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The Intracellular Domain of the Frazzled/Dcc Receptor is a Transcription Factor Required for Commissural Axon Guidance

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Abstract
During embryonic development, conserved families of attractive and repulsive cues steer axons by signaling through receptors that are expressed on axonal growth cones. In the canonical model of axon guidance receptor signaling, ligand binding induces the formation of protein complexes on receptor cytoplasmic domains, which locally remodel the growth cone plasma membrane and underlying cytoskeleton. Many axons navigate to their final synaptic targets by passing through a series of intermediate targets, at which they switch their responsiveness to one or more guidance cues. My thesis research identified a new mechanism of axon guidance receptor signaling: the intracellular domain (ICD) of Frazzled (Fra), the Drosophila ortholog of the conserved guidance receptor Deleted in Colorectal Cancer (DCC), functions as a transcription factor to control axon responsiveness to the embryonic ventral midline. In Chapter 1, I introduce the embryonic ventral midline as a model system for studying how axons modulate their responsiveness to guidance cues at intermediate targets and I discuss mechanisms through which DCC transduces signals from its Netrin ligands into local membrane and cytoskeletal rearrangements. In Chapter 2, I present data supporting the idea that the Fra ICD functions as a transcription factor to regulate axonal responsiveness to Slit-Robo repulsion at the midline of the Drosophila embryo. In Chapter 3, I discuss questions that remain regarding the mechanism through which Fra functions as a transcription factor and I examine the implications of this work, focusing on the possibilities that Fra regulates other transcriptional targets and that other axon guidance receptors function as transcription factors.

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THE INTRACELLULAR DOMAIN OF THE FRAZZLED/DCC RECEPTOR IS A TRANSCRIPTION FACTOR REQUIRED FOR COMMISSURAL AXON GUIDANCE

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ABSTRACT

THE INTRACELLULAR DOMAIN OF THE FRAZZLED/DCC RECEPTOR IS A TRANSCRIPTION FACTOR REQUIRED FOR COMMISSURAL AXON GUIDANCE

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During embryonic development, conserved families of attractive and repulsive cues steer axons by signaling through receptors that are expressed on axonal growth cones. In the canonical model of axon guidance receptor signaling, ligand binding induces the formation of protein complexes on receptor cytoplasmic domains, which locally remodel the growth cone plasma membrane and underlying cytoskeleton. Many axons navigate to their final synaptic targets by passing through a series of intermediate targets, at which they switch their responsiveness to one or more guidance cues. My thesis research identified a new mechanism of axon guidance receptor signaling: the intracellular domain (ICD) of Frazzled (Fra), the *Drosophila* ortholog of the conserved guidance receptor Deleted in Colorectal Cancer (DCC), functions as a transcription factor to control axon responsiveness to the embryonic ventral midline. In Chapter 1, I introduce the embryonic ventral midline as a model system for studying how axons modulate their responsiveness to guidance cues at intermediate targets and I discuss mechanisms through which DCC transduces signals from its Netrin ligands into local membrane and cytoskeletal rearrangements. In Chapter 2, I present data supporting the idea that the Fra ICD functions as a transcription factor to regulate axonal responsiveness to Slit-Robo

repulsion at the midline of the *Drosophila* embryo. In Chapter 3, I discuss questions that remain regarding the mechanism through which Fra functions as a transcription factor and I examine the implications of this work, focusing on the possibilities that Fra regulates other transcriptional targets and that other axon guidance receptors function as transcription factors.

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Chapter 1

Introduction

During embryonic development, conserved families of attractive and repulsive cues steer axons by signaling through receptors that are expressed on axonal growth cones. In the canonical model of axon guidance receptor signaling, ligand binding induces the formation of protein complexes on receptor cytoplasmic domains, which locally remodel the growth cone plasma membrane and underlying cytoskeleton. Many axons navigate to their final synaptic targets by passing through a series of intermediate targets, at which they switch their responsiveness to one or more guidance cues. My thesis research identified a new mechanism of axon guidance receptor signaling: the intracellular domain (ICD) of Frazzled (Fra), the *Drosophila* ortholog of the conserved guidance receptor Deleted in Colorectal Cancer (DCC), functions as a transcription factor to control axon responsiveness to the embryonic ventral midline. In the first part of this chapter, I introduce the embryonic ventral midline as a model system for studying how axons modulate their responsiveness to guidance cues at intermediate targets. In the second part of this chapter, I review canonical DCC signaling: the mechanisms through which DCC transduces signals from its Netrin ligands into local membrane and cytoskeletal rearrangements.

Regulation of axon responsiveness at the ventral midline

Axons navigate a series of intermediate targets, or choice points, en route to their final synaptic targets. At each intermediate target, axons must switch their responsiveness to guidance cues, so that they are initially drawn to the intermediate target and subsequently repelled from it. The embryonic midline is an intermediate target for commissural axons in all bilaterally symmetric animals and precise navigation at the midline is essential for coordination of the left and right sides of the body. Commissural axons must first be directed across the midline and then be prevented from re-crossing in order to ensure proper midline connectivity. Here, I discuss studies of commissural axon guidance at the ventral midline of vertebrates and insects, paying particular attention to the insights they have provided into mechanisms growing axons use to modulate their responsiveness to cues as they navigate toward their final targets. Specifically, I discuss two populations of neurons: commissural interneurons in the spinal cord and in the *Drosophila* ventral nerve cord.

The cell bodies of spinal commissural neurons differentiate in the dorsal spinal cord and project their axons ventromedially toward the floor plate (Dodd et al., 1988; Figure 1.1A). These axons subsequently exit the floor plate on the contralateral side and turn anteriorly toward the brain (Bovolenta and Dodd, 1990; Figure 1.1C). Pre- and post-crossing axonal segments can be differentially labeled using antibodies that recognize cell adhesion molecules that are expressed in spatially restricted patterns (Figure 1.1A and B). In addition, the spinal cord can be opened at the roof plate to create an "open-book" preparation and commissural neurons and their axons can be labeled and

visualized with lipophilic dyes; this preparation is particularly useful for analysis of post-crossing axonal trajectories (Figure 1.1C).

The ventral nerve cord of the *Drosophila* embryo has a segmentally repeated structure. Each abdominal hemisegment contains approximately 270 interneurons, most of which extend axons across the midline in either the anterior or posterior commissure (Rickert et al., 2011). All axons in the central nervous system can be labeled by antibody staining (Figure 1.1D) and the fact that most abdominal interneurons project axons contralaterally (Rickert et al., 2011) facilitated forward genetic screens which identified genes that play key roles in commissural axon guidance (Seeger et al., 1993; Hummel et al., 1999). In addition, subpopulations of neurons can be labeled with genetically encoded *Gal4* elements, which can be used to drive the expression of axonal markers and other transgenes. This approach allows for quantitative comparison of axonal trajectories in wild-type and mutant backgrounds and also provides a powerful system in which to evaluate cell-specific and protein domain requirements in transgenic rescue experiments (Figure 1.1D). Comparison of these two systems has revealed remarkable similarity in many of the core molecules and mechanisms that direct axon guidance at the midline. A recurring theme of these studies is the diversity of mechanisms that have evolved to spatially and temporally restrict the activity of cell surface guidance receptors in order to ensure appropriate transitions in axon responsiveness.

Growth toward the midline

As commissural axons approach the midline, they are preferentially responsive to midline attractants while suppressing their responsiveness to midline repellents. The first

guidance cues to be implicated in commissural axon attraction were proteins of the Netrin family, which were initially identified for their roles in axon guidance and mesodermal cell migration in the nematode *C. elegans* (Hedgecock et al., 1990; Ishii et al., 1992). Netrins are secreted from the floor plate and ventral spinal cord, forming a ventral high to dorsal low gradient during the time when spinal commissural axons are growing toward the ventral midline (Kennedy et al., 1994; Serafini et al., 1996; Kennedy et al., 2006). *In vitro* assays demonstrated that Netrin-1 elicits outgrowth of axons from spinal cord explants (Serafini et al., 1994) and induces attractive turning responses (Kennedy et al., 1994). Netrins are thought to signal both outgrowth and attraction through the receptor DCC, which is expressed on commissural axons as they approach the midline (Keino-Masu et al., 1996). In the spinal cords of mouse embryos mutant for either *Netrin-1* (the only Netrin expressed in the mouse spinal cord (Serafini et al., 1996; Kennedy et al., 2006)) or *Dcc*, the ventral commissure is thin, but not absent; many axons stall before reaching the floor plate and commissural axons that normally project ventromedially in a tight bundle misproject laterally and are defasciculated (Serafini et al., 1996; Fazeli et al., 1997). The observation that the ventral commissure is thinner in *Netrin-1* mutants than in *Dcc* mutants (Xu et al., 2014) and *in vitro* data indicating that *Dcc* mutant spinal cord explants retain some Netrin-responsiveness (Ly et al., 2008; Xu et al., 2014) suggest that Netrin may promote midline crossing through both DCC-dependent and DCC-independent mechanisms.

One additional receptor through which Netrin might signal midline attraction is the DCC paralog, Neogenin (Neo), which is expressed on commissural axons (Xu et al., 2014). *Neo* mutants have no defects in commissural axon guidance, but in *Dcc, Neo*

double mutants, the ventral commissure is thinner than in *Dcc* single mutants and comparably thin to the ventral commissure in *Netrin-1* mutants (Xu et al., 2014). Chickens have a single member of the DCC/Neo family, which has greater homology to mouse Neo than to mouse DCC. RNAi knockdown of this gene produces defects in commissural axon guidance in the chicken spinal cord reminiscent of the mouse *Dcc* mutant phenotype (Phan et al., 2011). However, Neo-dependent outgrowth and/or turning responses of spinal commissural neurons in response to Netrin have yet to be demonstrated. Netrin can bind to Neo (Wang et al., 1999), but it does so with much lower affinity than Neo's canonical repulsive ligand Repulsive Guidance Molecule (RGMa; Rajagopalan et al., 2004). *RGMa* mRNA is broadly expressed in the spinal cord (Niederkofler et al., 2004), but its potential role in the guidance of spinal commissural axons has not been evaluated. Thus, it is not yet clear how Neo contributes to the establishment of the ventral commissure.

Down Syndrome Cell Adhesion Molecule (DSCAM) has also been proposed to function as an attractive Netrin receptor in spinal commissural neurons. Both insect and vertebrate Netrin proteins can bind to DSCAM in a variety of *in vitro* assays (Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009). Disruption of DSCAM function by RNAi or expression of dominant negative forms of DSCAM causes many commissural axons to fail to reach the floor plate in rat and chicken spinal cords (Ly et al., 2008; Liu et al., 2009). However, analysis of mice with *Dscam* null mutations suggests that DSCAM is not required for Netrin-dependent midline attraction (Palmesino et al., 2012). *Dscam* mutants have no defects in commissural guidance in the spinal cord and *Dscam, Dcc* double mutants have commissural guidance defects comparable to *Dcc* mutants. It is

conceivable that in *Dscam* null mutants – but not in animals subject to acute *Dscam* knockdown – a compensatory mechanism emerges to allow for normal midline crossing; notably, DCC and Neo mRNA and protein levels are unchanged in *Dscam* mutants (Palmesino et al., 2012), indicating that if there is compensation, it can not be explained by up-regulation of the expression of other known Netrin receptors. A less likely possibility is that DSCAM plays essential roles in Netrin-dependent midline attraction in rat and chicken that are not conserved in the mouse. Alternatively, the RNAi phenotypes may represent an artifact of some sort, underscoring the ideas that knockdown data should be interpreted with caution and that genetic nulls should be analyzed whenever possible. The advent of new methods for genome modification that bypass the need for ES cell targeting should facilitate the analysis of null alleles in vertebrates other than mice.

Netrins and DCC play conserved roles to promote midline axon crossing. Flies have two *Netrin* genes, *NetA* and *NetB*, which are expressed transiently in midline neurons and persistently in midline glia during embryogenesis (Harris et al., 1996; Mitchell et al., 1996) and only one ortholog of *Dcc*/*Neo*, *Fra*, which encodes a protein that is expressed on commissural axons in the ventral nerve cord (Kolodziej et al., 1996). In embryos lacking both fly *Netrin* genes (*NetAB)* and in *fra* mutants, commissures are thin, but not absent, with posterior commissures more sensitive to loss of Netrin or Fra than anterior commissures (Kolodziej et al., 1996; Brankatschk and Dickson, 2006). *fra* mutants display more severe commissural guidance defects than *NetAB* mutants, implying that Fra promotes midline crossing in part through a Netrin-independent mechanism (Garbe et al., 2007; Yang et al., 2009; see below for further discussion). In fly

embryos engineered so that the only Netrin protein is membrane-tethered, both the anterior and posterior commissures develop normally (Brankatschk and Dickson, 2006), suggesting that long-range diffusion of Netrins is not required for commissural axon attraction. In *C. elegans*, Netrin has been shown to polarize neurons in a DCC-dependent manner (Adler et al., 2006; Xu et al., 2009), but this output of Netrin-DCC signaling has yet to be implicated in midline crossing. In both insects and vertebrates, the extents to which defects in commissural axon guidance in *Netrin* and *Dcc* mutants reflect defects in outgrowth, attraction, polarization, and/or regulation of gene expression remain unknown. Identification and disruption of distinct cytoplasmic motifs or residues that are required for these diverse signaling outputs would allow the relative contributions of these pathways to be dissected *in vivo*.

DSCAM's role in midline crossing and its potential function as a Netrin receptor have also been investigated in *Drosophila. Dscam* mutants are phenotypically normal with respect to midline axon crossing, but *Dscam*, *fra* double mutants have more severe midline crossing defects than either *NetAB* or *fra* mutants alone. Overexpression of DSCAM in ipsilateral neurons induces ectopic midline crossing, even in *NetAB* mutant embryos (Andrews et al., 2008). These genetic data imply that DSCAM promotes midline axon crossing through a Netrin-independent mechanism, but they do not exclude the possibility that DSCAM also functions as an attractive Netrin receptor. Many axons cross the midline even in *Dscam*, *fra* double mutants, suggesting that midline attractive or lateral repulsive signaling pathways that guide commissural axons toward the midline remain to be identified. The fact that additional cues and receptors have not been isolated in mutagenesis screens that have approached genomic saturation (Seeger et al., 1993;

Hummel et al., 1999) suggests that these genes may have earlier roles in embryogenesis that preclude analysis of midline axon crossing phenotypes and/or that their functions may be redundantly encoded.

In vertebrates, parallel pathways that guide commissural axons toward the ventral midline have been studied in greater detail. The residual ability of *Netrin-1*-mutant floor plate tissue to elicit turning of commissural axons (Serafini et al., 1996; Charron et al., 2003) is partially blocked by cyclopamine, a pharmacological inhibitor of the Sonic Hedgehog (Shh) effector Smoothened (Smo), and turning assays performed on dissociated commissural neurons have provided direct evidence that Shh can act as a chemoattractive cue (Charron et al., 2003; Yam et al., 2009). Conditional deletion of *Smo* in commissural neurons in the dorsal spinal cord causes commissural axons to misproject laterally and defasciculate as they are growing toward the floor plate (Charron et al., 2003). Genetic ablation of the Shh receptor *Boc*, which is expressed in commissural neurons, produces a similar phenotype, and RNAi knockdown of *Boc* inhibits the turning of commissural neurons toward a source of Shh (Okada et al., 2006). Shh appears to signal chemoattraction without regulating gene expression, as neither pharmacological inhibition of transcription nor expression of a dominant repressor of Gli transcription factors blocks Shh-induced turning responses *in vitro* (Yam et al., 2009), but this question has not been investigated *in vivo*. Analysis of *Gli2* conditional knockouts in spinal commissural neurons would test whether canonical Shh signaling impinges on midline crossing.

Netrin-1 mutant floor plate retains some ability to elicit attractive turning even in the presence of cyclopamine, suggesting that the floor plate might produce additional

attractive guidance cues (Charron et al., 2003). Recently, Vascular Endothelial Growth Factor A (VEGF), which is expressed in the floor plate during commissural axon guidance, was identified as a chemoattractant for commissural neurons (Ruiz de Almodovar et al., 2012). In embryos that are floor plate haplodeficient for *Vegf* or in which the VEGF receptor *Flk-1* has been conditionally deleted from spinal commissural neurons, commissural axons misproject laterally and are defasciculated as they grow toward the floor plate. Dissociated commissural neurons turn toward a source of VEGF *in vitro*, and this turning response is antagonized by the presence of function-blocking antibodies against Flk-1.

Finally, in the spinal cord, roof plate-derived repellents collaborate with floor plate-derived attractants to guide commissural axons toward the ventral midline. In the absence of a floor plate, commissural axons navigate normally through the dorsal part of the spinal cord before stalling (Bovolenta and Dodd, 1991; Hatta et al., 1991; Placzek et al., 1991; Yamada et al., 1991), suggesting that cues from another source must guide these axons during the early part of their trajectories. Roof plate tissue repels axons from spinal cord explants and this activity can be mimicked by cell aggregates expressing members of the Bone Morphogenetic Protein (BMP) family, BMP7 and GDF7, which are expressed in the roof plate during commissural axon outgrowth (Augsburger et al., 1999). BMPs likely signal repulsion through BMP receptor IB (BMPRIB), as roof plates from *Bmp7* of *Gdf7* mutants lack the ability to repel commissural axons (Augsburger et al., 1999; Butler and Dodd, 2003) and spinal cord explants from *BmprIb* mutants are unresponsive to roof plate-induced repulsion (Yamauchi et al., 2008). However, spinal cords from *Bmp7*, *Gdf7*, and *BmprIb* mutants display only modest defects in commissural

axon guidance, with commissural axons occasionally invading the roof plate or taking an aberrant medial trajectory (Butler and Dodd, 2003; Yamauchi et al., 2008), suggesting that other factors – potentially the complement of floor plate-derived attractants – can compensate for the loss of roof plate repulsion.

Exit from the midline

After commissural axons have reached the midline, they switch their responsiveness to midline cues, so that they can exit the midline and proceed toward their synaptic targets on the contralateral side of the embryo. During this phase of axon guidance, commissural axons are preferentially responsive to repellents expressed at the midline. The prototypical midline repulsive cues are Slit proteins, which signal repulsion through Roundabout (Robo) receptors. Slits and Robos were initially implicated in midline axon repulsion through forward genetic screens in *Drosophila* (Seeger et al., 1993; Hummel et al., 1999). Slit is expressed in midline glia throughout embryogenesis (Rothberg et al., 1990; Kidd et al., 1999); Robo is expressed on axons and shows a striking localization to longitudinal connectives, but is largely excluded from commissural segments (Kidd et al., 1998; Kidd et al., 1999). In *robo* mutant fly embryos, ipsilateral axons ectopically cross the midline and both ipsilateral and commissural axons re-cross the midline (Seeger et al., 1993; Kidd et al., 1998). *slit* mutants have an even more dramatic phenotype, in which all axons collapse on the midline (Kidd et al., 1999). Flies have three genes encoding Robo receptors and the observation that embryos mutant for both *robo* and *robo2* are phenotypically indistinguishable from *slit* mutants with respect to midline crossing (Rajagopalan et al., 2000a; Simpson et al., 2000b) suggests

that these two Robo receptors signal midline repulsion in response to Slit and that Slit-Robo signaling accounts for all midline repulsion in the fly. (Robo3 is not required for midline repulsion, but it plays an important role in mediolateral positioning of ipsilateral and post-crossing commissural axons (Rajagopalan et al., 2000b; Simpson et al., 2000a)).

Subsequent analysis of mouse mutants has confirmed that Slit-Robo signaling plays a conserved role in midline repulsion. In mice, three *Slit* genes are expressed in the floor plate (Holmes et al., 1998; Brose et al., 1999; Li et al., 1999; Yuan et al., 1999) and function redundantly to repel post-crossing commissural axons. In mice lacking all three *Slit* genes (*Slit 1/2/3*), many commissural axons stall at the floor plate and some turn back toward the ipsilateral side (Long et al., 2004). Mice express four *Robo* genes, three of which are involved in midline repulsion, while *Robo4* is specifically expressed in the vascular system (Park et al., 2003). Robo1 and Robo2 proteins are expressed at low levels on pre-crossing commissural axons and are up-regulated post-crossing (Long et al., 2004). Spinal commissural axons in *Robo 1/2* double mutants stall at the floor plate, but these defects are not as frequent as in *Slit 1/2/3* mutants, and *Robo 1/2* mutants never display re-crossing errors (Jaworski et al., 2010), implying the existence of another repulsive Slit receptor. Robo3 seems to repel post-crossing commissural axons but also plays a key role in preventing premature Slit responsiveness in pre-crossing commissural axons (Sabatier et al., 2004; Chen et al., 2008; see below for further discussion).

