The planar cell polarity regulator Flamingo cooperates with Netrin/Frazzled signaling during axon targeting in the *Drosophila* embryonic CNS

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Ai miei genitori

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Abbreviations

Abl	Abelson Tyrosine Kinase
AC	Anterior Commissure
BMP	Bone Morphogenetic Protein
BSC	Bloomington Stock Center
cAMP	Cyclic Adenosine Monophosphate
CAMs	Cell Adhesion Molecules
CNS	Central Nervous System
COMM	Commissurreless
CRD	Cysteine-rich Domain
DCC	Deleted in Colorectal Carcinoma
DGO	Diego
Dsh	Dishevelled
Dscam	Down Syndrome cell adhesion molecule
ECM	Extracellular Matrix Molecules
EGF	Epidermal Growth Factor
Egl	Eagle
Elav	embryonic lethal abnormal vision
Eph	Ephrin receptor
Esn	Espinas
FasII	Fasciclin II
Fmi	Flamingo
Fn	Fibronectin
Fra	Frazzled
Fz	Frizzled
F-actin	Fibrillar-actin
GAP	GTPase Activating Protein
Gcm	Glia cell missing

GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescence Protein
GPCRs	G-Protein Coupled Receptor
GPI	glycophosphatidylinositol
HRM	Hormon Receptor Domain
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
KD	Kinase Dead
LRR	Leucine-rich Repeat
MAM	Meprin/A5-protein/PTPmu
MLC	Myosin Light Chain
MT	Microtubule
Net	Netrin
N-cad	N-cadherin
NGS	Normal Goat Serum
PAK	p21-activated kinase
PC	Posterior Commissure
PCP	Planar Cell Polarity
Pk	Prickle
RBD	Raf-like Ras-binding domain
Repo	Reverse Polarity
ROBO	Rondabout
SAM	Sterile Alpha Motif
Sim	Single-minded
S2	Schneider 2 Cells
Sema	Semaphorin
Shh	Sonic Hedgehog
TM	Transmembrane
TSP	Thrombospondin

UAS	Upstream Activating Sequence
UNC	Uncoordinated
Vang	Vang Gogh
VNC	Ventral Nerve Cord
Wnt	Wingless and Int
WT	Wild-Type

Summary

Accurate target selection and proper synaptic connectivity are crucial for the functionality of neuronal circuits. In order to construct these networks, axons from developing neurons have to dynamically respond to complex guidance signals within the environment and from neighboring cells. These mechanisms have been intensily studied in the *Drosophila* embryonic midline, where several conserved families of guidance cues and receptors play a role in axon pathfinding. Here, signaling of Netrin and its receptor Frazzled (Net/Fra) is important for midline crossing of commissural axons, which sense the attractive cues NetA and NetB on the midline through the Fra receptor on their growth cones. Despite the importance of these molecular pathways, lack of *Net* or *fra* affects only some commissures, suggesting that other molecules are acting redundantly in order to fulfill this function. Such redundancy has been shown in vertebrates, where the morphogen Sonic Hedgehog (Shh) mediates midline attraction together with Netrin-1; however, no gene other than Net has been described to play such a role in *Drosophila*.

Recently, molecules belonging to the “core” planar cell polarity (PCP) proteins have been implicated in axon guidance in both vertebrate and invertebrates systems; in particular, evidences about roles in midline crossing have been accumulating. This thesis work demonstrates that the atypical cadherin Flamingo (Fmi) plays a guidance role in the embryonic CNS. Fmi is one of the “core“ PCP genes in *Drosophila*, and is also involved in dendrite morphogenesis and sensory neuron pathfinding. In *Net/fmi* or *fra/fmi* double mutants the majority of commissures in the *Drosophila* embryonic CNS are affected. In particular, most of the posterior commissural axon tracts appear to be lost completely. Moreover, the longitudinal axon tracts show a strongly disorganized fasciculation. Notably, these defects are not observed in *fmi* single mutants, suggesting a partial redundancy between Net/Fra pathway and Flamingo. Rescue experiments indicate that Fmi is required in neurons, and exclude a possible function in glia or midline cells. In addition, protein domain analysis revealed that Fmi acts as signaling molecule in this system. Cell-autonomous intracellular signaling is also supported by the observation that overexpression of Fmi in longitudinal neurons

can elicit ectopic midline crossing, whereas overexpression in a specific subset of commissural neurons is sufficient to partially suppress the *fra* mutant phenotype, suggesting that Fmi and Fra might signal through the same downstream pathway. This study also investigates the possible molecular pathway required for Fmi function in midline pathfinding. First, the analysis rules out the involvement of known cytoplasmic interacting proteins, such as Espinas or the Net/Fra downstream effector Abl, Instead, implicates the RhoGTPase Rac1 as important effector of Fmi-mediated midline crossing. Together with the analysis of other “core” PCP genes and Wnt family members the present work proposed that Fmi acts through a novel PCP signaling-independent mechanism during the formation of the *Drosophila* midline.

Taken together, the data presented in this work suggest that Fmi as a signaling molecule acts cooperatively with Fra signaling during specific aspects of *Drosophila* midline targeting, supporting the idea that polarity-regulating genes can play important roles not only in stationary systems such as epithelial sheets, but also in highly motile structures such as axonal growth cones. Moreover it reveals an important relationship between members of the PCP polarity system and other cardinal guidance systems.

1 INTRODUCTION

What makes the nervous system such an astonishing structure and distinguishes the brain from the other organs is not only the tremendous number of cells, but also their capability of communication in a precisely structured manner within functional neuronal networks. The establishment of this complex connectivity can only be achieved when each neuron, during its development, finds and targets the appropriate post-synaptic cell, choosing it over many other similar ones. How can each neuronal cell accomplish this complicated task? Developing neurons are equipped at the tip of their axons with a highly specialized structure, the growth cone, which is capable of extending and exploring the surrounding environment, eventually finding the synaptic partner. During this targeting process, a specific repertoire of receptors located on the membrane of the growth cone allows them to sense guidance cues. Guidance cues are molecules presented by cells in the environment of a growing neurite. Understanding and elucidating the cellular strategies used during neuronal pathfinding, the mechanisms involved in axon guidance *in vivo* and *in vitro* as well as the underlying molecular pathways are central goals in neurobiology. Valuable information has been obtained in the last three decades, which will be briefly reviewed in the following paragraphs.

1.1. Molecular and cellular mechanisms of axon guidance

For most of the developing neurons, targeting means navigating over long distances, encountering along the path many different types of cells which, in turn, provide different kinds of signals. To “simplify” and guarantee precise axonal targeting, long distances are usually broken into shorter steps, normally defined by the presence of intermediate targets. Those intermediate targets are usually choice points where the axons have to turn or change their direction. These can be either neurons or glia cells. Interaction of an outgrowing axon with intermediate targets, neurons and glia is based on signaling pathways activated by guidance cues and their receptors. Guidance cues can be divided into four categories: chemo-attractants, chemo-repellents, contact-attractants and contact-repellents (Figure 1-1, Tessier-Lavigne & Goodman, 1996). Long-range

cues are usually secreted proteins that can diffuse over long distances; they can induce either attraction, like Netrins (Rajasekharan & Kennedy, 2009), or repulsion, like Slits (Rajagopalan et al., 2000). Short range cues normally act through contact-mediated mechanisms, and are usually membrane-bound proteins (for example Ephrins) or extracellular matrix (ECM) molecules, like Cadherins. These short range cues usually regulate axon-axon interactions, such as fasciculation/defasciculation processes. In the following paragraphs, I'll describe the main axon guidance protein families.

