gives it the potential of developing into neural cells (Garcia-Bellido, 1979; Garcia-Bellido and Santamaria, 1978). In addition, lateral inhibition of *Notch* (N) and *Delta* (De) and the suppression of the ac/sc genes in surrounding cells allow only one of the ectodermal cells to commit to the neuroblast (NB) fate (Heitzler et al., 1996; Heitzler and Simpson, 1991). Approximately, five hundred neuroblasts that delaminate from the ectodermal layer arrange in a repeated fashion of approximately 40 NBs per segment, thus, forming the neural cells (Hartenstein et al., 1992; Prokop and Technau, 1991). Succinctly, a
series of asymmetric divisions in the mitotic NBs generate the ganglion mother cells (GMC). Each GMC expresses temporal gene networks that establish distinct neuronal fates to produce a pair of neurons or glia. (Chu-LaGraff and Doe, 1993; Isshiki et al., 2001; Parras et al., 1996; Skeath and Thor, 2003). Precise cell intrinsic and cell – cell signaling cascades are required for the differentiation into discrete axon bundles and the correct targeting of these axons to form the ladder-like ventral cord of the *Drosophila* embryo (Hidalgo et al., 1995; Menne et al., 1997; Spana et al., 1995).

**Axon pathway formation in Drosophila**

The central nervous system of *Drosophila melanogaster* is a bilaterally symmetrical structure consisting of the left and right hemispheres that are separated by specialized midline cells. The nerve cord is ladder-like consisting of the longitudinal connectives and commissural neurons. The combined effort of the pioneering ipsilateral neurons pCC and vMP2 that extend anterior and MP1 and dMP2 that project posterior along the A-P axis form the longitudinal connectives. These neurons never cross the midline and serve to form the intersegmental connective on either side of the midline (Jacobs and Goodman, 1989; Thomas and Crews, 1990). In contrast, each segment consists of another set of pioneering neurons that cross the midline to form a posterior commissure (Spana et al.) and anterior commissure (AC), the rungs of the ladder-like scaffold (Fig. 1.2A). Once the commissural axons cross to the contralateral side, they turn and extend longitudinally and never re-cross (Jacobs and Goodman, 1989; Spitzweck et al., 2010). The precision and accuracy required during neuronal
development occurs through the concerted effort of several signaling molecules expressed both at the midline and on the migrating axons. In general, the axon pathway formation near the midline is a competition between two major receptor pathways: Netrin-Frazzled and Slit-Roundabout. Recently, there has been evidence of other receptors that also help in forming the axon scaffold. This thesis focuses on the Netrin dependent Frazzled/DCC signaling both \textit{in vivo} and \textit{in vitro}.

\textbf{Netrin: Midline Attraction Vs Repulsion}

Netrin/Unc-6 was first discovered in \textit{C. elegans} in a screen for developmental defects (Ishii et al., 1992). To date, five isoforms of Netrin have been discovered in mammals - three secreted Netrins (1,3,4) and two glycosyl phosphatidylinositol (GPI) – anchored membrane proteins, Netrins G1 and G2 (Rajasekharan and Kennedy, 2009; Serafini et al., 1994). In \textit{Drosophila}, Netrin A and Netrin B located on the X-chromosome are strongly expressed in the CNS and share 55% similarity with each other (Harris et al., 1996). The functional similarity between invertebrates and vertebrates is attributed to the evolutionary conservation between the proteins, wherein Netrin A and B share 51% and 54% similarity respectively to chick Netrin-1. Characteristic of the super family of laminin, in its amino terminal end, the secreted and the tethered Netrins have laminin gamma-1 chain and laminin beta-1 chain respectively (Rajasekharan and Kennedy, 2009). This is followed by a globular domain VI and domain V consisting of three EGF repeats that Netrin may use to bind to its receptors in order to initiate downstream signaling pathways (Kruger et al., 2004).
Netrins are conserved bi-functional cues that affect both short range and long range signaling during nervous system development (Kennedy, 2000). The receptors that respond to Netrin include the super immunoglobulin family of receptors namely, DCC/Frazzled/Unc-40 and Neogenin, Unc5 and the recently discovered Down Syndrome Cell Adhesion Molecule (DSCAM). Netrin attracts commissural neurons across the midline via DCC/Fra/Unc-40 and DSCAM (Dorsten and VanBerkum, 2008; Round and Stein, 2007). In C.elegans, ectopic expression of Unc-5 in the neurons that express Unc-40 cause these axons to move away from the source of Unc-6 (Hamelin et al., 1993). Similarly, Xenopus spinal neurons expressing DCC is attracted towards Netrin but switches to repulsion when Unc-5 is expressed in these neurons in vitro (Hong et al., 1999; Keleman and Dickson, 2001; Kennedy, 2000). In Drosophila, loss of both the netrin genes cause thinning of commissures and prevent many posterior commissures from crossing over the midline confirming its role in midline attraction [Fig. 1.2 B; (Harris et al., 1996; Mitchell et al., 1996)]. Netrin attractive signaling is counter balanced by the Slit repulsive signaling that prevents the axons from crossing the midline.

Overview of Axon Guidance

All axons must make the initial decision to cross or not to cross the midline in order to pattern the ladder-like scaffold. This critical decision is made by the expression of Roundabout (Robo) family of receptors in all the axons (Kidd et al., 1998a) and another gene, Commissureless (Comm) that is expressed only in the subset of
commissural axons (Keleman et al., 2002; Kidd et al., 1998b). Axons that express the chemorepulsive receptor, Robo, respond to its ligand, Slit and do not cross the midline; instead these axons travel ipsilaterally to from the longitudinal connectives. Reduction of repulsion by either removal of both copies of robo or slit leads to multiple ectopic crossing or collapse of the neurons at the midline respectively [Fig 1.2 D-E; (Bashaw et al., 2000)]. Conversely, Fra activation in the commissures guides these axons across the midline. Furthermore, Fra also positively regulates the transcription of Comm which in turn down regulates Robo by receptor endocytosis, allowing attractive signaling to take place in the commissural axons (Keleman et al., 2002; Keleman et al., 2005; Myat et al., 2002; Yang et al., 2009). While attractive and repulsive signaling has been considered as independent events, there is now evidence of interdependency between the two guidance systems. At the transcriptional level, a single T-box protein Midline directs the control of fra, robo and slit expression and may be responsible for balancing the levels of the two systems (Liu et al., 2009b). In addition, there are other receptors such as DSCAM that act in a Netrin dependent and independent manner during axon pathway formation (Andrews et al., 2008). For example, in chick spinal cord explants, inhibition of DSCAM by short interfering (siRNA) abolishes axon outgrowth in response to Netrin stimulation. Subsequent re-expression of wild type DSCAM rescues the axonal outgrowth suggesting that it works as an attractive receptor like Fra. However, triple knockout of the two functionally redundant dscam genes and fra disrupt longitudinal axon tracts and allow very few anterior or posterior commissural axons to form (Andrews et al., 2008). These defects are more severe than a netrin mutant alone, where most of the anterior commissures are still intact (Harris et al., 1996; Mitchell et
al., 1996). This suggests that in addition to responding to Netrin attraction, DSCAM may also function in a Netrin independent manner. Recently, mutations in *turtle* have been described to exhibit missing commissures, similar to the phenotype from loss of chemotactic molecules like Netrin, Fra and DSCAM, thus, providing evidence of other Netrin independent mechanisms during midline guidance (Al-Anzi and Wyman, 2009). Nevertheless, Netrin signaling initiated through Fra at the midline is a major signaling event involved during axon guidance.

![Fig. 1.2 Loss of guidance receptor genes leads to axon scaffold defects.](image)

**Fig. 1.2 Loss of guidance receptor genes leads to axon scaffold defects.** This panel of pictures is exported from various papers and modified. A-C: Andrews et al., 2008. BP102 antibody stains all the axons in the CNS to show a ladder-like structure in Drosophila embryo. In the wild type, two parallel tracts of axon run ipsilaterally to form the longitudinal connectives. The anterior and posterior commissures (AC, PC) cross the midline to form the rungs the ladder (A). In the absence of either *fra* or *netrin*, the mutant embryos display scaffolding defects such as loss of commissures (B,C). While *fra* mutants show specific loss of posterior commissures (C), loss of netrin A and netrin B affects the formation of both AC and PC (B). In contrast, a *robo* mutant causes the axons to repeatedly cross the midline. These ectopic crossovers lead to the classic roundabout phenotype (*D; Fan et al., 2003*). Moreover, mutations in the ligand – *slit* leads to a complete collapse of the axons at the midline as depicted in panel E (Rajagopalan et al., 2000).

**Frazzled – Chemoattractive Midline Guidance Receptor**

Frazzled, initially discovered in an enhancer trap screen for mutations that affect nervous system development is expressed abundantly in the commissural axons
(Kolodziej et al., 1996). *Drosophila* embryos deficient in *fra* display gaps in the longitudinal connectives and loss of posterior commissural axons; a phenotype that is similar to but not as severe as loss of both *netrin* genes [Fig 1.2 B, C; (Dorsten and VanBerkum, 2008; Forsthoefel et al., 2005; Harris et al., 1996; Kolodziej et al., 1996)].

The two isoforms of Fra share an overall 43% sequence identity with vertebrate DCC (Kolodziej et al., 1996). Fra/DCC/ Unc-40 is a single pass transmembrane immunoglobulin receptor that has four C2 type immunoglobulin domains and six fibronectin type III repeats in its extra cellular domain (Cho et al., 1994; Kolodziej et al., 1996; Vielmetter et al., 1994). The fourth and fifth fibronectin domains interact with ligand (Kruger et al., 2004; Rajasekharan and Kennedy, 2009) to initiate a downstream signaling cascade through the intracellular tail, specifically using three evolutionarily conserved regions called P1, P2 and P3 motifs (Dorsten and VanBerkum, 2008; Garbe et al., 2007; Gitai et al., 2003; Kolodziej et al., 1996).

**Role of the conserved P-motifs in midline guidance**

Studies in both vertebrates and invertebrates indicate that the P-motifs recruit a scaffold of intracellular proteins that may act in serial or parallel pathways to regulate the cytoskeleton (Bashaw and Goodman, 1999; Dorsten et al., 2007; Gitai et al., 2003; Li et al., 2004; Ren et al., 2004). These P-motifs are also critical for the interaction of Fra with other receptors such as Unc5 and Robo (Bhat, 2005; Hong et al., 1999; Keleman and Dickson, 2001; Stein and Tessier-Lavigne, 2001).
In *C. elegans*, tethering the cytoplasmic domain of Unc-40 to the membrane by myristoylation resulted in a constitutively active form of the receptor [Unc-40^Myr (Gitai et al., 2003)]. Overexpression of the myristoylated receptor disrupts the circumferential trajectories of AVM neurons that normally migrate ventrally. Instead, these neurons are misguided away from the ventral side and display additional axon branches. Expressing Unc-40^Myr mutants with P1 or P2 motif deleted reduced the frequency of the axon migration defects implying that these motifs are required for Unc-40 mediated axon guidance. Further, downstream pathways were established by identifying genes that suppressed the dominant Unc-40^Myr gain of function phenotype. This study suggests that Unc-40 initiates parallel signaling mechanisms to regulate actin dynamics underlying axon guidance. The P1 motif genetically interacts with Ena and the P2 motif is required in the Ced10 (a Rac GTPase) and Unc-115/AbLIM (a putative actin binding protein) pathway (Gitai et al., 2003). Consistently, these effectors also interact with Fra and DCC receptors during commissure formation and neurite outgrowth (Dorsten et al., 2010; Forsthoefer et al., 2005; Shekarabi et al., 2005). However, certain differences exist between the model systems, probably indicating a level of specificity between species. To demonstrate this point, in *Drosophila*, gain of function studies indicate that the P1 motif has an inhibitory effect as deletion of this motif enhances the ectopic midline cross over defects. In contrast, expression of a Fra mutant with the P2 motif deleted rescued longitudinal connectives defects of a *fra* mutant as well as the wild type Fra (Dorsten and VanBerkum, 2008; Garbe et al., 2007). The role of the P3 motif has not been addressed in *C.elegans*; however, this motif is critical to both Fra and DCC function. In *Drosophila*, the P3 motif is required in a subset of commissural neurons and
for the formation of the longitudinal tracts. However, unlike in vertebrates, the P3 motif does not mediate a Netrin dependent multimerization of the receptor (Garbe et al., 2007; Stein and Tessier-Lavigne, 2001). This again suggests that despite evolutionary conservation, Fra may use a different mechanistic approach to mediate attraction in the CNS.

In general, the intrinsic machinery to establish the nerve cord structure involves three critical steps: First, the axons and their neighboring cells should have the complete combination of receptor and ligand system. Second, these receptors should be localized at the tip of the axonal growth cone. Finally, the axon should be able to initiate and direct signaling mechanisms that will transduce to the cytoskeleton, ensuring directed movement of the growth cone. Since the goal of this thesis is to understand how Fra activation may use Abl to drive the arrangement of actin cytoskeleton, a generic model of the cytoskeletal dynamics involved in growth cone movement is discussed before delving deeper into the mechanism of Fra or Abl signaling.

The Growth Cone: A Structural View

The extending tip of an axon, the growth cone (Ramón y Cajal, 1890) is capable of detecting and responding to environmental cues to direct axon extension along a given pathway. The growth cone forms specialized actin-based structures such as web-like lamellipodia and finger-like projections called filopodia that express receptors to explore the extracellular environment for secreted guidance cues.
The movement of the growth cone is initiated as a response to attractive and repulsive signaling. This includes a) exploration, involving extension and retraction of actin filaments, b) adhesion of the new extensions to the extracellular matrix and c) generation of traction force to advance the growth cone. Attractive cues initiate signaling that establishes focal adhesion points thus engaging a ‘clutch’ to the extracellular matrix. After the attachment to the substrate, microtubules move into the peripheral domain of the growth cone assisting the coupling of the actin cytoskeleton to the extracellular matrix. Now, the force generated from myosin contractility leads to the forward movement of the growth cone. However, repulsive cues disengage this ‘clutch’ from the extracellular matrix and now, the force from the contraction of the ATP driven

**Fig. 1.3: Growth Cone movement using the clutch mechanism.** The leading edge of the axon responds express surface receptors such as Fra, that respond to midline cues (e.g. Netrin) to initiate intra cellular signaling. This allows the growth to disengage the old adhesion site in favor of new adhesion on the extra cellular matrix. This formation of the clutch is accompanied with activation of actin and microtubule polymerization. The force generated here propels the cell to move forward.
myosin retracts the F-actin bundles from the membrane leading to growth cone retraction and collapse [Fig.1.3; (Jay, 2000)]. Thus, membrane receptors could potentially regulate three major networks producing movement in a growth cone: 1) coordinating myosin activity with actin and microtubule polymerization, 2) Activation and de-activation of cytoskeletal regulators such as Rho GTPases and 3) affecting phosphorylation levels, an event that is critical to the function of many proteins that modulate actin dynamics such as Abi and Sra-1/Kette.

**Actin and Myosin dependent movement of the growth cone**

Actin is a globular ATPase (G-actin) consisting of an outer and inner domain, each having two sub-domains, namely, outer sub domains 1 and 2 and inner sub domains 3 and 4. The ATP binding site is present in the center of the molecule in the largest cleft between sub domains 2 and 4 (Graceffa and Dominguez, 2003; Holmes et al., 1990; Lee and Dominguez, 2010). ATP hydrolysis regulates transition between G actin and filamentous actin [(F-actin); Fig. 1.4 A]. Kinetically asymmetrical actin goes through tread milling where actin monomers are constantly being added in the ATP bound state and removed from the minus end in the ADP-bound state [(Pollard et al., 2000); Fig 1.4A-B]. This process of actin tread-milling is a concerted effort of many proteins such as the Ena, Profilin, Cofilin, WAVE, Wasp and Arp2/3 to affect actin filament assembly (Lee and Dominguez, 2010; Pollard and Borisy, 2003; Rafelski and Theriot, 2004; Zigmond, 2004). To enable the net movement of actin in the growth cone, actin has to be retracted from the lamellipodia and filopodia, a phenomenon called
retrograde actin flow. The retrograde actin flow requires not only actin tread milling but also transient formation of adhesion points and myosin contractility (Lin et al., 1997; Medeiros et al., 2006).

Several isoforms of myosin exist in the growth cone but the conventional myosin II is thought to regulate actin dynamics. Myosin II is a hexameric protein consisting of a head, neck and tail region (Warrick and Spudich, 1987). The globular head of the heavy chains interacts with actin and utilizes the energy from ATP hydrolysis to generate traction force to ‘walk’ along the actin filaments (Fig. 1.4C). In addition, the tail end serves as a molecular transport vehicle to cargo the required proteins to the distal tip. The neck region consists of pairs of essential light chains (ELC) and regulatory light chains (RLC). Myosin activity is dependent on the phosphorylation state of the RLC. This phosphorylation can be attained by both calcium dependent and independent mechanisms (Bearer et al., 1996). To demonstrate this point, the Myosin Light Chain kinase (MLCK) which is a Calmodulin dependent enzyme (Calmodulin is a calcium binding protein) directly activates myosin by phosphorylating its regulatory light chains in response to calcium signals. In contrast, Rho Kinase regulates myosin activity both by inhibiting myosin phosphatase and phosphorylating myosin light chains in a calcium independent manner (Hirata et al., 2009; Kim and Chang, 2004; Schmidt et al., 2002).

The family of small Rho GTPases (Rho, Rac and Cdc42) is strongly implicated as regulators of actin-myosin dynamics. These proteins serve as molecular switches that alter between active and inactive states through the binding of guanine nucleotides.
Fig. 1.4: Actin Polymerization and movement of myosin on actin filaments. A. Actin monomers exist in a globular form called G-actin. Each of the molecules in panel A represents G-actin. Hydrolysis of ATP is the main factor that regulates the state change from G-actin to F-actin (filamentous actin). B. G-actin can form dimers and trimers, leading to the formation of F-actin. Immediately after the G-actin is incorporated into the F-actin chain, GTP inside the monomer gets hydrolyzed and remains trapped as ADP in the F-actin. C. The globular head of myosin binds to actin (1) and ATP (2). Myosin uses the energy derived from ATP hydrolysis (Jung et al.) to generate a power-stroke such that it moves towards the positive end of actin (4).

In the active state (GTP bound), GTPases transduce extracellular signals from membrane receptors into the cell and to the cytoskeleton. Two proteins facilitate this switching mechanism: Guanine Exchange Factors (GEFs) which increase Rho GTPase
activity by stimulating the exchange of GDP for GTP while *GTPase Activating Proteins* (GAPs) decrease their activity by stimulating intrinsic GTPase activity, hydrolyzing GTP to GDP (Bishop and Hall, 2000). The Rho GTPases regulate actin dynamics by either recruiting downstream effectors such as *Rac* associated 1 (Sra1) to the membrane or by trigging the activation of nucleation promoting factors (NPF) such as Wiskott Aldrich Syndrome protein (WASp), thereby activating Arp2/3 (*Takenawa and Suetsugu, 2007*).

Vertebrate cell culture demonstrates that Rac induces the formation of lamellipodia and membrane ruffling while activation of Cdc42 drastically increases the number of filopodia that form adhesive contacts in fibroblasts, thereby promoting motility of the cells (Allen et al., 1997; Machesky and Hall, 1996). Furthermore, knockdown of the Rho effector, Rho associated Kinase increases actin stress fibers and focal contacts in cultured cells (Nobes and Hall, 1999). The distinct global effects on the organization of the actin cytoskeleton are also regulated by other cytoplasmic proteins like Abelson Tyrosine Kinase, Enabled and Profilin to ultimately allow cell movement (Reinhard et al., 2001; Sohn and Goldschmidt-Clermont, 1994).

More importantly, Rho GTPases are known to work downstream of guidance receptors. For example, Fra uses its P3 motif to regulate myosin activity through Rho and Abl during the development of the *Drosophila* nerve cord (Dorsten et al., 2007). It is also suggested that Fra regulates actin dynamics underlying commissure formation (Dorsten et al., 2010; chapter 2). The binding of DCC P3 motif to myosin X further strengthens the concept that activation of membrane receptors such as Fra/DCC/Unc-40 at the growth cone initiate signaling pathways to couple actin and microtubule
assembly with myosin activity to promote axon steering events towards a target (Zhu et al., 2007).

The coupling of Actin and Microtubules dynamics in the Growth Cone

Microtubules are long, hollow unbranched cylinders mainly composed of alpha and beta tubulin. Microtubule dynamics are regulated by two main events: First, alpha tubulin is synthesized with a carboxyl tyrosine residue which is post-translationally removed by tubulin carboxypeptidase that acts specifically on polymerized tubulin [Fig. 1.5 A; (Arce and Barra, 1985; Barra et al., 1988; Brown et al., 1992; Bulinski and Gundersen, 1991)]. Thus, the microtubule assembly is dependent on the tyrosinated state of tubulin. Second, with the exchange of guanidine triphosphate (GTP) - both alpha and beta tubulin binds to GTP. Dimers can then bind to each other to form a heterodimer chain, also called as the protofilament. Hydrolysis of GTP at the end of the protofilament acts as a switch for the release of native tubulin heterodimer from the alpha-beta tubulin complex [Fig. 1.5 B; (Davis et al., 1994; Tian et al., 1999)]. Dynamic microtubules transiently explore the peripheral (P) growth cone domain following the F-actin assembly at the leading edge. The retraction of the microtubules simultaneously with the actin retrograde flow suggests a coupling mechanism between actin and microtubules (Dehmelt et al., 2003; Salmon et al., 2002; Waterman-Storer et al., 1998; Yvon and Wadsworth, 2000). Pharmacological inhibition of microtubule assembly resulting in its disappearance from the P domain is also accompanied by the growth cone turning away from a source of chemoattractive cues (Gordon-Weeks, 2004; Suter
et al., 2004), indicating its importance in growth cone steering (Marcos et al., 2009; Rajnicek et al., 2006; Schaefer et al., 2002). Mice hippocampus neurons deficient in MAP1B - a neuron-specific microtubule-associated protein that is implicated in the crosstalk between microtubules and actin show reduced chemoattractive response towards Netrin (Del Rio et al., 2004).