The absence of Slit signaling in the mouse does not lead to a complete loss of midline repulsion (Long et al., 2004), suggesting that vertebrates require other midline repellents to collaborate with Slits to prevent ectopic midline crossing and to facilitate midline exit. The class 3 secreted Semaphorin, Sema3B, is expressed in the floor plate

and ventral spinal cord during the period of commissural axon guidance (Zou et al., 2000) and its co-receptors Neuropilin-2 (Nrp2) and Plexin-A1 (PlexA1) are expressed on commissural axons, with PlexA1 expression enriched on axons during and after crossing (Nawabi et al., 2010). In mouse embryos mutant for *Sema3b*, *Nrp2*, or *PlexA1*, many commissural axons fail to exit the midline (Zou et al., 2000; Nawabi et al., 2010; Delloye-Bourgeois et al., 2015). In addition, PlexA1 can function as a repulsive Slit receptor, as Slit2 binds to PlexA1 and can induce growth cone collapse of *Robo 1/2* mutant spinal commissural neurons in a PlexA1-dependent manner (Delloye-Bourgeois et al., 2015). This observation suggests that PlexA1 may be the additional Slit receptor implied by the difference in the strengths of the *Robo 1/2* and *Slit 1/2/3* mutant phenotypes. Compound mutants in which Slit and Sema3B signaling are perturbed in combination have yet to be analyzed, so it is not clear whether additional repulsive signaling pathways facilitate floor plate exit and prevent inappropriate midline crossing.

Regulation of responsiveness to midline cues

How do commissural neurons regulate their sensitivity to midline cues so that they are preferentially responsive to midline attractants pre-crossing, but preferentially responsive to midline repellents post-crossing? The persistence of Netrin-1 and Shh expression in the floor plate past the time when commissural axons have crossed the midline (Kennedy et al., 1994; Kennedy et al., 2006; Yam et al., 2012) suggests that commissural neurons might actively silence their attraction to midline cues once they have reached the floor plate. The idea that commissural neurons may repress their attraction to Netrin in response to Slit exposure has emerged from a series of *in vitro*

experiments (Stein and Tessier-Lavigne, 2001). Dissociated *Xenopus* spinal neurons turn toward a source of Netrin in culture and even though these neurons are not repelled by Slit, exposure to Slit blunts their attraction to Netrin. In this context, Slit triggers a physical interaction between the cytoplasmic domains of DCC and Robo1 and Slit's ability to silence responsiveness to Netrin depends on DCC-Robo1 binding. The cytoplasmic motifs in these receptors that mediate the interaction are required for the silencing response and artificially restoring these receptors' abilities to interact with each other also restores Slit's ability to block attraction to Netrin. This model has yet to be tested *in vivo*.

Like Netrin, Shh continues to be expressed in the floor plate throughout spinal cord development, but commissural axons switch the polarity of their responsiveness to Shh after they reach the floor plate (Figure 1.2). After exiting the midline, most spinal commissural axons turn anteriorly. However, disruption of Shh signaling through a variety of pharmacological and genetic approaches causes post-crossing commissural axons to choose an anterior or posterior trajectory at random (Bourikas et al., 2005; Parra and Zou, 2010; Yam et al., 2012). Shh mRNA and protein are expressed in the spinal cord in a posterior high to anterior low gradient (Bourikas et al., 2005; Yam et al., 2012), suggesting that Shh might signal repulsion in post-crossing commissural neurons. When dissociated spinal commissural neurons are cultured and exposed to a gradient of Shh, the polarity of their response depends on their age (Yam et al., 2012). Neurons that have been cultured for a short time are attracted to Shh, while neurons that have been cultured for longer are repelled by Shh, consistent with the idea that as they are growing toward the midline, commissural neurons are attracted to floor plate-derived Shh, but after crossing

the midline, they are repelled by the high concentration of Shh in the posterior spinal cord. This switch is mediated by 14-3-3 adaptor proteins, which are preferentially expressed in post-crossing commissural axons and whose expression increases over time in cultured commissural neurons. *In vitro*, Shh-dependent repulsion can be blocked in aged neurons by pharmacological manipulations that antagonize 14-3-3 and can be mimicked in young neurons by premature expression of 14-3-3 or by manipulations that induce 14-3-3 activity independent of Shh. In the spinal cord, treatment with 14-3-3 inhibitors randomizes anterior-posterior turning after midline crossing, but has no effect on midline attraction, suggesting that 14-3-3 is specifically required for post-crossing commissural axon guidance in response to Shh. As *14-3-3* mRNA expression patterns have not yet been described in pre- and post-crossing commissural neurons, it is not clear at what level 14-3-3 expression is regulated to switch on repulsion in response to Shh.

Shh-dependent repulsion appears to be Smo-dependent, as conditional deletion of Smo from spinal commissural neurons leads to randomization of anterior-posterior turning (Yam et al., 2012). In chickens, mRNA for *Hedgehog-interacting protein* (*Hhip*), an inhibitor of Shh signaling, is transiently expressed in commissural neurons once their axons have reached the midline and RNAi knockdown of *Hhip* causes both midline stalling and aberrant posterior turns of post-crossing commissural axons (Bourikas et al., 2005). These defects have been interpreted as evidence that Hhip is a receptor through which Shh signals repulsion in post-crossing commissural neurons (Bourikas et al., 2005; Wilson and Stoeckli, 2013). However, Hhip has not been shown to signal in response to Shh in any context and is instead thought to antagonize Hh signaling by sequestering Hh proteins and restricting their diffusion (Chuang and McMahon, 1999; Chuang et al.,

2003; Jeong and McMahon, 2005). A requirement for Hhip in mediating Shh-dependent repulsion has yet to be established through turning or collapse assays, and mice mutant for *Hhip* do not display anterior-posterior turning defects (Yam et al., 2012). The observation that *Hhip* knockdown causes midline stalling (Bourikas et al., 2005) suggests the alternative possibility that Hhip may be transiently expressed in commissural neurons to blunt attraction to floor plate-derived Shh as commissural axons are exiting the midline. Functional studies assessing the potential contributions of the Shh receptors Boc, Cdo, and Gas1 in the guidance of post-crossing spinal commissural axons have not been reported. Gas1 repels enteric axons from gut-derived Shh (Jin et al., 2015), raising the possibility that Gas1 may signal Shh-dependent axon repulsion in other contexts.

In addition, considerable evidence has emerged indicating that commissural neurons actively inhibit their responsiveness to midline repellents while they are growing toward the midline. Vertebrates limit Robo repulsion in pre-crossing commissural axons through Robo3 (Figure 1.3). In mouse embryos mutant for *Robo3*, all spinal commissural axons fail to cross the midline (Sabatier et al., 2004). While floor plate tissue elicits outgrowth of axons from wild-type spinal cord explants, *Robo3* mutant axons fail to grow out of explants when exposed to wild-type floor plate tissue. Blockade of Slit activity with a soluble Robo2 ectodomain restores the ability of *Robo3* mutant explants to respond to floor plate-derived outgrowth signals. Likewise, a combination of Netrin-1 and Slit2 induces axonal outgrowth from wild-type, but not *Robo3* mutant explants, suggesting that the endogenous function of *Robo3* in pre-crossing commissural axons is to prevent precocious Slit responsiveness (Sabatier et al., 2004). Genetic data support the idea that the failure of *Robo3* mutant commissural axons to reach the floor plate *in vivo* is

due to excessive Slit repulsion through Robo1 and Robo2, as reduction in *Robo1*, *Robo2*, or *Slit* gene dosage partially rescues *Robo3* mutants (Sabatier et al., 2004; Jaworski et al., 2010). However, even the complete loss of *Robo1* and *Robo2* fails to fully rescue midline crossing defects in *Robo3* mutants, suggesting that Robo3 promotes midline crossing in part through Robo1- and Robo2-independent mechanisms (Jaworski et al., 2010). *Robo3*, *Slit 1/2/3* compound mutants have not been analyzed, so it is not yet clear whether this mechanism is Slit-dependent. In light of the observation that Robo1 and Robo2 are expressed at very low levels on pre-crossing commissural axons (Long et al., 2004), these genetic data imply that the activity of this small pool of Robo1 and Robo2 must be antagonized to prevent premature repulsion. A recent study suggests that Robo3 may promote midline attraction in addition to antagonizing midline repulsion (Zelina et al., 2014). *Robo3* mutant spinal cord explants display a reduced outgrowth response when exposed to Netrin-1 and, although Robo3 does not directly bind to Netrin, it does form a complex with DCC. Rescue experiments with a form of DCC that cannot bind to Robo3 would test whether Netrin-DCC attraction depends on this Robo3-DCC complex.

Robo3 is alternatively spliced, yielding two variants that differ in their cytoplasmic domains (Chen et al., 2008). One splice variant, Robo3.1, is specifically expressed on pre-crossing commissural axons, while the other, Robo3.2, is specifically expressed on post-crossing commissural axons. Isoform-specific rescue and RNAi experiments suggest that Robo3.1 is required to facilitate midline crossing, while Robo3.2 contributes to midline repulsion in post-crossing commissural axons. Notably, Robo3.2 knockdown in a *Robo1*, *Robo2* background causes occasional re-crossing of commissural axons, a phenotype observed in *Slit 1/2/3* mutants, but not in *Robo1*, *Robo2*

mutants. It has been speculated that perhaps Robo3.1 acts as a Slit sink, preventing Robo1 and Robo2 from binding to Slit, but lacking the ability to signal repulsion, while Robo3.2 functions as a classical Robo receptor, signaling repulsion in response to Slit (Sabatier et al., 2004; Chen et al., 2008). However, this possibility seems unlikely in light of reports that mammalian Robo3 proteins do not bind Slit (Camurri et al., 2005; Mambetisaeva et al., 2005; Li et al., 2014; Zelina et al., 2014), leaving the questions of how Robo3.1 antagonizes Robo1 and Robo2 activity and how Robo3.2 signals midline repulsion unresolved. In addition, the mechanisms regulating the alternative splicing of Robo3 remain unknown. Thus, many aspects of Robo3 function in commissural neurons both before and after midline crossing warrant further exploration.

In addition to limiting their responsiveness to Slits, pre-crossing commissural neurons suppress their responsiveness to Sema3B, in part through proteolytic degradation of PlexA1 (Nawabi et al., 2010; Figure 1.4). As commissural axons are growing toward the midline, they express only a low level of PlexA1, but PlexA1 expression is up-regulated on commissural axons after they have reached the midline. Spinal commissural neurons display increased PlexA1 expression upon exposure to floor plate-conditioned media, suggesting that the floor plate produces soluble factors that promote PlexA1 expression. PlexA1 is a substrate for calpain cleavage and blunting calpain activity either by RNAi knockdown or with pharmacological inhibitors causes spinal commissural neurons, which are ordinarily unresponsive to Sema3B, to undergo growth cone collapse when exposed to Sema3B. When mouse spinal cords are treated with calpain inhibitors, commissural axons stall at the floor plate, consistent with a role for calpain proteolysis in sensitizing commissural neurons to floor plate-derived repellents. Experiments with a

calpain-insensitive variant of PlexA1, which would be predicted to be active pre-crossing and therefore to prematurely signal midline repulsion, could validate the model that calpain proteolysis of PlexA1 is indeed responsible for limiting Sema3B responsiveness in pre-crossing commissural neurons.

Exposure to floor plate-conditioned media antagonizes calpain activity in dorsal spinal cord tissue (Nawabi et al., 2010), implying the existence of soluble floor plate-derived factors that block calpain activity. Glial cell line-derived neurotrophic factor (GDNF) is expressed in the floor plate during commissural axon guidance and it mimics the abilities of floor plate-conditioned medium to sensitize commissural neurons to Sema3B-induced growth cone collapse and to reduce both calpain activity and the abundance of PlexA1 proteolytic fragments in the spinal cord (Charoy et al., 2012). Medium conditioned by floor plate tissue from *Gdnf* mutant mice has reduced ability to sensitize commissural neurons to Sema3B-induced growth cone collapse. In embryos mutant for *Gdnf* or its receptor Neural Cell Adhesion Molecule (NCAM), spinal commissural axons frequently stall in the floor plate, consistent with GDNF's proposed function in promoting midline repulsion (Charoy et al., 2012). Other floor plate-derived factors that sensitize commissural neurons to Sema3B repulsion have been identified, including Shh (Parra and Zou, 2010) and Neuronal Cell Adhesion Molecule (NrCAM), which is cleaved to release a soluble ectodomain (Nawabi et al., 2010). NrCAM inhibits PlexA1 expression (Nawabi et al., 2010), but it is not clear whether either of these soluble factors regulates calpain activity.

In flies, there is no evidence that commissural neurons modulate their responsiveness to midline attractants. However, like vertebrates, flies inhibit Slit-Robo

repulsion in pre-crossing commissural neurons, but through a different mechanism (Figure 1.5). The endosomal protein Commissureless (Comm) binds Robo and prevents its trafficking to the growth cone, instead targeting it for lysosomal degradation (Keleman et al., 2002; Keleman et al., 2005). Expression of *comm* mRNA is tightly spatiotemporally controlled so that *comm* is specifically expressed in commissural neurons as they are sending their axons across the midline, but not before or after, and *comm* is rarely expressed in ipsilateral neurons (Keleman et al., 2002). This pulse of *comm* expression in commissural neurons reduces their responsiveness to Slit during midline crossing. *comm* mutants have a dramatic phenotype in which no axons cross the midline (Seeger et al., 1993; Tear et al., 1996) and analysis of *robo*, *comm* double mutants indicates that *robo* is epistatic to *comm* (Seeger et al., 1993). Surprisingly, embryos in which the endogenous *robo* gene is replaced with a mutant version that cannot be sorted by Comm are phenotypically normal (Gilestro, 2008), suggesting that Comm can regulate Slit-Robo repulsion through an additional mechanism.

comm expression is regulated, in part, by Fra. In *fra* mutants, *comm* expression is reduced in commissural neurons, but this output of Fra is Netrin-independent, as *comm* expression is unaffected in *NetAB* mutants (Yang et al., 2009). It is not clear whether Fra's ability to regulate *comm* is ligand-dependent or at what level it is regulated to produce the appropriate temporal pattern of *comm* expression. Fra appears to regulate *comm* transcription rather than the stability of *comm* mRNA, as *comm* pre-mRNA is reduced in *fra* mutants (Yang et al., 2009). *fra* mutants have much milder midline crossing defects than *comm* mutants, suggesting that parallel mechanisms must exist to regulate *comm*.

Conclusion

In the past two decades, many cues and receptors that attract commissural axons toward and repel them away from the ventral midline have been identified, and loss of function genetic data suggest that additional cues and receptors that regulate commissural axon pathfinding still await discovery. Both intrinsic (i.e. 14-3-3) and extrinsic (i.e. GDNF) factors that enable axons to modulate their responsiveness to midline cues have been identified, but our understanding of the cellular mechanisms that allow axons to switch their responsiveness to cues is incomplete. Regulation of receptor expression appears to be a common mechanism through which axonal sensitivity to cues can be gated. Recent reports that axon guidance receptors themselves can regulate both transcription (Taniguchi et al., 2003; Goldschneider et al., 2008; Yang et al., 2009) and translation (Tcherkezian et al., 2010) raise the intriguing possibility that guidance receptors may be able to directly regulate their own expression or the expression of other receptors.

Local Netrin-DCC signaling

As discussed above, precise regulation of axon guidance receptor expression is critical for the establishment of appropriate neural connectivity, both at the midline and elsewhere in the developing nervous system. But once these receptors are properly expressed, how do they signal to regulate growth cone guidance? The observation that the cytoplasmic domains of many axon guidance receptors, including DCC, do not contain obvious catalytic motifs led to the idea that these receptors might function as docking sites for protein complexes that catalyze local growth cone remodeling in response to

their ligands (reviewed in Huber et al., 2003). In this way, guidance receptors are thought to transduce gradients of cues into asymmetrical changes in the membrane and underlying cytoskeleton of growth cones, to steer them toward sources of attractive cues and away from sources of repellents. Here, I review the mechanisms through which Netrin-DCC signaling has been shown to regulate local growth cone dynamics and highlight ways in which these mechanisms exemplify signaling strategies that are broadly used by classical axon guidance receptors (DCC, Robo, Plexin, Eph).

Rho GTPases and upstream regulators of Rho GTPase activity

Members of the Rac homology (Rho) family of small GTPases, which are well-established regulators of cell morphology that can remodel the cytoskeleton through many different effectors, have been broadly implicated in axon guidance receptor signaling, including Netrin-DCC signaling (reviewed in Hall and Lalli, 2010). Netrin stimulation recruits the Rho GTPases Rac1 and Cdc42 to DCC and activates Rac1 and Cdc42 in DCC-expressing cells, including cultured spinal commissural neurons (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). In addition, Netrin activates Rac1 in embryonic mouse brains (Briançon-Marjollet et al., 2008). Rho GTPase activation is required for many cellular responses to Netrin, as pharmacological inhibition of Rho GTPase activity partially blocks Netrin-dependent outgrowth of spinal cord explants and expression of dominant negative forms of Rac1 and Cdc42 inhibits Netrin-DCC-dependent changes in cell morphology in a variety of cell lines (Li et al., 2002; Shekarabi and Kennedy, 2002). Expression of dominant negative forms of Rac and Cdc42 also prevents Netrin-induced growth cone expansion and filopodial formation in

cultured spinal commissural neurons (Shekarabi et al., 2005). Rho GTPases play pleiotropic roles in embryonic development and have been implicated in the signaling outputs of many different axon guidance receptors, confounding interpretation of their mutant phenotypes (Jin and Strittmatter, 1997; Kuhn et al., 1999; Vastrik et al., 1999; Vikis et al., 2000; Wahl et al., 2000; Driessens et al., 2001; Hu et al., 2001; Zanata et al., 2002; Fan et al., 2003; Oinuma et al., 2004a; Oinuma et al., 2004b; Turner et al., 2004; Gallo, 2006; Uesugi et al., 2009).

How is Rho GTPase activity spatially and temporally regulated to direct growth cone navigation? GTPases cycle between an active, GTP-bound state and an inactive GDP bound state. GTPase activating proteins (GAPs) stimulate GTP hydrolysis, inactivating GTPases, while guanine nucleotide exchange factors (GEFs) exchange GDP for GTP, restoring GTPases to their active state. Physical and genetic interactions between members of all families of classical axon guidance receptors and specific Rho GAPs and Rho GEFs have been demonstrated and in some cases the recruitment of these GAPs and GEFs to the receptors has been shown to be ligand-dependent, suggesting a potential mechanism through which axon guidance cues can modulate the local activity of Rho GTPases (Shamah et al., 2001; Aurandt et al., 2002; Driessens et al., 2002; Perrot et al., 2002; Swiercz et al., 2002; Lundström et al., 2004; Barberis et al., 2005; Cowan et al., 2005; Forsthoefel et al., 2005; Hu et al., 2005; Sahin et al., 2005; Toyofuku et al., 2005; Yang et al., 2006; Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007; Briançon-Marjollet et al., 2008; Li et al., 2008; Demarco et al., 2012).

The Rho GEF UNC-73/Trio physically interacts with the cytoplasmic domains of Fra and DCC (Forsthoefel et al., 2005; Briançon-Marjollet et al., 2008). Addition of recombinant Netrin to mouse brain extracts does not enhance this binding (Briançon-Marjollet et al., 2008), suggesting that Trio may be constitutively bound to DCC, rather than recruited in a ligand-dependent manner; however, Trio-DCC binding has not been investigated in *Netrin* mutant tissue. Netrin-dependent Rac1 activation is abolished in *Trio* mutant mouse brains and Netrin-dependent axon outgrowth is reduced in both spinal cord explants and dissociated cortical neurons from *Trio* mutants, indicating that *Trio* is required for Netrin responsiveness *in vitro* (Briançon-Marjollet et al., 2008). Worms, flies, and mice mutant for *Trio/unc*-73 have defects in axon growth and guidance, but to what extent these defects reflect a loss of Netrin-DCC signaling remains unclear. In *C. elegans*, many axons are guided toward the ventral nerve cord through opposing gradients of ventrally expressed UNC-6/Netrin, which signals attraction through UNC-40/DCC receptors, and dorsally expressed SLT-1/Slit, which signals repulsion through SAX-3/Robo receptors (Hedgecock et al., 1990; Ishii et al., 1992; Chan et al., 1996; Wadsworth et al., 1996; Zallen et al., 1998; Hao et al., 2001). Null mutants for any of these genes have partially penetrant defects in ventral axon guidance, providing a simple system in which to analyze the contribution of any gene to either the Netrin-DCC pathway or the Slit-Robo pathway. Disruption of a gene that acts in the Netrin-DCC pathway should enhance the defects caused by ablation of Slit or Robo function, and vice versa; indeed, *slt-1, unc-6* double mutants have more frequent defects in ventral axon guidance than either single mutant (Yu et al., 2002). *unc-73* mutants have defects in axon growth and guidance, including failure to grow ventrally (Hedgecock et al., 1987;

Siddiqui, 1990; Siddiqui and Culotti, 1991; McIntire et al., 1992; Steven et al., 1998), but it is not clear whether these defects reflect a loss of ventral attraction or a loss of dorsal repulsion. Loss of function mutations in *unc-73* do not suppress excessive axon outgrowth caused by neuronal expression of a myristoylated UNC-40 cytoplasmic domain (Myr-UNC-40), suggesting that UNC-40 can promote outgrowth independent of *unc-73* (Gitai et al., 2003). However, the relevance of this finding to Netrin-DCC signaling in general and to Netrin-dependent attraction in particular is not obvious, as Myr-UNC-40 is a constitutively active form of the receptor that does not require Netrin to signal and only promotes outgrowth, without directing guidance. Analysis of animals with mutations in *unc-73* and either *unc-6* or *unc-40* would reveal whether *unc-73* is required for Netrin-DCC-dependent axon guidance in the worm, but these phenotypes have not been reported. In the fly, *trio* mutants display many different axon guidance defects, including breaks in longitudinal fascicles, motor axon stalling, and mistargeting of photoreceptor and mushroom body axons (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). However, these phenotypes do not overlap with those of *Netrin* or *fra* mutants (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Brankatschk and Dickson, 2006; Timofeev et al., 2012). Specifically, *trio* mutants do not have defects in commissural axon guidance (Liebl et al., 2000; Forsthoefel et al., 2005) and have qualitatively different defects in motor axon targeting (Mitchell et al., 1996; Bateman et al., 2000). The class of photoreceptors with reported targeting defects in *trio* mutants does not express Fra and the class of photoreceptors with defects in *Netrin* and *fra* mutants has not been examined in *trio* mutants (Newsome et al., 2000; Timofeev et al., 2012). Heterozygosity for *trio* enhances loss of midline crossing in both *Netrin* and

fra mutants in the fly, consistent with a function for *trio* in a parallel pathway that promotes midline crossing (Forsthoefel et al., 2005). Analysis of brains and spinal cords of mouse *Trio* mutants revealed axon guidance defects in the spinal cord and brain that are similar to, but for the most part milder than, those reported in *Netrin-1* or *Dcc* mutants (Serafini et al., 1996; Fazeli et al., 1997; Briançon-Marjollet et al., 2008). It is not clear whether these defects reflect a requirement for Trio in Netrin-DCC signaling. Analysis of *Trio*, *Netrin-1* or *Trio*, *Dcc* double mutants would help resolve this question.