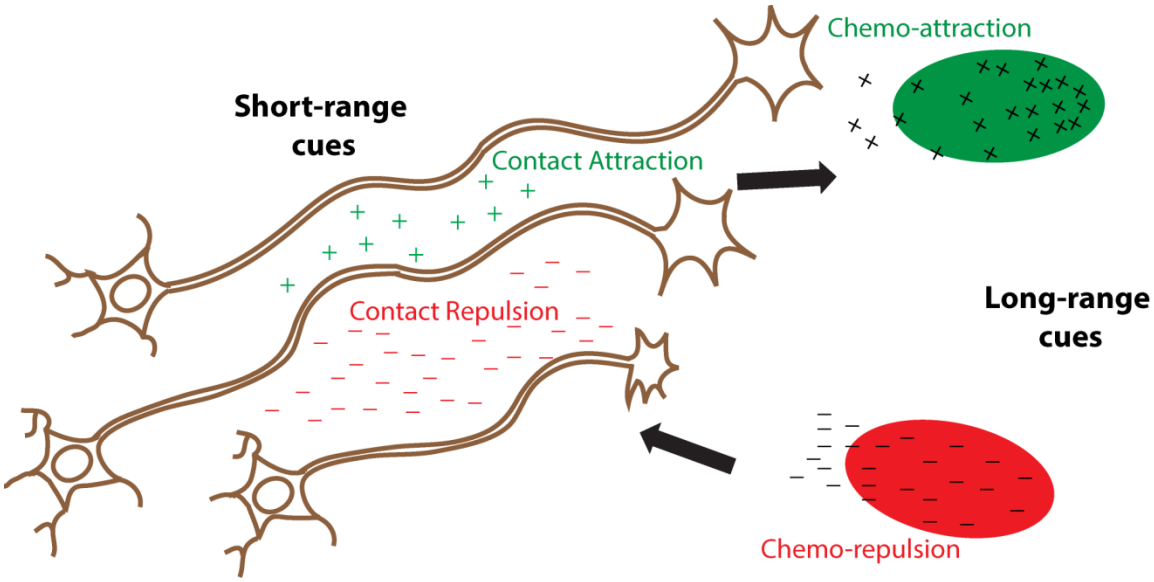


Figure 1-1 Axon guidance modalities elicited by guidance cues.

Developing axons are guided during their targeting process by molecules acting through four different modalities: chemo-attraction, chemo-repulsion, contact attraction and contact repulsion. Axons usually extend toward a source of attractant cues (green), while avoiding the source of repellent cues (red). These long-distance mechanisms are usually mediated by molecules capable of diffusing far away from their origin, thus forming gradients. While extending, they encounter other cells (neurons, glia, etc.,) that can express contact-attractants (green +), favoring fasciculation, or contact-repellents (red -), causing defasciculation. Adapted from (Tessier-Lavigne & Goodman, 1996)

Netrins are a small family of secreted guidance cues. They were originally identified in *C.elegans,* where the Netrin homologue *Unc-6* was shown to be required for guidance of migrating cells and pioneering axons along the dorso-ventral axis of the epidermis (Hedgecock et al., 1990). Here, Netrin is located at the ventral midline (Wadsworth et al. 1996). However, the existence of a midline chemotropic axon guidance factor was already revealed thanks to pioneer experiments on explants of embryonic rat floor plate. In these studies, it was shown that ventral midline floor plate cells promote outgrowth of dorsal spinal commissural axons, when cultured at a distance (Tessier-lavigne et al., 1988). Shortly after, it was shown that the mammalian Netrin-1 mediates this process also *in vivo* (Kennedy et al., 1994; Serafini et al., 1994, Serafini et al., 1996). Analogous functions were shown for the *Drosophila* Netrins (Harris et al., 1996; Mitchell et al., 1996). Netrins can act as both long-range signals, diffusing far away from their source (Kennedy et al., 1994; Kennedy et al., 2006), but also as short-range permissive cues (Brankatschk & Dickson, 2006; Deiner et al., 1997). Netrins are bifunctional, capable of attracting some neurons and repelling others. Genes of the UNC5 family encode for Netrin receptors that mediate repulsion (Hong et al., 1999; Keleman & Dickson, 2001; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992), whereas attraction is mediated by receptors of the DCC family, including UNC-40 in *C.elegans* (Chan et al., 1996), DCC in vertebrates (Keino-Masu et al., 1996) and Frazzled in *Drosophila* (Kolodziej et al., 1996). Dscam has been proposed to also mediate Netrin-dependent attraction (Andrews et al., 2008; Ly et al., 2008). Apart from organizing midline crossing of several classes of neurons, Netrins are also required for targeting of neurons in other systems. For instance, Netrin-1 is required for the axons of retinal ganglion cells to exit the retina and enter the optic nerve (Deiner et al., 1997), for the projection of dopaminergic neurons in the ventral midbrain (Lin et al, 2005) as well as for the formation of thalamo-cortical projections (Braisted et al., 2000).

Slits are large secreted proteins acting as repulsive guidance cues. They were initially identified as embryonic midline repellents in *Drosophila* and vertebrates (Kidd et al., 1999; Li et al., 1999). Receptors of the Robo family mediate Slit dependent repulsion (Kidd et al., 1998; Zallen et al., 1998). Slit/Robo signaling also controls midline crossing of olfactory sensory neurons in *Drosophila*

(Jhaveri et al., 2004) and, in vertebrates, of retinal axons (Hutson & Chien, 2002; Plump et al., 2002), olfactory bulb axons (Nguyen-Ba-Charvet et al., 2002) and cortical axons (Bagri et al., 2002). Additionally, Slit/Robo mediates topographic map formation in both visual and olfactory systems in *Drosophila*, regulating segregation of lamina cells and lobula cells (Tayler et al., 2004) and positioning of olfactory sensory axons within the antennal lobes (Jhaveri et al., 2004), respectively. Robo also mediates Slit-dependent axonal and dendritic branching (Ma & Tessier-Lavigne, 2007; Whitford et al., 2002).

The **Semaphorin** protein family includes both secreted and transmembrane proteins. The first Semaphorin identified was the grasshopper transmembrane protein Semaphorin-1a which is required for correct pathfinding of pioneer sensory axons in the developing grasshopper limb (Kolodkin et al., 1992). Sema3A was the first vertebrate Semaphorin identified and was purified from brain extracts as a factor functioning as axonal repellent *in vitro* (Luo et al., 1993). Around 20 different family members have been discovered in vertebrates, and all of them contain a common Semaphorin domain of 500 amino acids crucial for the association with their receptors, which belong to the Plexin family (Tamagnone & Comoglio, 2000). However, some secreted vertebrate Semaphorins do not bind directly to Plexins, but to their obligate co-receptors Neuropilin-1 and Neuropilin-2; Neuropilins then form an active holoreceptor complex with Plexin receptor (Kawasaki et al., 2002; Tran et al., 2007). Although Semaphorins usually have repulsive activity; some Semaphorins are also bifunctional, capable of mediating both attraction and repulsion. For instance, Sema3C promotes growth of cortical axons (Bagnard et al., 1998) and Sema3F stimulates growth in olfactory bulb axons (de Castro et al., 1999). Additionally, transmembrane Semaphorins can also act as receptors, for example regulating dendritic targeting in the *Drosophila* olfactory system (Komiyama et al., 2007) or guiding photoreceptors in the *Drosophila* visual system (Yu et al., 2010).