**Fig. 1.5: Model for regulating the dynamic instability of microtubules.** A. Initial step in the process of microtubule polymerization is the post-translation modification of α-tubulin. Here, the enzyme – tubulin carboxypeptidase catalyzes the removal of a tyrosine residue from α-tubulin. B. This α-tubulin binds to β-tubulin to form a dimer that exists in a GTP bound state (1). Dimers can elongate to give rise to a heterodimer chain. Once the dimer gets incorporated, the GTP gets hydrolyzed to GDP and remains trapped in the protofilament (2). Furthermore, hydrolysis of GTP at the end of the protofilament forms an unstable complex. This leads to dissociation of the dimer from the chain. The resulting α-β tubulin undergoes GDP-GTP exchange and cycles back in the highly dynamic polymerization-depolymerization process.
Genetic evidence of Frazzled mediated regulation of the cytoskeleton

Mutations in genes that regulate cytoskeletal dynamics such as trio - a Rac GEF, and abl enhance, while mutations in ena suppress the loss of commissure phenotype caused by the loss of fra (Dorsten et al., 2007; Dorsten et al., 2010; Forsthoefer et al., 2005) suggesting a role of Fra in regulating cytoskeletal dynamics. In fact, studies from our lab have linked Fra signaling to the regulation of both actin and myosin dynamics. These studies suggest the requirement of P3 in Fra signaling to myosin through a Rho/Abl pathway and to actin via Abl where Cdc42 and Rac also participate in axon pathway formation (Dorsten et al., 2007 and 2010). Abl could be regulating actin by either phosphorylating Fra (Forsthoefer et al., 2005) or by negatively regulating Enabled downstream of Fra (Li et al., 2005; Martin et al., 2005). Further, association of Drosophila Rho GEF2 with the tips of microtubules and evidence of actin-microtubule regulation by Rac and Cdc42 (Daub et al., 2001; Rogers et al., 2004) suggests that Fra regulates growth cone movement by initiating signaling events that regulate actin, myosin and microtubule separately. However, most of the understanding of this signaling mechanism stems from vertebrate cell culture research.

Understanding Fra/DCC signaling mechanism using cell culture

The central theme is that midline guidance receptors promote rearrangement of the cytoskeleton to dictate growth cone steering and motility. DCC promotes cell spreading and filopodia formation by activating Cdc42 and Rac; conversely inhibition of Rho and Rho Kinase increases these membrane protrusions (Li et al., 2002b;
Inhibition of Src kinase abolishes Netrin stimulated neurite outgrowth in commissural neurons from rat spinal explants. Other studies have also indicated that activation of specific kinases such as focal adhesion kinase (FAK), Src and Fyn Kinases is essential to Netrin dependent DCC induced neurite outgrowth and orientation (Li et al., 2004; Meriane et al., 2004). Similar to Fra, P3 motif is important to the function of DCC and binds to FAK which stimulates DCC phosphorylation required for axon attraction (Li et al., 2004; Ren et al., 2004). However, Drosophila mutants with an intact LD motif which is critical to FAK association are not able to rescue the P3 phenotype suggesting that FAK may not be important for Fra mediated axon guidance (Garbe et al., 2007). This indicates that Fra may utilize different downstream effectors to interact with the cytoskeleton. The probable differences in the molecular mechanism also illustrate the validity of using cell culture to systematically study how Fra downstream signaling modulates the cytoskeleton.

Therefore, in this thesis, Drosophila S2 cells are used to understand the native molecular mechanism underlying Fra activation and its effect on the actin cytoskeleton. Although DCC uses Src kinases, all the genetic data (previous and next section) in Drosophila suggest that Fra manipulates Abl to correctly form the axon scaffold. Thus, this thesis will focus on the structure/function analysis of Abl during Fra signaling.

**Role of Tyrosine Phosphorylation in Growth cone movement**

Several lines of evidence including pharmaceutical studies implicate the need for tyrosine phosphorylation during the dynamic translocation of the growth cone.
from one point to another (Goldberg and Wu, 1996). Over 87% of *Xenopus* spinal neuron growth cones are tyrosine phosphorylated at focal points suggesting that tyrosine phosphorylation is a key step in the clutch mechanism of growth cone movement (Robles et al., 2005). Genetic manipulations of kinase (e.g. Abl) and phosphatase (e.g. DLAR) genes in *Drosophila* leads to severe structural defects of the nerve cord indicating a fine regulatory interplay between these two major classes of proteins (Song et al., 2008; Wills et al., 1999a).

There are two kinds of tyrosine phosphatases, namely, receptor protein tyrosine phosphatases [RPTP, e.g. Leukocyte-antigen-related-like protein (LAR) and PTP10D] and nonreceptor phosphatases. The loss of LAR and PTP10D disrupts longitudinal tracts and motor axon formation in mammals and *Drosophila* (Desai and Purdy, 2003; Jeon et al., 2008; Schindelholz et al., 2001; Stepanek et al., 2005; Uetani et al., 2006; Wills et al., 1999a).

Cytoplasmic kinases are key molecules that antagonize the effect of phosphatases and integrate signaling networks that occur during numerous processes including neuronal development. Consistent with the role of Src kinases in Netrin mediated DCC attractive signaling, inhibition of Src abolishes DCC dependent neurite extension and orientation in rat embryonic explants. Similarly, impaired neurite outgrowth in Fyn deficient explants may reflect the loss of its selective interaction with DCC at tyrosine 1420 (Meriane et al., 2004; Rajasekharan et al., 2009; Ren et al., 2004). Src Kinases also interact with the cytoplasmic domain of the repulsive receptor Unc-5 in a phosphorylation dependent manner (Li et al., 2006). These kinases can interact not only with the receptors but also other downstream actin effectors such as
AFAP120 (a nervous system specific actin cross linking protein) to regulate axon extension (Harder et al., 2008).

The dramatic decrease of the commissures in a fra mutant by both Abl loss and gain of function implies that an optimal dose of Abl is required by Fra to mediate midline crossing of the commissural axons (Dorsten et al., 2010; Forsthoefel et al., 2005). This steady state balance may be achieved by the activity of phosphatases in the migrating neurons. Several studies have demonstrated that mutations in Abl genetically interact with mutations in phosphatases (e.g. Dlar)(Wills et al., 1999a). For example, Drosophila Leukocyte-antigen-related (Dlar) protein and its ligand, syndecan (Sdc) is required for motor neuron pathway formation. Here, Dlar mutant display ISNb motor neuron bypass phenotype, which is also observed with Abl overexpression. Moreover, a reduction of abl alleviates the Dlar ISNb bypass phenotype suggesting that these two gene products have opposing roles during axon scaffold formation (Wills et al., 1999a). Abl and Dlar also reciprocally phosphorylate and dephosphorylate each other in vitro indicating a direct co-regulation (Lanier, 2000). Mutations in abl also suppress midline crossing over and motor neuron defects seen in another phosphatase mutant, Ptp69D, indicating that Abl is an important kinase that regulates the formation of the Drosophila ventral nerve cord (Song et al., 2008). Other phosphatases such as the PTP61F also act in concert with Abl to regulate the recruitment, localization and expression levels of actin modifiers like Abl interacting protein (Abi) and Kette (Huang et al., 2007; Ku et al., 2009).

The indispensable role of Abl during Frazzled signaling in the gross development of the nervous system accompanied by little direct evidence of the mechanism that occurs at the cellular level has established the foundation of my thesis project.
Structural and functional domains of Abelson Kinase

The non receptor Abelson Tyrosine Kinase (Abl) was discovered as a proto oncogene for its role in chronic myelogenous leukemia (CML). However, now it is also acknowledged as one of the key molecules that link upstream receptor signaling to the cytoskeleton. The Abelson protein family is strongly conserved between species and includes two alternate myristoylated or non-myristoylated isoforms of vertebrate Abl, Arg, dAbl in Drosophila and Abl-1 in C.elegans.

Overall, Drosophila and human Abl share 62% amino acid sequence conservation. The amino-terminus of Abl has four sequentially arranged regions [Fig. 1.6]: The N-Cap domain is followed by two Src-homology regions SH3 and SH2 that share 86% with the human Abl. These domains interact with actin nucleation promoting factors (NPF) such as cortactin and N-WASp to modulate the activity of Arp2/3 complex in actin polymerization (Miller et al., 2010). Unlike Src kinase, dAbl kinase domain is in the N-terminal region with 87% similarity to the human Abl (Henkemeyer et al., 1988; Kruh et al., 1990). The carboxyl end of Abl consists of unique functional domains such as the proline rich (PXXP) regions to facilitate interactions with proteins containing the SH3 – SH2 domains, and Enabled/VASP Homology (EVH1) domain that it uses to directly bind to and regulate Enabled activity. At the extreme end of the C-terminus is a F-actin binding domain (FABD) via which Abl physically associates with filamentous actin (Bradley and Koleske, 2009).
**Structural role for regulation of kinase activity**

Abl kinases regulate their enzymatic activity by a ‘latch-clamp-switch’ mechanism (Harrison, 2003). The N-terminal cap, SH3 and SH2 domains fold back and latch over the catalytic domain by an intramolecular binding between the SH2 region and a conserved phosphotyrosine residue in the carboxyl tail. This latch is further tightened with the SH3 domain interacting with a proline in the linker region between the SH2 and kinase domain (Fig. 1.6 A). In fact, reports confirm that mutations in the SH3 regions lead to deregulated Abl kinase activity possibly due to the disruption of the auto inhibitory conformation (Barila and Superti-Furga, 1998; Bradley and Koleske, 2009; Brasher and Van Etten, 2000; Franz et al., 1989; Nagar et al., 2003; Tanis et al., 2003). Activation of the enzyme requires the de-coupling of the physical interaction between the amino and carboxyl ends. However, if we create a chimera such as the BcrAbl protein where the N-terminal regions of the human Bcr gene is fused to C-terminus region of *Drosophila* Abl, it disrupts the endogenous auto inhibitory mechanism (Fogerty et al., 1999). In chapter 3, we used this fusion protein as a constitutively active kinase to better understand the interaction between Abl and Frazzled. *In vivo*, autoinhibitory conformation is relieved by phosphorylation at two key tyrosine residues which is achieved by either of two mechanisms: In one, Abl auto phosphorylates in trans, but in the other, non receptor tyrosine kinases such as Src phosphorylate the tyrosine residues at the linker region and in the activation loop of the kinase domain, which allows access to its substrates (Fig 1.6 A-C)
Fig. 1.6: The structural regulation of Abl kinase activity. A. Abl exists in an inactive state when the N-terminus loops over and latches on the kinase domain. B. Abl kinase can get activated when cytoplasmic proteins bind to SH3/SH2 domain relieving the inhibitory interaction. C. Abl can also be activated by trans auto phosphorylation or phosphorylation by other cytoplasmic kinases.

Localization of Abelson in cultured cells

Considering the role of Abl in the actin cytoskeleton, it is not surprising that immunostaining of cells consistently reveals high level of Abl localizing to dynamic actin structures such as filopodia, dorsal membrane ruffles and focal adhesions in migrating macrophages, fibroblasts and neurons in response to upstream stimulation (de Arce et al., 2010; Jin and Wang, 2007; Woodring et al., 2004). The expression pattern of Abl and its co-localization with effectors of the cytoskeletal dynamics leads to the
speculation that Abl may activate and inactivate its substrates at different time points and morphological states. Fluorescent resonance energy transfer (FRET) based biosensors have been designed specifically to detect the activity of tyrosine kinases, such as Abl, which recognize substrates that have consensus phosphorylation sequence AYXXP, where ‘A’ is a polar residue and ‘P’ is a small side chain (Ting et al., 2001; Woodring et al., 2003). For example, this method has been used to observe the precise decoupling of the adaptor protein, Crk and its effector Crk associated substrate (CAS) when a kinase active Abl is reconstituted in abl-/- arg -/- embryonic mouse fibroblasts to inhibit migration (Kurokawa et al., 2001). Thus, this approach can shed light upon the spatial and temporal interaction of Abl with its substrates and consequently, the biological relevance of such an interaction in live cells.

**Regulation of Actin dynamics by Abelson Kinase**

While Abl promotes F-actin microspikes and filopodia formation, it also inhibits cell spreading on fibronectin indicating that Abl differentially regulates processes that are integral to actin polymerization. The accurate and in depth knowledge of the mode of action, by which Abl and Arg modulate actin remodeling still remains elusive. However, it can be safely predicted that these kinases play a critical role in (a) regulating actin binding proteins thereby promoting actin nucleation, (b) stabilizing nascent focal adhesion preventing retraction or collapse of growth cones and (c) directly interacting with F-actin.
Abl may directly or indirectly affect Arp2/3 activation, the molecular machine that nucleates actin monomers and is controlled by two major activators: the auto-inhibited WASp and WAVE (WASp-family verprolin-homologous) protein. Binding of small GTPases such as Cdc42 either directly or through adaptor proteins like Nck release this autoinhibited conformation of WASp allowing it to interact via the VCA domain to Arp2/3, promoting formation of actin filaments (Antoku et al., 2008; Rohatgi et al., 1999). Abl binding to WASp and consequent Wasp phosphorylation increases actin polymerization possibly by preventing its retraction to the inhibitory conformation (Burton et al., 2005; Miller et al., 2010). Furthermore, the co-localization of Abl and WASp at the membrane protrusions is dependent on this interaction. Alternatively, Abl mediated phosphorylation of cortactin stabilizes the actin filaments formed by N-WASP and Arp2/3 complex (Pollard, 2007).

Unlike WASp, WAVE exists in a pentameric inhibitory complex consisting of Sra1, Abi, Kette/Nap1 and HSPC300 in the cytosol (Eden et al., 2002). Abl interacts intimately with the individual components of the complex releasing WAVE to promote actin polymerization. It is observed that the adaptor protein Nck and GTPase Rac binds to Nap1 and Sra-1 respectively thus removing Nap1 and Sra1 from the complex (Eden et al., 2002; Steffen et al., 2004). Whether Abi is completely removed from the complex or if it is still bound to WAVE remains unclear but WAVE phosphorylation is essential to its activation of Arp2/3 (Innocenti et al., 2004). It is possible that Abi provides a link between WAVE and Abl creating a positive feedback loop that allows a continuous phosphorylation of WAVE (Bradley and Koleske, 2009; Leng et al., 2005; Stuart et al., 2006). Another angle to this mechanism exists in the interaction between the Abl's
proline rich regions (PXXP) and the SH3 domain of Crk II, an activator of Rac. Abl indirectly inhibits Rac activity by inactivating CrkII through reciprocal phosphorylation which allows both proteins to maintain a balanced level of activity (Antoku et al., 2008; Ren et al., 1994). However, the localization of active Rac with actin positive membrane structures and its association with the regulation of actin dynamics (Steffen et al., 2004) suggests the presence of other mechanisms also affecting WAVE activation.

During development, migrating axons or fibroblasts and even arterial vessels extend out filopodia and form nascent focal adhesions that need to be stabilized to continue a directed movement. Abl can stabilize these focal adhesion points by differentially regulating focal adhesion proteins (e.g. CAS), structural proteins (e.g. vinculin, that connect actin filaments to the transmembrane effectors) and paxillin that signal to remodel the cytoskeleton (Kain and Klemke, 2001; Lewis and Schwartz, 1998; Salgia et al., 1995).

Finally, Abl and F-actin can reciprocally regulate each other by direct binding (McWhirter and Wang, 1993). Mutation in the F-actin binding region of Abl reveals that actin binding actually inhibits Abl activity by further tightening its auto inhibitory conformation (Woodring et al., 2001; Woodring et al., 2002), which is released when cells attach to the extra cellular matrix or a substrate in vitro, increasing the Abl activity.

**Interactions with other regulators of actin polymerization**

In addition to the discussed modes of regulation, Abl interacts with various modifiers like Ena/VASP, Profilin and Cofilin that coordinate polymerization and depolymerization
of actin in motile cells. *Drosophila* Enabled was identified in a genetic screen as a dominant suppressor of lethality of *abl* mutations (Gertler et al., 1995; Gertler et al., 1990). Enabled dependent formation of dendritic branches, actin-rich microspikes of dendritic arborization (DA) and the inability of *ena* mutants to form neurites can be attributed to its anti-capping ability at the barbed end, responsible for elongating actin filaments (Bear et al., 2001; Tahirovic and Bradke, 2009). Abl may affect the interaction of Ena with other proteins by regulating its phosphorylation state (Comer et al., 1998). For instance, Abl positively regulates Ena by enhancing its interaction with lamellipodin (Lpd) upon Netrin activation in primary vertebrate neurons (Michael et al., 2010). In the absence of Abl, both Enabled and Arp2/3 are mis-localized, leading to excess actin polymerization in the apical microvilli and decreased actin in pseudocleavage and cellular furrows in the early stages of *Drosophila* development (Grevengoed et al., 2003). Consistently, over-expression of Abl and Ena/VASP also increases filopodia formation in immortalized vertebrate cells (Lebrand et al., 2004; Radha et al., 2007; Woodring et al., 2002).

The loss of Profilin (chickadee/chi) disrupts actin dependent processes such as oogenesis, bristle development and axon guidance (Hopmann and Miller, 2003; Kim et al., 2001; Manseau et al., 1996; Verheyen and Cooley, 1994). The dosage sensitive interactions of Profilin and Abl in motor neuron outgrowth (Wills et al., 1999b) coupled with the genetic interaction of Abl and Ena during Frazzled signaling (Forsthoefel et al., 2005) suggest that Enabled provides a link between Abl and profilin such that these effectors cooperatively work in the same pathway.
Lastly, Cofilin promotes actin de-polymerization by severing ADP-bound actin from old actin meshwork thus facilitating recycling of actin in the cell. Abl regulates cofilin activity indirectly by phosphorylating cortactin, which relieves the inhibition on Cofilin and thus facilitating its severing activity. Conversely, Cortactin dephosphorylation inhibits actin severing thus stabilizing the actin nucleation event (Boyle et al., 2007; Head et al., 2003; Oser et al., 2009).

To summarize, Netrin dependent Frazzled signaling is a major chemoattractive system during the development of the central nervous system in *Drosophila melanogaster*. Loss of *frazzled* prevents posterior commissural axons to form correctly. In this thesis, firstly the interactions between loss of *fra* and overexpression of Abl during the formation of commissures have been studied. Here, Abl overexpression in homozygote *fra* mutants enhances the loss of commissural axons. Furthermore, expression of RacV12 and Cdc42 but not MLCK phenocopy the genetic interaction of *fra* and gain of *abl* function, suggesting that Fra regulates actin dynamics underlying the commissure formation. Therefore, the second half of this thesis focuses on the molecular mechanisms of Fra and Abl interactions and how this may manipulate actin cytoskeleton leading to morphological changes in *Drosophila* S2 cells.
CHAPTER 2

In the Absence of Frazzled overexpression of Abelson Tyrosine Kinase disrupts
commissure formation in the Drosophila embryonic CNS

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Tyrosine Kinase Disrupts Commisure Formation and Causes Axons to Leave the 
Embryonic CNS’

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The biochemistry experiments demonstrating the lack of P-motif dependence of 
Fra to AblWT and BcrAbl and the phosphorylation of Fra were performed by me. This 
data gives valuable understanding of the genetic interaction between Fra and Abl.

In this thesis, this chapter is presented as an abridged version of the above Plos 
One paper. Dr. Dorsten was involved in the experimental design and scoring data of the 
genetic analysis. Ms. Stephanie Karmo assisted during the genetic data collection by 
staining, preparing and dissecting embryos from all the indicated genotypes in this work.
Abstract

In the *Drosophila* embryonic nerve cord, the formation of commissures requires both the chemoattractive Netrin receptor Frazzled (Fra) and the Abelson (Abl) cytoplasmic tyrosine kinase. Abl binds to the cytoplasmic domain of Fra and loss-of-function mutations in *abl* enhance *fra*-dependent commissural defects. To further test Abl’s role in attractive signaling, we over-expressed Abl or BcrAbl in Fra mutants anticipating rescue of commissures. Surprisingly, it leads to further decrease in the formation of both anterior and posterior commissures. Re-expressing wild-type Fra or Fra mutants with a P-motif deleted display normal AC and PC or fuzzy commissures as observed when Abl or BcrAbl respectively is expressed on its own indicating that the requirement of Fra independent of a specific P-motif. The lack of P-motif dependence is further seen in the physical binding of Abl or BcrAbl to Fra. Pan-neural over-expression of actin cytoskeletal modulators, activated Rac or Cdc42 in a *fra* mutant also induced a significant loss in commissures, but axons did not exit the CNS.