Another Rho GEF that physically interacts with DCC is Dock180 (Li et al., 2008). Dock180 and DCC are constitutively bound in embryonic cortical neurons, and Netrin stimulation increases this association; Dock180-DCC binding has not been analyzed in *Netrin* mutant tissue. Disruption of Dock180 function by RNAi or expression of a dominant negative form of Dock180 inhibits both Netrin-dependent activation of Rac1 and Cdc42 and Netrin-induced outgrowth and turning of dissociated cortical neurons and spinal cord explants. RNAi knockdown of Dock180 also causes spinal commissural neurons to stall before reaching the floor plate, consistent with a role for Dock180 in promoting outgrowth and/or attraction in response to midline-derived Netrin, but mutant phenotypes have not been analyzed. Additionally, genetic interaction experiments to determine whether this Dock180 loss-of-function phenotype reflects compromised Netrin-DCC signaling are lacking. There is no evidence that Dock180 plays a role in Netrin-DCC signaling in invertebrates.

A third Rho GEF, *tiam-1*, genetically interacts with *unc-6* and *unc-40* in *C. elegans* (Demarco et al., 2012), although physical interactions between this GEF and DCC have not been demonstrated. *tiam-1* mutants do not have defects in ventral axon

guidance, but *tiam-1*, *unc-73* double mutants do, suggesting that these two GEFs may function redundantly in ventral axon guidance. Mutations in *tiam-1* suppress Myr-UNC-40-induced ectopic axon outgrowth and do not enhance ventral axon guidance defects produced by RNAi against *unc-40*, suggesting that TIAM-1 may be required for Netrin-DCC signaling in the worm. The potential contribution of TIAM-1 to Netrin-DCC signaling in flies or vertebrates has not been investigated.

Src family kinase (SFK) activity has also been suggested to regulate Rho GTPase activity in response to Netrin. The SFK Fyn phosphorylates Trio, and this phosphorylation is required for Netrin-dependent Rac1 activation and axon outgrowth (DeGeer et al., 2013). Netrin also induces tyrosine phosphorylation of DCC through Fyn and Fyn-dependent phosphorylation of a particular cytoplasmic tyrosine residue on DCC is required for Netrin-DCC-dependent Rac1 activation and neurite outgrowth *in vitro* (Meriane et al., 2004). Interestingly, *in vitro* experiments have implicated SFK activation in all three vertebrate midline attractive pathways: Netrin-DCC, Shh-Boc, and VEGF-Flk-1 (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Yam et al., 2009; Ruiz de Almodovar et al., 2012). Exposure to Netrin, Shh, or VEGF activates SFKs and pharmacological or genetic inhibition of SFK activity blunts the abilities of these cues to elicit turning responses in a variety of assays. These data suggest that SFK activation may be an intracellular signaling event on which multiple chemoattractive pathways converge. Commissural axon guidance has not been closely studied in mice deficient for one or more SFKs, but the large number of vertebrate SFKs and the abilities of SFKs to functionally compensate for each other in other contexts (Stein et al., 1994) caution that it may be difficult to evaluate whether SFK activation is indeed a requisite step for midline

chemoattraction in intact vertebrate embryos. To date, the question of whether SFK activity is required for midline axon crossing has only been investigated *in vivo* in flies (O'Donnell and Bashaw, 2013), which have only two genes encoding SFKs. In *Drosophila*, reduction in SFK gene dosage causes ipsilateral axons to ectopically cross the midline and suppresses commissural axon guidance defects in genetic backgrounds in which midline attraction is disrupted. These data are consistent with a requirement for SFKs in midline repulsion, but not midline attraction, raising the possibility that flies and vertebrates use SFKs in opposite ways with respect to midline crossing. Alternatively, the vertebrate *in vitro* data implicating SFK activation in Netrin responsiveness may not reflect the *in vivo* functions of SFKs. In addition, a Fra receptor in which all cytoplasmic tyrosines are replaced with phenylalanines is capable of transducing responses to Netrin in commissural and motor neurons *in vivo*, arguing that tyrosine phosphorylation of DCC is not a requisite step for Netrin responsiveness in the fly (O'Donnell and Bashaw, 2013).

Downstream effectors of Rho GTPase activity

How does Rho GTPase activation transduce Netrin signals into cytoskeletal changes? Several actin-regulatory proteins have been implicated in this process, including Enabled (Ena), which binds barbed ends of actin filaments and antagonizes filament capping, promoting filament elongation (Bear et al., 2002; Barzik et al., 2005; Breitsprecher et al., 2008; Hansen and Mullins, 2010; Breitsprecher et al., 2011; Winkleman et al., 2014). Ena is concentrated at the leading edge of lamellipodia (Gertler et al., 1996; Reinhard et al., 1992; Rottner et al., 1999) and at filopodial tips in growth cones (Lanier et al., 1999), where it colocalizes with its binding partner Lamellipodin

(Krause et al., 2004). When Ena is mis-targeted to mitochondria, filopodial formation and extension and Netrin-dependent changes in growth cone morphology are blocked *in vitro*, implying that peripherally localized Ena is required for these processes (Lebrand et al., 2004). Mitochondrial mis-targeting of Lamellipodin induces the mis-localization of Ena, but mitochondrial mis-targeting of Ena has no effect on Lamellipodin localization, suggesting that Lamellipodin may recruit Ena to the growth cone periphery (Krause et al., 2004).

Studies of axon polarization, outgrowth, and guidance in *C. elegans* have provided evidence that Lamellipodin is required for Netrin-DCC-dependent signaling downstream of Rac. Neurons with ventrally projecting axons are polarized, such that their axons extend from the ventral side of their cell bodies, and this polarization is disrupted in both *unc-6* and *unc-40* mutants. MIG-10/Lamellipodin accumulates at the ventral edge of these neurons and its localization is disrupted in *unc-6* mutants, suggesting that a localized source of Netrin can induce the local accumulation of MIG-10 (Adler et al., 2006). Mutations in *mig-10* suppress Myr-UNC-40-induced ectopic axon outgrowth and enhance ventral axon guidance defects in *slt-1* mutants, suggesting that *mig-10* is required for UNC-6-UNC-40-dependent axon outgrowth and guidance (Chang et al., 2006; Quinn et al., 2006). Lamellipodin binds to a constitutively active form of Rac, but not to a constitutively inactive form of Rac and MIG-10 localization is disrupted in worms with mutations in the Rac gene *ced-10* (Quinn et al., 2008). Both *mig-10* and *ced-10* single mutants display ventral axon guidance defects and the frequency of these errors is not increased in the double mutants, suggesting that these two genes act in a common pathway. Moreover, mutations in *mig-10* suppress ectopic axon outgrowth induced by

expression of a constitutively active form of CED-10. Together, these data imply that Netrin stimulates the recruitment of Lamellipodin to activated Rac, which, in light of Lamellipodin's ability to recruit Ena, suggests a mechanism through which Netrin could stimulate localized F-actin elongation.

However, *in vivo* evidence that Ena is an effector of Netrin signaling remains scarce. *C. elegans unc-34/ena* mutants have mild defects in ventral axon guidance, but mutations in *unc-34* do not enhance the frequency of ventral axon guidance errors in *slt-1* mutants (Yu et al., 2002). However, mutations in *unc-34* suppress Myr-UNC-40-induced ectopic axon outgrowth (Gitai et al., 2003), suggesting that UNC-34 may signal downstream of UNC-40 in some contexts, but not others. Although Lamellipodin has been shown to recruit Ena in other contexts (Krause et al., 2004), ventral localization of UNC-34 in ventrally projecting neurons in *C. elegans* has not been demonstrated and UNC-34 localization in *unc-6*, *unc-40*, or *mig-10* mutants has not been assessed. Evaluation of Ena's contribution to Netrin-DCC signaling in the fly is confounded by the observation that Ena positively regulates Slit-Robo midline repulsion (Bashaw et al., 2000). However, heterozygosity for *ena* suppresses the gain-of-function phenotype produced by overexpression of a chimeric receptor consisting of the Robo ectodomain and the Fra cytoplasmic domain, which signals attraction in response to Slit (Bashaw et al., 1999), suggesting that Ena may play a role in DCC signaling in the fly (Forsthoefel et al., 2005). Mice have three Ena-like genes and animals null for all three fail to initiate axons (Kwiatkowski et al., 2007), preventing the analysis of axon guidance phenotypes. Mice mutant for just one of these genes, *Mena*, have defects in formation of the corpus callosum (Lanier et al., 1999), a phenotype that is also seen in *Netrin-1* and *Dcc* mutants

(Sabatier et al., 1996; Fazeli et al., 1997), but it is not clear whether the acallosal phenotype of *Mena* mutants reflects a loss of Netrin-DCC signaling. Thus, the contribution of Ena to Netrin-dependent axon attraction in vertebrates has not been determined *in vivo*.

Another cytoskeletal regulator that has been implicated downstream of Rho GTPases in Netrin-DCC signaling is UNC-115/actin-binding LIM (abLIM), which binds F-actin *in vitro* (Roof et al., 1997; Struckhoff and Lundquist, 2003) and contains a villin headpiece domain, which has been shown to induce the polymerization of G-actin to F-actin in other proteins (Friederich et al., 1992). *unc-115* mutants have axon guidance defects, including mild defects in ventral axon guidance (Wightman et al., 1997; Lundquist et al., 1998; Struckhoff and Lundquist, 2003). Mutations in *unc-115* enhance the ventral axon guidance defects caused by mutations in the Rac genes *ced-10* and *mig-2*, but not those caused by RNAi knockdown of a third Rac gene *rac2*. In addition, mutations in *unc-115* suppress ectopic axon formation induced by expression of a constitutively active form of RAC-2, but not constitutively active forms of CED-10 or MIG-2, suggesting that UNC-115 and RAC-2 act in a common pathway to regulate axon outgrowth and guidance (Struckhoff and Lundquist, 2003). Mutations in either *ced-10* or *unc-115* partially suppress ectopic axon outgrowth induced by Myr-UNC-40, but these defects are not further suppressed in *ced-10*, *unc-115* double mutants, suggesting that these two genes can regulate UNC-40-dependent axon outgrowth in the same pathway (Gitai et al., 2003). These genetic data imply that UNC-115 regulates axon growth and guidance downstream of Racs, but further work is needed to determine whether UNC-115 is truly an effector of Netrin-DCC signaling.

Local plasma membrane dynamics

As the cytoskeleton is expanding in the direction of axonal growth, the surrounding plasma membrane must grow to accommodate it. Recent work has suggested that Netrin signals through DCC to stimulate local exocytosis and asymmetrically expand the plasma membrane (Cotrufo et al., 2011). DCC directly interacts with the t-SNARE component Syntaxin-1 (Sytx1) and forms a complex with both Sytx1 and the v-SNARE tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP). DCC-Sytx1 binding is stimulated by Netrin-1 treatment *in vitro* and is weaker in brains from *Netrin-1* mutant mice. Experiments using botulinum toxins that specifically cleave different combinations of v-SNAREs revealed that Netrin-1-dependent membrane addition and directed axonal outgrowth toward a source of Netrin-1 depend on Sytx1. In addition, expression of a dominant negative form of Sytx1 or RNAi knockdown of either Sytx1 or TI-VAMP causes some spinal commissural axons to stall before reaching the midline, but it is it is not clear whether this phenotype reflects a loss of Netrin-dependent attraction. Nevertheless, this study establishes a mechanism through which Netrin binding to DCC can trigger polarized membrane insertion to accommodate asymmetrical growth cone expansion.

Conclusion

A host of *in vitro* studies have identified cytoplasmic proteins that can interact with axon guidance receptors, including DCC, and have suggested mechanisms through which guidance cues can direct local asymmetric changes in growth cone morphology. Testing these models *in vivo* remains a challenge for the field. Specifically, *in vivo*

genetic evidence linking particular cytoskeletal and membrane regulators to Netrin-dependent axon guidance is lacking.

In the following chapter, I present data supporting the idea that, in addition to signaling locally to regulate growth cone dynamics, Fra regulates axon guidance through a very different mechanism: it is cleaved to generate a soluble ICD that functions as a transcription factor to regulate axonal responsiveness to midline cues in the fly embryo. I also discuss the possibility that other axon guidance receptors may be able to signal through similar mechanisms.

Acknowledgements

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Figure 1.1. Commissural interneurons in the embryonic spinal cord of mouse and ventral nerve cord of *Drosophila*.

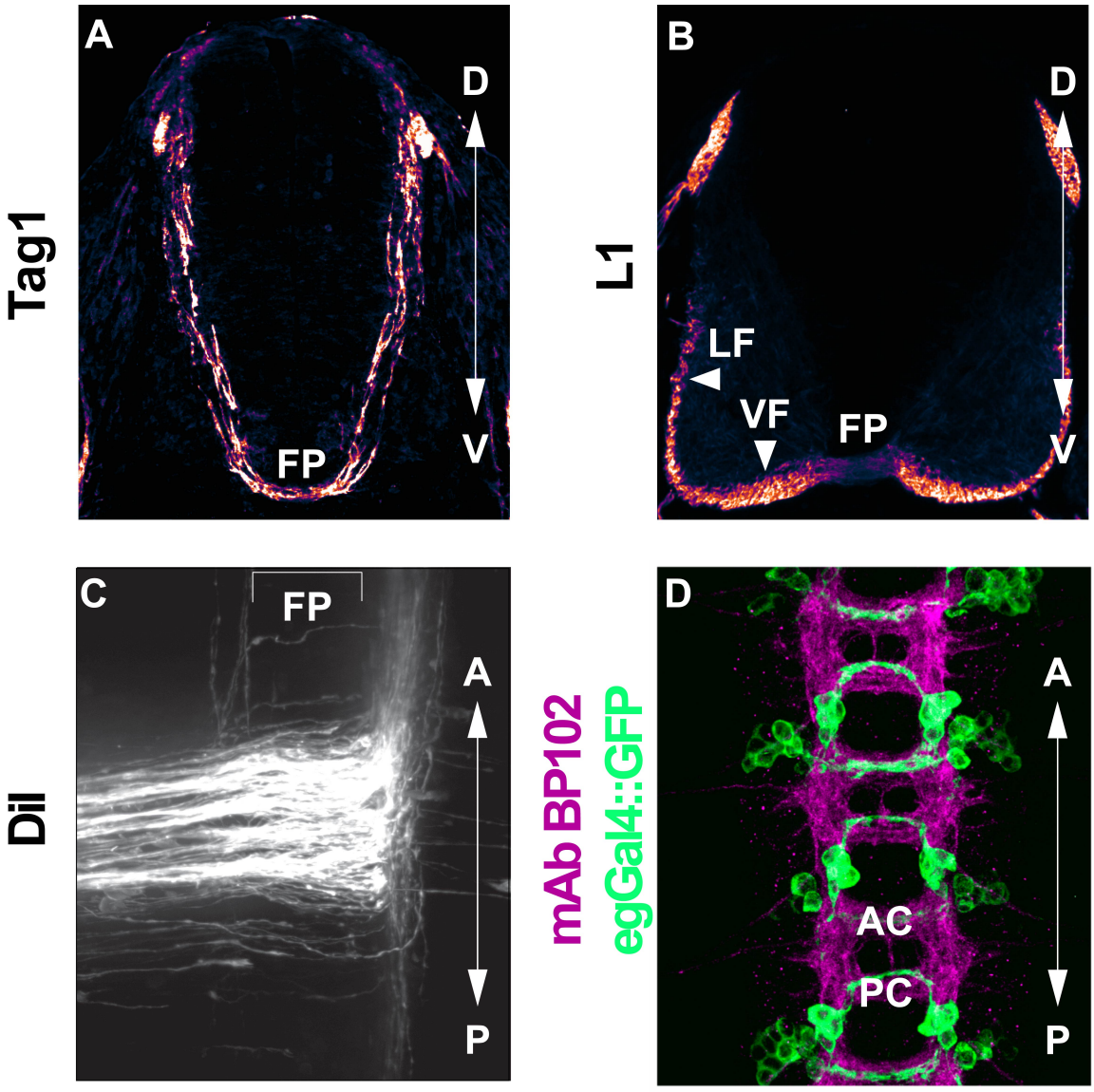

Figure 1.1. Commissural interneurons in the embryonic spinal cord of mouse and ventral nerve cord of *Drosophila*.

A) Transverse section of the mouse spinal cord at embryonic day 11.5. Pre-crossing spinal commissural neurons navigate ventromedially and express the cell adhesion molecule Tag1.

B) Transverse section of the mouse spinal cord at embryonic day 11.5. Post-crossing commissural neurons express the cell adhesion marker L1.

C) Open book preparation of the mouse spinal cord at embryonic day 11.5. Commissural neurons are labeled by DiI injection into the dorsal spinal cord. The majority of post-crossing commissural axons turn anteriorly. The bracket indicates the position of the floor plate.

D) Three segments of the *Drosophila* ventral nerve cord at stage 16. MAb BP102 (magenta) labels all axons in the central nervous system. *eg-Gal4* drives GFP expression (green) in a subset of commissural neurons.

FP, floor plate. LF, lateral funiculus. VF, ventral funiculus. AC, anterior commissure. PC, posterior commissure.

Note: the images in panels A-C were shared by Patricia Yam and Frédéric Charron (Institut de Recherches Cliniques de Montréal) and are used here with their permission.

Figure 1.2. Commissural axons switch the polarity of their response to Shh.