Ephrins are the last family of classical guidance cues. Similarly to Semaphorins, ephrins are also membrane-bound ligands. There are two subfamilies: class A ephrins are tethered to cell surface via GPI linkage, whereas class B Ephrins are transmembrane proteins. They interact with Class A Eph receptors and Class B Eph receptors respectively (Gale et al., 1996). Eph receptors constitute the

largest family of receptor tyrosine kinases. In the inactive form, Eph kinase activity is auto-inhibited through binding to their juxtamembrane domain. Upon ephrin activation, two tyrosine residues in the same juxtamembrane domain are phosphorylated, relieving inhibition of the kinase domain and, consequently, causing a conformational change that allows initiation of the downstream signaling (Klein, 2012). Similarly to Semaphorins, Ephrins are also capable of eliciting bidirectional signaling, activating downstream pathways in both receptor and ligand cells (Kullander & Klein, 2002). Activation of both forward and reverse signaling requires formation of high order clusters of Eph/ephrin complexes (Himanen et al., 2010). They have been initially described as important regulators of topographic map organization in the vertebrate visual system (Brennan et al., 1997; Marcus et al., 1996), where it was shown that complementary gradients of ephrins and Eph receptors are established within the tectum and the retina, thus directing the targeting of RGC axons through a repulsive, gradient-dependent mechanism. In particular, ephrins A and EphA receptors elicit pure repulsive response, whereas ephrins B and EphB receptors can elicit either repulsion or attraction, depending on the reciprocal abundance (Klein, 2012). They play a similar role in guiding the formation of a topographic map in the olfactory bulb, but the mechanism here is concentration-dependent rather than gradient-dependent. Olfactory neurons and target cells express different ephrinA and different EphA receptors (Cutforth et al., 2003); additionally, olfactory neurons that express different olfactory receptors also present different ephrinA levels. These specific olfactory receptor classes combined with different ephrinA levels are important for specifying the identity of the glomeruli. Accordingly, alteration of the ephrinA levels causes errors in the glomerular map (Cutforth et al., 2003). The ephrinA/EphA system is also required for correct wiring within the accessory olfactory bulb (AOB). Here vomeronasal axons which express differential levels of EphrinA5, target within the AOB, which in turn expresses a gradient of Eph6 receptor. However in this case neurons expressing high levels of ligand, project to an AOB area where high levels of receptor are present, arguing against a repellent signaling mechanism (Knöll et al., 2001). Together with other observations, these results suggest that Ephs and ephrins act mostly as repulsive contact-dependent cues, but can in some cases also mediate adhesion.

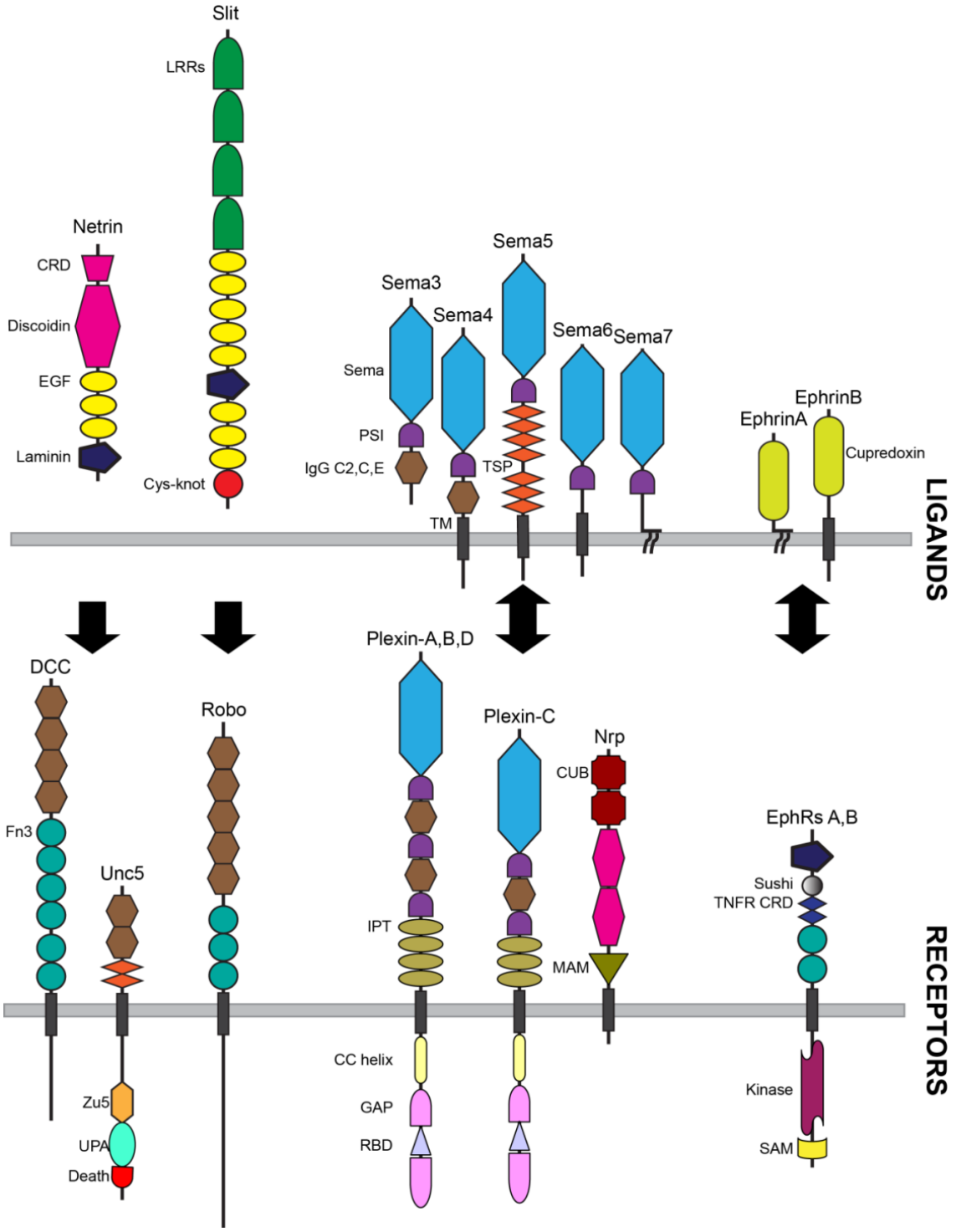


Figure 1-2 Families of axon guidance molecules and receptors.

Four major classes of conserved guidance cues depicted with their receptors. Arrow direction reflects the signaling direction (Semaphorins and ephrins elicit forward and reverse signaling). Main conserved domains are depicted.

Non-classical axon guidance factors. Apart from these classical axon guidance molecules, morphogens like some of the Wnts, Hedgehog (Hh) and bone morphogenetic protein (BMP) family members have been shown to act as axon guidance cues. Among the Wnt morphogen family, Wnt5 is important for selection of the appropriate commissure in the *Drosophila* embryonic CNS (Yoshikawa et al., 2003), whereas in mammals, spinal commissural axons are attracted by a decreasing anterior-to-posterior gradient of Wnt4 (Lyuksyutova et al., 2003). Sonic Hedgehog also plays an axon guidance role in vertebrates, acting as repellent for some retinal ganglion cells (Trousse et al., 2001) and as attractant for spinal cord commissural axons (Charron et al., 2003). TGFβ/BMP family members are also important for commissural axons guidance, repelling them away from the dorsal midline by activating canonical BMP receptors (Augsburger et al., 1999; Yamauchi et al., 2008).