Taken together, these data suggest that Fra activity is required to precisely regulate Abl-dependent actin cytoskeletal dynamics underlying commissure formation. In absence of Fra, Abl may be incorrectly utilized by other guidance receptors, thereby leading to excessive commissure loss.
Introduction

Attractive and repulsive guidance cues originating at or near the midline assist the formation of the *Drosophila* embryonic nervous system. The growth cone expresses membrane receptors for these cues, which upon activation initiate intracellular signaling pathways to govern axon outgrowth and steering as well as formation of dendritic branching patterns (Furrer et al., 2003; Garbe and Bashaw, 2004; Seeger et al., 1993). Commissures form as axons integrate information from chemoattractive Netrins guiding them towards the midline and Slit-dependent repulsion preventing them from crossing (Brose et al., 1999; Harris et al., 1996; Kidd et al., 1999; Mitchell et al., 1996). Ectopic misexpression studies indicate that these attractive and repulsive systems mostly work independently of each other (Garbe and Bashaw, 2007) but there is evidence of a fine interplay between systems at the transcriptional level (Butler and Tear, 2007; Yang et al., 2009) and downstream of receptors.

In *Drosophila*, Netrins are midline attractants detected by Frazzled, a receptor expressed on most CNS neurons and in its absence many posterior commissures fail to cross the midline (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Fra may also have a non-cell autonomous effect guiding selected neurons at the segmental boundary (Hiramoto and Hiromi, 2006). A second Netrin receptor, the Down's syndrome Cell Adhesion Molecule, Dscam (Schmucker et al., 2000), is also expressed on most neurons, and mutations in *fra* and *Dscam* interact to further reduce commissure formation (Andrews et al., 2008; Liu et al., 2009a). Since some commissures still form in *Netrin* null embryos, the presence of an additional Netrin-independent attractive
system has been proposed (Andrews et al., 2008; Kidd et al., 1999). This is supported by evidence that Dscam may respond to a non-Netrin cue (Andrews et al., 2008) and the recent identification of the gene, *turtle* that also interacts with mutations in *Netrin* or *fra* to further reduce commissure formation (Al-Anzi and Wyman, 2009).

Like most cell surface receptors, Fra functions via conserved motifs within its cytoplasmic domains to signal information to the cytoskeleton. Fra has three evolutionarily conserved P-motifs (P1, P2 and P3) in its cytoplasmic domain and it appears that P3 is particularly important for Fra signaling *in vivo* (Dorsten and VanBerkum, 2008; Garbe et al., 2007). Signaling pathways originating from these motifs are thought to govern key aspects of the cytoskeletal dynamics underlying axon extension and maneuvering (Dorsten et al., 2007; Forsthoefel et al., 2005; Garbe et al., 2007; Gitai et al., 2003; Guan and Rao, 2003). Of particular interest here is the Abelson tyrosine kinase signaling pathway, as both Abl itself and some of its key substrates such as Enabled have been implicated in both repulsive (Robo) and attractive (Fra) signaling at the midline.

Abl is a multifunctional tyrosine kinase that serves to link membrane receptors to actin dynamics underlying cell movement (Bradley and Koleske, 2009; Pendergast, 2002). In fact, *Drosophila* Abl is implicated as a key downstream effector in both Robo and Fra signaling. Zygotic loss-of-function mutants of *abl* display mild defects in the axon scaffold including ectopic midline crossing errors (Bashaw et al., 2001; Hsouna et al., 2003; Wills et al., 1999a; Wills et al., 2002; Wills et al., 1999b). Several guidance defects including crossover defects and fused commissures are also observed when *abl* mutants are combined with mutations in a variety of genes involved in the regulation of
cytoskeletal dynamics (Fritz and VanBerkum, 2000; Jin and Wang, 2007; Wills et al., 1999a; Wills et al., 1999b; Yang and Bashaw, 2006). In cultured cells, Abl interacts with small GTPases such as Rac, to promote actin remodeling involved in the formation of membrane ruffling and lamellipodia (Jin and Wang, 2007). The observation that Abl binds to and phosphorylates the cytoplasmic tail of Robo led to the suggestion that Abl is a key regulator of actin dynamics during the transduction of midline repulsive cues (Bashaw et al., 2001; Hsouna et al., 2003). However, other data suggest Abl’s role is not confined to the transduction of midline repulsion. Eliminating zygotic and maternal contribution of abl gives rise to missing commissures and gaps in the longitudinal connectives (Grevengoed et al., 2001). Increasing levels of Abl activity also interact with heterozygous robo mutants to induce ectopic crossovers, and overexpression of Abl in a comm mutant, experiencing high levels of midline repulsion, actually improves commissure formation (Hsouna et al., 2003). This suggests that increasing Abl activity could enhance midline attraction.

Indeed, Abl has been linked to the transduction of midline attractive cues. In GST-pull down and immunoprecipitation assays, Abl binds to the cytoplasmic tail of Fra and when Abl is expressed in S2 cells with Fra, the tyrosine phosphorylation levels of Fra increase (Forsthoefel et al., 2005). In vivo, very few commissures form in fra, abl double mutants (Forsthoefel et al., 2005). Interestingly, abl mutations also enhance the degree of commissure loss observed in combination with mutations in Dscam (Andrews et al., 2008), turtle (Al-Anzi and Wyman, 2009), amalgam and its receptor neurotactin (Liebl et al., 2003), fasciclin I (Elkins et al., 1990), and midline fasciclin (Hu et al., 1998). Together, this suggests that Abl plays multiple roles at the midline as it aids in the
transduction of both Netrin dependent and independent attractive cues. To further elucidate Abl’s role, we sought to elevate Abl activity in embryos that are null for fra. It was reasoned that if Abl is working with Fra to potentiate attractive signaling and a loss of abl in a fra mutant enhances commissural defects, then over-expression of Abl in a fra mutant should partially rescue commissure formation. In contrast to this prediction, we observed the fra commissural defects are dramatically enhanced as both anterior and posterior commissures fail to form and this is associated with a large number of CNS axons projecting beyond the CNS/PNS border. To investigate the mechanism by which Abl may be causing this paradoxical interaction with fra, we manipulate genes known to regulate cytoskeletal dynamics. Our results suggest that in order to respond correctly to midline attraction, Fra is required to regulate Abl activity, in part, to direct the cytoskeletal dynamics underlying movement across the midline. In the absence of Fra, Abl appears to perform these functions downstream of other guidance receptors to induce a variety of projection errors, including trajectories away from the CNS.

**Materials and Methods**

**Stocks:** Stocks were raised on conventional cornmeal and molasses based media at room temperature or 25°C. All of the stocks used in this study have been fully characterized in previous studies. The Fra deletion mutant transgenes (UAS-Fra\(^{\Delta P1}\), UAS-Fra\(^{\Delta P2}\), UAS-Fra\(^{\Delta P3}\) and UAS-Fra\(^{WT}\)) and the 1407-Gal4 driver on chromosome II were described (Dorsten and VanBerkum, 2008). Other constructs were described as follows: Abl\(^{WT}\), Abl\(^{KN}\) and BcrAbl (Hsouna et al., 2003), constitutively active MLCK (Kim
et al., 2002) and Rac$^{V12}$ and Cdc42$^{V12}$ (Fritz and VanBerkum, 2002) and P-element (Bloomington Stock Center; (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b). The phenotypes generated by these transgenes when expressed in wild type embryos have been previously described. Here, all phenotypes are compared to a fra heterozygote with a 1407-Gal4 driver to control for the presence of this driver chromosome and emphasize the change that occurs in the homozygote. Any differences between wild type and a fra heterozygote are briefly described in the text. Conventional breeding strategies or recombination experiments are used to combine gene mutations, the Gal4 driver or UAS transgenes in the same fly. Stocks were confirmed by complementation tests and/or PCR screening for P-elements as described previously (Dorsten and VanBerkum, 2008). To identify the genotypes of stained embryos, β-galactosidase (β-gal) marked balancer chromosomes are present in all final stocks.

**Histology:** Standard methods are used to collect eggs at 25°C and stain them with mAb BP102 or mAb and the HRP-linked goat anti-mouse secondary antibody. The genotype of the embryos was established by X-Gal staining that identified balancer chromosomes. Mutant phenotypes in the axon scaffold were scored and at least three replicates were analyzed using Student’s t-test. Loss of commissures were assessed as previously described (Dorsten et al., 2007), while AEP defects were scored based on the presence of a BP102 stained axon beyond the CNS/PNS boundary. Normally BP102 stains only CNS axons, and while motor nerve routes within CNS neuropile may be lightly stained, they are usually devoid of staining once in the PNS. Thus, BP102 staining of axons beyond the CNS/PNS boundary is a readily quantifiable phenotype.
**Expression constructs:** HA epitope tagged $\text{Fra}^{\text{WT}}$ and $\text{Fra}^{\Delta C}$ transgenes cloned into the metallothionine vector (pMT) were described previously (Forsthoefel et al., 2005). Individual P-motifs were deleted as defined in Kolodziej et al. (1996). pMT-$\text{Fra}^{\Delta P1}$ is deleted for PPDLWHHDQMEKLKD; pMT-$\text{Fra}^{\Delta P2}$ is deleted for TIESSKRGHPLKSFSVPPPTGATPKHTP; pMT-$\text{Fra}^{\Delta P3}$ is deleted for ELNQEMANLEGLMKDLSAITANE. All three P-motif deletion constructs contain a C-terminal HA epitope tag. To make myc tagged versions, we first modified a commercially available S2 cell metallothionine vector (pMT-V5His, Invitrogen) to include a hygromycin resistance cassette (pMT-Hygro), and then introduced a myc epitope tag and tobacco etch virus protease site (TEV) just before this vector’s V5 epitope and His tags. Then, using the HA-tagged versions as templates, we PCR amplified $\text{Fra}$ wild-type ($\text{Fra}^{\text{WT}}$) and P-motif deletions ($\text{Fra}^{\Delta P1}$, $\text{Fra}^{\Delta P2}$, $\text{Fra}^{\Delta P3}$) and cloned them into the unique SpeI and PvuI sites of this parental vector.

**S2 cell culture and immunoprecipitation studies:** *Drosophila* S2 cells were maintained in Schneider’s media supplemented with 10% fetal bovine serum (complete media) at 27°C as per the conditions in *Drosophila* Expression System (Invitrogen). Approximately $2 \times 10^6$ cells were transfected (Effectene, Qiagen) with either myc- or HA-tagged $\text{Fra}^{\text{WT}}$, $\text{Fra}^{\Delta P1}$, $\text{Fra}^{\Delta P2}$, and $\text{Fra}^{\Delta P3}$ using the procedure recommended by the manufacturer. For the HA-tagged version, selection was achieved by co-transfection with pCo-Hygro. Stable lines were selected over several weeks using 300 $\mu$g/ml hygromycin (Invitrogen). Fra protein expression was induced with 700 $\mu$M copper sulfate, and, 24 hours later, Fra was activated by adding a mixture of Netrin A and
Netrin B for 30 minutes at $27^0\text{C}$. The cells were harvested by centrifugation at 13000 rpm and lysed in NP-40 buffer (Forsthoefel et al., 2005). Cell extracts were pre-cleared by incubating with goat anti-mouse IgG magnetic beads (Pierce) for 30 minutes at $4^0\text{C}$. Following two-hour incubation with 1mg mouse anti-HA antibody (Sigma), Fra immunocomplex was precipitated using the magnetic IgG beads. The bead-protein complex was boiled in 1X SDS sample buffer and loaded onto a 7.5% poly-acrylamide gel. Protein was transferred to PVDF membrane (Millipore) following standard procedures and probed with antibody. Fra pull down was confirmed by blotting against the epitope tag using rabbit anti-HA or anti-myc (Sigma) at 1:10000. After stripping (50 mM Tris (pH 6.8), 2% SDS and 100 $\mu$M b-mercaptoethanol at $50^0\text{C}$ for 30 minutes), membrane was re-probed with either rabbit anti-"Drosophila" Abl (kindly provided by David VanVactor) or guinea pig anti-"Drosophila" Abl (kindly provided by Mark Peifer) or rabbit anti-Bcr (Cell Signaling) at 1:1000 dilution. The amount of Fra pull down was confirmed using a rabbit anti-myc or HA antibody (Sigma). For co-transfections with Abl transgenes, stable lines expressing full length Fra or mutants were transiently co-transfected with pMT GAL4 and either UASAbi$^{WT}$, UAS-Abi$^{KN}$, or UAS-BcrAbl (1:20). Twenty-four hours later, the cells were induced for protein expression and complexes were isolated as described above. To examine tyrosine phosphorylation of Fra and its P-motif deletions, the above procedure was used except that Frazzled expressing cells were treated with pervanadate (Forsthoefel et al., 2005) before Netrin was added. Phosphotyrosine was detected using PY-20 (Upstate) at 1:1500 and 1:20000 goat anti-mouse-HRP (Jackson).
Results

Previous studies showed that loss-of-function mutations in abl enhance the degree of commissure loss in a fra mutant (Forsthoefel et al., 2005) while over-expression of a constitutively active form of Abl (Bcr\(^{210}\)Abl, herein just BcrAbl) induces axons to ectopically cross the midline especially when wild-type Fra (Fra\(^{wt}\)) is co-expressed (Dorsten et al., 2007). Thus, it seems possible that Abl helps mediate midline attractive signaling, and if so, a logical prediction would be that over-expressing wild-type Abl, or BcrAbl in a fra homozygote would partially rescue fra-dependent defects in commissure formation. Accordingly, using the GAL4–UAS system and previously characterized stocks (Dorsten et al., 2007; Garbe et al., 2007), we over-expressed UAS-Abl\(^{WT}\) (U-Abl\(^{WT}\)) or UAS-BcrAbl (U-BcrAbl) in all neurons of a homozygous fra mutant (fra\(^{3}/fra^{4}\)) embryo and assessed commissure formation using the monoclonal antibody BP102. This antibody stains the anterior and posterior commissures (AC and PC, respectively) as well as longitudinal connectives running on either side of the midline (Figure 2.1A). We were surprised to find that, rather than rescuing fra\(^{3}/fra^{4}\) commissural defects, elevating Abl activity actually enhanced the loss of commissures (Figure 2.1E, F black arrowhead, PC; white arrowhead, AC). Another striking defect encompassed large bundles of axons exiting the CNS, often extending beyond the CNS/PNS boundary [(Figure 2.1E, F arrows) (herein designated AEP, Axons Exiting to Periphery)].

*Fra interacts with Abl to form commissure*
One of the predominant defects in fra^3/fra^4 mutants is thinning and missing posterior commissures, with nearly a quarter (24%) of segments showing this defect (Figure 2.1D, black arrowhead; graphed in 2.1K; (Dorsten and VanBerkum, 2008; Kolodziej et al., 1996). Over-expression of BcrAbl significantly (P < 0.001) enhances these defects nearly threefold with 76% of segments having thin or missing posterior commissures (Figure 2.1E, black arrowhead; quantified in K). Additionally, the anterior commissures, which are virtually unaffected in fra^3/fra^4 homozygote was either thin or missing in 41% of segments (P < 0.01; Figure 1E, white arrowhead; graphed in 2.1K). This reduction in commissural axons crossing the midline was particularly surprising since expression of BcrAbl in a wild-type embryo results in fuzzy commissures, a defect caused by extra axons crossing the midline (Dorsten et al., 2007). Fuzzy commissures remain evident in a fra^3 heterozygote (Figure 2.1B, black arrowhead). To test whether this result was unique to BcrAbl, we expressed wild-type Abl (U-Abl_{WT}) in a fra^3/fra^4 mutant background. In wild-type embryos, expression of Abl_{WT} alone is innocuous (Hsouna et al., 2003), although a rare commissure may be malformed in a fra^3 heterozygote (see arrowhead Figure 2.1C). When Abl_{WT} is expressed in a fra^3/fra^4 mutant, the phenotype is strikingly similar to that caused by expression of BcrAbl, with a three-fold (P < 0.01) enhancement in PC defects and the emergence of AC defects (Figure 2.1F, note that AEP defects are also present, black arrow; quantified in 2.1K). The enhancement in commissure defects partially requires Abl kinase activity as the pan-neural expression of a kinase dead version of Abl (U- Abl^{KN}) either wild-type or fra heterozygous embryos produces no phenotype or defect. However, while its expression in a fra^3/fra^4 mutant still significantly (P < 0.05) increases the frequency of thin and
missing PC defects, it is only about half that observed with over-expression of either Abl\textsuperscript{WT} or BcrAbl. Moreover the anterior commissures form correctly in the presence of Abl\textsuperscript{KN} (Figure 1K). Thus, both increasing and decreasing Abl activity in the absence of Fra reduces the ability of commissural axons to cross the midline.

It is intriguing that such a striking phenotype is uncovered when Fra is removed since pan-neural expression of Abl\textsuperscript{WT} on its own has very few defects and pan-neural expression of BcrAbl actually causes axons to erroneously cross the midline (Fogerty et al., 1999; Hsouna et al., 2003). This is also true when Abl signaling is increased in heterozygous fra mutants (Figure 2.1B, C). This phenotype depends on the presence of Fra protein, as re-expression of Fra\textsuperscript{WT} in the mutants reverts the phenotype back to that seen when either Abl\textsuperscript{WT} or BcrAbl is expressed alone: normal AC and PC formation is observed with Abl\textsuperscript{WT}, and the fuzzy commissures seen with BcrAbl return (Figure 2.1G, I).

**The three P-motifs of Fra are not required to interact with Abl at commissures**

In a previous gain-of-function assay, expression of BcrAbl in a subset of FasII expressing neurons induces ectopic crossovers, and these are enhanced by co-expression of Fra as long as the P3-motif is intact (Dorsten et al., 2007). Therefore, we tested whether a specific P-motif is also required for Fra to revert the loss in commissural axon crossing. Essentially, we compared the ability of Fra\textsuperscript{WT} to revert the commissural defects with our previously described series of Fra mutants in which the individual P-motifs have been deleted (U-Fra\textsuperscript{AP1}, U-Fra\textsuperscript{AP2} and U-Fra\textsuperscript{AP3}; Dorsten et al., 2007). All of the Fra P-motif deletion mutants were able to significantly restore
commisure formation in fra\(^3\)/fra\(^4\) mutants expressing Abl\(^WT\) (Figure 2.1J, L). In fra\(^3\)/fra\(^4\) mutants expressing BcrAbl, deletion of either Fra\(^\Delta P1\) or Fra\(^\Delta P2\) revert the commissure loss observed in this mutant and in fact restore the fuzzy commissure defects similar to that observed with Fra\(^WT\) (Figure 2.1L). While expression of U-Fra\(^\Delta P3\) also restores commissure formation, the frequency of fuzzy commissures is significantly (P < 0.001) reduced compared to Fra\(^WT\), as many segments now exhibit a distinct AC and PC (Figure 2.1H, black arrowhead, Figure 2.1L).

Expression of Abl in a fra mutant also results in dozens of axons exiting toward the periphery (AEP defects), and surprisingly, this defect is readily observed using BP102. Generally considered a marker of all CNS axons, BP102 clearly labels AC, PC and LC axons, and lightly stains ISN and SN nerve routes within the CNS, but it does not normally stain axons beyond the CNS/PNS boundary. Therefore, in mutant embryos, dense BP102 staining of axons beyond the CNS/PNS boundary clearly delineates a novel phenotype, herein termed an AEP defect (Axon Exiting to Periphery). Consistent with the known BP102 staining pattern, in wild-type and fra\(^3\)/fra\(^4\) mutant embryos we do not observe any AEP defects [Figure 2.1 D]. When BcrAbl is expressed in a fra heterozygote, we observe minimal AEP defects and none in the same background with Abl\(^WT\) [Figure 2.1 B and C]. However, several hemi-segments in embryos expressing BcrAbl or Abl\(^WT\) in the absence of Fra exhibit AEP defects as many
Fig. 2.1: Increasing Abl activity in a fra mutant reduces commissure formation. Stage 16 embryos stained with mAb BP102 are depicted with anterior up. Indicated UAS- (U) transgenes are expressed pan-neurally using the 1407-Gal4 (1407-G4) driver line recombined onto a fra3 chromosome. For this reason, phenotypes are compared to heterozygous fra3 rather than a wild-type embryo; in most cases the heterozygote is similar to wild-type. [A] Normal anterior commissures (AC), posterior commissures and longitudinal connectives are observed in a fra3 1407-GAL4 heterozygote stained with BP102. [B] Over-expression of BcrAbl in a fra3 heterozygote causes fuzzy commissures in most segments while [C] expression of AblWT causes only a rare fuzzy commissure (black arrowheads). [D] Homozygous fra3/fra4 mutants display thinning and missing PC’s (black arrowhead) in some segments but the AC remains unaffected (white arrowhead). Over-expression of either [E] BcrAbl or [F] AblWT in a fra3/fra4 background enhances the degree of thinning and missing PC’s (black arrowhead), and many AC’s (white arrowhead) disappear. [K] Quantification of defects in the AC and PC are graphed with percent of commissures missing in a fra3 1407-GAL4 heterozygote (white, highlighted with ‘w’), fra3 1407-Gal4/fra4 homozygote and fra3 1407-Gal4/fra4 homozygotes expressing the indicated Abl transgene. All three Abl transgenes significantly enhance the loss of PCs and both BcrAbl and AblWT affect AC formation (*P, 0.05; **P, 0.01; ***P, 0.001). [G] Co-expression of a FraWT transgene restores the fuzzy commissure defects caused by expression of BcrAbl but [H] with the FraP3 transgene the AC and PC commissures are more distinct. Expression of [I] FraWT or [J] FraP3 transgenes also rescues commissure formation when AblWT is overexpressed in a homozygous fra mutant. [L] The distribution of segments containing fuzzy or distinct commissures in fra3/fra4 embryos co-expressing BcrAbl and each of the Fra P-motif deletion mutants is graphed.
axons expressing the BP102 epitope actually extend past the CNS/PNS boundary ([Figure 2.1 E and F], black arrows).