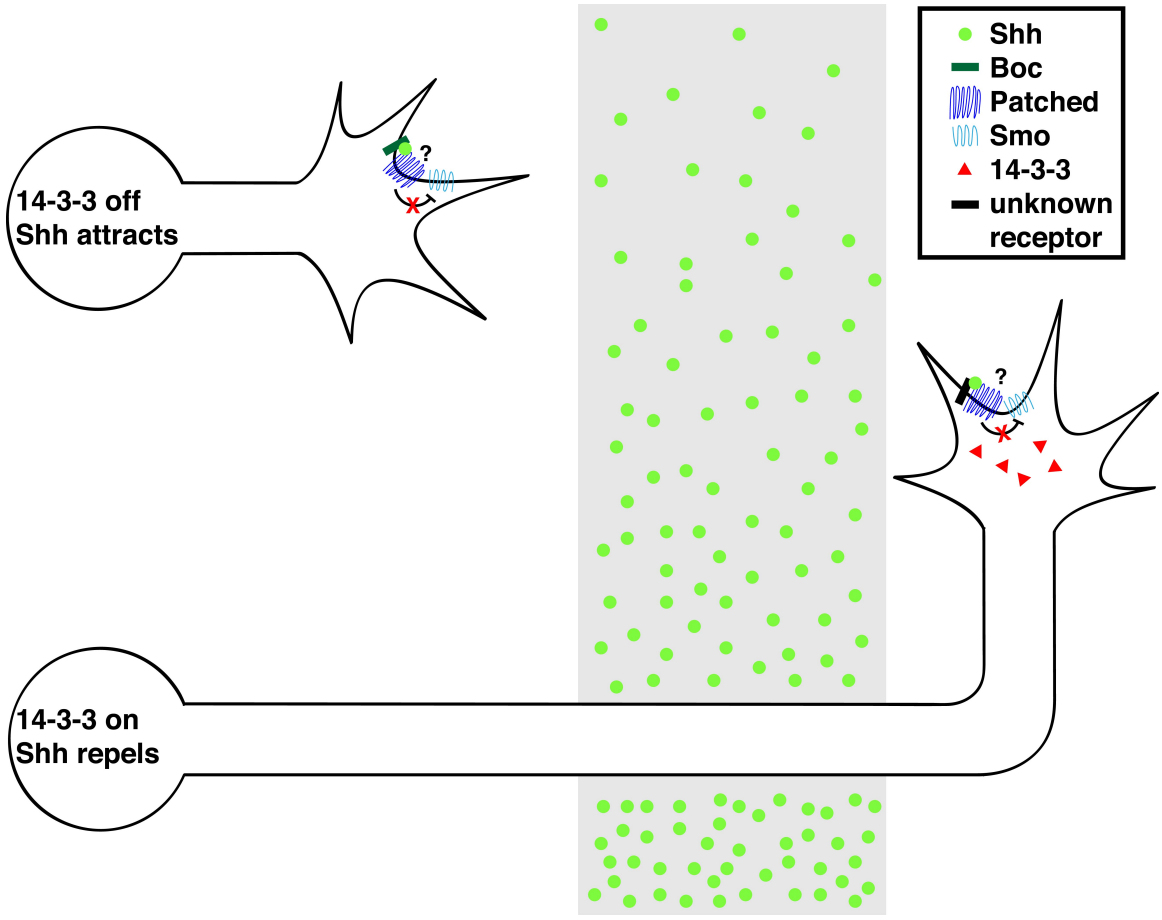

Figure 1.2. Commissural axons switch the polarity of their response to Shh.

In pre-crossing spinal commissural neurons, the receptor Boc signals attraction to midline-derived Shh. These neurons turn anteriorly after they have crossed the midline, in response to a posterior high to anterior low gradient of Shh, but the relevant Shh receptor is not known. 14-3-3 is specifically expressed in post-crossing commissural neurons and is required for Shh-dependent repulsion, but not attraction. Both attractive and repulsive Shh signaling depend on Smo, but it is not clear whether the Shh co-receptor Patched, which relieves repression of Smo to permit Shh signaling in other contexts, is required for Shh-dependent attraction or repulsion.

Figure 1.3. Robo3 regulates Slit responsiveness of commissural axons.

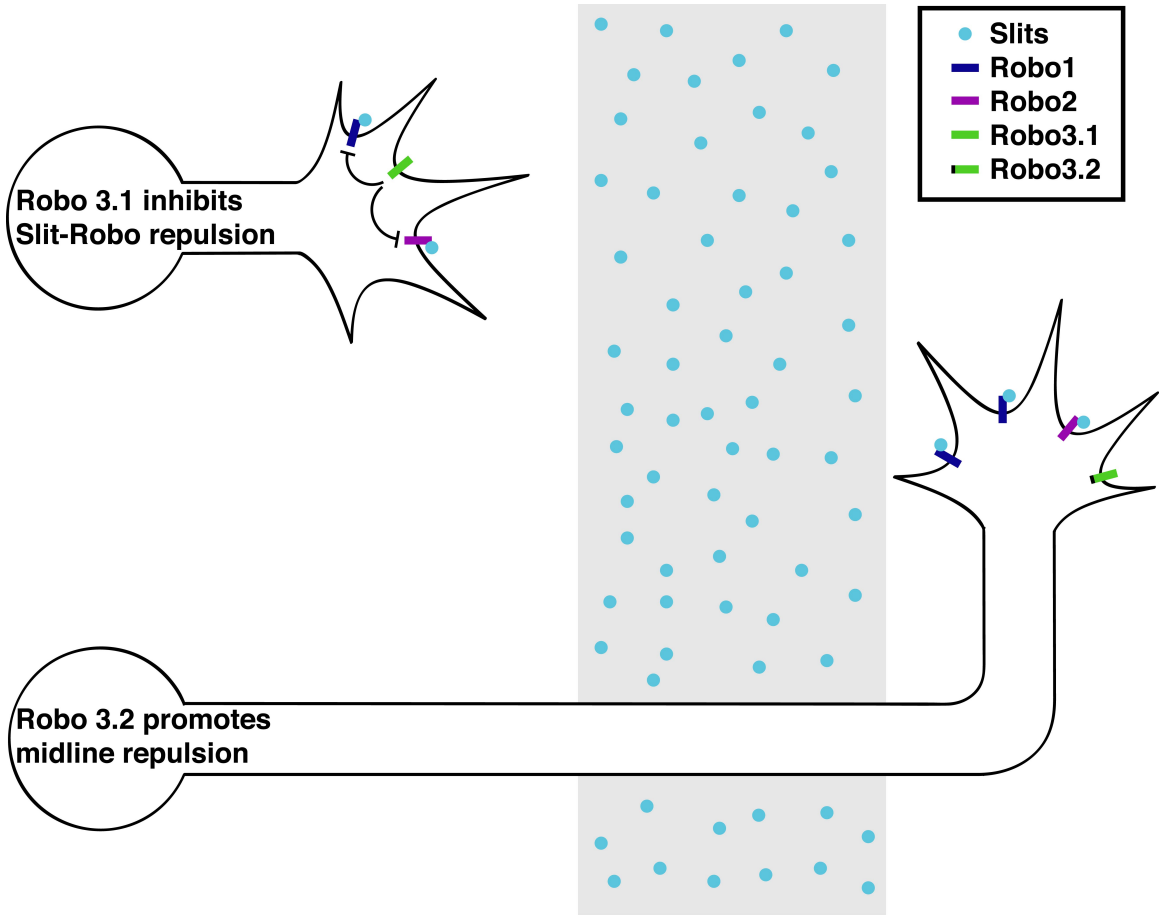

Figure 1.3. Robo3 regulates Slit responsiveness of commissural axons.

In pre-crossing spinal commissural neurons, Robo3.1 inhibits Slit repulsion through Robo1 and Robo2. After crossing, Robo3.1 is no longer expressed and Robo3.2 collaborates with Robo1 and Robo2 to signal midline repulsion.

Figure 1.4. GDNF modulates Sema3B responsiveness by regulating PlexA1 proteolysis.

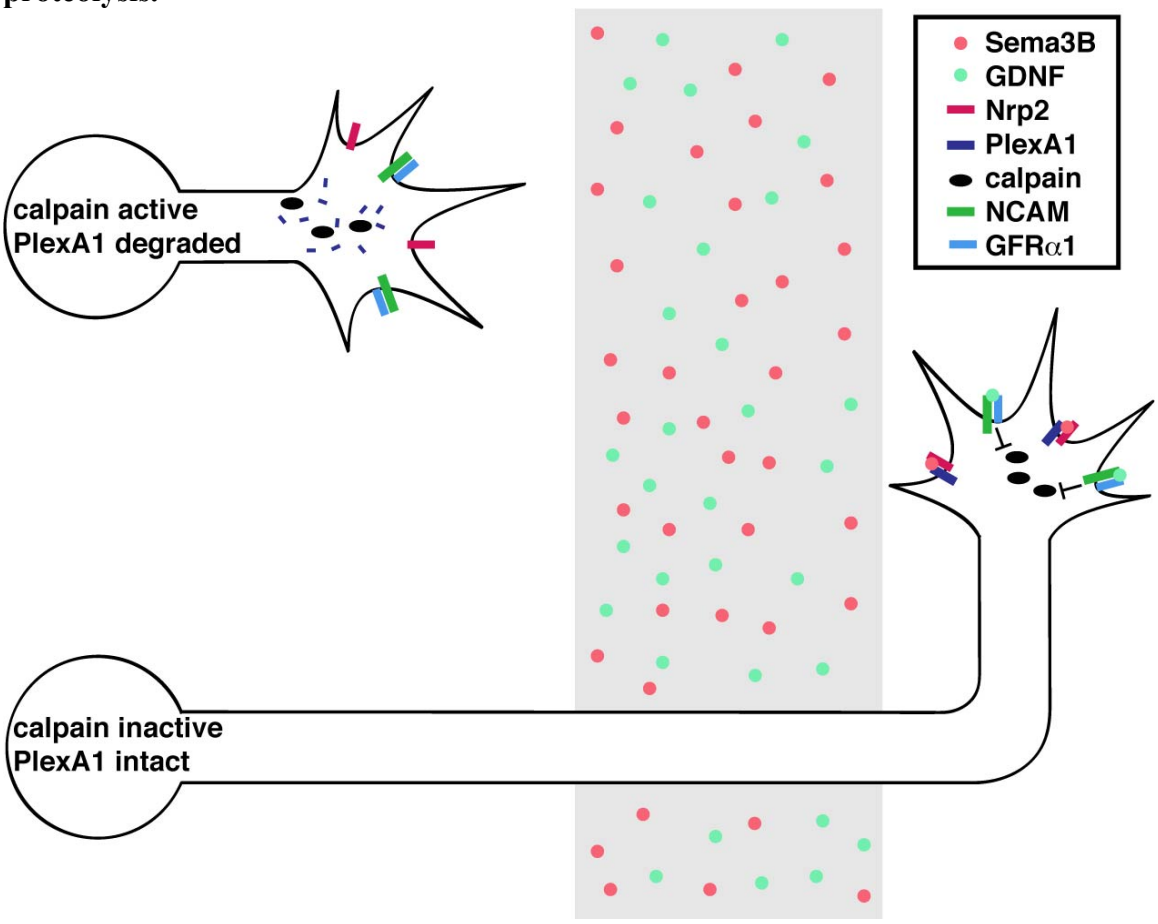

Figure 1.4. GDNF modulates Sema3B responsiveness by regulating PlexA1 proteolysis.

As spinal commissural axons are growing toward the midline, calpain cleaves the Sema3B receptor PlexA1 to reduce sensitivity to Sema3B. When these neurons reach the midline, GDNF signals through NCAM and its co-receptor GFRα1 to reduce calpain activity. Sema3B then signals repulsion through Nrp2 and PlexA1.

Figure 1.5. Comm regulates Slit responsiveness by inhibiting trafficking of Robo to the growth cone.

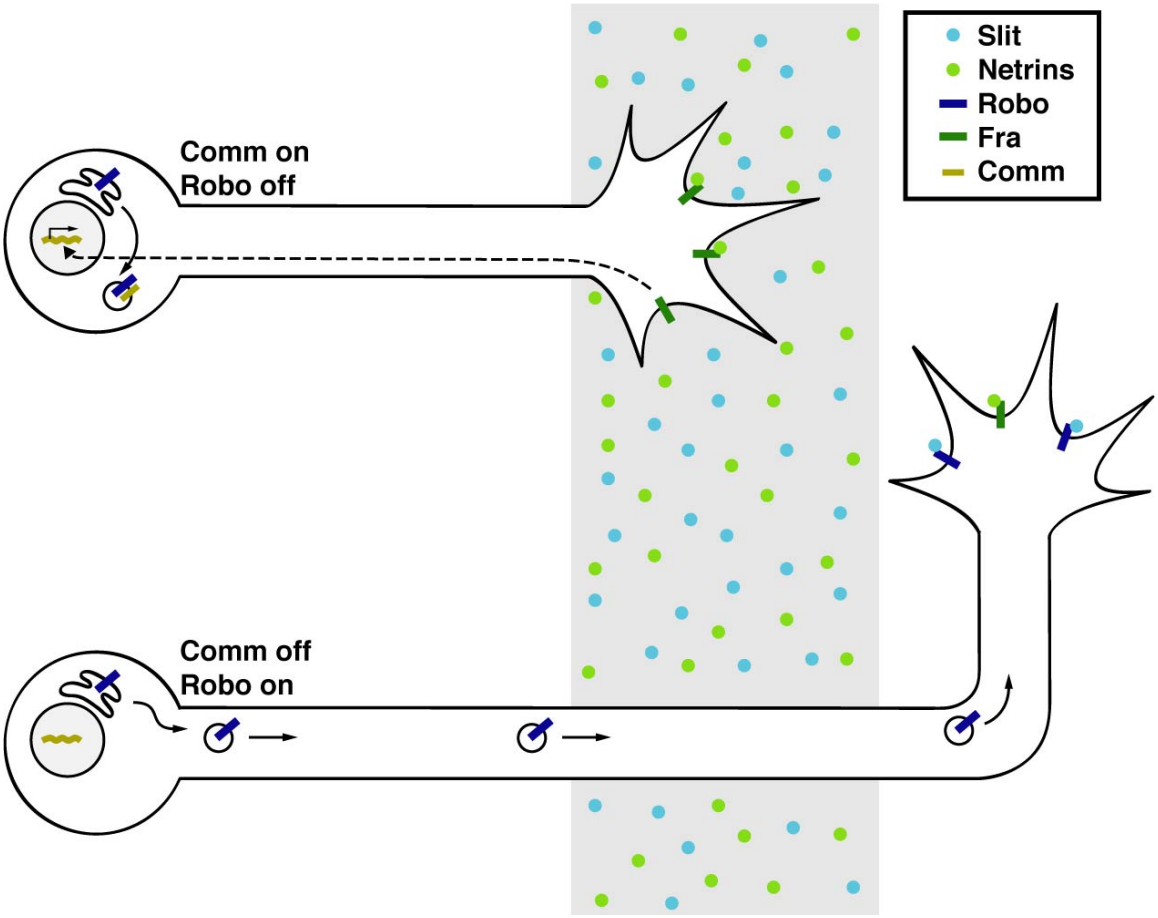

Figure 1.5. Comm regulates Slit responsiveness by inhibiting trafficking of Robo to the growth cone.

In *Drosophila*, as commissural neurons grow toward the midline, they express the endosomal protein Comm, which targets newly synthesized Robo for lysosomal degradation. Fra regulates *comm* transcription independent of its canonical ligands, Netrins. After crossing, Comm expression is extinguished and Robo is trafficked to the growth cone, where it signals repulsion in response to Slit.

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Chapter 2

The intracellular domain of the Frazzled/DCC receptor is a transcription factor required for commissural axon guidance

In commissural neurons of *Drosophila*, the conserved Frazzled (Fra)/Deleted in Colorectal Cancer (DCC) receptor promotes midline axon crossing by signaling locally in response to Netrin and by inducing transcription of *commissureless* (*comm*), an antagonist of Slit-Roundabout (Robo) midline repulsion, through an unknown mechanism. Here, we show that Fra is cleaved to release its intracellular domain (ICD), which shuttles between the cytoplasm and the nucleus, where it functions as a transcriptional activator. Rescue and gain-of-function experiments demonstrate that the Fra ICD is sufficient to regulate *comm* expression and that both γ-secretase proteolysis of Fra and Fra's function as a transcriptional activator are required for its ability to regulate *comm in vivo*. Our data uncover an unexpected role for the Fra ICD as a transcription factor whose activity regulates the responsiveness of commissural axons at the midline and raise the possibility that nuclear signaling may be a common output of axon guidance receptors.

Introduction

During the development of the nervous system, chemotropic cues serve as navigational signals for growing axons. These cues signal through axon guidance receptors, which are expressed on axonal growth cones. In the canonical view of axon guidance receptor signaling, ligand binding induces the formation of protein complexes on receptor cytoplasmic domains, which locally remodel the growth cone plasma membrane and underlying cytoskeleton. In this way, guidance receptors are thought to transduce gradients of cues into asymmetrical structural changes in growth cones, to steer them toward sources of attractants and away from sources of repellents (reviewed in O'Donnell et al., 2009). A particularly dramatic demonstration that local signaling is sufficient to execute some chemotropic responses comes from the observation that isolated growth cones that have been physically severed from their cell bodies remain capable of responding to guidance cues (Campbell and Holt, 2001).

Growing axons must also modulate their responsiveness to guidance cues in order to navigate intermediate targets on the way to their final synaptic partners. One of the best-studied examples of this phenomenon is the growth of commissural axons across the ventral midline of the embryonic central nervous system in bilaterally symmetric animals (reviewed in Dickson and Zou, 2010; Evans and Bashaw, 2010a). Throughout the period of time when commissural axons are crossing the midline, cells at the midline produce a host of chemotropic cues, including both attractants and repellents. In both insects and vertebrates, these include Netrins, which signal attraction through Frazzled (Fra)/Deleted in Colorectal Cancer (DCC) receptors (Serafini et al., 1994; Kennedy et al., 1994; Harris et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996;

Serafini et al., 1996; Fazeli et al., 1997; Brankatschk and Dickson, 2006), and Slits, which signal repulsion through Roundabout (Robo) receptors (Seeger et al., 1993; Holmes et al., 1998; Kidd et al., 1998a; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000; Long et al., 2004; Jaworski et al., 2010). As commissural neurons are growing toward the midline, their responsiveness to midline-derived repellents, including Slits, is suppressed. Once these axons have crossed the midline, they become responsive to Slits and other midline repellents, which facilitates midline exit and prevents re-crossing (Seeger et al., 1993; Kidd et al., 1998a; Kidd et al., 1998b; Zou et al., 2000; Keleman et al., 2002; Sabatier et al., 2004; Keleman et al., 2005; Chen et al., 2008; Nawabi et al., 2010; Parra and Zou, 2010; Charoy et al., 2012; Yam et al., 2012).

In *Drosophila*, while commissural axons are crossing the midline, the endosomal protein Commissureless (Comm) reduces sensitivity to Slit by inhibiting the trafficking of Robo to the growth cone plasma membrane (Keleman et al., 2002; Keleman et al., 2005). Expression of *comm* mRNA is tightly spatially and temporally regulated such that commissural neurons transiently express *comm* while their axons are crossing the midline, but not before or after. Ipsilateral neurons, whose axons do not normally cross the midline, rarely express *comm* (Keleman et al., 2002). Previously, we found that in addition to its canonical role in signaling Netrin-dependent outgrowth and/or chemoattraction, Fra has a second way of promoting midline axon crossing: independent of Netrins, Fra induces *comm* mRNA expression in commissural neurons (Yang et al., 2009). However, the mechanism(s) by which Fra regulates gene expression remain unknown.

Here, we report that Fra is cleaved by γ-secretase, releasing its ICD, which shuttles between the cytoplasm and the nucleus. This proteolysis is required for Fra's ability to regulate *comm* expression. In rescue and gain-of-function assays *in vivo*, the Fra ICD is sufficient to induce *comm* expression and midline crossing. In addition, the conserved P3 motif in the Fra ICD encodes a transcriptional activation domain. A point mutant variant of Fra that is specifically deficient for transcriptional activation, but is intact for other P3-dependent functions, cannot regulate *comm* expression *in vivo*. Moreover, *comm*-regulatory function can be restored to this receptor with a heterologous transcriptional activation domain, providing strong *in vivo* evidence for a requirement for Fra's transcriptional activation function. Thus, Fra acts in two different cellular compartments to control midline crossing: at the growth cone, Fra regulates local membrane and cytoskeletal dynamics in response to its canonical Netrin ligands, and in the nucleus, Fra functions as a transcription factor to modulate growth cone sensitivity to Slit-Robo repulsion.

Results

Fra is cleaved by γ-secretase

Fra's vertebrate orthologs, DCC and Neogenin (Neo) are substrates for metalloprotease-dependent ectodomain shedding and subsequent γ-secretase-dependent intramembrane proteolysis (Galko and Tessier-Lavigne, 2000; Taniguchi et al., 2003; Parent et al., 2005; Goldschneider et al., 2008; Bai et al., 2011; Okamura et al., 2011), prompting us to examine whether Fra also undergoes proteolytic processing. We pan-neurally expressed C-terminally-tagged *UAS-Fra-Myc* with *elav-Gal4* and probed

embryo lysates with an antibody against Myc (Figure 2.1A). We detected a ~200 kDa band corresponding to the full-length receptor, as well as smaller C-terminal fragments of approximately 50 kDa, 35 kDa and 25 kDa (ICD A, B, and C, respectively). We made a transgenic line that allowed us to express the ICD of Fra, without any extracellular or transmembrane residues, under *Gal4*/*UAS* control. When we expressed *UAS-Fra ICD-Myc* with *elav-Gal4*, we detected a doublet that corresponds in size to the largest of these C-terminal fragments, as well as the smaller C-terminal species (Figure 2.1A). To determine whether these C-terminal fragments are specific cleavage products of the Fra cytoplasmic domain, we replaced the Myc epitope with the smaller HA epitope and again examined the sizes of Fra ICD fragments. Consistent with our observations using the Myc-tagged receptor, we detected three C-terminal fragments in lysates from embryos pan-neurally expressing Fra-HA (Figure 2.1B). All three of these fragments are shifted to lower molecular weights (~45 kDa, 30 kDa and 20 kDa), commensurate with the decrease in the size of the epitope tag. We also examined lysates from embryos expressing a truncated, C-terminally HA-tagged Fra receptor that is missing its entire cytoplasmic domain (FraΔC-HA) and did not detect Fra ICD fragments (Figure 2.1B). Together these observations indicate that the Fra receptor can be processed to generate distinct C-terminal fragments.