Besides their requirement for axon fasciculation, cell-adhesion molecules have been shown to play axon guidance roles. For example, DSCAM, a Ig CAM in *Drosophila*, plays a role in axonal and dendritic-self avoidance (Hattori et al., 2009; Wojtowicz et al., 2007). L1, another member of Ig superfamily, acts as homophilic adhesion molecule, but stimulates axonal growth (Lemmon, Farr, & Lagenaur, 1989). Integrins, a family of heterodimeric receptors have been shown to be expressed in many types of developing neurons and to function as regulators of neuronal migration and axonal extension, thanks to the interaction with extracellular matrix molecules like laminin and fibronectin (Myers et al., 2012).

These complex molecular pathways ultimately exert their functions by changing the conformation of the navigating axons; to do so, they need to modulate neuronal cytoskeleton dynamics. In the following, I will briefly describe the signaling cascades connecting guidance molecules to the cytoskeleton.

1.2 Signal transduction on the growth cone cytoskeleton

As explained above, the outgrowth of axons during development, essential for precise neuronal wiring, is orchestrated by a myriad of guidance cues. Thus, a critical task of the axon is to navigate within such an information-enriched environment. Axonal processes therefore have tips equipped with a peculiar motile structure, called growth cone, that not only serves as sensor for environmental cues, but also as signal transducer and motility device (Vitriol & Zheng, 2012). Growth cone movement is based on polymerization/depolymerization of cytoskeletal components, such as microtubules and actinfilaments (Lowery & Van Vactor, 2009). The growth cone is composed of three main domains: the peripheral domain (P-domain), the transition zone (T-zone) and the central domain (C-domain) (Figure 1-3) (Dent, Gupton, & Gertler, 2011; Lowery & Van Vactor, 2009). The P-domain is the most distal and most motile part of the growth cone, bearing actin-reach structures such as lamellipodia and filopodia. Lamellipodia are membranous veil-like protusions with actin filaments organized in a loose meshwork, from which thin finger-like structures, the filopodia extend. These contain mainly actin filament bundles that constantly undergo elogantion/depolymeritazion cycles, thus conferring growth cone mobility (Lowery & Van Vactor, 2009). The T-zone, enriched in myosin and thus capable of contracting the actin network, separates the P-domain from the C-domain, a domain enriched in microtubules (MT) filaments composed of α/β tubulin heterodimers. Both actin filaments and microtubules are polarized (Zou, 2012). Actin filaments have a “barbed” end, where addition of actin monomers is favored, and a “pointed” end, where dissociation of ADP-actin happens (Pollard & Borisy, 2003). Several proteins are known to be associated with and to regulate actin filaments. For example, actin nucleators such as the Arp2/3 complex promote branching and *de novo* polymerization of actin filaments, necessary for extension of lamellipodia (Millard et al., 2004). Members of the formin protein family also nucleate actin filaments; additionally, their association with the barbed end enhances the polymerization rate and prevents the binding of capping proteins (Kovar, 2006). Acting depolymerizing factors (ADFs) and cofilins bind to the pointed end of the filaments, generating free ADP-actin fragments and increasing the number of

filaments ends through fragmentation and severing (Gungabissoon & Bamburg, 2003); both these functions ultimately promote actin polymerization. On the other hand, proteins such as gelsolin prevent actin polymerization by capping barbed ends, thus promoting depolymerization (McGough et al, 2003). MTs are also polarized, with a (+) and a (-) end. The (+) end, where tubulin subunits are added more rapidly, is usually oriented toward the tip of the growth cone, whereas depolymerization mainly occurs at the (-) end. MTs are more rigid compared to actin filaments and support the extension of axons and dendrites (Sakakibara et al., 2013); they also act as "rails" for the transport of organelles within the process (Hirokawa & Takemura, 2004).

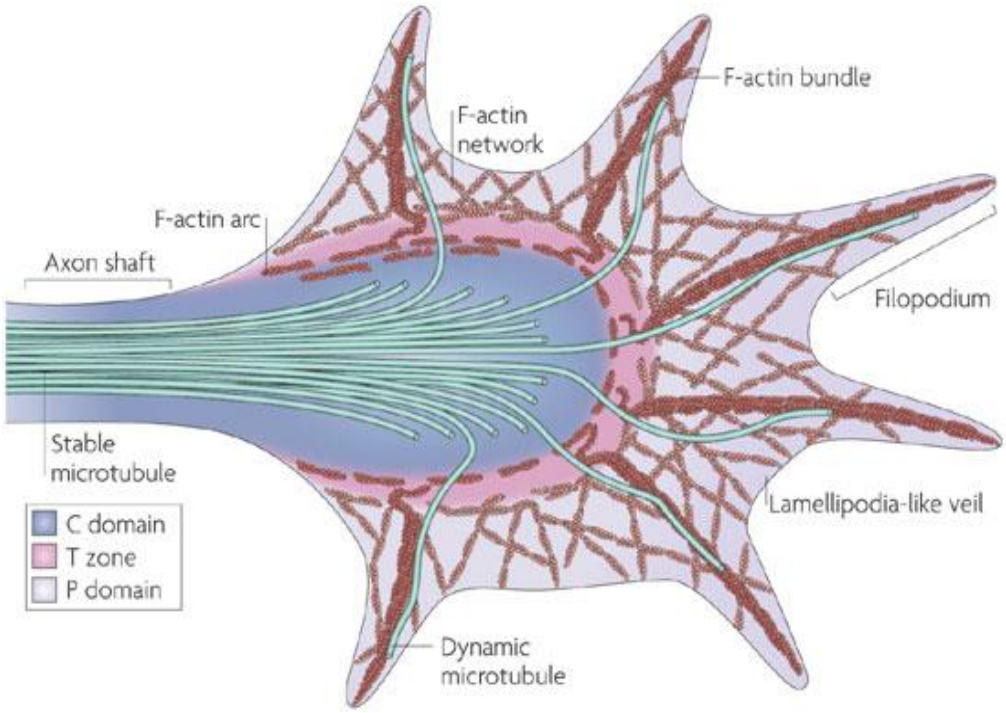


Figure 1-3 The growth cone cytoskeleton.

The growth cone can be structurally divided into three domains. The peripheral (P) domain contains long bundles of actin filaments (F-actin), which form the filopodia as well as actin filaments organized in a loose meshwork, which form lamellipodia. Additionally, individual microtubules (MTs) extend along filopodia. In the central (C) domain, stable bundles of MTs enter in the growth cone from the axon shaft, together with many organelles, vescicles and central actin bundles. The transition (T) zone is the interface between C and P domains, where actomyosin contractile structures form a hemicircumferential ring. Adapter from (Lowery & Van Vactor, 2009).