**Abl and BcrAbl bind to Fra independent of a specific P-motif**

Our genetic rescue data suggest that Fra regulates Abl activity independent of a specific P-motif, while the P3 motif may have a specific interaction with hyperactivated BcrAbl. To further address this, we asked whether these genetic interactions are reflected in a biochemical interaction between Abl and Fra. Forsthoefel et al. (2005) showed that Abelson binds to the cytoplasmic tail of Frazzled but did not determine if the binding required a specific P-motif. Using Drosophila S2 cells, we created two sets of stable lines expressing either HA- or myc-tagged wild-type or P-motif deletion mutants of Fra under the control of a metallothionine promoter. Consistent with studies on DCC, the vertebrate homologue ((Geisbrecht et al., 2003; Stein et al., 2001), we found that Netrin binds to Fra *in vitro* (data not shown). Further, when compared to the level of HA-tagged receptor pulled down in the immunoprecipitation assays, endogenous Abl continues to bind to Fra even when an individual P-motif (ΔP1, ΔP2 and ΔP3) is deleted (Figure 2.2A). Similar results were observed using the myc-tagged versions of wild-type Fra and its P-motif deletions. When co-expressed in S2 cells, both wild-type (AblWT) and kinase inactive (AblKN) transgenes bind to wild-type Fra as long as the cytoplasmic domain is present (Figure 2.2B), although it is noted that only a small fraction of the available protein is found in complex with each other.

Unlike wild-type Abl, BcrAbl specifically requires the presence of P3 motif of Fra to form the fuzzy commissures. To ask if this genetic interaction is reflected in a
specific binding of BcrAbl to the P3 motif of Fra, we transiently transfected S2 cell lines expressing myc-tagged versions of wild-type or P-motif deletion mutants of Fra with a BcrAbl transgene. Fra\textsuperscript{WT} or its P-motif deletions were immuno-precipitated using the C-terminal myc-tag and BcrAbl binding assessed using a commercial antibody against the Bcr component. Like wild-type Abl, BcrAbl clearly binds to Fra independently of a P-motif (Figure 2.2C). Thus, the selective genetic interaction between BcrAbl and the P3 motif of Fra is not reflected in differential binding properties.

Forsthoefel et al. (2005) demonstrated that increased expression of Abl in S2 cells also led to increased phosphorylation of Fra. Therefore, we asked if Fra phosphorylation is altered by removal of a P-motif. Fra or its P-motifs were immunoprecipitated from stable S2 cell lines using the C-terminal myc-tag and assessed for phosphotyrosine levels by western blot. Considering the amount of receptor precipitated, phosphorylation of Fra is largely unaffected by deletion of P1 or P2, while deletion of P3 may be increasing Fra phosphorylation (Figure 2.2D, top). In the blot shown, cells were pretreated with pervanadate (~30 min) to increase signal intensity and to demonstrate the lack of P-motif dependence. Phosphorylation is also observed in the absence of pervanadate treatment, although at a much lower level (see bottom panel Figure 2.2D). Phosphorylation of Fra\textsuperscript{WT} increases slightly if cells are transiently transfected with wild-type or BcrAbl transgenes, and it reverts to the basal level of S2 cells alone with Abl\textsuperscript{KN}, presumably reflecting the endogenous Abl activity. BcrAbl binding to Fra, as well as its hyperactivation, is confirmed in this blot by the presence of an autophosphorylated BcrAbl band at the top of the gel (arrowhead). Together, these immunoprecipitation assays indicate that both Abl and BcrAbl binding
Fig. 2.2: Both Ablwt and BcrAbl bind to Frazzled independently of a specific P-motif. [A] Stable Drosophila S2 cells were created that express an HA-tagged form of wild-type [wt] or P-motif deletion (ΔP1, ΔP2, ΔP3) mutants of Frazzled (Frax-HA). Frazzled was immunoprecipitated (IP) from cells using a mouse monoclonal anti-HA, and western blots were probed (IB) with guinea pig anti-Drosophila Abl (D-Abl; top) or rabbit anti-HA (bottom). Compared to the amount of Frazzled receptor pulled down, endogenous Abl binds to Frazzled independently of a P-motif. [B] Transiently transfected Ablwt or Abl\(^{KN}\) (kinase inactive) genes also bind to a myc-tagged version of wild-type Fra (Frawt-myc) but not to a HA-tagged receptor missing its cytoplasmic domain (Fra\(^{-C\text{-HA}}\)). [C] Activated BcrAbl, detected by a rabbit antibody against the Bcr region, also binds to Fra-myc even if one of the P-motifs is removed. No band is observed in mock IPs from S2 cells expressing only BcrAbl (far left). The amount of Fra pulled down (middle row), and the amount of BcrAbl available in lysates (bottom row) is shown. [D] In the top panel, S2 cells were pretreated for 30 min with pervanadate and then wild-type or P-motif deletions of Frazzled were immunoprecipitated using mouse anti-myc and the degree of tyrosine phosphorylation assessed by probing a blot with mouse anti-phosphotyrosine (a-pY; top row). Compared to the amount of receptor pulled down (bottom row), the level of tyrosine phosphorylation does not appear to be diminished when a P-motif is deleted. The bottom panel assesses Frawt phosphorylation when Abl transgenes are co-expressed in S2 cells that were not pretreated with pervanadate. Compared to the level observed in S2 expressing only Frawt-myc, phosphotyrosine levels increase slightly if cells are co-transfected with Abl\(^{wt}\) or BcrAbl, but not Abl\(^{KN}\) (bottom blot). In this blot, autophosphorylation of BcrAbl denoting its activated state is apparent (arrowhead). The position of molecular weight standards (in kDa) is indicated on the left side of each panel.

to Fra and the state of Fra phosphorylation are independent of any specific P-motif. This
is consistent with the ability of the deletion mutants to rescue commissure formation when Abl or BcrAbl is over-expressed in a fra mutant.

**Fra regulates the cytoskeletal dynamics during commissure formation**

Besides its interactions with axon guidance receptors at the midline, Abl is a key regulator of the cytoskeletal dynamics underlying growth cone advance and steering (Bradley and Koleske, 2009; Pendergast, 2002). Thus, we hypothesize that increasing Abl activity would alter the dynamics of this response and cause guidance defects. To test this idea, we sought to disrupt cytoskeletal dynamics in a fra mutant independent of Abl, but using gene mutations known to affect midline guidance.

Cdc42 regulates multiple elements of the cytoskeleton, and in *Drosophila*, expression of a constitutively active form of the protein (U-Cdc42V12) in a specific subset of FasII positive neurons leads to ectopic crossovers, the frequency of which are halved by a heterozygous fra mutation (Dorsten et al., 2007; Fritz and VanBerkum, 2002). Like that observed in wild-type embryos, pan-neural expression of Cdc42V12 in a fra³ heterozygote also causes severe errors in axon pathway formation as commissures fuse and large gaps appear in the LC (Figure 2.3A, arrowhead). When Cdc42V12 is expressed in a fra³/fra⁴ homozygote most commissures are absent in nearly every segment, and LC formation improves as fewer gaps are now observed (Figure 2.3A, D). It is also noted that over-expression of Cdc42V12 in a fra³/fra⁴ mutant caused a dramatic loss in commissures that is even more severe than over-expression of BcrAbl or AblWT. These data confirm the importance of Fra signaling during formation of commissures and point to a key role for Abl in regulating cytoskeletal elements.
The severity of these Cdc42\textsuperscript{V12} phenotypes prompted us to test another protein, Rac, which also regulates actin dynamics and a constitutively active form of Rac (Rac\textsuperscript{V12}) also causes FasII expressing axons to ectopically cross the midline (Dorsten et al., 2007; Fritz and VanBerkum, 2002). When Rac\textsuperscript{V12} is pan-neurally expressed in wild-type embryos or a fra\textsuperscript{9} heterozygote (Figure 2.3B), commissures and longitudinal connectives appear thin and slightly disorganized. Interestingly, overexpression of Rac\textsuperscript{V12} in a homozygous fra embryo also yields a major reduction in commissure formation (Figure 2.3E). However, unlike BcrAbl and Abl\textsuperscript{WT} the axons in these mutant embryos never cross the CNS/PNS boundary even though some axons appear to orient away from the midline (Figure 2.3E, arrow). In order to determine whether the Fra and Abl interaction observed was a result of its regulation of actin or myosin dynamics, we sought to selectively elevate myosin activity in a fra mutant using a constitutively active Myosin Light Chain Kinase (U-ctMLCK) that increases myosin activity by phosphorylating the Regulatory Light Chain of myosin II (Kim et al., 2002). Expression of ctMLCK in a subset of FasII positive axons induces ectopic midline crossing errors, which, like Rac\textsuperscript{V12} and Cdc42\textsuperscript{V12}, are sensitive to heterozygous removal of fra (Dorsten et al., 2007). Pan-neural expression of ctMLCK in wild-type embryos leads to ectopic crossovers (i.e. fuzzy commissures) in almost every segment (Kim et al., 2002). While the frequency of this defect is somewhat diminished in a fra heterozygote, many segments still display fuzzy commissures (Figure 2.3C, arrow). Unlike Cdc42 or Rac, however, expression of ctMLCK in fra homozygous embryos does not reduce commissure formation beyond that normally observed in a fra mutant alone (Figure 2.3F). Similar results were observed for all three transgenes (U-Cdc42\textsuperscript{V12}, U-Rac\textsuperscript{V12}...
Fig. 2.3: Expression of RacV12 and Cdc42V12 reduces commissure formation in a fra mutant.
Stage 16 embryos stained with the mAb BP102 are depicted with anterior up. UAS (U) transgenes are expressed pan-neurally using the 1407-GAL4 (1407-G4) driver line recombined onto the fra\textsuperscript{3} chromosome, and phenotypes are compared to heterozygous fra\textsuperscript{3}. Phenotypes in heterozygous fra\textsuperscript{3} embryos tend to be somewhat milder than that observed in a wild-type embryo (see text). [A] Expression of Cdc42\textsuperscript{V12} in a fra\textsuperscript{3} heterozygote background results in fused commissures [arrowhead] and large gaps in the longitudinal connectives. [B] Expression of Rac\textsuperscript{V12} in a fra\textsuperscript{3} heterozygote causes a thinning of PC (black arrowhead) and AC (white arrowhead) as well as LC (black arrow). [C] Expression of ctMLCK in a fra\textsuperscript{3} heterozygote results in fuzzy commissures (black arrowhead). Expression of both [D] Cdc42\textsuperscript{V12} and [E] Rac\textsuperscript{V12} in a fra\textsuperscript{3}/fra\textsuperscript{4} mutant significantly reduces commissure formation (black arrowheads) and, with expression of Rac\textsuperscript{V12}, some axons may orientate towards the periphery (arrow in E), but they are not observed to exit the CNS. [F] Expression of ctMLCK in a fra\textsuperscript{3}/fra\textsuperscript{4} mutant does not significantly alter the PC (arrowhead) and LC defects normally observed in a fra mutant.
and U-ctMLCK) when the pan-neural elav-Gal4 driver line was utilized (data not shown). Together, the Rac and Cdc42 data suggest that, in the absence of Fra, unilateral perturbation of actin cytoskeletal dynamics is sufficient to reduce commissure formation. These data continue to highlight the critical role Fra plays in regulating cytoskeletal dynamics during guidance events at the midline, and implicate all three molecules Abl, Rac and Cdc42 in this process.

**Discussion**

Frazzled and Abelson Tyrosine kinase activity clearly cooperate during the formation of embryonic commissures. In the absence of Fra, detection of Netrin-dependent chemoattraction is compromised and many posterior commissures fail to cross the midline. Both anterior and posterior commissures are absent if fra and abl activity is lost. This presumably reflects the ability of abl mutations to interact with a second Netrin receptor, Dscam, as well as Netrin independent receptors (e.g. Turtle) known to be important for commissure formation (Al-Anzi and Wyman, 2009; Andrews et al., 2008). Finally, Abl itself is required for commissure formation as most commissures are also lost when both maternal and zygotic contributions of Abl are genetically removed (Grevengoed et al., 2001). Given these different observations, it seemed plausible that over-expressing Abl in fra null embryos would improve commissure formation. However, instead of an improvement, we clearly document a major decrease in both anterior and posterior commissures and the induction of a novel phenotype whereby axons normally confined to the CNS now project into the periphery (AEP defects). It is worth
emphasizing that these phenotypes occur even with the over-expression of a wild-type Abl transgene that retains its autoinhibitory domain and must be activated by endogenous mechanisms (Hsouna et al., 2003; Pendergast, 2002). These phenotypes completely dependent on the absence of Fra but not any particular P-motif and also occur in the absence of Netrins. The latter again emphasizes the possibility that Abl also works downstream of novel receptors (Al-Anzi and Wyman, 2009; Andrews et al., 2008). Interestingly, the loss of commissures is also observed when activated Rac or Cdc42 GTPases are over-expressed in a homozygous fra mutant. Taken together, we propose that during exploration of the midline, Fra is a key regulator of Abl activity and helps determine how the cytoskeletal machinery utilizes Abl. In the absence of Fra, axon outgrowth does not simply stall; but rather, axons follow a variety of aberrant trajectories away from the midline. This suggests that Fra normally competes with several other receptor systems to dictate how Abl functions to regulate the cytoskeletal machinery. While competitors undoubtedly include other midline guidance cues, the emergence of AEP defects suggests that Fra also competes with guidance systems not normally associated with the midline. In the absence of Fra, these other receptors appear to utilize the extra Abl to alter cytoskeletal dynamics at a variety of choice points, ultimately preventing commissure formation and directing some axons out of the CNS.

The Abl gain-of-function phenotype described herein occurs only if fra is absent. In other words, commissures form correctly in a heterozygous fra mutant or when partially active Fra transgenes with a single P-motif deleted are re-expressed with Abl. In S2 cell immunoprecipitation experiments, both Abl and BcrAbl bind to the cytoplasmic tail of Fra independent of any specific P-motif. While surprising given the conservation
of these P-motifs and their known importance to Fra function (Dorsten and VanBerkum, 2008; Garbe et al., 2007), the lack of P-motif specificity is consistent with our genetic rescue experiments. All three P-motif deletion mutants rescue commissure formation and the AEP defects elicited by over-expression of either wild-type Abl or BcrAbl in fra embryos. The ectopic midline crossovers (fuzzy commissures) observed with only BcrAbl also depends on Fra and specifically the P3-motif, confirming a previous report (Dorsten et al., 2007). However, BcrAbl is not an endogenous Drosophila protein and, as discussed by others (Stevens et al., 2008), the human Bcr domain may induce neomorphic phenotypes. Because BcrAbl does not preferentially bind to the P3 motif, and wild-type Abl does not elicit crossover defects, we now suspect that the ectopic crossovers are an example of a neomorphic phenotype, a hypothesis that will be extensively addressed in future work.

Fra initiates a downstream signaling event that may recruit Abl to its cytoplasmic domain (Forsthoefel et al., 2005). Fra\textsuperscript{WT} immunoprecipitation assays reflect a direct or indirect association between Fra and Abl\textsuperscript{WT} or BcrAbl. We cannot rule out a direct binding as it has been shown that the cytoplasmic tail of Fra fused to glutathionine-S-transferase (GST) directly binds to \textit{in vitro} translated Abl (Forsthoefel et al., 2005). It is possible that our failure to observe P-motif dependence may be because Abl acts as a scaffold in a protein complex that is associated with more than one P-motif or at regions in between the P-motifs.

The substoichiometric binding observed between Fra and Abl suggest that a transient substrate enzyme interaction may be occurring. In fact, in S2 cells, tyrosine phosphorylation of Fra is not affected by removal of the P1 or P2 motif. Taking into
account that vertebrate Arg and Abl use the conserved SH3 and SH2 domains in the N-terminal region to interact with other proteins, it can be speculated that *Drosophila* Abl SH3 may be interacting with the PXXP region in between P1 and P2 motifs (Antoku et al., 2008; Cao et al., 2008). The presence of tyrosine residues in the close proximity of this region, two of which are conserved with vertebrate DCC further strengthen this idea. It is intriguing that removal of P3 motif may actually increase Fra phosphorylation because this motif is also critical to Fra signaling *in vivo* (Dorsten and VanBerkum, 2008; Garbe et al., 2007).

Immunoblots of Fra phosphorylation in the absence of pervanadate pretreatment also suggest the steady-state level of tyrosine phosphorylation may be relatively low, or

| DCC     | LLVIIVTVGVITVLYL...I-CTRRS-SAQQKKRAT--HSAG  |
| FRA     | LLIIIATAVLVLLV...RKKRQQGTVKQKNNVGV          |
| DCC     | RKGS---KDLR...FRA PK----PPDLWIHDQ...FRA PK--PPDL WIH HDQ... |
| FRA     | YMTTSNNG----TM----P1----VTPVC |
| DCC     | QDLTPVSHS...FRA YMTTSNNG------TMERPEY----PRTQYSHQNRSHQ |
| FRA     | PVPFP...PVPFPTLR...FRA YMTTSNNG------TMERPEY----PRTQYSHQNRSHQ |
| DCC     | AKLMIPMDAQSNNPAV...FRA YMTTSNNG------TMERPEY----PRTQYSHQNRSHQ |
| FRA     | PVRSG...QQSLTQPSNSMAQTPEHDG |
| DCC     | SS--EEAPRT...FRA GDANFCNAGN--AAAGNGCVSTIESKRGSFLKSFSVPG---PPPT |
| FRA     | P2--SS--EEAPRT...FRA GDANFCNAGN--AAAGNGCVSTIESKRGSFLKSFSVPG---PPPT |
| DCC     | SA--IE--PFKBP...FRA GAPVPEKTPVATIRPQNS--PKKPFSAAATPRLQ--GGG |
| FRA     | P3--SA--IE--PFKBP...FRA GAPVPEKTPVATIRPQNS--PKKPFSAAATPRLQ--GGG |
| DCC     | APFVSEESHKP...FRA VVHSTDEIQRLAPSTST--EELNQ...NANLEGMKQLSAITANEFEC |
| FRA     | P3--APFVSEESHKP...FRA VVHSTDEIQRLAPSTST--EELNQ...NANLEGMKQLSAITANEFEC |

**Fig. 2.4: Some tyrosine residues are conserved between Fra and DCC.** The amino acid sequences of vertebrate DCC and *Drosophila* Abl have been blasted using pubmed BLAST. The red rectangle encloses the conserved P-motifs (P1, P2 and P3) and red stars represent the conserved tyrosine residues between DCC and Fra. There are also other tyrosine residues (green stars) that are not conserved between the two species but may be critical for Fra phosphorylation.
highly dynamic. This may be due to the presence of tyrosine phosphatases such as PTP61F that antagonize Abl activity for some substrates (Ku et al., 2009; Schindelholz et al., 2001), and antagonistic action between Abl and several phosphatases during nerve cord development has been documented (Jeon et al., 2008; Schindelholz et al., 2001; Song et al., 2008; Wills et al., 1999a; Wills et al., 1999b). Since tyrosine phosphorylation of vertebrate DCC is required for attractive responses, axon outgrowth and orientation of the axon (Li and Hua, 2008; Li et al., 2004; Li et al., 2002a; Li et al., 2002b; Meriane et al., 2004; Ren et al., 2004), it will be important to systematically assess how Fra and Abl physically interact to regulate each other’s activity during midline guidance. Even though the kinase activity and the F-actin binding domain of Abl are not required for its physical association with Frazzled, it may be essential to how Frazzled signaling modulates the actin cytoskeleton (Fig 2.2, and chapter 3). In chapter 3, we have studied the role of both the kinase activity and F-actin binding domain of Abl during Fra signaling in S2 cells.