γ-secretase cleaves its substrates in the membrane, releasing their ICDs, which can signal intracellularly in a variety of ways (reviewed in Haapasalo and Kovacs, 2011). The largest C-terminal peptide generated by proteolysis of Fra is approximately the size of the Fra ICD, suggesting that this fragment might be a product of γ-secretase proteolysis. To investigate whether Fra is cleaved by γ-secretase, we examined lysates

from embryos in which γ-secretase function was reduced. Presenilin (Psn) is the catalytic subunit of γ-secretase (Wolfe et al., 1999), a multi-protein complex that also includes Aph-1, Nicastrin, and Pen-2 (Yu et al., 2000; Francis et al., 2002; Goutte et al., 2002; Edbauer et al., 2003; Fraering et al., 2004). We analyzed lysates from genetically heterogeneous populations of embryos in which C-terminally epitope-tagged *UAS-Fra* transgene expression was pan-neurally driven by *elav-Gal4* only in *psn* or *aph-1* mutant embryos. To restrict *UAS-Fra* expression to mutant embryos, we used flies in which the *Gal4* and *UAS* elements were recombined onto mutant chromosomes or we used flies in which the chromosomes bearing the mutations were maintained as heterozygotes with balancer chromosomes ubiquitously expressing the Gal4 repressor Gal80 (see Figure 2.1C-D for details). As γ-secretase components are maternally deposited (Ye et al., 1999; Hu et al., 2002), we analyzed late stage 17 embryos (20-24 hours) in order to minimize the amount of Psn or Aph-1 present. In these embryos, Psn or Aph-1 function is likely strongly reduced, but not absent. In either lysates or immunoprecipitates from psn^{12} or $aph\text{-}1^{D35}$ mutant embryos, the abundance of both the Fra ICD and the smaller C-terminal fragments of Fra is reduced (Figure 2.1E-H), suggesting that the Fra ICD is a product of γ-secretase proteolysis. In addition, these experiments suggest that even though the smaller fragments are not likely to be directly generated by γ-secretase proteolysis, subsequent processing of the ICD depends on γ-secretase cleavage. DCC and Neo are cleaved approximately in the middle of their ICDs by caspases and this proteolysis is required for the abilities of these receptors to induce apoptosis (Mehlen et al., 1998; Matsunaga et al., 2004). The caspase cleavage site in DCC and Neo is not conserved in

Fra, but there are several aspartate residues in the Fra ICD that are candidate caspase cleavage sites.

The Fra ICD is sufficient to induce comm *expression*

Fra promotes midline crossing of commissural axons both by signaling outgrowth and/or chemoattraction in response to Netrins and by promoting *comm* transcription, independent of Netrins, to inhibit Slit-Robo midline repulsion. We reasoned that if the Fra ICD is regulating *comm* by acting as a transcription factor, it should be sufficient to perform the aspects of Fra's function that are due to its regulation of *comm*, but not the aspects that are due to its ability to transduce Netrin signals. To test this idea, we examined the ability of the Fra ICD to rescue *fra* loss-of-function phenotypes. The *eg-Gal4* element is expressed in a subset of neurons in the embryo, including three commissural EW interneurons per abdominal hemisegment (Dittrich et al., 1997). We used *eg-Gal4* to drive the expression of *UAS-Tau-Myc-GFP*, a marker that labels the axons and cell bodies of the EW neurons and facilitates quantitative evaluation of axonal trajectories. We combined this labeling with fluorescent *in situ* hybridization, using a probe that recognizes *comm* mRNA, so that we could score *comm* expression in each individual EW neuron. In embryos that are wild-type for *fra* or heterozygous for fra^3, axons of the EW neurons have reached the midline by stage 14, the time when these neurons express maximal amounts of *comm* mRNA (Figure 2.2A-C; Keleman et al., 2002; Yang et al., 2009). In fra^3 mutants, these neurons often fail to express *comm* and their axons fail to cross the midline at the appropriate time (Figure 2.2A-C; Yang et al., 2009). These midline crossing and *comm* expression defects can be rescued by expression

of a full-length *UAS-Fra* transgene with *eg-Gal4* (Figure 2.2B-C; Yang et al., 2009). In addition, expression of *UAS-Fra ICD* with *eg-Gal4* partially rescues midline crossing defects and fully rescues *comm* expression in the EW neurons of fra^3 mutants (Figure 2.2A-C).

We also examined the Fra ICD's ability to regulate *comm* expression in a subset of ipsilateral neurons, using a similar approach. The *ap-Gal4* element is expressed in three ipsilateral interneurons per abdominal hemisegment (the ap neurons; O'Keefe et al., 1998), which stochastically express *comm* at stage 17 (Figure 2.3A-C; Keleman et al., 2002; Yang et al., 2009). Expression of either full-length *UAS-Fra* or *UAS-Fra ICD* with *ap-Gal4* induces ectopic midline crossing of ap axons and ectopic expression of *comm* in the dorsal ap neuron (Figure 2.3A-C; Yang et al., 2009). These effects are dose-dependent, as expressing two copies of either *UAS-Fra* or *UAS-Fra ICD* produces more frequent midline crossing events than single copy expression (Figure 2.3B). The Fra ICD is a less potent inducer of ectopic midline crossing than full-length Fra, but the full-length receptor and the ICD are comparable in their abilities to induce *comm* expression (Figure 2.3A-C). Together, these rescue and gain-of-function genetic data support the idea that the Fra ICD is sufficient to carry out the transcriptional regulatory component of Fra's activity, but not the local, Netrin-dependent component.

γ-secretase proteolysis of Fra is required for Fra to regulate comm *expression*

We used this gain-of-function assay to test whether Fra's ability to regulate *comm* expression depends on its proteolysis by γ-secretase. When we analyzed embryos in which *UAS-Fra* was misexpressed with *ap-Gal4* in *psn* mutants, we found that Fra's

ability to induce *comm* expression is fully suppressed in two different *psn* mutant backgrounds (Figure 2.3C), suggesting that γ-secretase proteolysis of Fra is required for Fra's ability to regulate *comm*. Interpretation of midline crossing phenotypes in these experiments is confounded by several factors, including reports that proteolysis of DCC can antagonize canonical Netrin-DCC signaling (Galko and Tessier-Lavigne, 2000; Bai et al., 2011); the observation that Robo activity, which plays a key role in preventing the ap neurons from crossing the midline, is regulated by metalloprotease-dependent ectodomain shedding (Coleman et al., 2010), an event which is typically followed by γ-secretase proteolysis; and the likelihood that ectopic crossing events induced by full-length Fra are primarily a consequence of Netrin-dependent attraction (Figure 2.6D; O'Donnell et al., 2013).

The Fra ICD shuttles between the cytoplasm and the nucleus

If Fra regulates *comm* expression by functioning as a transcription factor, its ICD should be localized in nuclei. We initially investigated the subcellular localization of Fra in *Drosophila* S2R+ cells expressing C-terminally epitope-tagged Fra ICD or full-length Fra. In these experiments, we labeled nuclei by staining cells with an antibody against nuclear lamin, a component of the nuclear envelope. Under control conditions, the Fra ICD appears to be excluded from the nucleus. However, when nuclear export is blocked, either pharmacologically, with leptomycin B, an inhibitor of CRM1-dependent nuclear export, or genetically, by deleting P3, which encodes Fra's nuclear export signal (NES), the Fra ICD accumulates in the nucleus (Figure 2.4A), suggesting that the Fra ICD normally shuttles between the nucleus and the cytoplasm. When we expressed full length

Fra in S2R+ cells, we could not detect its C-terminus in the nucleus, even when nuclear export was blocked (Figure 2.4A and data not shown), suggesting that one or more components required for the receptor proteolysis that we observe *in vivo* are not present or active in our S2R+ cell cultures or, alternatively, that the amount of nuclear ICD generated from full-length receptor is too low to detect in this assay.

To examine the subcellular localization of the Fra ICD *in vivo*, we expressed *UAS-Fra ICD-Myc* with *ap-Gal4*, which allows for single cell resolution of nuclear localization. We observed some cells in which the Fra ICD is enriched in the nucleus and others in which the Fra ICD is mostly cytoplasmic (Figure 2.4B). When we expressed *UAS-Fra ICD-ΔP3-Myc* with either *eg-Gal4* or *ap-Gal4*, we detected its expression in the nucleus in every cell we examined, suggesting that the Fra ICD shuttles between the nucleus and cytoplasm *in vivo* and indicating that the NES we mapped *in vitro* appears to have the same activity *in vivo* (data not shown). Using a variety of *Gal4* drivers, we were never able to detect the C-terminus of full-length Fra in nuclei *in vivo*, even using FraΔP3, which lacks a NES (data not shown).

Our inability to detect the C-terminus of full-length Fra in nuclei is reminiscent of reports that the C-terminus of full-length Notch cannot be detected in the nucleus by conventional immunostaining (Fehon et al., 1991; Lieber et al., 1993; Rebay et al., 1993), despite the finding that nuclear localization of the Notch ICD is necessary for its function (Struhl and Adachi, 1998). We reasoned that the nuclear abundance of the C-terminus of full-length Fra might, likewise, be too low at any given time for us to detect by conventional methods. Therefore, we designed a reporter for Fra nuclear localization, in order to label cells in which Fra has entered the nucleus, with the expectation that this

approach should be more sensitive than immunostaining (Figure 2.4C). We generated a transgene (*UAS-Reporter*) in which a transcription factor consisting of a DNA-binding domain from the bacterial transcription factor LexA (LexA DBD) and an activation domain from the yeast transcription factor Gal4 (Gal4 AD) is inserted at the very C-terminus of full-length Fra (Figure 2.4C). The LexA DBD that we used to make this reporter has mutations that abolish its intrinsic ability to enter the nucleus (mutLexA DBD; Rhee et al., 2000; Marshall et al., 2007) and the Gal4 AD does not localize to the nucleus (Silver et al., 1988). We pan-neurally expressed this transgene with *elav-Gal4* in embryos in which a membrane-bound GFP is under the control of *lexAop* (*lexAop-mCD8-GFP*). Thus, GFP should only be expressed if the Fra ICD has access to the nucleus. Indeed, we detected GFP expression throughout the CNS in these embryos, including on commissural axons (Figure 2.4D), indicating that Fra can translocate to nuclei of commissural neurons *in vivo*.

We attempted to make a variant of the Fra ICD that lacks the ability to enter the nucleus, in order to test whether nuclear localization of the Fra ICD is required for its ability to regulate *comm*. We made serial deletions across the entire Fra ICD and tested the localization of these variants in S2R+ cells. Using this assay, we did not identify a sequence that is required for nuclear localization (data not shown). We also used a reporter assay in yeast to test which sequences within the Fra ICD are sufficient to confer nuclear localization. We used a strain of yeast in which a *lexAop* insertion upstream of the *ADE2* gene disrupts endogenous *ADE2* expression, causing the cells to accumulate a red pigment. In this strain, *ADE2* is under the control of *lexAop*, so expression of a transcriptional activator with a LexA DBD causes the yeast to turn white (Figure 2.5A).

We fused a series of sequences spanning the entire Fra ICD to the mutLexA DBD-Gal4 AD transcriptional activator and expressed these fusion proteins in *ADE2* reporter yeast. We identified three different regions of the Fra ICD that are sufficient to confer nuclear localization (Figure 2.5B). This redundancy prevented us from generating a Fra ICD variant that is defective for nuclear localization.

The Fra ICD encodes a transcriptional activation domain

The ICDs of DCC and Neo have been shown to function as transcriptional activators in reporter assays *in vitro*; however, whether these ICDs function as transcription factors *in vivo* and what, if any, is the biological significance of their transcriptional outputs is unknown (Taniguchi et al., 2003; Goldschneider et al., 2008). To determine whether the Fra ICD, like its vertebrate orthologs, contains an activation domain, we returned to the *ADE2* reporter yeast strain. For these experiments, we took advantage of the fact that expression of a transcription factor consisting of a LexA DBD (fused, in this case, to a strong nuclear localization signal (NLS)) and any activation domain drives expression of *ADE2*, causing the yeast to turn white (Figure 2.6A). Expression of a LexA DBD-Fra ICD fusion produces white yeast, indicating that the Fra ICD can function as a transcriptional activator (Figure 2.6B). A fusion between a LexA DBD and a Fra ICD lacking the conserved P3 motif (Fra ICDΔP3) fails to drive reporter expression, while a fusion between LexA DBD and P3 functions as a transcriptional activator, indicating that P3 is necessary and sufficient for Fra's transcriptional activation function (Figure 2.6B).

Fra regulates midline axon crossing and comm expression by functioning as a transcriptional activator

To determine whether Fra's ability to regulate commissural axon guidance and *comm* expression depends on its function as a transcriptional activator, we examined whether FraΔP3, which lacks Fra's activation domain, could rescue *fra* loss-of-function phenotypes. Expression of *UAS-FraΔP3* with *eg-Gal4* fails to rescue *comm* expression in the EW neurons of fra^3 mutants (Figure 2.7). To more directly test whether this lack of rescue is a consequence of the loss of Fra's activation domain or reflects other defects in the receptor, we performed a domain replacement experiment using the VP16 AD. Expression of *UAS-FraΔP3-VP16AD* with *eg-Gal4* does not rescue *comm* expression in fra^3 mutants (Figure 2.7).

This result could either mean that Fra's function as a transcriptional activator is not required for its ability to regulate *comm* or that P3 has an additional function in Fra's *comm*-regulatory pathway besides its function as an activation domain. To distinguish between these possibilities, we attempted to make mutations in Fra that specifically abrogate its transcriptional function, while leaving P3, which forms an alpha helix (Hirano et al., 2011; Wei et al., 2011), structurally intact. We performed an alanine mutagenesis scan across P3 and determined whether each point mutant had a functional activation domain and NES (Figure 2.6C). We used the presence of a functional NES as a proxy for the structural integrity of P3, as leucine-rich NESs, such as the one in P3, are alpha helices and this structure, rather than primary sequence, is the basis for their recognition by the nuclear export karyopherin CRM1 (Dong et al., 2009). Therefore, we reasoned that mutant ICDs that lacked functional activation domains, but retained functional NESs were good candidates to have specific deficits in transcriptional

activation without deficits in other P3-dependent functions. To determine whether a point mutant ICD had a functional activation domain, we fused it to LexA DBD and expressed it in *ADE2* reporter yeast. To determine whether a point mutant ICD had a functional NES, we fused it to a C-terminal epitope tag and examined its localization in S2R+ cells. Using this approach, we identified two point mutants, L1351A and E1354A, that are deficient for transcriptional activation, but are normally exported from the nucleus (Figure 2.6C) and we selected E1354A for further study. When we misexpressed *UAS-FraE1354A* with *ap-Gal4*, we found that it induces ectopic midline crossing almost as effectively as wild-type Fra, suggesting that this mutant is able to carry out canonical Netrin signaling (Figure 2.6D). In contrast, expression of *UAS-Fra-ΔP3* with *ap-Gal4* causes a much weaker ectopic crossing phenotype (Figure 2.6D).

Having defined specific mutations that disrupt transcriptional activation without disrupting other P3-dependent activities of the receptor, we next tested whether FraE1354A is able to rescue Fra's midline guidance and transcriptional regulatory activities. Expression of *UAS-FraE1354A* with *eg-Gal4* fails to rescue the loss of *comm* expression in EW neurons of fra^3 mutants, strongly suggesting that Fra's transcriptional activation function is required for this activity (Figure 2.8B). We were surprised to find that FraE1354A provides no rescue of midline crossing (Figure 2.8A), even though this receptor is likely intact for Netrin-dependent signal transduction (Figure 2.6D). In fact, we found that expression of FraE1354A antagonizes midline crossing in embryos heterozygous for fra^3 (Figure 2.9), suggesting that FraE1354A acts as a dominant negative with respect to midline crossing. To rigorously test whether FraE1354A's inability to rescue midline crossing and *comm* expression stems from the disruption of

Fra's activation domain, we generated a *UAS-FraE1354A* transgene with the VP16 AD fused to its C-terminus and evaluated its ability to rescue midline crossing and *comm* expression in fra^3 mutants. Strikingly, we found that addition of a heterologous VP16 AD to the FraE1354A receptor restores its ability to rescue both midline crossing and *comm* expression, providing compelling *in vivo* evidence that Fra's function as a transcriptional activator is required for its ability to promote midline crossing and regulate *comm* (Figure 2.8A-B).

Discussion

In this study, we identify the Fra ICD as a transcription factor that regulates the expression of *comm*, a key modulator of axonal responsiveness at the midline. γ-secretase proteolysis of Fra releases its ICD, which is capable of nuclear translocation and is sufficient to promote midline crossing and regulate *comm* expression in rescue and gain-of-function assays *in vivo*. The conserved P3 motif within the Fra ICD functions as a transcriptional activation domain and this activity is required for Fra's regulation of *comm* expression. Thus, in addition to its canonical role signaling locally to regulate growth cone dynamics, Fra functions as a transcription factor to regulate axonal responsiveness at the midline.

Regulation of Fra's function as a transcription factor

comm is expressed in commissural neurons with exquisite temporal specificity (Keleman et al., 2002). How might the transcriptional activity of the Fra ICD be regulated to contribute to *comm*'s expression pattern? γ-secretase proteolysis is typically

the second cleavage event in a proteolytic cascade, preceded by ectodomain shedding. Indeed, pharmacological experiments suggest that DCC's ectodomain is shed as a result of metalloprotease cleavage and that this proteolytic event is required for subsequent γ-secretase-dependent processing (Galko and Tessier-Lavigne, 2000; Bai et al., 2010). Metalloprotease-dependent ectodomain shedding is often ligand-dependent, while subsequent γ-secretase processing depends on the shape of the membrane-tethered metalloprotease cleavage product. For example, metalloprotease-dependent shedding of the Notch ectodomain is stimulated by the binding of Notch ligands (Brou et al., 2000; Mumm et al., 2000), and the subsequent γ-secretase cleavage of the membrane-tethered ICD is constitutive (Struhl and Adachi, 2000). As Fra regulates *comm* independent of Netrins (Yang et al., 2009), Fra ectodomain shedding may occur in response to the binding of a different ligand. Alternative ligands for DCC have been identified, including the vertebrate-specific proteins Draxin (Ahmed et al., 2011) and Cerebellin 4 (Haddick et al., 2014). In addition, the secreted protein MADD-4 physically associates with the *C. elegans* ortholog of Fra/DCC, UNC-40, and guides sensory neurons and muscle arms in an UNC-40-dependent manner (Seetharaman et al., 2011; Chan et al., 2014). The function of the *Drosophila* ortholog of MADD-4, CG31619, has not been investigated, nor has its ability to bind to Fra.

It seems unlikely that the transcriptional activity of the Fra ICD is controlled at the level of nuclear localization. When we express Fra ICDΔP3 (lacking a NES) in the commissural EW neurons *in vivo*, it accumulates in the nucleus at the earliest developmental stages we can observe (data not shown), suggesting that the Fra ICD is constitutively imported into the nucleus. We observe nuclear accumulation of full length

Fra ICD (with a NES) only occasionally (Figure 2.4B and data not shown), implying that after the Fra ICD translocates to the nucleus, it is rapidly exported. The fact that Fra's NES and activation domain are both encoded by P3 raises the possibility that when Fra is engaged in transcriptional activation, the association of co-activators with P3 might prevent it from associating with nuclear export machinery, coupling Fra's nuclear activity to its nuclear retention.

Mechanism of Fra's function as a transcription factor

Our finding that Fra's ability to regulate *comm* expression depends on its function as a transcriptional activator implies that the Fra ICD can associate with chromatin, but the Fra ICD does not contain an obvious DNA-binding domain. A Neo DNA-binding domain has not been identified either, but chromatin immunoprecipitation experiments have demonstrated that the Neo ICD associates with chromatin *in vitro* (Goldschneider et al., 2008). The Fra ICD's DNA-binding activity and specificity likely arise from associations between the Fra ICD and DNA-binding partners, as is the case with Notch. The Notch ICD has no DNA-binding activity of its own and associates with DNA as part of a complex including an obligate CSL (CBF1/RBPjκ, Su(H), Lag-1) DNA-binding partner (Nam et al., 2006; Wilson and Kovall, 2006). If the Fra ICD can associate with multiple DNA-binding proteins, it might allow the Fra ICD to regulate the expression of many different target genes, depending on which of its DNA-binding partners are expressed in particular cell types or developmental contexts.