1.2.1 Downstream molecular cascades transducing guidance signals.

In order to direct growth or retraction of a developing axon, guidance cues and receptors must activate proper signaling pathways to steer the growth cone. The pathways act in a focal manner on actin cytoskeleton dynamics within the growth cone (O'Donnell et al, 2009). Activation of specific molecular pathways can promote attraction or repulsion, resulting in growth cone collapse or extension. Several molecules and second messengers act downstream of the major axon guidance receptors to regulate growth cone dynamics. Here I will present some of the most important, starting with Rho GTPases. Rho-family GTPases are a subgroup of the Ras superfamily of GTPases, and are known regulators of cytoskeletal structures (Hall, 1998). These proteins cycle between an inactive, GDP-bound state and an active, GTP-bound state, where they interact with specific effectors and transmit downstream signaling events (Hall & Lalli, 2010). Their activity is influenced by the GTP/GDP ratio present in the cell, as well as by different regulatory molecules. For example, Guanine nucleotide exchange factors (GEFs) are capable of activating GTPases by increasing the exchange of GDP for GTP. GAPs (GTPase activating proteins) act in opposite direction, and enhance the rate of GTP hydrolysis (Govek et al, 2005). Among the Rho GTPases family, Rac, Cdc42 and RhoA have been implicated downstream of many signaling receptors. In general, Rho appears to act antagonistically to Rac/Cdc42: RhoA is usually associated with repulsive cues and growth cone collapse, whereas Rac and Cdc42 are associated with attractive cues and growth cone extension. However, many exceptions have been found to this rule. Guidance receptors can regulate Rho GTPases directly; for example, Plexin-B was shown to bind Rac directly, probably sequestering Rac from its effector Pak, and thereby blocking the signaling cascade (Vikis et al. 2000; Vikis et al., 2002). However, in most of the cases, guidance receptors act indirectly on GTPases, through GEFs and GAPs. Identification of these specific regulatory molecules is challenging, due to redundancy or requirement of the same molecules downstream of different pathways. However, it was shown that α-chimaerin is an essential mediator of EphrinB3/EphA4 pathway in vivo (O'Donnell et al., 2009a). α-chimaerin mutants show a similar phenotype to ephrinB3-/- or EphA4-/-

mutants in spinal cord interneurons. Furthermore, it is necessary for EphrinB3/EphA4-induced growth cone collapse in cultured neurons (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). α-chimaerin Rac-GAP activity increases upon EphA4 phosphorylation, leading to growth cone collapse (Shi et al., 2007). Another example of a GAP directly linked to a signaling is Vilse/crGAP, which was identified in Drosophila as a regulator of Slit-dependent guidance decisions in both CNS axons and tracheal cells (Hu et al., 2005; Lundström et al., 2004). Vilse/crGAP acts as positive regulator of Slit repulsion, since its loss of function enhances axon guidance defects in animals with partial loss of Slit or Robo. Vilse specifically antagonizes Rac function: its overexpression in the eye selectively suppresses the gain of function phenotype of Rac, but not of Rho, and enhances midline axon guidance defects caused by overexpression of a dominant-negative version of Rac, but leaves unaffected the phenotype cause by expression of dominant negative Cdc42 (Hu et al., 2005). Vilse/CrGAP can directly bind to Robo, supporting direct linking of the receptor with GAP regulation of Rac (Hu et al., 2005).

Rho GTPase activity is also required for Netrin-mediated attraction: DCC-dependent neurite outgrowth requires activity of both Rac and Cdc42 (Li et al., 2002). The Trio GEF and the CZH family GEF DOCK180 have been both implicated in Netrin-mediated axon guidance. Trio positively contributes to midline attraction in the *Drosophila* embryonic CNS and can physically interact with Frazzled (Forsthoefel et al. 2005) and the mammalian DCC (Briançon-Marjollet et al., 2008). Loss of *Trio* in cortical neurons prevent Netrin-dependent Rac activation (Briançon-Marjollet et al., 2008) and *Trio*$^{-/-}$ mice display similar axon guidance defects to *DCC*$^{-/-}$, although in a milder version, indicating that additional factors must be present in order to elicit Netrin-dependent midline attraction. DOCK180 may fulfill this function, being required for cortical neuron outgrowth in response to Netrin as well as for commissural neurons turning in explant assays (Li et al., 2008).

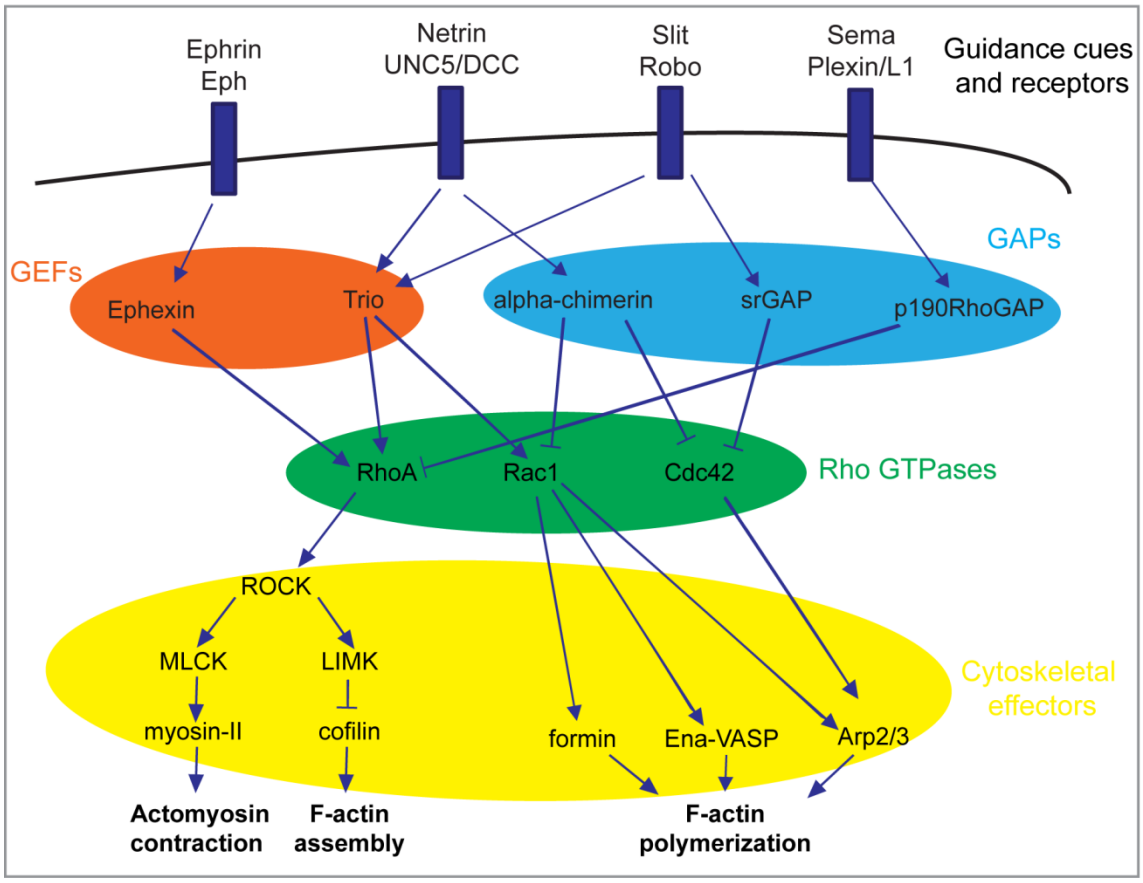


Figure 1-4 Regulation of cytoskeleton dynamics by guidance cues.