Our data using activated forms of Rac and Cdc42 suggest that the primary defect underlying the loss of commissures in fra mutants expressing Abl lies in the inability of growth cones to properly regulate actin dynamics. This could involve both attractive and repulsive systems. Proper axon guidance also requires concerted regulation of the cytoskeletal dynamics underlying axon outgrowth and steering (Huber et al., 2003). Like most guidance receptors, Fra, or its vertebrate and C. elegans homologues are known to initiate signaling pathways affecting cytoskeletal dynamics (Dorsten et al., 2007; Gitai et al., 2003; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Abl is also a key regulator of actin dynamics in vertebrate cells (Bradley and Koleske, 2009; Pendergast,
2002; Woodring et al., 2001, 2003; Woodring et al., 2002) and of the development of the
Drosophila nervous system. Mutations in abl interact with several cytoskeletal regulators
to affect axon pathway formation: kette (Bogdan and Klambt, 2003), capulet (Wills et
al., 2002), chicadee [Profilin; (Wills et al., 1999b], enabled (Bashaw et al., 2000; Gates
et al., 2007) and trio (Bateman et al., 2000; Forsthoefer et al., 2005; Liebl et al., 2000).
Thus, in the absence of Fra-dependent regulation, does elevated Abl activity affect the
cytoskeletal machinery to indirectly cause a reduction in commissures? Here, we tested
this basic concept by expressing in fra mutants other key regulators of cytoskeletal
dynamics known to affect midline guidance (Dorsten et al., 2007; Fritz and VanBerkum,
2000; Kim et al., 2002). Surprisingly, over-expression of activated Rac and Cdc42 in a
fra mutant replicates the loss of commissure phenotype observed with Abl. The Cdc42
result is most intriguing as expression in a wild-type or heterozygous fra embryo results
in fused commissures and gaps in the longitudinal connectives. Yet, upon complete
removal of Fra, commissures do not form and the longitudinal connectives reform. Thus,
in the absence of Fra, commissure formation appears to be particularly sensitive to
manipulation of actin-based processes. It is possible that our manipulation of Cdc42 and
Rac activity in a fra mutant is affecting shared processes related to actin polymerization.
For example, in vertebrate studies, Cdc42 and Abl work in parallel to regulate actin
polymerization (Burton et al., 2003; Leng et al., 2005; Stuart et al., 2006) and Abl may
activate Rac in response to cell adhesion (Burton et al., 2005; Hernandez et al., 2004;
Zandy et al., 2007). If so, our data suggest that in the absence of Fra activity these key
regulators are being used by other surface receptors to regulate actin dynamics in a
manner that ultimately prevents commissure formation. This is certainly consistent with
the number of guidance systems that have been linked to these regulators (Guan and Rao, 2003; Heasman and Ridley, 2008; Round and Stein, 2007; Zandy and Pendergast, 2008) and the scope of guidance defects detected in our embryos. At the very least, Cdc42 and Rac data continue to highlight the degree and importance of Fra-dependent regulation of cytoskeletal dynamics, especially actin-based processes, during commissure formation. It seems likely that Fra is competing with several other receptor systems whose presence (but not identity) has been uncovered by over-expression of Abl, Rac or Cdc42 in homozygous fra embryos. In addition to fra and Dscam (Andrews et al., 2008; Forsthoefel et al., 2005), loss-of-function mutations in abl interact with the cell-cell adhesion molecules neurotactin and amalgam (Liebl et al., 2003), fasI (Elkins et al., 1990), midline-fasciclin (Hu et al., 1998) and turtle (Al-Anzi and Wyman, 2009) to reduce commissure formation and some of these are fairly ubiquitously expressed in the nerve cord. Abl has also been linked to the regulation of cell-cell adhesion molecules alone or in combination with receptor systems such as Notch (Crowner et al., 2003; Zandy and Pendergast, 2008).

In summary, the data suggest a model whereby Fra activity initiates key signaling events that dictate when and how Abl activity is utilized during commissure formation. Rac and Cdc42 are probably also involved in this process, and together with Abl, help regulate key aspects of actin dynamics underlying commissure formation. In the absence of Fra other midline guidance systems are still functioning well enough to form most commissures, but they are clearly sensitive to perturbation of intracellular signaling pathways regulating cytoskeletal dynamics. Hence, when Fra is removed, other guidance systems appear to recruit Abl, Rac or Cdc42 activity to misdirect axon
outgrowth, ultimately preventing commissure formation. Thus, in a normal embryo, Fra must be sending information that allows it to compete very well against these other guidance receptors to properly regulate axon outgrowth and steering during commissure formation. While the identity and specific role of these guidance systems awaits discovery, the sensitivity of the CNS axon scaffold to Abl over-expression will be an important tool for identifying these competing pathways.

Overall, it can be speculated that Fra signaling interacts with Abl in order to regulate actin dynamics underlying commissure formation. To determine if this hypothesis is true, a reduced cell culture system can be used as it allows us to specifically activate Fra and assay the ensuing changes in actin based cell morphology. The next chapter describes our development of such an assay and the role Abl plays during this process.
Chapter 3

Frazzled and Abelson interact to regulate actin dependent Drosophila S2 cell morphology
Abstract

Activation of a guidance receptors initiate signaling pathways that regulate the actin dynamics required for growth cone movement and steering. In this study, we have developed a *Drosophila* S2 cell assay to study how the Netrin chemoattractive receptor Frazzled (Fra) initiates signals to alter actin dependent cell morphology. When S2 cells are plated on a Concanavalin A substrate they usually form a lamella structure, but if Fra is present, it utilizes all three P-motifs in its cytoplasmic tail to preferentially promote the formation of filopodia. In particular, P1 and P3 motifs are the most important to form filopodia, yet even in their absence S2 cells display membrane ruffling and/or serrations. Using RNAi techniques, we determined that Fra requires endogenous Abl to extend filopodia in S2 cells, and that loss of Abl can be rescued by a wild type Abl. Using a dual RNAi strategy that differentiates between an Abl transgene and endogenous Abl, we determined that a kinase inactive mutant (Abl\textsuperscript{KN}) acts as a dominant negative mutant preventing Fra from forming filopodia. Deleting the F-actin binding domain (FABD) of Abl (Abl\textsuperscript{AF}) prevents it from localizing to the cell periphery of an S2 cell, but in the presence of Fra, to which it can bind, it does localize to the membrane and supports filopodia formation. Removing the FABD from Abl\textsuperscript{KN} relieves the dominant negative effect of Abl\textsuperscript{KN} as many more Fra-dependent filopodia form. Thus, we propose that Fra activation regulates Abl activity, possibly by recruiting it to its cytoplasmic tail. Abl then appears to be used as a scaffold protein to presumably phosphorylate and regulate other actin binding proteins required to form filopodia. Future work will need to identify how Fra and Abl bind and what regions of Abl participate in filopodia formation.
Introduction

Regulation of actin dynamics is an integral part of various pathways that culminate in motility, morphogenesis and axon path finding (Bristow et al., 2009; Dent et al., 2004; Roh-Johnson and Goldstein, 2009). Receptors expressed at the growth cone surface respond to extracellular cues and orchestrate actin dynamics that lead to directed steering of the axon (Dorsten et al., 2010; Forsthoefel et al., 2005; Gitai et al., 2003; Shekarabi and Kennedy, 2002). Generally, receptor activation by attractive cues promote the extension of filopodia to facilitate the forward movement of an exploring axon, while repulsive cues signal the retraction of these protrusions leading to growth cone collapse.

The family of Deleted in Colorectal Cancer (DCC) receptors that include Frazzled (Fra) in Drosophila and Unc40 in C. elegans respond to the attractive cue, Netrin, to mediate axon outgrowth and turning. Each of these receptors initiate signaling via three evolutionarily conserved P-motifs in their cytoplasmic tail to modulate the cytoskeletal dynamics (Dorsten et al., 2007; Dorsten and VanBerkum, 2008; Dorsten et al., 2010; Gitai et al., 2003; Li et al., 2002a; Li et al., 2002b(Dorsten, 2007 #2480; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Binding of FAK (focal adhesion kinase) and Fyn kinase to DCC; and also the physical interaction between Abl or Trio with Fra support the idea that intracellular proteins either get activated or recruited to the P-motifs in response to receptor activation (Dorsten et al., 2010; Forsthoefel et al., 2005; Li et al., 2004).
In *Drosophila*, two isoforms of Netrin (Netrin A and Netrin B) present themselves to their receptor - Fra, during the axon pathfinding. Homozygous mutations in either *fra* or both of the *netrin* genes either prevent commissural axons from crossing the midline or these axon tracts are malformed (Dorsten and VanBerkum, 2008; Kolodziej et al., 1996). Loss of *fra* specifically affects the formation of the posterior commissures in the developing embryo. Loss of function mutations in genes that regulate cytoskeletal dynamics, such as *trio* and Abelson Tyrosine Kinase (*abl*), further decrease the presence of commissural axons, preventing the formation of both anterior and posterior commissural axons (Forsthoefel et al., 2005) while Enabled (Ena) suppresses *fra* developmental defects (Forsthoefel et al., 2005). We are particularly interested in Abelson kinase because in *Drosophila fra* mutant embryos, both decreasing Abl levels or overexpressing wild type Abl exacerbates *fra* dependent loss of commissures (Dorsten et al., 2010; Forsthoefel et al., 2005). It is interesting to note here that either overexpression of Abl or treatment with low doses of cytochalasin, a drug that inhibits actin polymerization, leads to similar motor neuron pathfinding defects. This implies a role for Abl in regulating actin dynamics during growth cone movement (Kaufmann et al., 1998; Wills et al., 1999a). Thus, it is not surprising that mutations in *abl* also interact with several actin modulators, such as small GTPases, *ena, trio* and *profilin* to alter the normal targeting of the axons (Fox and Peifer, 2007; Grevengoed et al., 2003; Michael et al., 2010; Wills et al., 1999a; Wills et al., 1999b).

The importance of Abl may lie in its ability to tyrosine phosphorylate other proteins, as this event is known to be important for neurite outgrowth (Goldberg and Wu, 1995, 1996). In fact, the tips of filopodia in primary neurons and cultured cells are
enriched with phosphotyrosine residues (Wu and Goldberg, 1993; Wu et al., 1996) and several kinases such as the Src family are responsible for localization of phosphorylated receptor at the leading edge of a cell (Meriane et al., 2004; Ren et al., 2008). In fact, Src and Abl family of kinases share a similar N-terminal region. Most of the work on DCC signaling focuses on Src kinases and little is known about the cellular role of Abl downstream of DCC or Fra. Similar to Src's interaction with DCC, Abl binds to the cytoplasmic domain of Fra and there is an increased level of phosphorylated Fra in the presence of ectopic Abl (Dorsten et al., 2010; Forsthoefel et al., 2005). These data suggest that the signaling mechanism of Abl's function downstream of Fra might be similar to that of Src in DCC signaling.

The Abelson Kinase family of proteins is conserved between species. It includes Abl and Abl related gene (Arg) in vertebrates, dAbl in Drosophila, and Abl-1 in C.elegans. While vertebrate Abl shuttles into the nucleus, Arg and Drosophila Abl display only cytoplasmic localization, indicating that these two proteins may share similar functions in the cell. Structurally, the Abl kinases have four sequentially arranged regions starting with the N-Cap, which is followed by two Src-homology (SH) regions, (SH3 and SH2) and the kinase domain (Bradley and Koleske, 2009). The feature that distinguishes Abl from Src family of kinases is the presence of a unique F-actin binding domain in its carboxyl terminus.

Expression of Abl in several cultured cells promotes membrane ruffling, filopodia or lamellipodia (Miller et al., 2004; Radha et al., 2007; Stuart et al., 2006). These cellular morphologies represent different aspects of regulated actin dynamics. Although the difference in the morphological readout may be indicative of different cell types, it also
supports the view that Abl receives upstream signals to regulate actin dependent cell movement and migration (Michael et al., 2010; Miller et al., 2004; Radha et al., 2007; Wang et al., 2001; Woodring et al., 2002; Woodring et al., 2004). Pharmacological inhibition of Abl kinase activity abolishes Abl dependent filopodia formation and deletion of the F-actin binding domain (FABD) induces microspikes (Woodring et al., 2001; Woodring et al., 2002; Woodring et al., 2004). Moreover, a C-terminus deletion also prevents Arg from localizing to the periphery of fibroblasts indicating that binding of actin may have an inhibitory role on Abl function (Miller et al., 2004).

Given that Abl binds to Fra and genetically interacts with it, and regulates actin, it is reasonable to hypothesize that Abl provides a key link between upstream receptor and the actin cytoskeleton. While genetic studies have suggested that Fra regulates actin remodeling during growth cone movement, no study has yet demonstrated that Fra activation actually promotes the formation of actin rich membrane protrusions. However, it has been shown that upon Netrin activation, DCC increases filopodia number and surface area of HEK293T cells and other neuronal cells (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Therefore, to understand the molecular nuances of Fra signaling and shed light upon its molecular interaction with Abl, it is important to establish an *in vitro* cell culture assay, similar to those utilized for vertebrate studies (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). In this paper, we describe a *Drosophila* S2 cell culture assay which allows us to document the immediate effects of Fra activation on actin based cell morphology. Wild type Fra promotes the formation of F-actin rich filopodia in S2 cells when plated on Con A and this reaction specifically requires Abl. Knockdown of Abl by RNA interference reduces Fra’s ability to
extend filopodia, and this can be rescued by over-expression of wild type Abl but not a kinase inactive form. Removal of the F-actin binding domain from the C-terminus of Abl reduces but does not eliminate Fra-dependent filopodia formation, consistent with the ability of this mutant to still be recruited into a Fra complex at the membrane.

**Materials and Methods**

*Expression constructs:* The Myc-tagged versions of wild type Fra and mutants with individual P-motifs deleted used in this chapter are the same as that described in chapter 2. To overexpress FLAG-tagged Abl in S2-Fra cells, the pMT-V5His vector (Invitrogen) was modified to create another parental vector termed as the pMT-FLAG-Blast. This vector includes a blasticidin (Blast) resistance cassette. Further, T4 polynucleotide phosphorylated Flag sense (5' GCC CGC GAC TAC AAG GAC GAC GAT GAC AAA GAT TAC AAA GAT GAC GAC GAC AAG TAA TAG TTT 3') and anti-sense oligos (5' AAA CTA TTA CTT GTC GTC ATC ATC TTT GTA ATC TTT GTC GTC CTT GTA GTC GC 3') were annealed and ligated into pMT-Blast using NotI and Pmel restriction sites. A stop codon was added after the FLAG tag to terminate translation of the His tag. Abl wild type (Ablwt) and Abl\textsuperscript{KN} templates for PCR amplification were obtained as NotI fragments from pFtz-Abl\textsuperscript{WT} and pFtz-Abl\textsuperscript{KN} respectively (Hsouna et al., 2003). As described in Hsouna et al (2003), Abl\textsuperscript{KN} has a Lysine (K) converted to Asparagine (N) at amino acid 417 giving rise to a unique Hpal site. The full length Ablwt and Abl\textsuperscript{KN} (~4917 base pairs) was PCR amplified and cloned into pMT-FLAG-Blast vector in between SpeI and NotI restriction sites. To delete the F-
actin binding domain (FABD), we designed a reverse primer (5’ TAT AGC GGC CGC TCT GTG TGG AGC TGG 3’) that annealed immediately before the FABD (4586 to 4570 bp). Then, we used this reverse primer and a forward primer starting at ATG of Abl sequence to amplify Abl\(^\Delta F\) and Abl\(^{K\Delta F}\) fragments to clone into pMT-FLAG-Blast.

**S2 cell culture and transfection:** *Drosophila* S2 cells were maintained in Schneider’s media supplemented with 10% fetal bovine serum (FBS, complete media) and 50µg/ml gentamicin at 25\(^{\circ}\)C. Approximately 2x10\(^6\) cells were transfected (Effectene, Qiagen) with Myc-tagged Fra\(^{WT}\), Fra\(^{\Delta P1}\), Fra\(^{\Delta P2}\), and Fra\(^{\Delta P3}\), or FLAG-tagged Abl\(^{WT}\), Abl\(^{KN}\), Abl\(^{\Delta F}\), Abl\(^{K\Delta F}\). 300µg/ml hygromycin (Invitrogen) was used to select for stable Fra lines while Abl expressing cell lines were obtained through selection in 5µg/ml blasticidin (Invitrogen). Dual lines expressing Frawt myc and Abl\(^{WT}\), Abl\(^{KN}\), Abl\(^{\Delta F}\) or Abl\(^{K\Delta F}\) were selected and maintained in 300µg/ml hygromycin and 5µg/ml blasticidin. We noted that the expression levels of both Fra and Abl decreased over time after several passages in culture.

In order to have a continuous source of Netrin, we made stable S2 lines expressing either Myc-tagged NetA3 or NetB3 in a pMT vector. These plasmids were kindly provided by Dr. Mark Seeger. The Netrins are truncated and consists only of the C terminal portion [(Net A: 524-727bp; NetB: 649-793); Harris et al., 1996]. Since the C-terminal portion is not similar to laminin, the fusion protein does not stick to the cell membrane and instead is secreted out into the media. After inducing the cells for Netrin expression for 24 hours, we collect the media and centrifuge at 10000 rpm to pellet the
cells. The supernatant containing each Netrin is mixed in 1:1 ratio and aliquoted into tubes for future use.

**Reverse Transcription PCR:** To determine if S2 cells expressed either Netrin A or Netrin B, we used reverse transcription PCR (RT-PCR) to detect the levels of netrin mRNA expression in S2 cells. Specific primers were designed to differentiate between Netrin A and Netrin B. The sequence of the primers are as follows: Netrin A forward primer – 5’ CTGTTTACTCTGATCCTG 3’ and Netrin A reverse primer – 5’ AATGCGGCAAATGCA 3’; Netrin B forward primer – 5’ TGATGCAGGGTGCCA 3’ and Netrin B reverse primer – 5’ TGTGCCAGTGCAACG 3’. RT-PCR reaction was performed using the protocol as directed by the RT-PCR kit instructions (Invitrogen SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity; Cat. No. 12574-035).

**Immunoprecipitation Studies:** Fra protein expression was induced with 700 µM copper sulfate and 24 hours later, Fra was activated with exogenous Netrin A and Netrin B for 30 minutes. Cell were lysed in NP-40 buffer [(50mM Tris pH8, 100mM NaCl, 1mM MgCl2, 1% NP-40, 10mM NaF, 2mM Na3VO4, 5µg/ml Aprotinin and 5µg/ml Leupeptin); Forsthoefel et al., 2005] by 5X 10 seconds sonication pulses or by passing it 20 times (check again) through 21 gauge needle-syringe system, followed by 45 minutes incubation on ice. Mouse anti-myc was used to co-precipitate Fra and Abl immunocomplex using an established protocol (Dorsten et al., 2010; see Chapter 2).
The immunocomplex was separated on a 7.5% SDS poly-acrylamide gel after which the proteins were transferred to PVDF membrane at 45V for 4 hours. For immunoblotting, the membrane was blocked in 5% milk-TBST (0.05% tween) for an hour, followed by incubation in primary antibody (anti-myc or anti-flag) at 1:5000 dilution. Then, the excess antibody was washed off using 0.05% TBST and incubated with secondary antibody (1:5000). After washing away any unbound antibody, the membrane is probed with ECL reagent (GE-Amersham) to detect the HRP conjugated complex.

**RNA interference:** To knockdown Abl in Drosophila S2 cells, we primarily used the dsRNA that was designed by the O'Farrell lab [(herein referred as cds RNAi); (Foley and O'Farrell, 2004)], and was kindly provided to us by Dr. Russ Finley. In addition, we also designed a primer set with flanking T7 sequence to target the 5’untranslated region (UTR). The PCR amplified DNA templates is approximately 700 base pairs. The primer sequences are as follows: T7–forward 5’Abl UTR rna: 5’ TAA TAC GAC TCA CTA TAG GGA GAC CAC GGG CGG GT TAT CGG TAT GC. T7–reverse 5’Abl UTR rna: 5’ TAA TAC GAC TCA CTA TAG GGA GAC CAC GGG CGG GTG GTT TTT CGC 3’.

PCR products serve as templates for *in vitro* transcription of the double stranded RNA (dsRNA) using the MEGASCRIPT T7 kit (Ambion). The dsRNA is cleaned by phenol chloroform precipitation, before loading approximately 1.5 µg (0.5µl) to confirm the size on agarose gel. The remaining dsRNA is stored at -80°C.

To knock down the expression of Abl in S2 cells, a slightly modified protocol of (Pile et al., 2003) was used. Briefly, 3x10⁶ cells were plated in 4ml complete
Scheinder’s media in a 60mm culture dish for an hour at room temperature. The media was then carefully removed and replaced with 2ml of serum free media and the cells are incubated for 30 minutes with approximately 50µg of dsRNA. Then, 4ml of complete media was added and incubated for 4-5 days at 25°C. On day 5, a second dose of dsRNA was added to the media such that the cells are exposed to dsRNA for a total of seven days. On the sixth day, 700uM copper sulfate is added to the media to drive protein expression. The knockdown efficiency was determined by SDS-PAGE and western blotting using guinea pig antibody against endogenous Abl (kindly provided by Mark Peifer), Anti-alpha tubulin (Cell Signaling) was used at 1:1000 dilution to control for gel loading. The secondary antibodies, namely, anti-guinea pig HRP and anti-rabbit HRP was used at 1:5000 dilutions (Jackson laboratories).

**Cell morphology assay and immunostaining:** In order to assay the cell morphologies of *Drosophila* S2 cells, nitric acid washed glass cover slips (22x22; Fischer) were coated with 250µg/ml Concanavalin A (Sigma). After 2 hours incubation at 37°C, the cover slips were rinsed with autoclaved distilled water and stored at 4°C. The assay was initiated by plating approximately 2x10^6 cells in 60mm plates. Then, expression of transgenes was induced with 700µM copper sulfate for approximately 24 hours, and Fra is activated by adding Netrin to the media (300µl/4ml). Approximately, 10^6 cells were plated on Concanavalin A (Con A) and allowed to spread for 30 minutes at 25°C (room temperature) after which it is fixed with pre-warmed (at 37°C) 4% paraformaldehyde for 10 minutes. The plating time was increased to approximately 40 minutes in the dual RNAi experiments. Following three phosphate buffered saline (PBS)
washes, the cells are permeabilized with 0.1% PBS – Triton-X 100 (PBST) for 20 minutes and then blocked with 5% BSA – PBST for an hour. Actin was visualized using Texas Red conjugated Phalloidin (Invitrogen) at 1:50 dilution (0.8units). Expression and localization of Fra and Abl proteins was determined using either mouse or rabbit anti-myc and mouse anti-flag (both from Sigma) respectively, at 1:500 dilution. Antibodies against endogenous proteins, namely, anti-Ena (5G2), anti profilin (chi1J), anti-Trio (9.4A) and anti-Rho (p1D9) were purchased from the Developmental Hybridoma Studies Bank (DHSB) and were used at 1:200 dilutions. The fluorescent secondary antibodies anti-mouse rhodamine, anti-mouse FITC, anti-rabbit FITC and anti-rabbit Texas Red were all used at 1:200 dilution (Jackson laboratories). The immuno-stained slides were mounted with vectashield containing DAPI (Vector Laboratories). The cell morphologies were manually blind scored using Zeiss Imager Z.1 - Axio Cam microscope under 100X magnification. The images obtained were also obtained at this same magnification to finally give a scale of 1-10µm. The images were then put into a panel using adobe Photoshop. Statistical analysis was performed using student t-test in graph pad and Microsoft Excel software.