The observation that a structurally intact P3 is required for Fra-dependent transcription (Figure 2.7) suggests that P3 plays another role in Fra's transcriptional

output besides its function as an activation domain. One possibility is that P3 is required for Fra's association with chromatin, perhaps by functioning as a binding interface for Fra's DNA-binding co-factors. This idea is supported by our observation that FraE1354A antagonizes midline crossing in both *fra* mutants and heterozygotes, while FraΔP3 has only a mild effect (Figures 2.8A-B and 2.9). Perhaps the ICD of FraE1354A inhibits midline crossing by occupying chromatin sites that are normally targets of both Fra and other transcriptional activators that act in a parallel pathway; the ICD of FraΔP3 would not have this effect if P3 is required for Fra's association with chromatin. Fra E1354A is not likely to be inhibiting endogenous Fra in our rescue experiments, as fra^3 is either a strong hypomorphic or null allele (Kolodziej et al., 1996; Yang et al., 2009). This model predicts that Fra has other transcriptional targets in EW neurons that are relevant for commissural axon guidance. It will be informative to identify additional transcriptional targets of Fra both in embryonic commissural neurons and in other cell types. In the retina, R8 photoreceptor axons have targeting defects that are much milder in *Netrin* mutants than in *fra* mutants (Timofeev et al., 2012), raising the possibility that the Netrin-independent output of Fra signaling in this system might be through the transcriptional pathway we have identified.

Proteolytic regulation of axon guidance receptor signaling

Cleavage of axon guidance receptors has been shown to regulate the activities of these receptors in a number of different ways. Degradation of axon guidance receptors can provide temporal control of axonal sensitivity to guidance cues. In vertebrates, this mode of regulation controls axonal responsiveness to members of the class 3 family of secreted Semaphorins (Sema3s), which signal repulsion through Neuropilin (Nrp)/Plexin

(Plex) co-receptors. Calpain proteolysis of PlexA1 in pre-crossing spinal commissural neurons reduces their sensitivity to Sema3B, which is expressed in the ventral spinal cord as these axons are growing toward the ventral midline (Nawabi et al., 2010). ADAM metalloprotease cleavage of Nrp1 reduces the sensitivity of proprioceptive sensory axons to Sema3A allowing them to terminate in the ventral spinal cord, where Sema3A expression is high (Romi et al., 2014). In addition, γ-secretase proteolysis of DCC in vertebrate motor neurons inhibits their responsiveness to midline-derived Netrin, preventing them from ectopically projecting toward the midline (Bai et al., 2011).

Proteolytic processing has also been implicated as a requisite step in local repulsive Robo signaling in *Drosophila* (Coleman et al., 2010). The Robo ectodomain is cleaved by the ADAM metalloprotease Kuzbanian and this proteolytic event is required for Robo's ability to transduce repulsive signals *in vivo* and for Slit-dependent recruitment of effectors of local Robo signaling *in vitro*. As γ-secretase-dependent intramembrane proteolysis is typically constitutive following ectodomain shedding, and occurs subsequent to metalloprotease processing of the human Robo1 receptor (Seki et al., 2010), it is likely that *Drosophila* Robo is cleaved to produce a soluble ICD. The observation that Robo proteolysis is required for local Slit-Robo signaling does not exclude the possibility that the Robo ICD may also have a nuclear function that contributes to axon guidance in the fly, but this possibility has not yet been explored.

Proteolysis has also been identified as a regulator of contact-mediated axonal repulsion. Eph receptors signal repulsion in response to their transmembrane ephrin ligands; ephrins can also function as receptors, signaling repulsion in response to Eph binding. Metalloprotease and subsequent γ-secretase cleavage of both Ephs and ephrins

have been demonstrated, providing a mechanism through which adhesive interactions can be broken to allow for repulsive signaling (Hattori et al., 2000; Janes et al., 2005; Tomita et al., 2006; Litterst et al., 2007; Lin et al., 2008; Gatto et al., 2014). The importance of this mode of regulation for axon targeting has not yet been established *in vivo* and a recent study using an EphA4 variant that is insensitive to metalloprotease cleavage suggests that EphA4 proteolysis is not required for EphA4-dependent motor axon targeting (Gatto et al., 2014).

Here, we have identified a new way in which axon guidance receptor proteolysis can influence axon responsiveness to guidance cues. γ-secretase-dependent processing of Fra releases its ICD, which translocates to the nucleus, where it functions as a transcription factor to regulate the guidance of commissural axons (Figure 2.10). We propose that the ability to signal from the nucleus may be a common property of axon guidance receptors and may serve as a general mechanism through which axon guidance receptors regulate their own activity or the activities of other receptors. Human Robo1 is processed by sequential metalloprotease and γ-secretase cleavage and its ICD localizes to the nucleus *in vitro* (Seki et al., 2010). It remains to be seen whether the ICDs of Ephs, ephrins, and Neuropilins, which are cleaved by γ-secretase, and of Plexins, which are proteolytically processed, but have not yet been identified as γ-secretase substrates, translocate to the nucleus as well. It will also be interesting to determine whether the ICDs of other axon guidance receptors signal from the nucleus to regulate aspects of neuronal morphogenesis and function besides axon pathfinding. Finally, recent work indicating that the cleaved C-terminus of the *Drosophila* Wnt receptor Frizzled translocates to the nucleus and contributes to the establishment of postsynaptic structures

by regulating RNA export (Mathew et al., 2005; Mosca and Schwarz, 2010; Speese et al., 2012) serves as a reminder that the trafficking of cell surface receptor fragments to the nucleus may allow these fragments to signal not only by regulating transcription, but in other ways as well.

Experimental Procedures

Molecular biology

The Fra ICD (amino acids 1098-1375) was amplified by PCR from pUAST-Fra-Myc (Garbe and Bashaw, 2007) and cloned as an EcoRI/NotI fragment into pUAST to generate pUAST-Fra ICD-Myc. Fra ICDs containing P motif deletions were amplified by PCR from full-length Fra constructs containing these deletions (Garbe et al., 2007) and Fra ICDs containing deletions of non-P motifs or containing point mutations were generated using serial overlap extension PCR, using pUAST-Fra ICD-Myc as a template. All Fra ICD constructs were cloned as EcoRI/NotI fragments into pUAST and start codons were added to all constructs. Myc-tagged Fra ICD was also cloned as an EcoRI/NotI fragment into a pUAST vector containing 10x UAS and an attB site for ϕC31-mediated targeted integration (p10UASTattB).

For yeast nuclear localization assays, untagged Fra ICD fragments were amplified by PCR from pUAST-Fra ICD-Myc and cloned into pNIA-CEN (Marshall et al., 2007) as EcoRI/BamHI fragments. For yeast activation assays, untagged full-length Fra ICD, Fra ICDΔP3, and P3 were amplified by PCR from pUAST-Fra ICD-Myc and cloned as EcoRI/BamHI fragments into pEG202-NLS. Point mutant variants of the Fra ICD were

generated using serial overlap extension PCR using pEG202-NLS-Fra ICD as a template and cloned as EcoRI/BamHI fragments into pEG202-NLS.

To generate the Fra nuclear localization reporter, a fusion between the C-terminus of Fra and a LexA DBD-Gal4 AD transcription factor was generated by serial overlap extension PCR using p10UAST-Fra-Myc and pNIA-CEN as templates. This fusion was cloned as a PshAI/NotI fragment into p10UAST-Fra-Myc. During this process, the C-terminal Myc tag was removed from Fra.

Full-length wild-type and mutant Fra constructs used for rescue experiments in Figures 2.7, 2.8, and 2.9 were cloned into a p10UASTattB. These constructs include identical heterologous UTR and signal sequences (from the *wingless* gene) and N-terminal 3x HA tags. Untagged Fra and FraΔP3 were amplified by PCR from p10UAST-Fra-Myc without a signal sequence and cloned as SpeI/KpnI fragments into pUAST-HA-Robo (Evans and Bashaw, 2010b), which had been cut with NheI and KpnI to remove Robo. HA-tagged Fra and FraΔP3 were cut from the resulting plasmid and cloned as NotI/KpnI fragments into p10UASTattB to generate p10UAST-HA-Fra and p10UAST-HA-FraΔP3. The Fra C-terminus was amplified from pEG202-NLS-FraE1354A ICD and cloned as a PshAI/KpnI fragment into p10UAST-HA-Fra to generate p10UAST-HA-FraE1354A. The VP16 activation domain was fused to the C-terminus of FraΔP3 and FraE1354A by serial overlap extension PCR using p10UAST-HA-FraΔP3, p10UAST-HA-FraE1354A, and pTol2-LexA-VP16 (from Jonathan Raper) as templates. During this process, the VP16 activation domain was mutated to destroy a PshAI site. These fusions were then cloned as PshAI/KpnI fragments into p10UAST-HA-Fra.

All constructs were sequenced to ensure that mutations were not introduced during PCR amplification.

Genetics

The following mutant alleles were used in this study: fra^{3} (Kolodziej et al., 1996); psn^{12} (Lukinova et al., 1999); psn^{145} (Annette Parks, personal communication to FlyBase); $aph1^{D35}$ (Hu and Fortini, 2003); eg^{MZ360} (*eg-Gal4*; Dittrich et al., 1997); ap^{Gal4} (*ap-Gal4*; Benveniste et al., 1998).

The following published transgenic lines were used in this study: P{UAS-Fra-Myc} 86Fb (O'Donnell et al., 2013); P{UAS-Fra-HA} #2 (Garbe et al., 2007); P{UAS-FraΔC-HA} #2 (Garbe et al., 2007); P{13XLexAop2-mCD8::GFP}attP2 (Bloomington Stock Center); P{UAS-Tau-Myc-GFP} (Callahan et al., 1998); P{Gal4-elav.L}3 (elav-Gal4; Bloomington Stock Center).

The following transgenic lines were generated: P{UAS-Fra ICD-Myc} 86Fb; P{UAS-Fra ICD-Myc} #26; {UAS-Fra nuclear localization reporter} 86Fb; P{UAS-HA-Fra} 86Fb; P{UAS-HA-FraΔP3} 86Fb; P{UAS-HA-FraΔP3-VP16AD} 86Fb; P{UAS-HA-FraE1354A} 86Fb; P{UAS-HA-FraE1354A-VP16AD} 86Fb. Transgenic flies were generated by BestGene Inc. (Chino Hills, CA), using ϕC31-directed targeted integration into the same landing site for all constructs (at cytological position 86Fb) to ensure comparable mRNA expression levels between lines. In addition, standard P-element transformation was used to generate UAS-Fra ICD-Myc lines and one of these, P{UAS-Fra ICD-Myc} #26, was used for rescue experiments scored in Figure 2.2B.

All crosses were performed at 25ºC. Immunostained embryos were genotyped using a combination of balancer chromosomes with embryonically expressed markers and linked, epitope-tagged transgenes.

Immunostaining of embryos

Embryo fixation and staining were performed as described (Kidd et al., 1998a). The following primary antibodies were used: rabbit anti-Myc (Sigma, C3956, 1:500), rabbit anti-GFP (Life Technologies, A11122, 1:250), chick anti-βgal (Abcam, 9361, 1:1000), mouse anti-βgal (DSHB, 40-1a, 1:50), mouse anti-nuclear lamin (DSHB, ADL84.12, 1:20), Alexa 647-conjugated goat anti-horseradish peroxidase (Jackson, 123-605-021, 1:250). The following secondary antibodies were used: Cy3 goat anti-rabbit (Jackson, 115-165-003, 1:1000), Alexa-488 goat anti-mouse (Life Technologies, A11001, 1:500), Cy3 goat anti-chick (Abcam, 97145, 1:500), HRP goat anti-rabbit (Cell Signaling, 7074S, 1:500). For the nuclear localization reporter experiments in embryos, the GFP signal was enhanced using the Tyramide Signal Amplification kit (Perkin Elmer) according to manufacturer's instructions.

Fluorescent in situ *hybridization*

Fluorescent *in situ* hybridization was performed as previously described (Labrador et al., 2005) and antisense, digoxigenin-labeled *comm* probes were generated as previously described (Yang et al., 2009).

Imaging and phenotypic analysis

Images were acquired using a spinning disk confocal system (Perkin Elmer) built on a Nikon Ti-U inverted microscope using a Nikon OFN25 60x objective with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head with Volocity imaging software. Images were processed using ImageJ. When scoring EW crossing, a segment was considered to have a crossing defect if one or both bundles of EW axons (one bundle per hemisegment, two bundles per segment) failed to reach the midline. When scoring ap crossing, a segment was considered to have an ectopic cross if it contained at least one continuous projection that extended all the way across the midline and reached the lateral bundle of ap axons on the contralateral side. *comm* expression was scored using Volocity imaging software. Embryos expressed *UAS-Tau-Myc-GFP* and EW or ap neurons were identified by anti-Myc immunostaining. If the cell body of a neuron could be detected by the *in situ* signal, that neuron was scored as positive. Crossing and *comm* expression were scored in EW neurons at stage 14 and in ap neurons at early stage 17. For all analyses, segments A1-A7 were scored. Midline crossing phenotypes and *comm* mRNA expression were scored blind to genotype whenever possible.

Biochemistry

To generate embryonic lysates, approximately 100 µl of dechorionated embryos were lysed in 0.5 ml of TBS-V (10 mM Tris (pH 8), 150 mM NaCl, 1 mM Na_3VO_4) supplemented with 1% Surfact-AMPS NP-40 (Thermo Scientific), complete protease inhibitor cocktail (Roche), and 1 mM PMSF by manual homogenization using a plastic

pestle. For the experiments in *psn* and *aph-1* mutants, 20-24 hour staged collections of embryos were generated. After homogenization, embryos were gently rocked at 4ºC for 10 minutes and centrifuged in a pre-chilled rotor for 10 minutes at 14,000 rpm. An aliquot of the total lysate was removed for analysis by Western blot. The remainder of the soluble phase was removed and incubated with 1-2 µg of rabbit anti-Myc antibody (Millipore) or rabbit anti-HA antibody (Covance) for 45 minutes with gentle rocking at 4ºC. 50 µl of a 50% slurry of protein A and protein G agarose (Invitrogen) were added to the tubes and samples were incubated for an additional 30 minutes with gentle rocking at 4ºC. Samples were washed three times in lysis buffer and then boiled for 10 minutes in 50 µl of 2x Laemmli SDS Sample Buffer. Proteins were resolved by SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose for subsequent overnight incubation at 4ºC with mouse anti-Myc (DSHB, 9E10, 1:1000) or mouse anti-HA (Covance, 16B12, 1:1000) in PBS supplemented with 5% dry milk and 0.1% Tween-20. Secondary antibodies (HRP goat anti-mouse, Caltag, 6920-100, 1:25,000) were applied for 1 hour at room temperature. Signals were detected using either ECL 2 or ECL Prime (Amersham) according to manufacturers instructions. Western blots were quantified using the gel analysis tool in ImageJ.

Cell culture and immunostaining

Drosophila S2R+ cells were maintained at 25ºC in Schneider's medium containing 10% fetal calf serum. Cells were seeded on poly-L-lysine-coated coverslips and transfected with 0.5 µg of the appropriate UAS-containing plasmid and 0.5 µg of pRmHa-3-Gal4 (Klueg et al., 2002) using Effectene (Qiagen) according to

manufacturer's instructions. After 24 hours, Gal4 expression was induced with 1 mM $CuSO_4$. 24 hours post-transfection, cells were treated with either 1 μM Leptomycin B (Cell Signaling) or the equivalent volume of vehicle (ethanol) for 6 hours, washed with PBS, and fixed with 4% paraformaldehyde/PBS for 15 minutes at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100/PBS for 5 minutes, blocked with 0.1% Triton X-100/PBS + 5% normal goat serum for 5 minutes, incubated with primary antibodies at room temperature for 1 hour and secondary antibodies at room temperature for 30 minutes. The following primary antibodies were used: rabbit anti-Myc (Sigma C3956, 1:500), mouse anti-nuclear lamin (DSHB, ADL84.12, 1:20). The following secondary antibodies were used: Cy3 goat anti-rabbit (Jackson 115-165-003, 1:1000), Alexa-488 goat anti-mouse (Life Technologies A11001).

Yeast transformations

The yeast strain used for both nuclear localization and activation reporter assays was Y860 [*α his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 ura3-1::URA3:lexAop-ADE2*] (a gift from Erfei Bi). Yeast cells were grown overnight at 30ºC in liquid YPD media until log phase (OD_{600} = 0.4-0.6). The PEG/lithium acetate method was used to transform yeast (Ito et al., 1983). Yeast were then plated onto solid SD media lacking histidine and grown at 30ºC for 2-3 days.

Acknowledgements

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Figure 2.1. Fra is cleaved by γ-secretase.

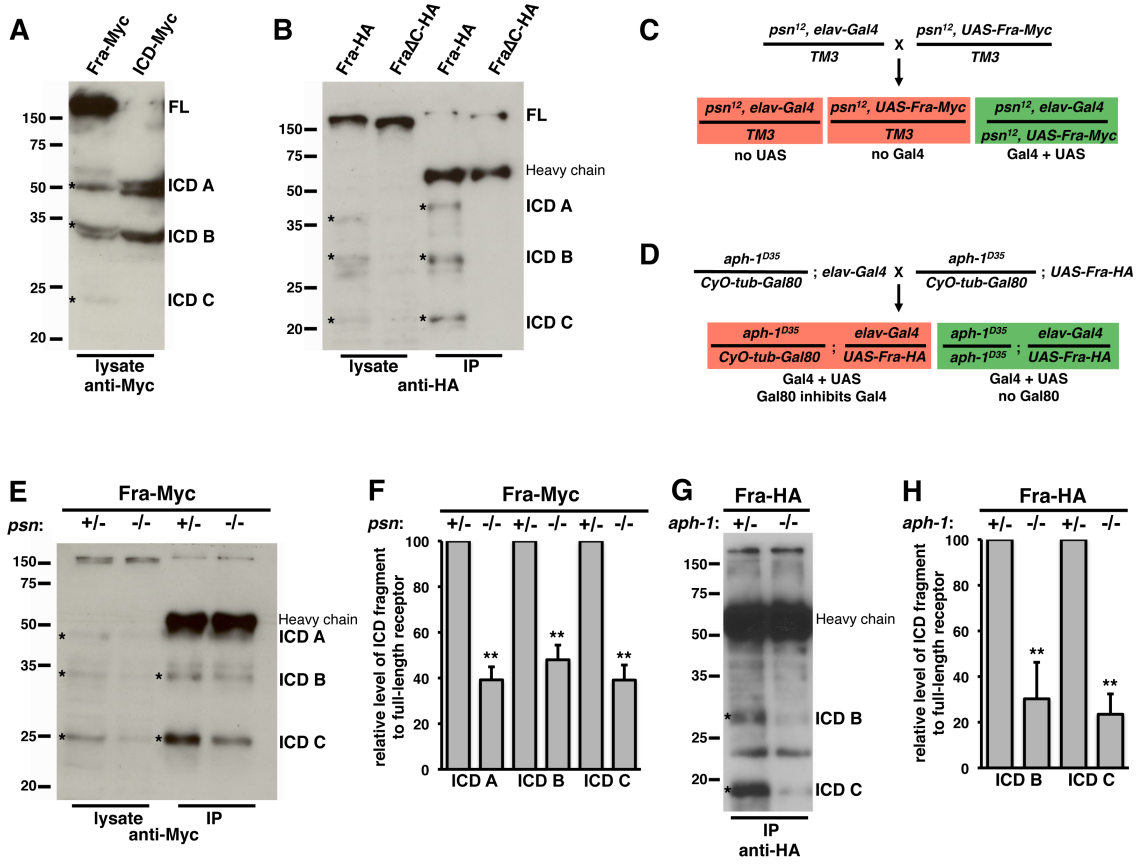


Figure 2.1. Fra is cleaved by γ-secretase.

A) Protein extracts were made from embryos pan-neurally expressing either a full-length Fra receptor with a C-terminal 6x Myc tag (first lane) or a similarly tagged Fra ICD (second lane). Proteins were resolved by SDS-PAGE and Western blots were performed with anti-Myc antibody. We detected full-length receptor at approximately 200 kDa (FL) and several C-terminal fragments, including species at approximately 50 kDa, 35 kDa, and 25 kDa (ICD A, ICD B, and ICD C; indicated by asterisks).

B) Protein extracts were made from embryos pan-neurally expressing either a full-length Fra receptor with a C-terminal 3x HA tag (first lane) or a Fra receptor missing its cytoplasmic domain (second lane) and HA-tagged proteins were immunoprecipitated from these extracts with anti-HA antibody (third and fourth lanes). Proteins were resolved by SDS-PAGE and Western blots were performed with anti-HA antibody. The 3x HA tag is smaller than the 6x Myc tag and, accordingly, ICD A, ICD B, and ICD C are shifted to smaller sizes of approximately 45 kDa, 30 kDa, and 20 kDa in both total protein extracts and immunoprecipitates (first and third lanes, indicated by asterisks). We did not detect these species in extracts or immunoprecipitates from embryos expressing FraΔC (second and fourth lanes). The position of the IgG heavy chain is indicated in the lanes that contain immunoprecipitates.

C) Schematic of strategy used to express *UAS-Fra-Myc* with *elav-Gal4* specifically in *psn* mutants.

D) Schematic of strategy used to express *UAS-Fra-HA* with *elav-Gal4* specifically in *aph-1* mutants.