Upon binding with their ligands, guidance receptors activate signaling pathways that ultimately results in cytoskeleton rearrengements. In particular, they activate GEFs (Guanine nucleotide exchange factors), which activate RhoGTPases by promoting the exchange of GDP to GTP, and/or GAPs (GTPases activity proteins), which act in the opposite direction. Rho GTPases in turn act directly or indirectly on cytoskeletal effectors, such as Cofilins, Formins etc, causing rearrengements on the actin cytoskeleton. Adapted from (Blakely et al., 2011)

Rho GTPases–dependent cytoskeletal modulation usually requires effector proteins. For example, one of the best characterized is the dual Cdc42/Rac effector p21-activated kinase, PAK (Bokoch, 2003). PAK activation results in inhibition of the actin depolymerizing factor cofilin by activating its inhibitor, LIM kinase (Dan et al, 2001). PAK phosphorylation also inhibits the myosin activator myosin light chain kinase (MLCK) and the microtubule destabilizing protein, Op18/stathmin (Daub et al. 2001; Sanders, 1999). *In vivo*, PAK is required for *Drosophila* photoreceptor axon targeting (Hing et al. 1999; Newsome et al., 2000) as well as in *Drosophila* olfactory neurons for proper glomerular axon targeting (Ang et al., 2003). Stimulation of RhoA results in activation of Rho

kinase (ROCK), serine-threonine kinases that regulate LIMK. Additionally, Rho kinases can regulate myosin activity through the phosphorylation of myosin light chain (MLC), which in turn increases actin-myosin contractility (O'Donnel et al., 2009b). Sema3A dependent growth-cone collapse in mouse DRG neurons is correlated with a rapid increase of phosphorylated Cofilin, thus stimulating actin depolymerization (Aizawa et al., 2001). This effect is mediated by LIMK.

Several *in vivo* and *in vitro* models have been employed to assess the molecular and cellular basis of axon guidance; among those, the Drosophila embryonic CNS has been particularly powerful, especially for the possibility of performing large scale genetic screenings. New genes involved in many aspects of axonal development have thus been identified and characterized (Seeger et al., 1993).

1.3 The *Drosophila* CNS midline

The switch from radial to bilateral symmetry in the nervous system was a key event in animal evolution: without such a divergence, the planet might be still populated by sea anemones, sponges and similar animals with rather simple nervous systems (Kidd, 2009). This bilateral symmetry created a distinct left and right side in the nervous system and in the whole body around its own axis of symmetry, the midline. In both vertebrates and invertebrates, the midline plays a fundamental role in wiring the nervous system, providing a variety of guidance cues essential for correct axonal growth and targeting of developing neurons (Dickson & Zou, 2010). Different types of neurons sense and interpret these signals in different ways and at different times, according to the different developmental stage. Both the vertebrate spinal cord and the invertebrate ventral nerve cord (VNC) are built up of an orthogonal array of axonal projections: commissural axons, the ones extending across the midline, are essential for communication and coordination between left and right side of the body; longitudinal axon tracts, running parallel to the midline, include axons that carry ascending and descending signals from and to the brain. For each class of neurons, the midline represents either an intermediate target, or a barrier that keep them on their own side of the CNS, or again a source of signals dictating specific anterior/posterior decisions as well as specific pathways selection.

The *Drosophila* embryonic CNS is a well-established tool for studying axon guidance: in particular, the stereotypy of projections and the availability of many genetic markers make it possible to study at cellular resolution the growth and targeting of identifiable neurons (Sánchez-Soriano et al., 2007). Here, commissural neurons grow toward the midline and cross it in one of the two main commissures in each segment. Once they cross, many of them turn anteriorly or posteriorly into a longitudinal pathway, where they join a smaller population of ipsilateral neurons. The next paragraphs will describe the molecular and cellular mechanisms important for correct embryonic CNS development.

1.3.1 Early phases of *Drosophila* embryonic axonogenesis.

Early development of commissural embryonic axons in the *Drosophila* ventral nerve cord is strictly dependent and related to migration and development of glial and neuronal midline cells (Jacobs & Goodman 1989; Klämbt et al. 1991). The pioneering of the two commissures takes place between 8 and 10 hours of embryonic development (stage 12, Figure 1-5A) (Klämbt et al., 1991). The first axons grow straight toward the pair of V cells, the most anterior of the ventral unpaired median (VUM) midline neurons. As they reach the midline, movements of the MP1 neurons create a space anteriorly to the V cells, which is soon occupied by the pioneer growth cones. They change direction growing anteriorly around the V cells, and once in contact, they fasciculate with their contralateral homologs at the midline (Klämbt et al., 1991). Although those pioneer neurons will later belong to the posterior commissures (PC), they transiently occupy at this stage the space of the future anterior commissure. Immediately after fasciculation, other neurons extend the growth cones and pioneer the future anterior commissures (AC). The commissures are now in contact, over the midline, with a straight AC and a V shapes PC. At the end of stage 12, MGM glia and MP1 neurons migrate and contribute to the separation of the commissures. During next embryonic stages, axonogenesis of follower neurons takes place. At the end of embryonic development, each abdominal neuromere contains a huge population of interneurons, estimated around 954 cells (Rickert et al., 2011), 36 motor neurons innervating body wall muscles (Landgraf et al., 1997; Landgraf et al., 2003) and 32 longitudinal glia cells originating from the NB lineage (Beckervordersandforth et al. 2008).

Within each segment, longitudinal tracts are pioneered by four neurons, which extend in pairs in opposite directions: pCC and vMP2, projecting anteriorly, and MP1 and dMP2, projecting posteriorly (Hidalgo & Brand, 1997; Jacobs et al., 1989; Lin et al., 1994) (Figure 1-5B). At the beginning of axonogenesis, their growth cones contact half way in the segment to establish a first single fascicle.