Results

As a guidance receptor, Fra is expected to regulate key aspects of actin dynamics and this can be often observed as changes in the morphology of cultured cells (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Drosophila S2 cells are a reliable model for biological assays of actin dynamics (Rogers et al., 2002; Rogers et al., 2003).
Therefore, we have developed a S2 cell assay to observe the alterations in actin-based cell morphology that occur in response to Netrin activation of Fra. S2 cells are typically plated on poly-lysine or Con A. Cell morphology assessed by staining with phalloidin, which highlights the F-actin distribution and also allows us to visualize the cellular structure.

Accordingly, we transfected S2 cells with a myc epitope tagged version of wild type Fra cloned into a pMT vector, modified to carry the hygromycin resistance gene. Stable populations of Fra expressing cells (hereafter S2-Fra) were isolated following standard protocols. Expression of Fra from the metallothionine promoter was induced with CuSO$_4$. Cells were then exposed to a mixture of recombinant Netrin A and B and processed either for western blot analysis of protein expression or plated onto coverslips to determine changes in cell morphology.

Western blot analysis confirmed expression of a full-length Fra protein within 4 hours of CuSO$_4$ induction and expression levels reached saturation within 24 hrs. There was also some leaky expression of the transgene in the absence of CuSO$_4$. Phalloidin staining allowed us to visualize changes in cell morphology and because ectopic Fra localizes to the plasma membrane of S2 cells, its distribution (using anti-myc staining) provides us with a secondary measure of cell morphologies [Fig. 3.1 G-I]. Compared to S2 cells alone, which preferentially exhibit a discoid or “fried-egg” like morphology when stained with phalloidin, S2-Fra cells exhibit a range of cell morphologies. To begin quantifying these Fra dependent changes, cell morphologies were classified into one of the following six categories: (1) Round: Cells that do not spread on the substrate and exhibit strong actin localization at the periphery [Fig.3.1A]. (2) Lamella: The
discoid/‘fried-egg’ like morphology as demonstrated previously for S2 cells plated on Con A [Fig.3.1B; (Rogers et al., 2003)]. These cells have uniform actin staining in the cell body and periphery. (3) Serrated: These cells spread out on Con A but have actin positive jagged membrane morphology in contrast to the smooth contour in lamella [Fig.3.1C], (4) Membrane ruffles: Cells with highly ruffled, curled or ‘wavy’ dorsal plasma membrane with some areas having more concentration of F-actin than others [Fig.3.1D; (Jin and Wang, 2007)]. Cells that have both ruffles and spiky protrusions have been included in this category. (5) Filopodia: Cells with finger-like projections greater than 2 µm long extending from the advancing edge of the cells and enriched for phalloidin stained F-actin [Fig.3.1E] and finally (6) Microspikes: Round cells with extremely small peripheral protrusions, generally less than 2 µm. These cells also show strong peripheral localization of actin. In some studies, these protrusions have been referred to as the precursors of filopodia [Fig.3.1F; (Kozma et al., 1995)].

When plated on poly-lysine coverslips for 2 hrs, S2 cells do not spread well and appear mostly round [see also (Rogers et al., 2003)]. Under these conditions, 26% of un-induced S2-Fra cells form filopodia. This increases to 33% once Fra expression is maximally induced (700uM CuSo$_4$, for 24 hrs), and to 43% after saturating amounts of recombinant Netrin (300µl/4ml) are added to the media [Fig. 3.2 B].

The high number of filopodial morphologies apparent before Netrin addition combined with the leaky expression of Fra led us to suspect that S2 cells may have a low level of endogenous Netrin expression. In the absence of a strong *Drosophila* antibody, we tested if S2 cells endogenously express Netrin by assaying the levels of Net A and Net B mRNA. Using specific sets of primers for Net A or B, RT-PCR analysis
Fig. 3.1: Distribution of morphologies when S2 cells are plated on Con A. When S2 cells or S2 cells expressing a transgene in plated on 250µg/ml Con A for approximately 30 minutes, it displays a range of cellular morphologies. A. Round cells with strong peripheral actin staining. B. Lamella: These cells spread extensively on Con A to form lamellar structure. C. Serrated: The cells spread and exhibit a jagged periphery. D. Dorsal Ruffling: The cells have a dorsal membrane ruffling. E. Filopodia: These cells extend long protrusions filled with F-actin. F: Microspikes: These cells appear round that has short F-actin protrusions. F-actin was visualized with phallolidin Texas-red after which the cells were mounted on vecta shield consisting DAPI. G-I: Immunostaining of S2-Fra cells reveal that Fra (green) is exhibited at the membrane (G) and both F-actin (red) and Fra (green) is localized in the filopodia (H-I).
confirmed that S2 cells express a low but detectable level of Netrin B mRNA but not Netrin A mRNA [Fig. 3.2 A]. It seems likely that this low level of Netrin expression partially activates Fra even in non-induced cells and supports our conclusion that Fra signaling selectively increases filopodia formation. These data are consistent with Netrin/DCC dependent increase in filopodia in HEK293T, NG108-15 and rat commissural neurons (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). In all subsequent experiments, to better isolate the Fra effect, we compared the distribution of Fra dependent morphologies to S2 cells alone, after exposing both types of cells to recombinant Netrin.

In contrast to poly lysine, S2 cells form lamella structures as they attach and spread on Con A coated coverslips [(Fig.3.2 B); (Rogers et al., 2003)]. Compared to S2 cells, S2-Fra cells produce a range of cell morphologies on Con A but filopodia still remains the most prominent phenotype as 40% of S2-Fra cells exhibit filopodia upon activation by exogenous Netrin [Fig. 3.2 B]. Filopodia is also the primary morphology observed when S2-Fra cells are plated on different concentrations of Con A (25µg/ml, 50µg/ml, 100µg/ml, 250µg/ml or 500µg/ml), or if Fra expression is induced for only 4 hours (data not shown). Thus, under a variety of conditions, activation of Fra in S2 cells selectively promotes the formation of filopodia.

In the above assays, cells were plated on Con A for 2 hrs before fixing. As a guidance receptor, Fra is expected to initiate several signaling pathways that alter cytoskeletal dynamics immediately upon ligand binding. Thus, we sought to investigate what effect shorter plating times might have on Fra dependent cell morphologies. S2-Fra cells were induced for expression for 24 hours, activated by recombinant Netrin as
described above and then plated on Con A coverslips for 15, 30, 45, 60 or 90 minutes prior to fixation and immunostaining. S2 cells exhibiting Fra dependent filopodia are most prevalent immediately after plating on Con A, as 70% of the S2-Fra cells exhibit filopodia at 15 minutes and this filopodia frequency tapers to 25% at 90 minutes [Fig. 3.2 C]. With increasing time, S2-Fra cells appear to spread more and many cells display lamella or serrated morphologies. The strong effect of the plating time is further evidence that Fra selectively promotes filopodia formation. In summary, we have modified existing S2 cell assays to uncover a Fra dependent increase in filopodia. Fra expression was induced for approximately 24 hours with 700 µM copper sulfate, and then $10^5$ cells were plated for 30 minutes on 250 µg/ml Con A coated glass cover-slips before fixing and immunostaining. Since we must compare all transgene expression to S2 cells alone to quantify the distribution of cellular morphologies, each of the following experiments was performed as a cohort that includes S2 cells alone as an internal comparison.

*Fra requires its P-motifs to form filopodia in S2 cells*

The conserved P-motifs in the cytoplasmic domain of Fra are required for its function *in vivo* (Dorsten and VanBerkum, 2008; Garbe et al., 2007). Consequently, we would expect that the ability of Fra to alter S2 cell morphologies would be compromised when an individual P-motif is deleted. To test this idea, mutant forms of Fra, where an individual P-motif (P1, P2 or P3) was deleted were expressed in S2 cells and the distribution of cell morphologies compared to that caused by S2-Fra cells. Confirming the importance of all three motifs to Fra function, deletion of individual P-motifs does
change the distribution of cell morphologies when compared to S2 cells alone [Fig. 3.2 D-E; (Dorsten and VanBerkum, 2008)]. As demonstrated in the previous section, Fra activation drastically decreased the frequency of lamella [83% to 3%; P<0.005] in favor of filopodia [8% to 77%; P<0.005]. In addition, we also see a significant increase in membrane serration [4% to 14%; P<0.05]. When compared to S2 cells, each P-motif deletion mutant remains active, however, it alters S2-Fra’s spectrum of cell

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**Fig. 3.2: Fra activation forms filopodia in a P-motif dependent manner.**

A. To determine if S2 cells expressed endogenous Netrin, we did RT-PCR on S2 cells mRNA using primers that would differentiate Netrin A from Netrin B. While Netrin A is absent in S2 cells, Netrin B is present in low levels. S2 cells were transfected with metallothionine-inducible vectors expressing myc-epitope tagged Fra wild type or Fra P-motif deletions. B. When S2-Fra cells are plated on Con A and poly-lysine, it forms filopodia. Fra expression induces filopodia which is further enhanced when activated with exogenous Netrin. C. The formation of filopodia is particularly sensitive to the time of plating the cells on Con A. Over 70% of the cells form filopodia within 15 minutes. D. Expression of Frawt primarily increases filopodia but also membrane serration is observed. E. Deletion of either P1 or P3 motif reduces formation of filopodia. Interestingly, deletion of P1 and P2 or P3 increases number of serrated cells and membrane ruffling respectively. ***/φφφ equals p-value less than 0.005 and */φ is p-value less than 0.05
morphologies [Fig. 3.2 D-E]. The predominant trend is to decrease filopodia and increase membrane ruffling and/or serrations [Fig 3.2 E]. For example, removal of either P1 or P3 reduces the frequency of cells with filopodia by approximately 20% [Fig. 3.3B; P<0.05] and now more cells have membrane serrations and/or ruffles. Deletion of P2 is least deleterious, and although there is a slight reduction in the frequency of cells exhibiting filopodia, this did not reach significance. The differential effects of each P-motif deletion on S2 cell morphology is consistent with the in vivo rescue data (Dorsten and VanBerkum, 2008; Garbe et al., 2007) and thus lends support to our conclusion that Fra increases filopodia in S2 cells.

Immunolocalization of actin modifiers in S2-Fra cells

Induction of filopodia by Fra implies that it initiates signaling pathways to rearrange the actin cytoskeleton. If true, immunolocalization of proteins known to be regulators of actin dynamics should support this idea in S2-Fra cells. To evaluate this, we used available monoclonal antibodies to compare the cellular localization of four actin-associated proteins (Enabled, Profilin, Trio and Rho GTPase) in S2 and S2-Fra cells after activation by Netrin and plating on Con A coverslips. In these experiments, Fra protein was visualized using an antibody to its myc epitope tag and localization of Fra to the membrane of S2 cells allowed us to assess the cell morphology [Fig 3.3 B, E, H and K inset]. In S2 cells, Enabled is localized in the cytoplasm and at the cell periphery as also observed in the Rogers’ study (2003). In S2-Fra cells, while we still observe cytoplasmic Ena, it is also localized to the Fra dependent filopodia as expected.
for an anti-capping protein [Fig. 3.3 A-C; (Gertler et al., 1996; Applewhite et al., 2008)].
In contrast, Profilin has a diffused central localization with no visible staining in the
periphery of both S2 [Fig. 3.3 D; (see also Rogers et al., 2003)] and S2-Fra cells [Fig. 3.3E-F]. Trio is evenly distributed in the cytoplasm and appears to be less intensely
stained at the periphery of lamella [(S2); Fig. 3.3 J] and filopodia [(S2-Fra); Fig. 3.3 K-L].
Consistent with this staining, ectopic expression of vertebrate Trio shows uniform
distribution in HeLa cells (Medley et al., 2000). Here, Rho has the most interesting
change in pattern. It is ubiquitously expressed in the cytoplasm and on the membrane of
S2 cells, but in S2-Fra cells Rho is also observed as a punctate pattern along the length

![Image of cellular localization of actin modulators](image)

**Fig. 3.3:** Fra activity induces differential localization of actin modulators in S2 cells. A. In S2
cells, Enabled is expressed both in the perinuclear region and throughout the lamella. D. However,
Profilin is observed primarily in the nuclear region. G. J. Rho and Trio are expressed across the
cytoplasm. On Frazzled activation (inset), Enabled localizes through the length of the filopodia in
Frazzled expressing cells and is also found on the tips of the filopodia (B-C). In contrast to that, there
is expression of Profilin and Trio appear to be diffused in the cytoplasm and is completely absent at
the tip (E-F). Meanwhile, Rho and Fra are seen in the filopodia (H-I).
of the filopodia [Fig. 3.3 G-I]. While we cannot definitively co-localize Rho to Fra, these staining patterns are consistent with Fra initiating intracellular signals that remodel the actin cytoskeleton.

**Abl knock down redistributes phosphotyrosine residues in S2 cells**

Abl tyrosine kinase is known to physically and genetically interact with Fra during embryonic development of the *Drosophila* nerve cord (Dorsten et al., 2010; Forsthoefel et al., 2005). Having established a cell culture assay for Fra activity, we proceeded to ask if Abl activity was required in S2 cells for Fra-dependent formation of filopodia. To deplete Abl from S2 or S2-Fra cells we used two non-overlapping RNA interference (RNAi) probes. The first RNAi probe targets the region between EVH and FABD, and is obtained from S2 dsDNA template library for RNAi and will be referred to as cds RNAi in the rest of the paper (Foley and O'Farrell, 2004). The second RNAi probe targets the 5' untranslated regions (UTR) and henceforth will simply be referred to as UTR RNAi. In these experiments, S2 or S2-Fra cells were cultured in Schneider’s media for an hour and then the cells were treated with dsRNA for 30 minutes in serum free media. After 3-4 days, a second dose of dsRNA was provided and the cells were re-incubated for a total of seven days before being assayed for protein expression and cellular morphology. It is important to note that the second dose of dsRNA and extended incubation time was required to obtain the >90% decrease in protein expression observed by western blot analysis [Fig. 3.4 A]. In S2 cells alone, knock down of Abl does not lead to a major change in the distribution of cell morphologies. Only a small,
but significant increase in the frequency of cells displaying microspikes is observed [Fig. 3.4 B; (9 to 15%; $P<0.005$)].

**Fig. 3.4: Effect of Abl knock down in S2 and S2-Fra cells.** A. Endogenous Abl was knocked down using dsRNA against the coding sequence region (Cds RNAi). Knock down was then confirmed using guinea pig anti-Abl antibody. B. Loss of endogenous Abl in S2 cells gives rise to a small but a significant increase in microspikes. C. Moreover, Abl is needed during Fra signaling as loss of Abl interferes with Fra’s ability to extend out long filopodia. **$P<0.005$** and * $P<0.05$. D. S2 cells spread on concanavalin A to form a flat lamellar structure. Here, the phospho-tyrosine residues are distributed throughout this lamella with very strong localization at its periphery. In addition, phosphorylation of tyrosine is also observed along the Fra dependent filopodia. Furthermore, some of these pY residues are observed with Fra at the tips of the finger-like extensions. E. When Abl is knocked down; there is a re-distribution of phospho-tyrosine residues. Here, over 60% of the cells are now no longer able to localize phospho-tyrosine residues to the edge of the cell membrane.
More interestingly, knock down of Abl in S2-Fra cells leads to a significant loss in the frequency of cells displaying filopodia [Fig. 3.4 C; (41% to 21% using cds RNAi probe); P<0.005] and an increase in the number of cells forming microspikes [9 to 15%; P<0.005] or remaining round [Fig. 3.4 C; 10% to 19%; P<0.05]. We observe a similar effect when Abl is knocked down using the UTR RNAi probe in S2 and S2-Fra cells (Table 1). This suggests that Abl is at least partially required by Fra to extend filopodia. The emergence of microspikes may reflect an alteration in levels of phosphorylated Fra as an increase in microspikes is also observed when we exposed S2 or S2-Fra cells to the generic tyrosine kinase inhibitor genistein. This drug is quite toxic to S2 cells, but at low sub-lethal doses (10-80 µg/ml media), both S2 and S2-Fra cells exhibit a major increase (20 to 80%) in the frequency of cells with microspikes (Appendix B).

To further investigate the role of tyrosine kinases, we examined the distribution of phosphotyrosine residues in S2 and S2-Fra cells plated on Con A by immunostaining. In S2 cells, phosphotyrosine residues are distributed uniformly in the cytoplasm with an obvious central localization and a much stronger staining at the periphery of the lamella [Fig. 3.4 E]. Interestingly, knockdown of Abl using either cds or UTR RNAi re-distributes the phospho-tyrosine residues into a uniform punctate pattern within the lamella with no obvious localization to the periphery of S2 cells [Fig. 3.4 E, H]. Similar results using both probes strongly support our contention that these data do not reflect non-specific off-target affects. In S2-Fra cells, an even punctate staining in the cytoplasm as well as the membrane, including along the length of the filopodia, is observed [Fig.3.4 D, F, and G].

Finally, we sought to globally increase phosphotyrosine levels in S2 and S2-Fra cells using pervanadate - a generic inhibitor of tyrosine phosphatases (Bugga et al.,
With pervanadate treatment, the number of S2 cells forming filopodia increases from 10% to 35%. Interestingly, in the presence of Abl RNAi, pervanadate treatment fails to promote filopodia in S2 cells, and instead, the cells exhibit microspikes (Appendix C). These data further strengthen the idea that Abl is a key kinase regulating actin based morphology in S2 cells, and is particularly important in the formation of filopodia. Inhibiting tyrosine phosphatases by treating S2-Fra cells with pervanadate also results in 67% of the cells forming filopodia. This increase in the frequency of filopodia in the presence of pervanadate is prevented by knock down of the endogenous Abl with cds RNAi. However, Fra is still able to induce filopodia formation in many cells (Appendix C). These data support the importance of Abl and Fra activity in the formation of filopodia but point to additional complexity.

**Co-expression of Fra and Abl wt in S2 and S2-Fra cells**

If Abl knock down inhibits Fra’s ability to project filopodia, then co-overexpression of wildtype Fra and Abl might be expected to increase the frequency of cells with filopodia. Accordingly, we determined the effect of over-expression of Abl in both S2 and S2-Fra cells by creating stable lines expressing FLAG tagged wild type Abl (Abl\textsuperscript{WT}) alone or in combination with myc-tagged Fra\textsuperscript{WT} (S2-Fra cells). In S2 cells, over-expression of Abl re-organizes the actin cytoskeleton by promoting dorsal ruffles [39 %; P<0.005] and filopodia [32%; P<0.05] at the expense lamella [10%; Fig. 3.5 A].

Dual staining against the myc and FLAG epitope tags for Fra and Abl, respectively, identified cells co-expressing Fra and Abl. As expected, expression of
Fra\textsuperscript{WT} alone significantly increases the frequency of cells forming filopodia [35%; P<0.05; Fig. 3.5 B]. While co-expression of Frawt and Abl\textsuperscript{WT} tends to further increase the frequency of cells expressing filopodia [35% to 43%; Fig. 3.5 B], this did not reach statistical significance in this experiment. There was no other change in the distribution of any other cell morphologies. Since immunostaining confirmed the presence of both Fra and Abl in these S2 cells, it seems likely that one or more of the other regulators of actin dynamics may be rate limiting.

To further address the role of Abl in Fra-dependent filopodia formation we set out to test whether Abl activity was required and what role the unique F-actin binding domain might play in these actin based morphologies. This required the development of a dual RNAi strategy that would allow us to differentiate between a recombinant Abl transgene and the endogenous Abl in S2 and S2-Fra cells. The dual RNAi strategy compares the cell morphologies using two different RNAi probes, a coding sequence (cds) RNAi probe and a UTR RNAi probe (Table 1). The cds probe targets both endogenous and recombinant Abl, while the UTR probe specifically knocks down only the endogenous Abl, allowing specific expression of Abl transgene from the metallothionine promoter of the plasmid. Thus, a comparison of cell morphologies elicited by cds or UTR RNAi probe will provide information on the activity of the Abl transgene.

In this new round of experiments, the cell morphologies of S2 and S2-Fra cells alone or in combination with Abl transgenes were compared under three different conditions: 1. No RNAi probe, which reveals changes due to simple over-expression of Abl, 2. A cds RNAi probe which knocks down both endogenous and transgene
expression, and 3. UTR RNAi, which preferentially knocks down only endogenous Abl. S2 or S2-Fra cells, with or without an Abl transgene were treated for a total of seven days with two doses of either RNAi probe. Fra and Abl transgene expression was induced by copper for 24 hours, and, if Fra was present, exogenous Netrin was added for 30 minutes for approximately 40 minutes due to increase in coverslips. Cells were then plated on Con A coverslips, and processed for immunostaining and quantitative analysis of cellular morphologies.