E) Protein extracts from embryos pan-neurally expressing Fra-Myc in *psn*^{12} mutants were resolved by SDS-PAGE and Western blots were performed with anti-Myc antibody. All three C-terminal fragments (indicated by asterisks) are reduced in abundance relative to full-length receptor in the total lysates (compare first and second lanes) and the two smaller fragments are reduced in abundance in immunoprecipitates (compare third and fourth lanes). ICD A is obscured in immunoprecipitates by the IgG heavy chain.

F) Quantification of Fra ICD fragments in total lysates relative to full-length receptor in *psn*^{12}/+ compared to *psn*^{12}/*psn*^{12}. Data were analyzed by Student's t-test. ** indicates $p<0.005$. Error bars indicate standard deviation. Data are from six independent experiments.

G) Protein extracts from embryos pan-neurally expressing Fra-HA in *aph-1*^{D35} mutants were made and HA-tagged proteins were immunoprecipitated with anti-HA antibody. Proteins were resolved by SDS-PAGE and Western blots were performed with anti-HA antibody. The two smaller fragments (indicated by asterisks) are reduced in abundance in immunoprecipitates. The largest fragment is obscured by the IgG heavy chain.

H) Quantification of Fra ICD fragments relative to full-length receptor in *aph-1*^{D35}/+ compared to *aph-1*^{D35}/ *aph-1*^{D35}. Data were analyzed by Student's t-test. ** indicates $p<0.005$. Error bars indicate standard deviation. Data are from four independent experiments.

Figure 2.2. The Fra ICD is sufficient to fully rescue *comm* expression and partially rescue midline crossing defects in commissural neurons of *fra* mutants.

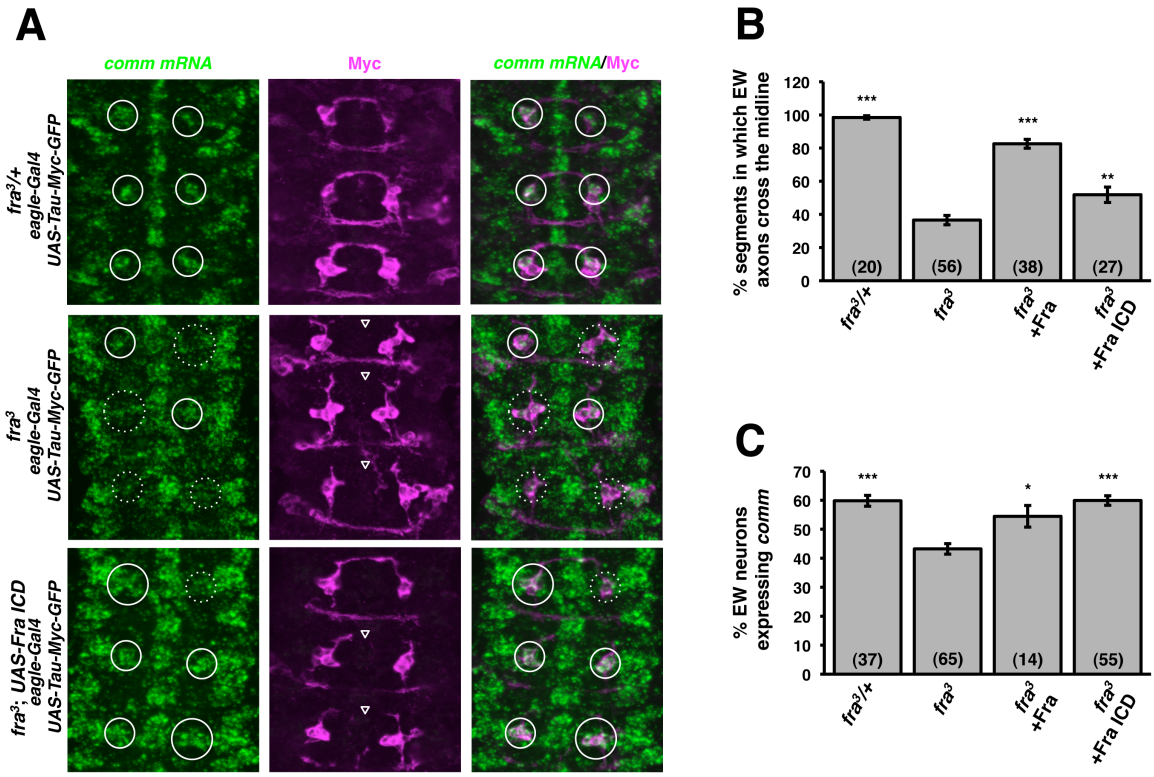


Figure 2.2. The Fra ICD is sufficient to fully rescue *comm* expression and partially rescue midline crossing defects in commissural neurons of *fra* mutants.

A) Fluorescent *in situ* hybridization for *comm* mRNA (green) in stage 14 embryos. Anterior is up. The cell bodies and axons of EW neurons are labeled with *eagle-Gal4* driving expression of *UAS-Tau-Myc-GFP*. Anti-Myc immunostaining is shown in magenta. White circles indicate the positions of EW neuron cell bodies. Solid circles indicate EW neurons that express *comm* and dotted circles indicate EW neurons that do not express *comm*. Open arrowheads indicate segments in which EW axons fail to cross the midline.

B) Quantification of EW axon crossing in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to *fra* mutants. ** indicates $p<0.005$, compared to *fra* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

C) Quantification of *comm* expression in EW neurons in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to *fra* mutants. * indicates $p<0.01$, compared to *fra* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

Figure 2.3. γ-secretase proteolysis of Fra is required for Fra's ability to regulate *comm* expression.

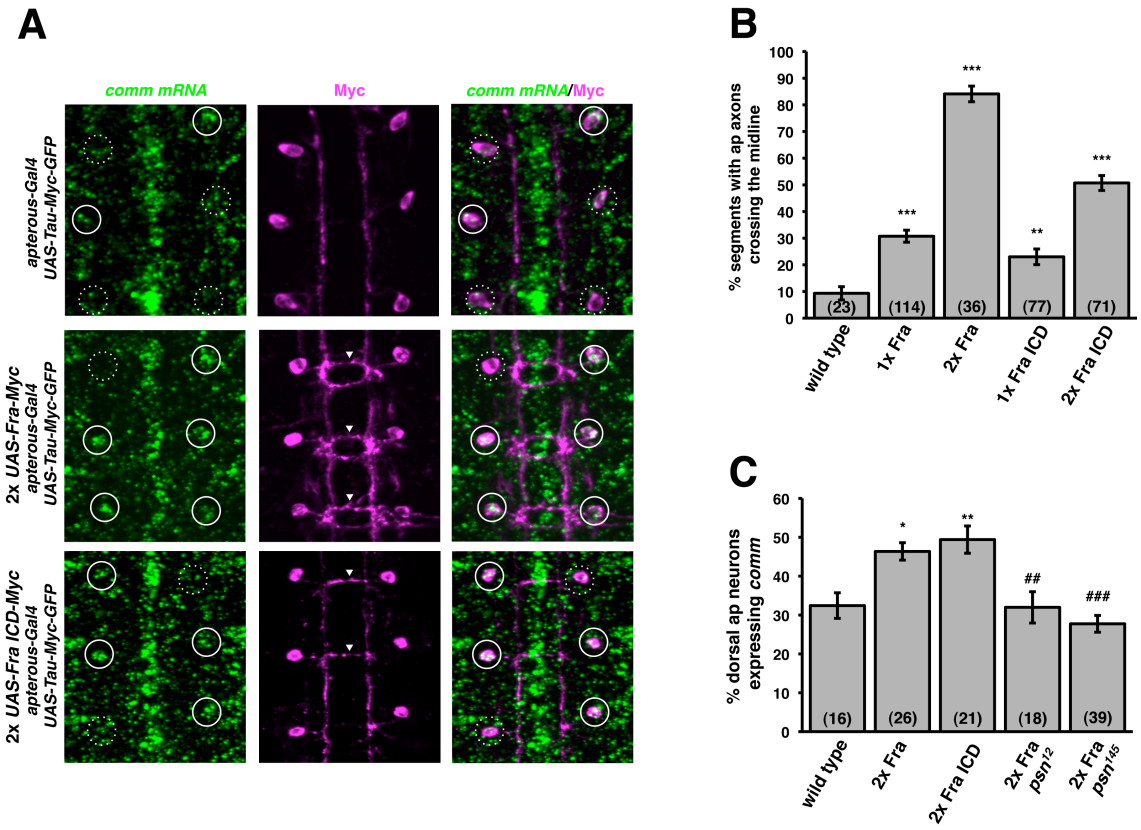


Figure 2.3. γ-secretase proteolysis of Fra is required for Fra's ability to regulate *comm* expression.

A) Fluorescent *in situ* hybridization for *comm* mRNA (green) in stage 17 embryos. Anterior is up. The cell bodies and axons of ap neurons are labeled with *apterous-Gal4* driving expression of *UAS-Tau-Myc-GFP*. Anti-Myc immunostaining is shown in magenta. White circles indicate the positions of dorsal apterous neuron cell bodies. Solid circles indicate ap neurons that express *comm* and dotted circles indicate ap neurons that do not express *comm*. Arrowheads indicate segments in which ap axons ectopically cross the midline.

B) Quantification of ap axon crossing in stage 17 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to wild type embryos. ** indicates $p<0.005$, compared to wild type embryos. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

C) Quantification of *comm* expression in dorsal ap neurons in stage 17 embryos. Data were analyzed by ANOVA, followed by Student's t-test. ** indicates $p<0.005$, compared to wild type embryos. * indicates $p<0.02$, compared to wild type embryos. ### indicates $p<0.0001$, compared to wild type embryos expressing two copies of Fra. ## indicates $p<0.002$, compared to wild type embryos expressing two copies of Fra. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

Figure 2.4. The Fra ICD shuttles between the cytoplasm and the nucleus.

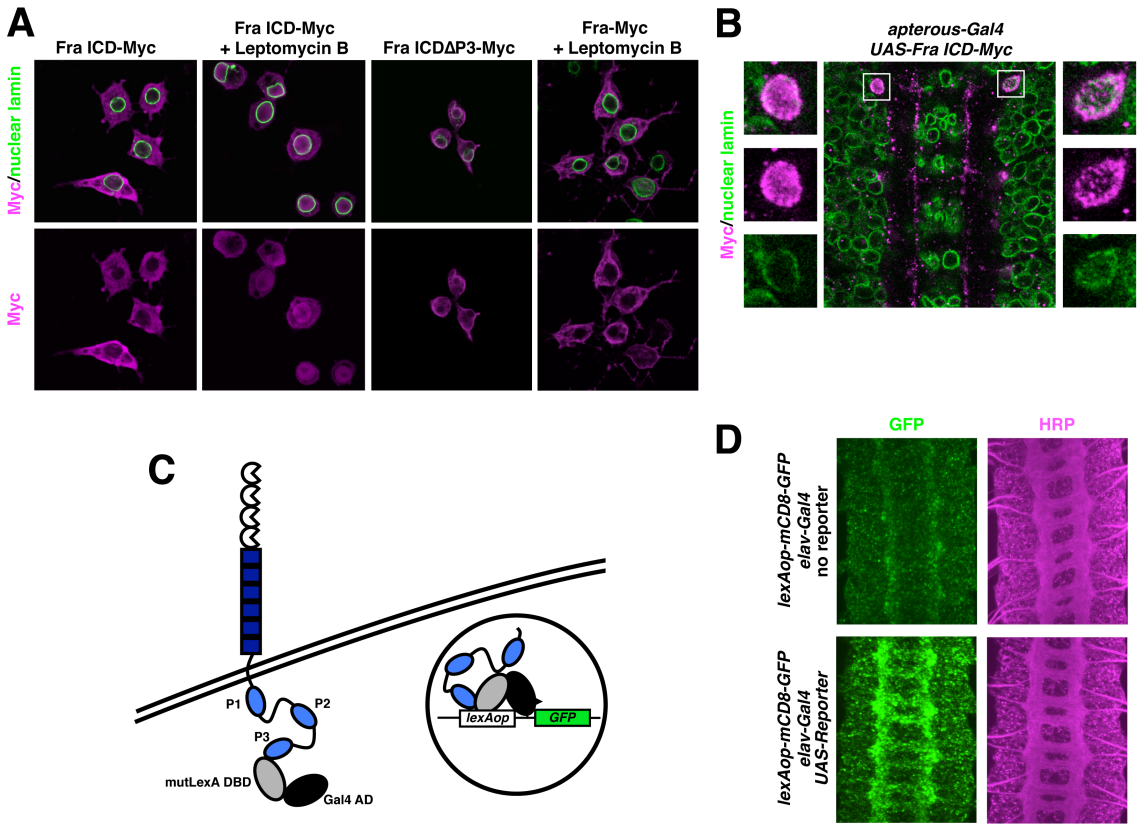


Figure 2.4. The Fra ICD shuttles between the cytoplasm and the nucleus.

A) S2R+ cells were transfected with the indicated Myc-tagged constructs and treated with Leptomycin B or vehicle, as indicated. Cells were immunostained with antibodies against Myc (magenta) and nuclear lamin (green). A single optical plane is shown.

B) Stage 16 embryo in which *apterous-Gal4* is driving expression of *UAS-Fra ICD-Myc*. Anterior is up. The embryo is stained with antibodies against Myc (magenta) and nuclear lamin (green). The regions inside the white boxes are enlarged to the sides of the main panel. Note that in the cell on the left, the Fra ICD is enriched in the nucleus, while in the cell on the right, the Fra ICD is largely excluded from the nucleus. A single optical plane is shown.

C) Schematic of nuclear localization reporter assay.

D) Stage 16 embryos stained with antibodies against GFP (green) and horseradish peroxidase (HRP; magenta). HRP immunostaining labels the axonal scaffold. Anterior is up.

Figure 2.5. Nuclear localization is redundantly encoded in the Fra ICD.

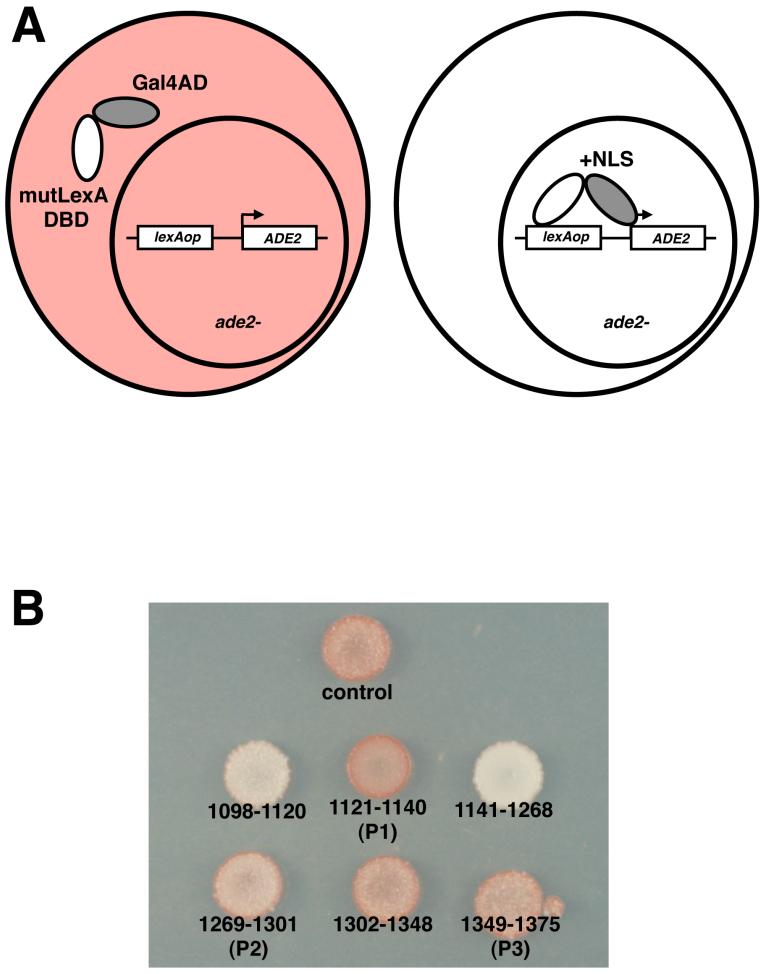
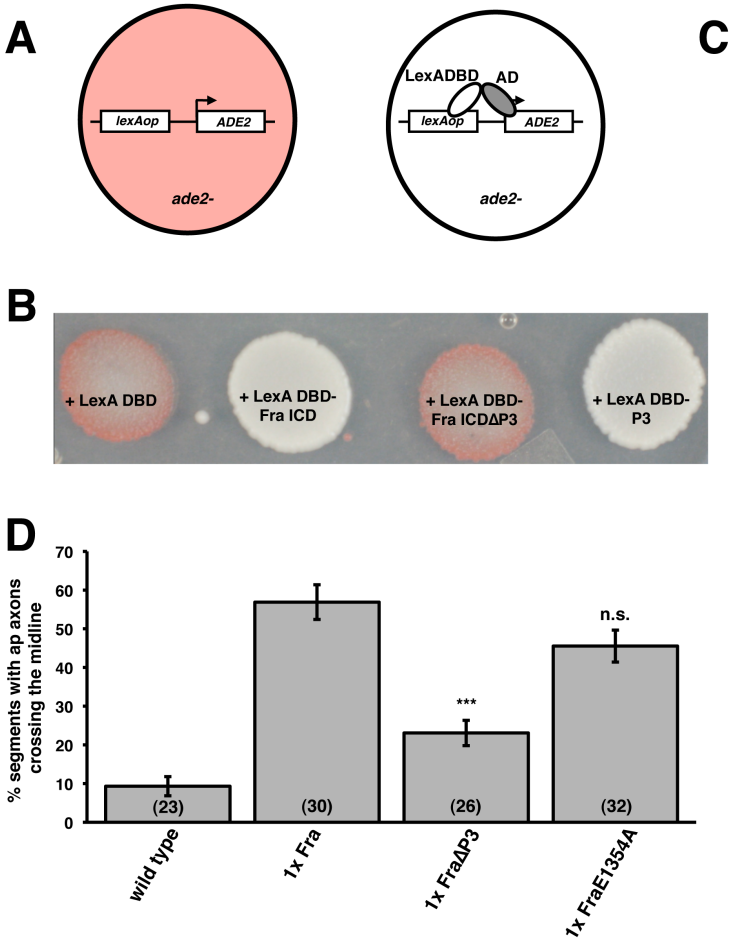


Figure 2.5. Nuclear localization is redundantly encoded in the Fra ICD.

A) Schematic of yeast nuclear localization assay.

B) Yeast were transformed with plasmids encoding fusions between the mutLexA DBD-Gal4 AD transcriptional activator and the indicated regions of the Fra ICD. Numbers indicate the amino acids included in each fragment. Note that 1098-1120 and 1141-1268 have strong activity and 1269-1301 (P2) has detectable, but weaker activity.

Figure 2.6. The Fra ICD encodes a transcriptional activation domain.



C

	export?	activation?
E1349A	Y	++
E1350A	Y	n.t.
L1351A	Y	++
N1352A	Y	+
Q1353A	Y	++
E1354A	Y	-
M1355A	Y	+
A1356	n.a.	n.a.
N1357A	Y	++
L1358A	N	-
E1359A	Y	++
G1360A	Y	++
L1361A	Y	-
M1362A	Y	+
K1363A	n.t.	++
D1364A	Y	+
L1365A	N	-
S1366A	Y	++
A1367	n.a.	n.a.
I1368A	Y	+
T1369A	Y	++
A1370	n.a.	n.a.
N1371A	n.t.	n.t.
E1372A	n.t.	n.t.

Figure 2.6. The Fra ICD encodes a transcriptional activation domain.

A) Schematic of yeast activation assay.

B) Yeast were transformed with plasmids encoding LexA DBD and the indicated forms of the Fra ICD. Note that P3 is necessary and sufficient for activation.

C) Summary of an alanine mutagenesis scan to identify point mutants within P3 that are specifically deficient for transcriptional activation. Data in the export column indicate whether the mutant ICD was exported from the nucleus in S2R+ cells. Y indicates that the ICD did not accumulate in the nucleus in the absence of Leptomycin B. N indicates that the ICD accumulated in the nucleus in the absence of Leptomycin B. Data in the activation column indicate whether the mutant ICD functioned as transcriptional activator in the yeast assay. ++ indicates that the yeast appeared white; + indicates that the yeast appeared light pink; - indicates that the yeast appeared dark pink. n.t. indicates that the mutant was not tested. n.a indicates alanine residues within P3. The mutants enclosed in the red boxes appear functional for nuclear export, but non-functional for transcriptional activation.

D) Quantification of ap axon crossing in stage 17 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to embryos expressing Fra. n.s. indicates $p>0.05$, compared to embryos expressing Fra. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

Figure 2.7. P3 is required for Fra's ability to regulate *comm* expression.