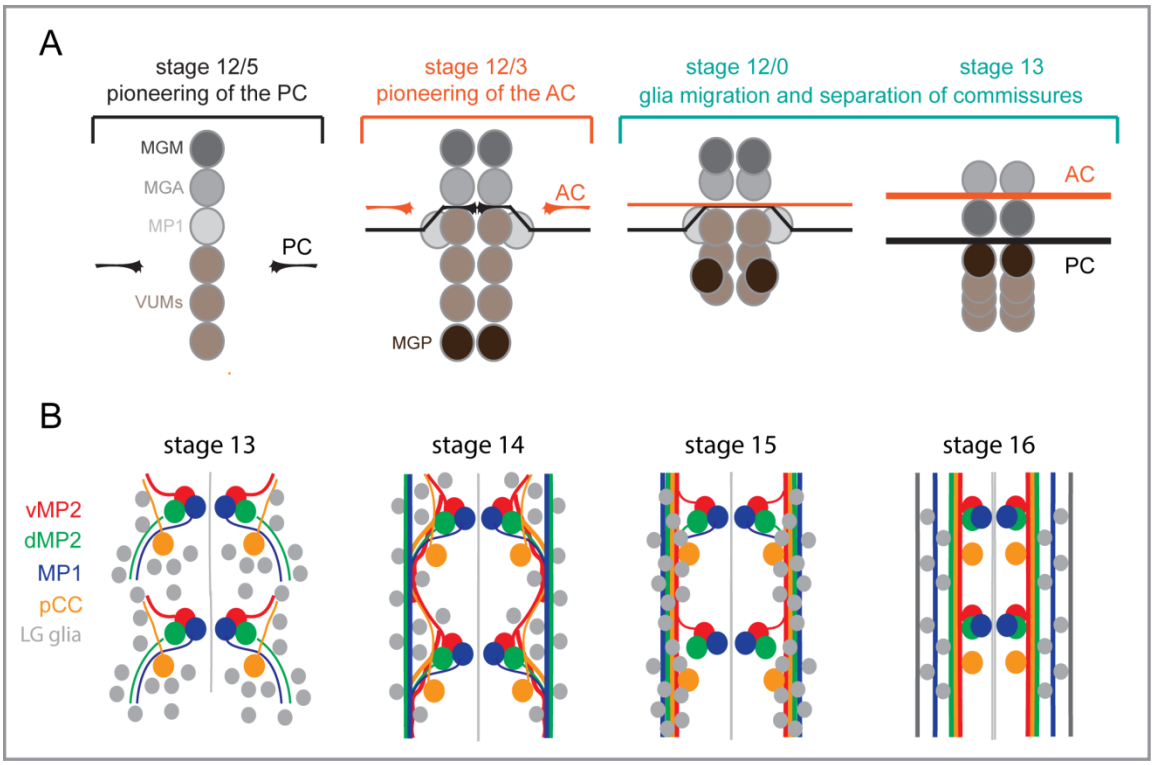


Figure 1-5 Pioneering of the commissures and the longitudinal tracts in the *Drosophila* VNC.

A. Extension of the first commissural axons happens during stage 12 of the embryonic development. Pioneer axons (depicted as black arrows) extend in the future PC toward the most anterior located VUM neuron. Movements of the MP1 midline neurons cause an anterior change in their direction, thus resulting in connection in the future AC. Axons of the future AC then extend and fasciculate, getting in contact with the PC. Finally, in stage 13, migration of glia cells causes separation of the commissures. **B**. Longitudinal tracts are pioneered by four neurons: at stage 13, MP1 and dMP2 extend posteriorly, whereas vMP2 and pCC extend anteriorly, contacting each other. During stage 14 and 15 axons defasciculate and fasciculate again, partially guided by longitudinal glia cells (grey circles). At final stages, vMP2, dMP2 and pCC axons are located in the innermost bundle, whereas MP1 traces the intermediate bundle.

Later, pioneer axons undergo a series of fasciculation and defasciculation events, that terminate with the formation of two fascicles at each sides of the midline: PCC fasciculate with dMP2 in a medial position, vMP2 runs also along this fascicle, but in a more ventral position, separated from pCC/dMP2 fascicles, and MP1 runs along a second fascicle, pioneering the intermediate of the three fascicles labeled with anti-FasII antibody (Hidalgo & Brand, 1997). The third most lateral fascicle establishes really late in development, and the neurons pioneering it are still not known. Importantly, longitudinal connectives do not form properly when pioneer neurons (Hidalgo & Brand, 1997) are ablated. Late-developing neurons use the axonal scaffold established by the pioneer neurons as rails, transiently fasciculating/defasciculating with and from the pioneer bundles (Tessier-Lavigne & Goodman, 1996). Additionally, longitudinal glia plays an important role in orienting pioneer growth cones and in directing fasciculation/defasciculation events during their extension (Hidalgo & Booth, 2000). Loss of longitudinal glia results as well in pathfinding defects of longitudinal tracts, leading to discontinuity in the bundle (Hidalgo & Brand, 1997).

1.3.2 Molecular pathways involved in embryonic midline patterning

Molecular pathways so far implicated in embryonic CNS patterning orchestrate different kinds of guidance decisions, such as midline crossing, anterior-posterior pathway and longitudinal bundle selection. Here, I will describe guidance cues playing a role at those choice points.

Midline cells exert their fundamental role for the formation of the embryonic CNS secreting guidance cues that direct crucial steps of axonal development of both commissural and ipsilateral neurons. Among those, Netrins and Slits are the most conserved in different species, and the major midline attractants and repellents, respectively. As mentioned before, in *Drosophila* there are two members of the Netrin protein family: NetrinA and NetrinB. They have an overlapping and redundant function in promoting midline crossing of commissural axons, since only when both are deleted there is a significant reduction in the amount of axons that cross the midline (Brankatschk & Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996). Similar defects are observed in mutant for *frazzled*, which encodes for a Netrin receptor from the DCC family

(Kolodziej et al., 1996). Recently, Dscam was shown to contribute to Net-dependent commissure formation, although it can also act in an independent manner (Andrews et al., 2008). In mice, *netrin-1* is expressed in dorsal-ventral gradient in the spinal cord, and is thought to act as a long-range chemoattractant for commissural axons (Kennedy et al., 1994; Kennedy et al., 2006). However in flies there is evidence that Netrins act as short-range permissive cues, promoting growth across the midline once the axons get there, rather than attracting axons over long distances (Brankatschk & Dickson, 2006). In particular it was shown that a version of NetrinB thetered to the cell membrane is fully functional, suggesting that graded expression might not be required (Brankatschk & Dickson, 2006). Indeed, commissural axons orient normally toward the midline in *NetA NetB* mutants (Brankatschk & Dickson, 2006). It is important to notice that in *Net/fra* mutants, many commissures are still able to form (Brankatschk & Dickson, 2006), suggesting that other molecules might be acting as chemoattractants. In mice it was shown that Sonic Hedgehog plays this role in addition to Netrin-1 (Charron et al., 2003); however there is no evidence that hedgehog or other molecules act as attractant at the fly midline (Dickson & Zou, 2010).

As mentioned before, not all the axons cross the midline: some approach the midline but then turn and continue on an ipsilateral pathway. Also, commissural axons usually cross the midline only once. Thus, they need to gain repulsiveness against the midline that they just crossed. The Slit/Robo molecular pathway plays a crucial role in repelling axons from the midline (Kidd et al., 1999; Kidd et al., 1998). In *Drosophila* there is only one *Slit*, which is secreted from the midline cells, and three *robo* genes: *robo*, *robo2* and *robo3* (Battye et al., 1999; Kidd et al., 1998; Rajagopalan et al. 2000; Rothberg et al., 1988; Simpson et al., 2000).

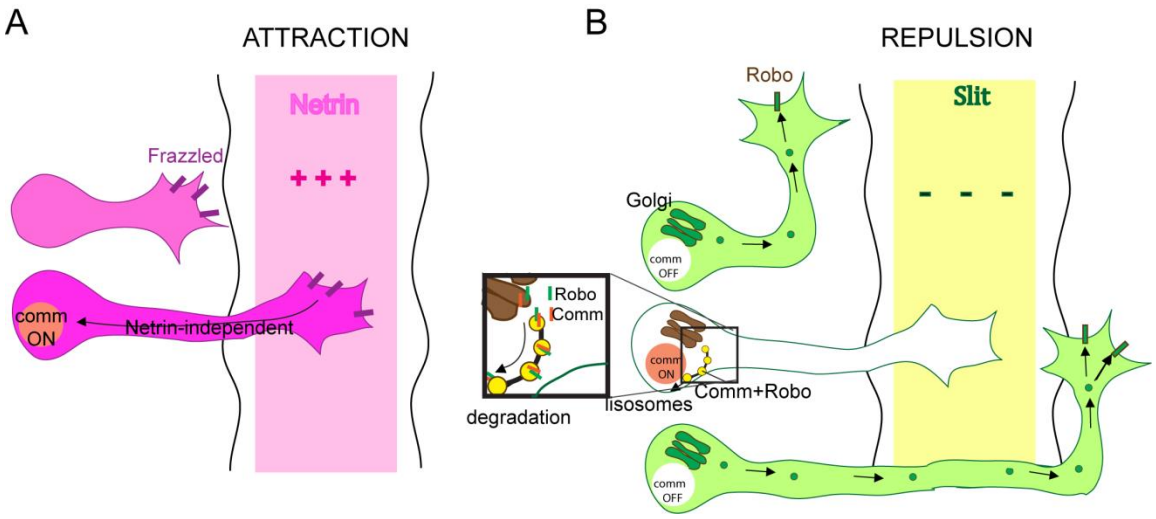


Figure 1-6 Netrins and Slits regulate commissure formation in the *Drosophila* VNC.