As expected, over-expression of wild type Abl in S2 cells results in fewer cells exhibiting lamella and many more cells with an increase in membrane ruffles [Fig. 3.5 C; compare dashed bars to white bars; P<0.005]. Knock down of both endogenous and recombinant Abl (cds RNAi) revert S2 cells back to predominantly lamella [Fig. 3.5 A; compare white and gray bars; P<0.05]. In contrast, treatment with UTR RNAi confirms the functionality of the Ablwt transgene as the ruffles remain prominent at the expense of cells forming only lamella [Fig. 3.5; compare gray and black bars]. However, it is noted that with UTR RNAi treatment fewer cells exhibit filopodia as compared with S2 cells over-expressing Abl, and more cells remain round [Fig. 3.5 C; compare white bar to black bars; P<0.005]. As expected, in S2-Fra cells the Ablwt transgenes remains active and helps form filopodia. In this round of experiments, approximately 37% of S2-Fra cells exhibit filopodia and this remained the same with over-expression of Ablwt [Fig. 3.5 D; compare dashed and white bars]. The cds RNAi probe significantly decreased filopodia formation [37% down to 8%; P<0.005; Fig. 3.5 D; compare white and gray bars) but many cells (25%) still exhibit filopodia when only endogenous Abl is knocked down using the UTR probe [Fig. 3.5 D; compare gray and black bars].
Reduction in both endogenous Abl and transgene levels (the cds probe) also tends to cause a small but significant increase in cells with microspikes [4% to 16%; P<0.05; Fig. 3.5 D; compare white and gray bars] and many more S2-Fra cells fail to spread, and instead remain round [13% to 40%; P<0.005; Fig. 3.5 D; compare white and gray bars]. Using the UTR probe does not cause an increase in microspikes or round cell morphologies when compared to S2-Fra cell alone [Fig. 3.5 D; compare gray and black bars]. It is noted that compared to other experiments, in this set of slides, we observed an unusual increase in the frequency of S2-Fra cells with dorsal ruffles, which did not change when Abl was co-expressed or cells were treated with either RNAi probe. As these slides were processed in parallel with the three Abl mutants discussed below, we suspect that this increase in ruffling may reflect the increase in plating time required to process all of the slides. Nevertheless, the Ablwt transgene is clearly functional and contributes to S2 and S2-Fra cell morphology, and in particular confirms that Ablwt is required during Fra dependent formation of filopodia. These data also confirm the validity of the dual RNAi approach.

Our ability to selectively alter endogenous levels with a UTR probe allows us to investigate the role of (a) Abl Kinase activity and (b) the F-actin binding domain (FABD) at its C-terminus. An absence of kinase activity does not prevent Abl from binding to Fra (Forsthoefel et al., 2005) and over-expression of a kinase dead version of Abl (AblKN) still leads to axon guidance defects in vivo (Dorsten et al., 2007; Hsouna et al., 2003). The C-terminus F-actin binding domain is the hallmark of Abl kinases and is believed to be important in the regulation of actin dynamics.
Fig. 3.5: Abl is required by Fra to specifically form filopodia. A. Over-expression of flag epitope tagged Abl decreases lamella formation in favor of membrane ruffles. It also significantly increases the frequency of cell exhibiting filopodia. B. However, when co-expressed with Fra, we do not observe any shift in the distribution of cellular morphologies when compared to Fra. C-D. To differentiate the role of the endogenous and recombinant Abl, we utilized a dual RNAi strategy, wherein, we targeted either the coding sequence (cds RNAi) or 5'UTR region. Knock down of both endogenous and recombinant Abl in S2 cells (C), promotes lamella formation (compared white and gray bars) and suppresses the membrane ruffling previously observed (compared white and gray bars). Specific expression of recombinant Abl in the absence of endogenous Abl (black bars - UTR RNAi) revert the formation of both lamella and ruffling. D. In S2-Fra cells, we do not observe any differential effect in the frequency of lamella or membrane ruffling. However, cds RNAi decreases the formation of filopodia and instead increases the frequency of microspike and round cells. Moreover, specific expression of Abl using UTR RNAi partially revert the formation of the filopodia.
Overexpression of Abl mutants in S2 and S2-Fra cells

To address the role of the Abl kinase activity in the regulation of actin based cell morphology, we created S2 and S2-Fra cell lines expressing a FLAG-tagged kinase inactive Abl (AblKN). Over-expression of AblKN in S2 cells still shifts cells away from lamella [5% to 26%; P<0.05; Fig. 3.6 A] and towards membrane ruffling [11% to 45%; P<0.05; Fig. 3.6 A]. Compared to wild type Abl, expression of AblKN does not yield filopodia [Fig. 3.6 B].

In S2-Fra cells, expression of AblKN significantly reduces filopodia formation [37% to 8%; P<0.0005; Fig 3.6 A] and many more cells remain round [32%; P< 0.005; table 3.1]. With AblKN expression many cells also display membrane ruffling although this frequency is not significantly different from S2-Fra cells expressing Ablwt [Fig. 3.6 B]. The increase in ruffles suggests that the AblKN transgene retains some functionality but that activity is important for the formation of filopodia. In fact, AblKN appear to have a dominant negative effect as its expression reduces filopodia formation even in the presence of endogenous Abl.

To examine the role of F-actin binding domain, stable S2 and S2-Fra cell lines were created expressing FLAG-Abl lacking the C-terminal F-actin binding domain (AblAF). In S2 cells, over-expression of AblAF still alters the distribution of cell morphologies as fewer cells exhibit lamella [29%; down from 65%; P<0.005; Fig. 3.6 A] and many undergo membrane ruffling [54%; increased from 11%; P<0.0005; Fig. 3.6 C] when compared to S2 cells alone. However, compared to Ablwt overexpression, AblAF does not induce an increase in filopodia [29% vs 11%; Fig. 3.6 A].
Fig. 3.6: Overexpression of Abl mutants in S2 and S2-Fra cells. A-C. S2 cells expressing Abl wt, AblKN, AblAF or AblKN,AF is plated on Con A as described in the methods. All the Abl transgenes display an increase in membrane ruffling primarily at the expense when compared to S2 cells alone (A,C). Expression of AblKN, AblAF or AblKN,AF significantly decreases Abl dependent filopodia (B). D-F. Upon co-expression with Fra, Abl mutants do not show any changes in the frequency of lamella and membrane ruffles (D,F). However, the frequency of the Fra dependent filopodia decreases if AblKN, AblAF or AblKN,AF is expressed in S2-Fra cells (E).
In S2-Fra cells, over-expression of Abl^{ΔF} reduces the frequency of cells exhibiting filopodia by 20% [37% to 17%; P<0.05; Fig. 3.6 E] but there was little effect on the distribution of other cell morphologies. Thus, when over expression in S2 or S2-Fra cells, deletion of the F-actin binding domain of Abl does not block all of its activity but does partially inhibit filopodia formation. Finally, we also deleted the FABD from a kinase inactive version of Abl (Abl^{KNΔF}). When Abl^{KNΔF} is over-expressed in S2 or S2-Fra cells, the distribution of morphologies is indistinguishable from the Abl^{KN} mutant alone. These data suggests that in the presence of both endogenous and recombinant Abl, the kinase inactive mutant (Abl^{KN}) behaves like a dominant negative even when the FABD is deleted.

In summary, these over-expression data indicate that eliminating kinase activity or the FABD does not completely destroy the functionality of Abl. Both Abl^{KN} and Abl^{ΔF} mutants alter cell morphologies although both also prevent filopodia formation, especially in response to Fra signaling. However, interpretation of these over-expression data is complicated by the continued presence of endogenous Abl. Accordingly, during this set of experiments, we also employed our dual RNAi strategy, exposing cells to either a cds or UTR RNAi probe and comparing the distribution of cell morphologies. The UTR probe will allow us to delineate the contribution of the transgene as only the expression of endogenous Abl is suppressed. Thus, comparing cds to UTR morphologies allows us to delineate the functionality of the Abl mutant. The complete data set is provided in Table 1, while the UTR data is also presented in Fig 3.7, with a comparison to Abl^{wt} morphologies.
**Role of the Abl mutant transgenes in S2 and S2-Fra cells**

In S2 cells where endogenous Abl is suppressed by the UTR probe, the distribution of cell morphologies observed when the three Abl mutants are expressed (Abl^{KN}, Abl^{AF}, Abl^{KNAF}) is similar to that observed when Ablwt transgenes is expressed (Fig 3.7 A). Each transgene is functional as the lamellar structure typically observed in S2 cells is shifted to an increase in ruffling but none of the transgenes sustain an increase in the frequency of filopodia. Abl^{KN} is the least capable of forming membrane ruffling (23%; P<0.05). One additional phenotype that often coincides with the absence of endogenous Abl is also observed, namely, an increase in the frequency of cells remaining round [21% (KN) and 18% (KNAF) up from 15% (Ablwt); Fig 3.7 A].

In contrast, however, in S2-Fra cells, the use of the UTR probe to knock down endogenous Abl levels did uncover an unusual role for the FABD (Fig. 3.7B). Compared to expression of a wild type transgene, when endogenous Abl levels are suppressed, expression of Abl^{KN} shows a strong inhibition of filopodia formation (25% to 8%; P<0.0005). This is similar to the loss of filopodia in S2-Fra cells that have reduced levels of both endogenous and transgene Ablwt (8%) and confirms that in this assay, Abl^{KN} has a strong dominant negative affect on filopodia formation. Most surprisingly, this dominant negative affect appears to depend on the presence of the FABD. Here, when endogenous Abl levels are selectively reduced with the UTR probe, expression of either the Abl^{AF} or the Abl^{KNAF} mutant still allows approximately 21% of S2-Fra cells to exhibit filopodia, a frequency similar to that obtained with the Ablwt transgene in parallel experiments [(25%); Fig. 3-7B]. Like Abl^{KN} expression, these two mutants also cause more S2-Fra cells to remain round [27%(AF); 34% (KNAF)]. The Abl^{KNAF} result is
particularly interesting as this mutant now allows more filopodia formation than the Abl\textsuperscript{KN} mutant on its own, suggesting that the dominant negative effect at least partially requires an intact FABD.

Fig. 3.7: Kinase activity and actin binding of Abl affects Fra dependent filopodia. In this experiment, we specifically expressed the Abl transgenes (wt, KN, ΔF, KNΔF) by using the UTR RNAi to preferentially target the endogenous Abl in S2 (A) and S2-Fra (B) cells. A. In S2 cells, all the transgenes lower the frequency of lamella while increasing the number of cells exhibiting membrane ruffling. In addition, all the three mutants reduce the frequency of filopodia in these cells. B. In S2-Fra cells, both lamella and ruffling remain unaffected when compared to S2-Fra by its own. However, Abl\textsuperscript{KN} significantly interferes with filopodia formation; an effect that is not seen as much when actin binding domain is deleted. Moreover, Abl\textsuperscript{KNΔF} allows S2-Fra cells to form filopodia similar to the co-expression of Abl\textsuperscript{ΔF} alone.
Table 1: Dual RNAi of S2 and S2-Fra cells using cds and UTR probes

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* P<0.05, ** P<0.005: Significant difference in S2 or Fra with RNAi when compared to S2 or Fra alone without RNAi respectively
† P<0.05, †† P<0.005: Significant difference of transgene overexpression (without RNAi) when compared to S2 alone (without RNAi)
# P<0.05, ## P<0.005: Significant difference of Abl mutants compared to wild type Abl or Fra alone.
φ P<0.05, φφ P<0.005: Significant suppression of Abl or Fra-Abl overexpression phenotype upon RNAi (compare w/o RNAi and cds/utr RNAi)
‡ P<0.05, ‡‡ P<0.005: Significant reversion of cds RNAi phenotype upon transgene expression in the absence of endogenous Abl
**Abl binds to Fra in the absence of its actin binding domain**

Since both Abl$^{\Delta F}$ and Abl$^{\Delta KN \Delta F}$ stimulate filopodia formation in S2-Fra cells, we sought to determine if these Abl mutants bind to Fra. Previously, we used co-immunoprecipitation to demonstrate that both wild type and kinase inactive Abl bound to Fra (Dorsten et al., 2010). To further test if the actin binding domain of Abl was required to bind to Fra, we used the myc tag to immunoprecipitate the Fra complex from S2-Fra cells and asked if Abl transgenes was present by immuno-blotting against its flag tag. As demonstrated in Figure 3.8 deleting the entire actin binding domain of Abl (amino acids 1524 – 1639) did not interfere with Abl’s ability to bind to Fra. This physical interaction between Fra and the Abl$^{\Delta F}$ mutants is consistent with the increase in filopodia formation we observed above. Is it possible that the physical association with Fra also affects localization of Abl in cells?

![Fig. 3.8: Abl can physically interact with Fra without its FABD.](image)

FRA WT - WT KN AF KNAF IP: ms anti-myc IB: rb anti-myc

FRA WT - WT KN AF KNAF Cell lysates IB: ms anti-myc IB: ms anti-flag

**Localization of Abl transgenes in S2 and S2-Fra cells**

To determine the localization of the recombinant Abl proteins in S2 and S2-Fra cells, we utilized the unique FLAG-tag at the C-terminal of our transgenes. When S2
cells are plated on Con A, recombinant wild type Abl is distributed in a punctate fashion throughout the cell, and is present at the membrane, including the tips of filopodia [Fig. 3.9 A-C]. Membrane localization is not dependent on its kinase activity as Abl\(^{KN}\) still localizes to the membrane of S2 cells [Fig. 3.9 D-F]. In contrast, in S2 cells, very little Abl\(^{ΔF}\) or Abl\(^{KNΔF}\) is localized to the membrane periphery [Fig. 3.9 G-I; J-L]. This localization is consistent with the failure of Abl related gene (Arg) to localize to the membrane.

![Figure 3.9: In the presence of Fra, Abl\(^{ΔF}\) translocates to the membrane.](image)

**Fig. 3.9: In the presence of Fra, Abl\(^{ΔF}\) translocates to the membrane.** Immunostaining against the epitope tag on Abl shows that both Abl\(^{wt}\) (A-C) and Abl\(^{KN}\) (D-F) are expressed in the entire cytoplasm and the membrane in a punctate fashion in S2 cells. Some of these molecules are seen along with actin staining (C; F). In S2-Fra cells, both Abl\(^{wt}\) (M-O) and Abl\(^{KN}\) (P-R) localize with Fra at the periphery of the cell membrane. However, unlike the Abl\(^{wt}\) (A) or the kinase inactive (D), Abl mutants that do not have the actin binding domain – Abl\(^{ΔF}\) (G-I) and Abl\(^{KNΔF}\) (J-L) is absent from the periphery of S2 cells. Interestingly, in the presence of Fra, these Abl mutants: Abl\(^{ΔF}\) (S-U) and Abl\(^{KNΔF}\) (V-X) can now translocate to the membrane. Mouse antibody against FLAG and secondary FITC identified S2 cells expressing Abl\(^{wt}\) (A), Abl\(^{KN}\) (D), Abl\(^{ΔF}\) (G) and Abl\(^{KNΔF}\) (J). Fra was detected using primary antibody against myc, followed by secondary FITC staining (M, P, S, V) and Abl was detected using mouse anti-flag and mouse anti-rhodamine (N, Q, T and W). Phalloidin Texas Red allowed us to visualize F-actin (B, E, H, and K).
periphery of vertebrate cells when its C-terminal F-actin binding domain is removed (Miller et al., 2004; Wang et al., 2001). These data suggests that Abl requires its actin binding motif to localize to the periphery of S2 cells.

In S2-Fra cells, both Frawt and Ablwt are expressed throughout the length of the filopodia, including the tips [Fig. 3.9 M-P]. Similar to S2 cells, the kinase inactive mutant Abl\textsuperscript{KN} still localizes to the cell periphery [Fig. 3.9 Q-S]. Surprisingly, unlike in S2 cells where removal of the F-actin binding domain prevents the Abl mutants from localizing to the periphery of S2 cells, in presence of Fra, both Abl\textsuperscript{AF} and Abl\textsuperscript{KNAF} mutants are found at the cell periphery [Fig. 3.9 T-V; W-Y]. This cellular localization, along with the co-immunoprecipitation data above, suggests that Fra is recruiting Abl to the membrane. Together these data support the hypothesis that Fra recruits Abl to the membrane to help regulate actin dynamics underlying filopodia formation.

**Discussion**

As an axon guidance receptor, Fra is expected to respond to its ligand, Netrin, to transduce intracellular signals that regulate the machinery underlying axon outgrowth and steering. Here, we confirm this expectation at the cellular level as Fra significantly alters the spectrum of actin dependent S2 cell morphologies. When plated on Con A coverslips, most S2 cells (>60%) spread radially to form an extensive lamella structure (Rogers et al., 2003). When Fra is expressed in S2 cells and activated by exogenous Netrin, a lamellar structure is only observed in a minority of cells (~10%). Instead, the most prominent morphology observed upon Fra activation is long finger like projections from the cell periphery, that we call filopodia as they are enriched with actin. In support,
these cells display other hallmarks of filopodia namely, the presence of Ena and Rho at the tips of the filopodia, but not profilin, and phosphotyrosine residues along the length of the filopodia including its tip.

Fra preferentially forms filopodia on two different substrates, poly-lysine and Con A, although the degree of filopodia formation is highly dependent on the plating time. With traditional plating times [>60 min; (Rogers et al., 2002)] filopodia are present in a significant proportion of cells expressing Fra (25% to 32%), but the frequency of cells exhibiting filopodia is much higher (to >70%) if cells are plated on Con A for only a short period of time (15 min). These data suggest that in S2 cells, the proximal signaling events initiated by Fra involve an alteration in actin-based dynamics leading to filopodia. In addition to filopodia, in later experiments, Fra expression induces membrane ruffling and may indicate the amount of protein expressed in these cells. These data are consistent with vertebrate studies where Netrin activation of DCC also leads to an increase in filopodia formation in rat commissural neurons, HEK293T and NG108-15 cells (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Furthermore, like DCC, Fra also localizes along the length and at the tips of the filopodia, and this coincides with increased phosphotyrosine levels, (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). DCC is tyrosine phosphorylated by Src family kinases - Fyn and FAK [Focal Adhesion kinase; (Li et al., 2006; Li et al., 2004; Ren et al., 2004)], and Fra is phosphorylated by Abelson (Dorsten et al., 2010; Forsthoefel et al., 2005). Together, these data emphasize the functional conservation of Fra/DCC/Unc-40 receptors.

Fra utilizes all three of the conserved P-motifs in its cytoplasmic tail to induce filopodia formation, however, P1 and P3 may be the most important as their removal
significantly reduces Fra dependent filopodia. This is consistent with in vivo genetic studies where both P1 and P3 motifs are required for the formation of longitudinal fascicles and adult viability (Dorsten and VanBerkum, 2008; Garbe et al., 2007). On the other hand, even deletion of these motifs does not prevent Fra from inducing other changes in the morphology of S2 cells, indicating that each deletion mutant remains partially active. This is consistent with the ability of each P-motif deletion mutant of Fra to still rescue the gross morphology of commissures (Dorsten and VanBerkum, 2008; Garbe et al., 2007). On the other hand, while genetic studies in Drosophila imply that P2 motif is inert, in our S2 cell assay, this P-motif appears to be active in regulating actin dynamics, contributing to membrane ruffling and serration. In C. elegans, the P2 motif genetically interacts with Ena to regulate circumferential axon migration (Gitai et al., 2003). Thus, the conservation of the P-motifs in the Fra/DCC/Unc40 family of receptors is consistent with their ability to regulate different aspects of actin dynamics, including membrane ruffling and serration, ultimately to promote filopodia formation. To do so, P-motifs are thought to regulate intracellular proteins by either activating or recruiting them to the cytoplasmic tail of Fra.

One such effector appears to be Abl Kinase, as it binds to Fra and may phosphorylate it since co-expression of Abl with Fra increases the level of phosphorylated Fra in S2 cells (Dorsten et al., 2010; Forsthoefel et al., 2005). Here, Fra requires Abl kinase to form filopodia in S2 cells, as filopodia are significantly reduced if endogenous Abl is reduced using several RNAi probes. One study, using larval wing imaginal discs, did report an increase in filopodia when Abl was knocked down (Gates et al., 2007). We suspect that a difference in cell plating conditions, or more likely, cell
types accounts for this discrepancy. S2 cells have an embryonic hemocytic lineage while Gates’ study used cells derived from larval wing imaginal discs. Moreover, is S2 cells Fra’s ability to form filopodia is compromised when the overall tyrosine kinase activity is inhibited with genistein and Fra dependent filopodia increase when phosphatases are inhibited with pervanadate. That Abl is directly involved is also supported by the ability of dominant negative effect of Abl\textsuperscript{KN} mutant on Fra dependent filopodia formation, where many cells remain round. Our data is also consistent with Abl’s role as a key regulator of actin dynamics in vertebrate cells (Master et al., 2003; Michael et al., 2010; Miller et al., 2010; Peacock et al., 2007; Radha et al., 2007) and the requirement for tyrosine kinase activity during Netrin dependent axon outgrowth (Meriane et al., 2004; Rajasekharan and Kennedy, 2009).