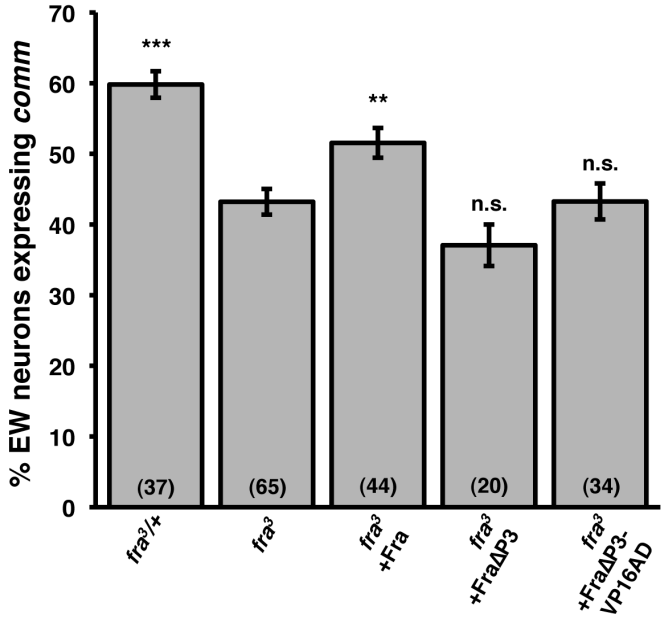


Figure 2.7. P3 is required for Fra's ability to regulate *comm* expression.

Quantification of *comm* expression in EW neurons in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to *fra* mutants. ** indicates $p<0.005$, compared to *fra* mutants. n.s. indicates $p>0.05$, compared to *fra* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

Figure 2.8. Fra's transcriptional activation function is required for its ability to regulate midline crossing and *comm* expression.

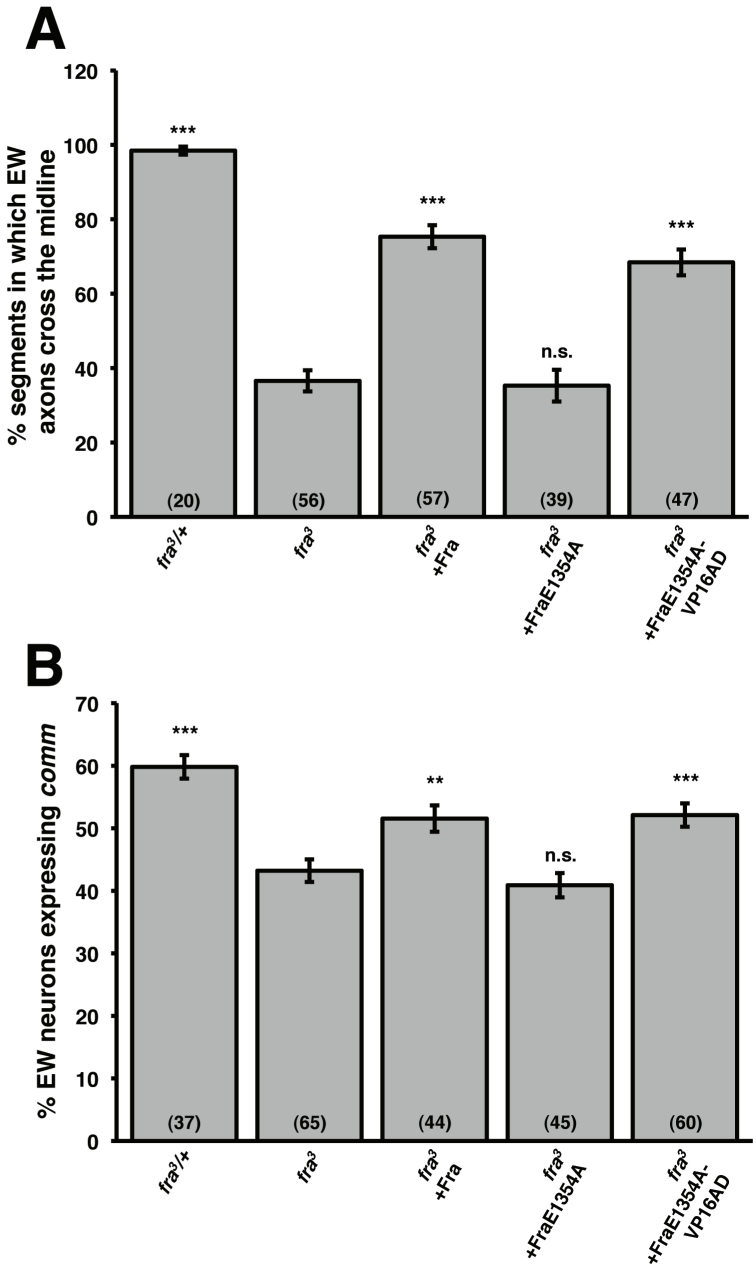


Figure 2.8. Fra's transcriptional activation function is required for its ability to regulate midline crossing and *comm* expression.

A) Quantification of EW axon crossing in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to *fra* mutants. n.s. indicates $p>0.05$, compared to *fra* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

B) Quantification of *comm* expression in EW neurons in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to *fra* mutants. ** indicates $p<0.005$, compared to *fra* mutants. n.s. indicates $p>0.05$, compared to *fra* mutants. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

Figure 2.9. FraE1354A antagonizes midline crossing in commissural neurons.

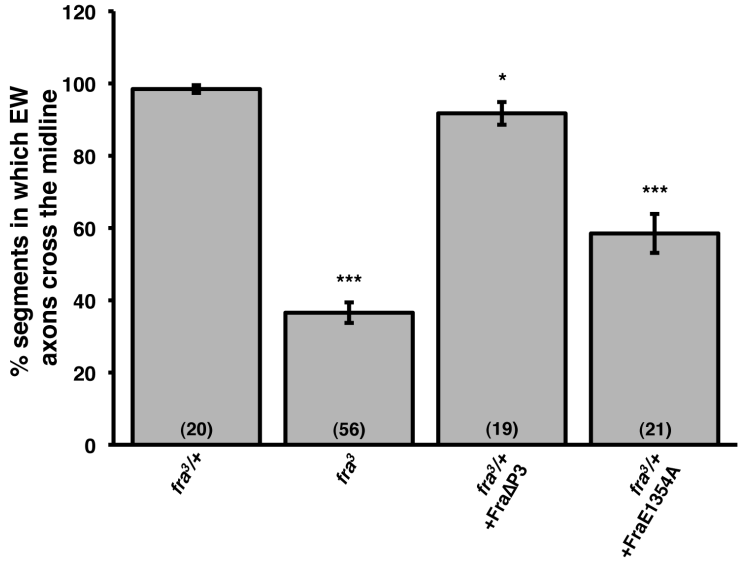


Figure 2.9. FraE1354A antagonizes midline crossing in commissural neurons. Quantification of EW axon crossing in stage 14 embryos. Data were analyzed by ANOVA, followed by Student's t-test. *** indicates $p<0.0001$, compared to *fra* heterozygotes. * indicates $p<0.05$, compared to *fra* heterozygotes. Error bars indicate SEM. Number in parentheses indicates number of embryos scored.

Figure 2.10. A model for Fra-dependent *comm* expression.

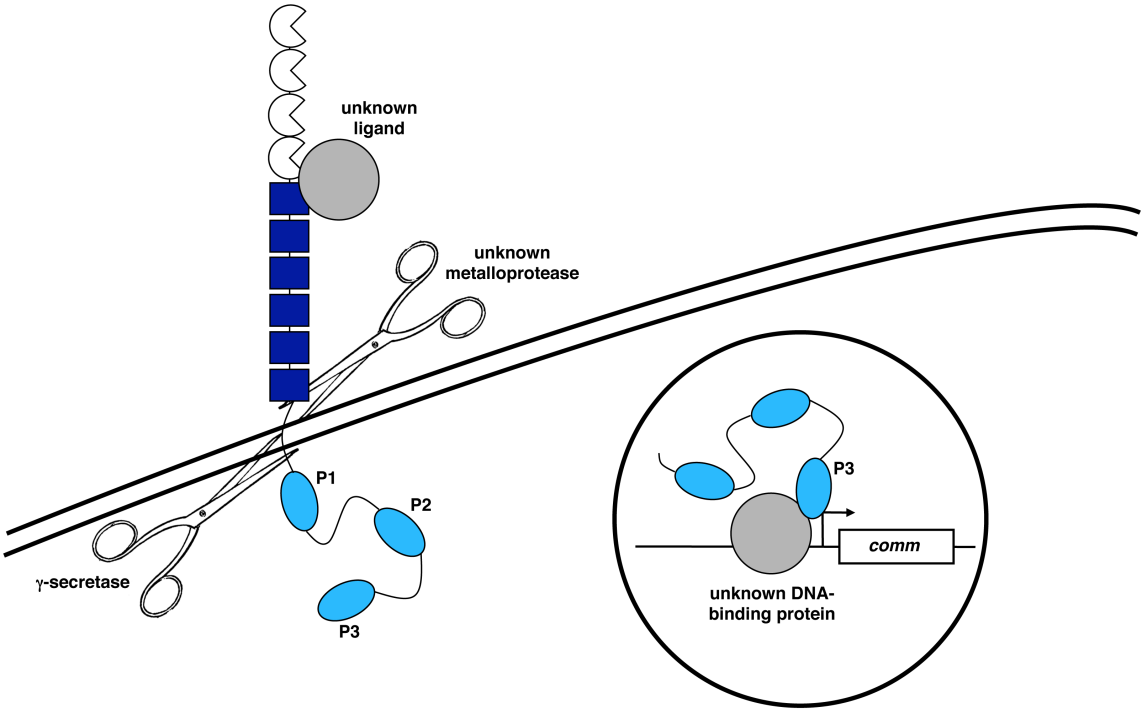


Figure 2.10. A model for Fra-dependent *comm* expression.

Full-length Fra is cleaved by γ-secretase, likely in response to an unknown ligand, which stimulates metalloprotease cleavage. The soluble ICD then translocates to the nucleus, where it functions as a transcriptional activator to induce *comm* expression, either directly or indirectly. The Fra ICD likely associates with DNA by interacting with one or more unknown DNA-binding proteins. P3 functions as a transcriptional activation domain.

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Chapter 3

Future directions

My thesis research found that, in addition to its canonical role signaling locally in the growth cone to promote axon outgrowth and attraction in response to Netrins, the axon guidance receptor Frazzled (Fra)/Deleted in Colorectal Cancer (DCC) is cleaved to release its intracellular domain (ICD), which functions as a transcription factor *in vivo* to regulate the expression of the Slit-Robo antagonist, *commissureless* (*comm*) and promote midline crossing of commissural axons in the *Drosophila* embryo. Several questions about the mechanism through which Fra regulates transcription remain unanswered, including what genes act upstream of Fra in the *comm*-regulatory pathway and how the Fra ICD interacts with DNA. In addition, my thesis work raised several broader questions, including whether this mechanism is conserved in other organisms, what other genes Fra and its orthologs might regulate in commissural neurons and other cell types, and whether other axon guidance receptor ICDs have nuclear functions *in vivo*. In this chapter, I discuss experimental approaches to address these questions, with a focus on experiments that can be performed in the *Drosophila* embryo.

Genes upstream of Fra that control Fra's ability to regulate transcription

Pharmacological experiments suggest that metalloprotease cleavage precedes γ-secretase cleavage of DCC (Galko and Tessier-Lavigne, 2000; Bai et al., 2011). It is not clear whether metalloprotease processing of DCC is Netrin-dependent, dependent on another ligand, or ligand-independent. As metalloprotease processing of many substrates is ligand-gated and Fra's transcriptional regulation of *comm* is Netrin-independent (Yang et al., 2009), it seems likely that another ligand regulates proteolysis of Fra. In addition, the metalloprotease that cleaves Fra prior to γ-secretase proteolysis has not been identified. Thus, genes that act upstream of γ-secretase to regulate Fra-dependent *comm* expression remain unknown.

One candidate ligand for Fra is CG31619, which is orthologous to MADD-4 in *C. elegans*. MADD-4 is a secreted protein that can bind to UNC-40/DCC and guides sensory neurons and muscle arms in an UNC-40-dependent manner (Seetharaman et al., 2011; Chan et al., 2014). *CG31619* mutants should be analyzed to determine whether they have defects in *comm* expression and EW axon crossing and biochemical experiments should be performed to determine whether CG31619 can bind to Fra. If CG31619 is not a ligand for Fra or if it binds Fra, but is not required for its ability to regulate *comm*, perhaps the relevant Fra ligand(s) could be identified in a screen. Soluble, alkaline phosphatase-tagged receptor ectodomains can be used to probe live-dissected embryos and detect ligands (Flanagan et al., 2000). This approach has been used in fly embryos over-expressing secreted and cell surface molecules to identify ligands for orphan receptors, and a panel of fly lines in which secreted and cell surface molecules are under *UAS* control already exists (Fox and Zinn, 2005; Kurusu et al., 2008).

The metalloprotease that cleaves DCC has not been identified, but Neogenin is reportedly cleaved by the metalloprotease Tumor Necrosis Factor-α–converting enzyme (TACE/ADAM17; Okamura et al., 2011). Flies have an orthologous gene (*tace*), but neither mutant phenotypes nor expression data have been reported. Flies have fewer than ten genes with confirmed or predicted metalloprotease activity, so examining each (with preference given to those with strong expression in the embryonic CNS) should not be prohibitive. To test whether candidate metalloproteases cleave the Fra ectodomain, Fra with an extracellular epitope tag could be expressed in metalloprotease mutants and the abundance of Fra's ectodomain in embryo lysates evaluated by Western blot, using approaches similar to those that we used to examine the generation of Fra ICD fragments in *psn* and *aph-1* mutant embryos (see Results section of Chapter 2 and Figure 2.1C-H).

For any candidate ligand or metalloprotease, mutants should be analyzed to determine whether they have defects in commissural axon guidance and, more importantly, *comm* expression. Analysis of midline crossing phenotypes may be complicated. For example, embryos with mutations in genes encoding additional Fra ligands might not have defects in midline crossing, as the Netrin-dependent outputs of Fra signaling will remain intact. Embryos lacking both fly *Netrin* genes (*NetAB*) and the ligand required for Fra proteolysis should have midline crossing defects comparable to those seen in *fra* mutants, unless Fra promotes midline crossing through yet another pathway. Metalloproteases have many targets, so analysis of midline crossing defects in metalloprotease mutants may be confounded by several different factors. For example, the ADAM metalloprotease Kuzbanian (Kuz) is required for Robo signaling, and *kuz* mutant embryos have a phenotype that reflects a loss of midline repulsion (Fambrough et

al., 1996; Schimmelpfeng et al., 2001; Coleman et al., 2010). This phenotype could mask a role for Kuz in promoting midline attraction. In addition, Kuz is maternally deposited (Fambrough et al., 1996; Rooke et al., 1996), so zygotic mutant embryos have residual Kuz protein. Other metalloproteases may also be maternally deposited, especially if they have substrates that play essential roles in the early embryo, and this maternal product could preclude observation of a midline crossing phenotype. Thus, analysis of *comm* expression will likely be much more informative.

Fra-binding proteins, including DNA-binding proteins

Fra does not have an obvious DNA-binding domain (DBD) and likely associates with chromatin through interactions with other proteins that have DNA-binding activity, as discussed in Chapter 2. The FraE1354A point mutant could be a useful reagent for identifying such proteins, as well as other proteins that interact with the Fra ICD. In a yeast two-hybrid screen, a bait protein is fused to a DBD, while a library of prey proteins is fused to an activation domain. The bait and prey are then co-expressed in yeast that have a reporter that will only be expressed if the bait and prey interact, bringing the DBD and AD together. Therefore, a yeast two-hybrid screen cannot be performed using bait that has an AD, because reporter expression will be driven even in the absence of prey. For this reason, it has not been possible to perform a yeast two-hybrid screen with the full-length Fra ICD. However, the FraE1345A ICD would be ideal for such a screen, as it is structurally intact, but lacks a functional AD (Figure 2.6C-D). This screen has the potential to identify nuclear Fra-binding proteins, including those that have DNA-binding activity. In addition, this approach may identify proteins that bind to Fra and participate

in canonical Netrin-Fra signaling. Co-activators that bind to P3 may not be identified in this screen, as the E1354A mutation prevents Fra from functioning as a transcriptional activator in a reporter assay (Figure 2.6C). For nuclear proteins identified in this screen, mutants should be analyzed for defects in commissural axon guidance and *comm* expression, as described for candidate ligands and metalloproteases.

Conservation in vertebrates

Vertebrates do not have an ortholog of *comm* and instead inhibit Slit-Robo repulsion in pre-crossing commissural axons through Robo3 (Sabatier et al., 2004). Nevertheless, it will be interesting to determine whether DCC ICD-dependent transcriptional activation is required for commissural axon guidance in the spinal cord, particularly in light of our observations suggesting that Fra may have multiple transcriptional targets in the *Drosophila* commissural neurons that are important regulators of midline crossing, as discussed in Chapter 2. DCC is required for the growth and guidance of spinal commissural neurons, as discussed extensively in Chapter 1, and axon guidance phenotypes in the embryonic spinal cord can be rescued in intact mouse embryos (Chen et al., 2008). The DCC ICD can function as a transcriptional activator in *in vitro* reporter assays (Taniguchi et al., 2003). For rescue experiments, a mouse DCC variant should be generated that is functionally analogous to FraE1354A; that is, it should have a specific deficit in DCC's transcriptional activation function. The E1354 residue in Fra is conserved in DCC, so the corresponding point mutant should be tested for transcriptional activation in yeast and for nuclear export in mammalian cells *in vitro*. If this mutant does not behave as expected, an alanine mutagenesis scan can be performed

in order to generate a suitable reagent for *in vivo* rescue experiments. DCC that is specifically deficient for its transcriptional activation function should then be introduced into *Dcc* mutant embryos and spinal commissural axon guidance should be evaluated.

Other transcriptional targets of Fra

The simplest way to identify genes that are regulated by Fra would be to extract RNA from wild-type and *fra* mutant embryos and analyze it in RNA-seq or cDNA microarray experiments. However, this approach would not distinguish between genes that are direct targets of Fra's transcriptional activity and those that are regulated through indirect mechanisms. Reports that Netrin's ability to elicit outgrowth *in vitro* depends on MAP kinase and calcineurin activity (Forcet et al., 2002; Graef et al., 2003) suggest that Fra may have the ability to indirectly regulate gene expression. The reagents I generated, specifically the *UAS-FraE1354A* and *UAS-FraE1354A VP16AD* transgenic lines, could be helpful in efforts to specifically identify genes that are regulated by Fra's transcriptional activity, rather than by an indirect Fra-dependent mechanism.

For example, RNA could be extracted from from embryos pan-neurally expressing *UAS-Fra*, *UAS-FraE1354A*, and *UAS-FraE1354A VP16AD*, as well as wild-type controls, and analyzed in RNA-seq or cDNA microarray experiments. Including a *UAS-GFP* marker in these experiments would facilitate cell sorting and allow neuronal RNA to be analyzed. Direct transcriptional targets of Fra are likely to be up-regulated when FraE1354A VP16AD, but not FraE1354A, is expressed. In contrast, indirect targets of Fra – genes that are regulated by Fra through mechanisms that do not depend on Fra's function as a transcriptional activator – are likely to be up-regulated when both

FraE1354A and FraE1354A VP16AD are expressed. Embryos expressing wild-type Fra would be a positive control, while wild-type embryos would be a negative control. A similar approach could be used in *fra* rescue experiments, but these would be more technically challenging, as mutant embryos would have to be sorted away from their siblings.

ICDs of other axon guidance receptors as transcription factors

The ICD of human Robo1 can enter the nucleus (Seki et al., 2010) and it is likely that the ICDs of other axon guidance receptors share this property, as discussed in Chapter 2. In addition, we have observed the ICDs of all three *Drosophila* Robo receptors in the nucleus in S2R+ cells (data not shown). The three *Drosophila* Robos play partially overlapping, but distinct, roles in axon guidance in the embryo. Robo and Robo2 signal midline repulsion (Seeger et al., 1993; Kidd et al., 1998; Rajagopalan et al., 2000a; Simpson et al., 2000b), Robo2 and Robo3 play important roles in specifying the mediolateral position of longitudinal axon tracts (Rajagopalan et al., 2000b, Simpson et al., 2000a), and Robo2 plays additional roles in promoting midline crossing (Simpson et al., 2000a, Spitzweck et al., 2010) and guiding motor axons (Santiago et al., 2014). Robo2 and Robo3 also regulate the differentiation of serotonergic neurons and, intriguingly, the expression of *SerT* mRNA is reportedly decreased in the serotonergic EW neurons in the embryo (Couch et al., 2004). In addition, Robo2 inhibits the expression of the transcription factor Prospero in intestinal stem cells to control cell fate, although it is not clear whether this regulation is at the mRNA or protein level (Biteau and Jasper, 2014). Thus, Robo receptors have been implicated in a number of different

biological processes in the fly embryo and *robo*, *robo2*, and *robo3* mutants have distinct and well-characterized phenotypes. Determining whether expression of any of the Robo ICDs can rescue these mutant phenotypes would be a good starting point for examining whether Robo ICDs can function as transcription factors *in vivo*.

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