It is interesting that expression of kinase inactive Abl (Abl\textsuperscript{KN}) or removal of the FABD still allows these Abl mutants to increase membrane ruffling of S2 cells. Abl does promote membrane ruffling in fibroblasts and neurons (Jin and Wang, 2007; Michael et al., 2010). Moreover, ruffling is accomplished by expression of each mutant as the frequency of ruffling drops when the cells are depleted of both endogenous and recombinant Abl, but remains if the expression of the transgenes continues. These data indicate that all the three Abl mutants are active and regulate certain aspects of actin dynamics, probably via some of its other domains, including the SH3 or SH2 in its N-terminal or the EVH domain in the C-terminus (Brasher and Van Etten, 2000; Nagar et al., 2003). Future work will have to address the role of these domains in Fra dependent formation of filopodia.
This study confirms previous work on the role of the FABD of Arg in localizing it to the cell periphery (Miller et al., 2004) as removal of the FABD from Drosophila Abl prevents this mutant from localizing to the cell membrane. Interestingly, however, in Fra cells, FABD deletion mutants now localize to the periphery and this coincides with their ability to bind to Fra, and support filopodia formation. Most intriguingly, the dominant negative effect of a kinase inactive mutant is suppressed if the FABD is also removed. This suggests that the negative effect of an Abl\textsuperscript{KN} mutant is associated with actin localization. Physical association of Abl with actin can regulate Abl function and Abl is also a key regulator of several proteins associated with actin polymerization (Miller et al., 2004; Mitra and Radha, 2010; Wang et al., 2001; Woodring et al., 2001; Woodring et al., 2002). It is possible that since Abl\textsuperscript{KN} can not phosphorylate target proteins, it traps a scaffold of actin binding proteins (e.g. Abi, Kette and WASP), which prevent filopodia formation. The ability of an Abl\textsuperscript{KNAF} mutant to still simulate filopodia formation with Fra suggest that Fra may still utilize Abl\textsuperscript{KNAF} as a scaffolding protein. However some of the proteins from the complex are now diassociated or their certain functional domains are exposed such that it can be phosphorylated by other kinases to recruit them to the membrane and form filopodia. Identifying these other proteins will be an important next step.

So, how might Abl function downstream of Fra? We hypothesize that activation of Fra initiates a signaling pathway that recruits Abl to its cytoplasmic tail. Once recruited, Abl may not only phosphorylate Fra (Dorsten et al., 2010; Forsthoefel et al., 2005), but also act as a scaffold protein to help form a Fra signaling complex that involves localization of actin binding proteins, and allow them to form filopodia in S2 cells.
Presumably similar mechanisms will regulate Fra dependent neurite outgrowth and axon tract formation. This model is supported by the genetic interaction of fra with actin modulators such as Ena, Trio and Rho (Dorsten et al., 2007; Dorsten et al., 2010; Forsthoefel et al., 2005), and the role of Abl as a key regulator of actin dynamics (Wang et al., 2001; Woodring et al., 2001; Woodring et al., 2002). Abl also regulates lamella formation by phosphorylation of Abi, which also controls Abi’s localization to the membrane in S2 cells (Huang et al., 2007). Abi localization and phosphorylation by Abl is antagonized by the tyrosine phosphatase PTP61F that interestingly, is also known to compete with Abl for the regulation of Kette, another actin binding protein, during Drosophila eye development (Ku et al., 2009). In this case, Kette phosphorylation via Abl is important for Kette localization to the membrane. Abi and Kette are an integral part of the pentameric WAVE/SCAR complex, regulating WAVE activity, stability and localization (Bogdan and Klambt, 2003; Bogdan et al., 2005; Kunda et al., 2003). Thus, Abl may be indirectly responsible for regulating actin dynamics by controlling the phosphorylation and localization of these proteins.

To summarize, an S2 cell assay of Fra signaling to the actin cytoskeleton has been developed. Using all three of the conserved P-motifs, Fra activity induces several alterations in actin-based cell morphology and preferentially promotes filopodia. Filopodia formation requires endogenous Abl activity, which appears to be recruited to the Fra complex. Abl activity is required and while the F-actin binding domain aids in filopodia formation, it is not essential. In addition to kinase activity, Abl also appears to have an important scaffold formation, presumably participating in complex formation at the conserved P-motifs. This new cell assay system will be instrumental in helping
understand how Fra signals information to the cytoskeleton downstream of its activation, and thus help us understand how Fra signaling contributes to axon pathway formation.
In this thesis, we have uncovered an important interaction between Fra and Abl both in vivo during axon pathway formation and in situ in S2 cell culture for actin dynamics. In Chapter 2, it has been demonstrated that Fra signaling is critical for commissure formation and this might be achieved by regulating actin dynamics. This theory is validated by the genetic interaction between fra and Abl kinase (Abl) and the GTPases, Rac and Cdc42 but not MLCK. Thus, the second part of the thesis (Chapter 3) focused on optimizing a cell culture system to study the how Fra utilizes Abl to alter actin dependent S2 cell morphologies. In this thesis, I demonstrated for the first time that Fra utilizes all three P-motifs to regulate actin dynamics and pre-dominantly promotes filopodia formation in S2 cells. Moreover, the kinase activity of Abl is critical to Fra dependent filopodia as most of the cells remain round when Fra is co-overexpressed with a kinase inactive mutant (Abl\textsuperscript{KN}). However, when Abl\textsuperscript{KNAF} is co-expressed with Fra, inhibitory effect observed of Abl\textsuperscript{KN} is relieved as many more cells can extend filopodia.

The data in this thesis suggest a model whereby Netrin activation of Fra initiates a signaling casacade that either recruits Abl to the cytoplasmic tail of Fra or activates Abl. In turn, Abl may act as a scaffold to recruit more intracellular effectors or it may simply phosphorylate its substrates including for example Ena, Abi and Kette [Fig. 4.1 A]. It is known that phosphorylation of these proteins by Abl translocates them to the membrane where it can regulate actin nucleation machinery involved in the formation of filopodia (Comer et al., 1998; Juang and Hoffmann, 1999; Huang et al., 2007; Ku et al.,...
Fig. 4.1: Proposed Model for Fra and Abl signaling. A. Netrin activation of Fra recruits Abl to the cytoplasmic tail of Fra (1-2). Abl in turn acts a scaffold to recruit other proteins (3) and also phosphorylates its substrates (4), thereby positively regulating actin dynamics used to signal filopodia formation (5). B. In the absence of the kinase activity, downstream effectors of Abl remains inactivated (6), which in turn leads to the inhibition of filopodia (7). C. However, deleting actin binding domain relieves this inhibitory effect. Furthermore, it may also allow other kinases to phosphorylate substrates of Abl (8), thereby at least partially activating the signaling to filopodia (9). Right bracket depicts physical binding of Fra and Abl and the non-labelled shaded boxes represent unknown proteins that may physically interact with Fra and Abl complex. Ena, Abi and Kette are known substrates of Abl kinase. In the absence of the kinase activity, these substrates of Abl remain unphosphorylated and therefore cannot signal actin polymerization. This would explain the inhibition of filopodia formation observed in our cultured S2-Fra cells [Fig. 4.1 B]. However, deleting the actin binding domain of Abl relieves the inhibitory effect, possibly by altering the conformation of Fra and Abl complex. The conformational
change may also allow other kinases to phosphorylate Ena, Abl and Kette, thereby inducing filopodia formation [Fig. 4.1 C]. Although several of the Abl substrates have been suggested above, none of these have been directly linked to the Fra and Abl pathway. Thus, future experiments need to focus on understanding the molecular, cellular and genetic interactions of Fra and Abl signaling in both cultured cells and the developing nerve cord of Drosophila. The following sections have outlined some of the remaining biological questions and suggest some experiments to address them.

1) **What domain of Abl is required for its interaction with Fra?**

In chapter 2, we demonstrated that Abl can bind to Fra independent of any particular P-motif. Mutations in the C-terminus of Abl (kinase inactivation or deletion of the actin binding domain) do not disrupt this physical binding of Fra and Abl. It is therefore possible that a functional domain in the N-terminus of Abl is required to bind to Fra. To identify this domain, GST-fusion proteins or flag epitope tagged version of Abl deletion mutants such as ΔN-Cap, ΔSH2, ΔSH3 and ΔEVH1 will be cloned in pMT vector. Fra and Abl will be co-immunoprecipitated and its interaction or lack thereof will be detected by SDS-PAGE, followed by immunoblotting against the epitope tags (Fra-Myc; Abl-Flag). It must be noted here that BcrAbl, a fusion protein of the human Bcr and Drosophila Abl, can bind to Fra. Given that this fusion protein lacks the N-cap region of Abl, it seems likely that a mutant Abl lacking its N-Cap region would be able to physically interact with Fra. However, it is possible that the stoichiometry of this binding may be compromised. On the other hand, based on studies on vertebrate Abl/Arg protein interactions and the presence of several proline residues in the cytoplasmic tail of Fra, I hypothesize that Drosophila Abl will use its SH3 or SH2 domain to bind Fra.
2) **Which domain of Fra is required for Abl binding?**

Although the cytoplasmic tail is required for Fra and Abl binding, deletion of individual P-motifs does not abolish this interaction. I hypothesize that the lack of P-motif dependence may be due to Abl binding to more than one P-motif. To test this idea, epitope tagged Fra mutants with different combinations of the P-motifs deleted, such as Fra\(^{ΔP2ΔP3}\), Fra\(^{ΔP1ΔP3}\) or Fra\(^{ΔP1ΔP2}\) will be created. The lack of binding of these Fra mutants to wild type Abl in co-immunoprecipitation assays will show P-motifs are important for Fra and Abl interaction. Conversely, use of Abl mutants (Abl\(^{ΔN-Cap}\), Abl\(^{ΔSH3}\), Abl\(^{ΔSH2}\) or Abl\(^{EVH1}\)) to pull down the various P-motif mutants will identify the domain(s) on both Fra and Abl that are important to the physical binding between these two proteins.

3) **Does the presence of Abl affect the formation of Fra immunocomplex?**

Abl/Arg (Abl related protein) can regulate actin dynamics by one of two ways: 1) by directly binding and phosphorylating actin nucleation proteins such as WASP and 2) by activating and translocating actin modifiers to the leading edge of the cell (Bogdan and Klambt, 2003; Bogdan et al., 2005; Kunda et al., 2003; Miller et al., 2010). For example, Abl binds and phosphorylates Ena (Comer et al., 1998), Abi (Juang and Hoffmann, 1999) and Kette (Ku et al., 2009), and also advances their recruitment to the membrane in cultured cells (Huang et al., 2007; Michael et al., 2010). Thus, it is not unlikely that Abl acts as a scaffold and recruits actin modifiers like Ena to the cytoplasmic domain of Fra. Ena is an interesting candidate for testing the scaffold mechanism as it suppresses the guidance errors caused by *abl* and *fra* but it binds solely to Abl and not to Fra *in vitro* (Comer et al., 1998; Forsthoefel et al., 2005; Gertler
et al., 1995). We speculate that Fra immunoprecipitation in the presence of overexpressed Abl would pull down Ena as a part of the immuno-complex. In addition to recruiting effector proteins to Fra, Abl may also work downstream of Fra to activate and localize Abi and Kette to the membrane. Indeed, if Abl does promote membrane localization of these proteins then immunostaining of Abi and Kette should reveal that the expected peripheral localization in S2-Fra cells will be lost in S2-Fra cells depleted for endogenous Abl.

4) **Can the effects seen in vivo be studied using primary neurons in culture?**

Expression of both Fra and Abl mutants in *Drosophila* nerve cord is predicted to disrupt the axon scaffold structure (section 1.5). However, the gross morphological defects may occur due to axon stalling, excessive growth or axon branch orientation errors. These questions can be answered by studying Fra and Abl mutants in tractable primary neurons from *Drosophila* embryos. Briefly, mutants described in Chapter 3, section 1. and 2 of this chapter will be cloned into pUAST vector which will be injected into *Drosophila* embryos. After the germline transformants have been established, cells will be dissociated from transgenic embryos and cultured on glass coverslips for further microscopic and phenotypic analysis (Sicaeros and O'Dowd, 2007).

5) **Does the in vitro interactions of Fra and Abl (1-4) also affect axon pathway formation?**

A detailed structure function analysis of Fra and Abl has never been done in *Drosophila* nerve cord. The transgenic lines from the above section (4) will be used to conduct parallel experiments to study their role in the formation of the axon pathways. We
expect each of the Abl mutants to genetically interact with Fra as each domain (N-Cap, SH3, SH2, EVH1 and FABD) has a unique function. Mutations in the N-Cap, SH3 or SH2 domains of Abl interfere with its auto-regulation. Thus, it is possible that these mutants will suppress the commissureless phenotype observed in the overexpression of Abl in a fra mutant. Deletion in the F-actin may deregulate Abl kinase activity (Woodring et al., 2001; Woodring et al., 2002) which may in turn increase the guidance defects but we cannot be entirely sure of this prediction.
### Table 2: Fra promotes filopodia formation in S2 cell via its P-motifs

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### Table 3: Effect of Abl knock down on Fra dependent filopodia

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<td>1.7</td>
<td>7.7</td>
<td>2.6</td>
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<td>Fra WT Abl WT</td>
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<td>43</td>
<td>9</td>
<td>16.7</td>
<td>9.6</td>
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<tr>
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<td>8.8</td>
<td>0.8</td>
<td>5.7</td>
<td>4.7</td>
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Table 4: Overexpression of Abl in S2 and S2-Fra cells

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<tr>
<th></th>
<th>N</th>
<th>Lamella</th>
<th>Filopodia</th>
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<th>M. Ruffles</th>
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<th>Microspikes</th>
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<tr>
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<td>47</td>
<td>10</td>
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<td>9</td>
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<tr>
<td>Std Dev</td>
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<td>1.5</td>
<td>3.7</td>
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<tr>
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<td>1264</td>
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<td>7</td>
<td>19</td>
<td>13</td>
<td>13</td>
<td>15</td>
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<tr>
<td>Std Dev</td>
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<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
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<tr>
<td>Fra wt myc w/o RNAi</td>
<td>1309</td>
<td>7</td>
<td>41</td>
<td>18</td>
<td>10</td>
<td>10</td>
<td>14</td>
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<tr>
<td>Std Dev</td>
<td></td>
<td>2.5</td>
<td>0.5</td>
<td>3</td>
<td>2.5</td>
<td>1.9</td>
<td></td>
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<tr>
<td>Fra wt myc w/ RNAi</td>
<td>1283</td>
<td>6</td>
<td>21</td>
<td>22</td>
<td>11</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Std Dev</td>
<td></td>
<td>3.6</td>
<td>5.7</td>
<td>2.9</td>
<td>0.7</td>
<td>0.6</td>
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Table 5: Fra and Abl primers used for molecular studies

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<tr>
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<th>GC%</th>
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<tr>
<td>1</td>
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<td>58.4</td>
<td>48</td>
<td>5’ GCCTACTAGTATGGCCATCACAACA 3’</td>
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<td>2</td>
<td>5’ SpeI Fra Cyto</td>
<td>61.7</td>
<td>46.8</td>
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<td>48.1</td>
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<tr>
<td>4</td>
<td>3’ PvuI modified FraΔCyto</td>
<td>63.5</td>
<td>50</td>
<td>5’ ATATAAACGATCGGCTTGGGCACGCAAC 3’</td>
</tr>
<tr>
<td>5</td>
<td>5’ SpeI Abl wt</td>
<td>65.8</td>
<td>62.9</td>
<td>5’ GACACTAGTATGGGGGCTCAGCAGGAGGC 3’</td>
</tr>
<tr>
<td>6</td>
<td>5’ SpeI Abl wt. new</td>
<td>62.7</td>
<td>62.5</td>
<td>5’ CCGACTAGTATGGGGGCTCAGCAGG 3’</td>
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<tr>
<td>7</td>
<td>5’ fwd_RT_Net A</td>
<td>49.5</td>
<td>47.3</td>
<td>5’ GTGTATTACTCTGTATCCGCGG 3’</td>
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<tr>
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<td>3’ rev RT Net A</td>
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<td>46.6</td>
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<td>54.1</td>
<td>60</td>
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<tr>
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* labelled differently on original sheet
Appendix A

Time dependent induction curve for Fra expression: S2 cells stably transfected with myc epitope tagged pMT-Fra using hygromycin as the selection agent. Here, 2x10^6 cells were plated in 60mm plates and induced for expression by adding 700uM CuSO4. As noted above, at zero time point, S2-Fra cells show leaky expression. The increase in Fra expression is directly proportional to the time the cells were induced. Immunoblotting using mouse anti-myc was used to detect the presence of Fra protein in the cells.
Genistein treatment of S2 and S2-Fra cells increase the frequency of microspikes: To determine the effect of kinase activity on filopodia formation, we chose to use a generic kinase inhibitor – genistein to inhibit overall kinase activity. S2 and S2-Fra cells were plated and induced for expression as described in the methods. Then, different doses of genistein (as indicated in the graph) were added to the media and the cells were incubated for 30 minutes. The cells were then plated on Con A and fixed as per the standardized protocol (see methods). Both S2 and S2-Fra cells were stained with phalloidin Texas red to detect F-actin while anti-Myc FITC allowed us to identify Fra positive cells. Overall, we observed that the frequency of microspikes begin to drastically increase as the concentration of genistein increases in both S2 (A) and S2-Fra cells (B).
Appendix C

**Effect of pervanadate treatment on S2 and S2-Fra cells in the absence and presence of Abl RNAi:**
Here, we sought to indirectly increase overall kinase activity by treating the cells with a phosphatase inhibitor – pervanadate. S2 and S2-Fra cells were plated, after which one set of cells were treated with cds RNAi to knock down the levels of endogenous Abl (see methods). On the day of staining, the cells were treated with pervanadate, along with Netrin addition for 30 minutes. A. In S2 cells, upon pervanadate treatment, more cells exhibit filopodia formation. However, the cells that were also targeted with cds RNAi cannot form filopodia; instead these cells show increased number of round or microspike cells. B. Similar, to S2 cells, we note a drastic increase in the frequency of cell forming filopodia in S2-Fra cells. However, unlike S2-cells, inhibition of Abl in the presence of pervanadate did not abolish the filopodia formation as still a high number of cells can form filopodia.
**Appendix D**

**A**

![Diagram of Coding Sequence of Abl]

**B**

![Image of blot with anti-DAbi and anti-tubulin antibodies]

**Rnai targets designed against Drosophila Abl sequence:** A. In addition to the generic Abl RNAi target (purple bar; O'Farrell Library; provided by Dr. Finley), four other regions of Abl were targeted to silence its message RNAi. dsRNA was synthesized against two regions in the coding sequence: 1) N-Cap domain (black bar on blue box) and 2) F-Actin binding domain (black bar on red box). Furthermore, Abl knockdown was also achieved by targeting the 5’UTR (orange bar) and 3’ UTR (green bar). B. Cells treated with the dsRNA for seven days. Then, these cells were lysed and immunoblotted with anti-Abl (Drosophila) to check for the efficiency of Abl knockdown. As demonstrated in the above blot, all five RNAi probes drastically reduce Abl expression (>90%) as compared to S2 cells untreated with Abl RNAi. The same blot was probed with anti-tubulin to control for equal loading of the total cell lysates.
Appendix E

A cartoon representation of the modified pMT-Hygro vector: The original pMT-His-V5 vector from invitrogen was modified to incorporate the hygromycin resistance gene under the pcopia promoter within the same vector. Further, 3X myc sequence was added along with a tobacco etch virus (TEV) site. Wild type Fra and its mutants were cloned into PmeI and PvuI site. There is a PacI site; however using it introduces a stop codon. This would not allow the myc to be translated in frame with the gene inserted at that site. This strategy allowed us to stably transfect Fra lines without co-transfecting with another plasmid carrying the resistance gene.
A cartoon representation of the modified pMT-blasticidin vector: The original pMT vector is modified to include a blasticidin resistance gene under the effect of the pcopia promoter (A). This vector was then modified to introduce 2X flag tags after the multi-cloning site (B). All Abl constructs were cloned into this vector within Spel and NotI sites.
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ABSTRACT

FRAZZLED AND ABELSON INTERACT TO REGULATE THE ACTIN CYTOSKELETON IN DROSOPHILA

by

BRIDGET ELSA VARUGHESE

May 2011

Advisor: Dr. Mark VanBerkum
Major: Biological Sciences
Degree: Doctor of Philosophy

Guidance receptors such as Frazzled affect cell shape and motility by directly or indirectly modulating the cytoskeleton. Fra is particularly needed for the formation of the posterior commissures in a developing Drosophila embryo. The cytoplasmic tyrosine kinase, Abelson Kinase (Abl) enhances the loss of commissures observed in fra mutant. Abl physically interacts with Frazzled to help guide commissural axons across the midline. Furthermore, the loss of commissural axons is only seen when the actin dynamics are perturbed. Abl is also known to regulate actin-dependent processes underlying formation of filopodia, microspikes and membrane ruffles. So, we established a Drosophila S2 cell culture paradigm to study how Frazzled and Abelson may co-regulate actin-dependent morphology. Ectopic expression of Frazzled in S2 cells produces long filopodia-like extensions in over 70% of the cells when plated on concanavalin A coated cover slips. These phalloidin stained filopodia are reduced when
either the P1 or P3 cytoplasmic motif is removed. Frazzled is localized to the tips of these filopodia, and is associated with an increase in phosphotyrosine levels as well as alterations in localization of proteins modulating actin dynamics and also known to interact with Abelson. Loss of Abl by RNA interference causes S2 cells to form peripheral microspikes as well as a decrease in phosphotyrosine staining at the edge of the cell. When Abl is knocked down in Fra expressing cells, they are unable to extend filopodia but microspikes persist. Moreover, overexpression studies reveal that while Abl<sup>ΔF</sup> may be still functional but the kinase activity of Abl is particularly critical for Fra dependent filopodia. Together, these data indicate that Fra may be regulating Abl to control the actin dynamics underlying the formation of the commissures.
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Education

Ph.D - Biological Sciences, Wayne State University, Detroit -MI
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- Loyola University Chicago Jesuit Community Research Fellowship for the year
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Publications

pLOs One: In the absence of Frazzled over-expression of Abelson tyrosine kinase
disrupts commissure formation and causes axons to leave the embryonic CNS.
(doi:10.1371/journal.pone.0009822)