A. Midline cells secrete Netrins, major attractants midline guidance cues. Frazzled-expressing neurons (magenta) are attracted toward the midline by Netrins. Additionally, Netrin-independent Frazzled signaling suppresses responsiveness of neurons toward Slit, thus inhibiting repulsion. Frazzled acts in this context regulating expression of Commissureless. **B**. Commissureless regulates Robo expression by diverting it from the Golgi to the endosomes, thus impairing its membrane localization. Interestingly, Comm is expressed only in midline crossing neurons, whereas in ipsilateral or post-crossing neurons is not expressed, thus allowing Robo localization (green neurons) on the growth cone membrane and thus, repulsion toward Slit expressing midline (yellow).

Loss of *slit* or *robo* results in excessive amount of neurons crossing the midline (Kidd et al., 1999; Kidd et al., 1998). Interestingly, *robo* seems to be pan-neuronally expressed in the embryonic CNS (Kidd et al., 1998); moreover, in embryos mutants for either *slit* or *robo* all axons are capable of growing toward the midline (Battye et al., 1999; Kidd et al., 1999; Seeger et al., 1993). Both these data suggest that commissural axons have some specific capability of transiently overcome Slit-dependent repulsion. The most crucial regulator of Robo activity is Commissureless (Comm), a small transmembrane protein that is expressed in commissural but not in ipsilateral neurons (Georgiou & Tear, 2002; Keleman et al., 2002). Comm was shown to be both necessary and sufficient for midline crossing (Keleman et al., 2002; Keleman et al., 2005; McGovern & Seeger, 2003), and the resulting mutant phenotype is the loss of virtually all the commissures (Seeger et al., 1993; Tear et al., 1996). Since double mutants for

comm and *robo* have *robo* mutant phenotypes (Seeger et al., 1993), it was proposed that Comm regulates midline crossing by antagonizing Robo. In fact, Comm regulates intracellular trafficking of Robo, diverting it from the Golgi to the endosomes, thus preventing its expression on the growth cone membrane (Keleman et al., 2002; Keleman et al., 2005) (Figure1-6B). Transiently higher levels of Comm in crossing axons cause a reduction of Robo on the membrane, making them insensitive to Slit. Frazzled seems to be involved in regulating *comm* transcription in commissural axons in a Netrin-independent mechanism (Yang et al., 2009))(Figure 1-6A).

Formation of a correct orthogonal array of axonal tracts at the midline does not rely only on the decision whether to cross or not to cross the midline, but also on correct positioning along the anterior-posterior axis. For commissural axons, this involves making the correct anterior or posterior turn after crossing as well as choosing to cross the midline either in the anterior or the posterior commissure (Dickson & Zou, 2010). In flies, the choice between AC or PC is in part determined by the position of the cell body, with many axons simply crossing in the closest commissure (Rickert et al., 2011). However, for some commissural axons, this decision relies also on specific molecular signals that allow discrimination between the two bundles. It was shown that the secreted protein Wnt5 is predominantly expressed by cells near the PC, acting as a specific repellent signal for AC axons, thus keeping them from crossing in the PC (Yoshikawa et al., 2003). Absence of Wnt5 diverts some of the AC axons in the PC, whereas ectopic expression of Wnt5 in the entire midline prevents the formation of the AC (Yoshikawa et al., 2003). The selective effect of Wnt5 on AC axons relies on the specific expression of the Wnt5 receptor Derailed (Drl) on AC growth cones (Bonkowsky et al., 1999; Yoshikawa et al., 2003). Drl is a member of the Ryk family of atypical receptor tyrosine kinases. *Drl* mutant embryos have some AC axons crossing in the PC, and forced expression of Drl in the PC reroutes some of these axons in the AC (Bonkowsky et al., 1999), similarly to what was shown for *wnt5* (Yoshikawa et al., 2003). Longitudinal axons face anterior-posterior decision too, and also this decision, as the AC/PC selection, seems to rely on local cues rather than on long-range gradients as for vertebrates (Lyuksyutova et al., 2003). In particular, longitudinal axons will

repeatedly encounter the same cues as they extend from one segment to the other, due to the segmentally-repeated expression of many proteins. Therefore, they need to adjust their response differently whether their growth cones extend in their own segment or in others. Such a guidance decision has been described for dMP2 axons, which, as mentioned above, extend posteriorly to pioneer the medial FasII positive longitudinal pathway (Hidalgo & Brand, 1997). dMP2 is an ipsilateral neuron and therefore expresses *robo*. However in these neurons Robo is required not only to prevent dMP2 from crossing the midline, but also for its proper longitudinal extension into the next segment (Hiramoto & Hiromi, 2006). Accordingly, in *robo* mutants dMP2 diverts medially from its longitudinal trajectory, because of an ectopic response to a lateral patch of Netrin-enriched neurons (Hiramoto & Hiromi, 2006). This specific Netrin localization is required for proper lateral turning and pathfinding behavior of dMP2 (Hiramoto et al., 2000), but in order not to re-turn medially in the next segment, where the growth cone will encounter a similar Netrin-enriched axonal patch, dMP2 must become irresponsive to Netrin. Robo seems to mediate this suppression of Netrin attractiveness, being specifically expressed in dMP2 axons only after the Netrin-dependent turn (Hiramoto & Hiromi, 2006).

Lastly, during targeting the longitudinal axons have to select the proper fasciculation partners and the correct lateral position. Several molecules have been shown to act as markers for correct axon-axon interactions, such as typical cell adhesion molecules (CAMs) like Fasciclin II (Grenningloh et a.l, 1991; Lin et al., 1994), Fasciclin III (Snow et al., 1989), N-cadherin (Iwai et al., 1997), Neurotactin (Escalera et al., 1990; Hortsch et al., 1990) or axon guidance molecules, like Sema-1a (Yu et al., 1998). Lateral pathway selection has been shown to be Robo-dependent (Rajagopalan et al 2000b; Simpson et al., 2000): *Robo*, *robo2* and *robo3* are expressed in a specific pattern, defining three broad zones within the longitudinal connectives. Robo is expressed alone in the medial zone, Robo and Robo3 define the intermediate zone, whereas the lateral zone expresses all three *robo* genes (Rajagopalan et al., 2000b). This so-called Robo code is instructive for lateral positioning of axons, since loss of *robo2* or *robo3* leads to shift of lateral axons close to the midline. Conversely, miserexpression of either of them in medial axons causes lateral displacement (Rajagopalan et

al., 2000; Simpson et al., 2000). Recently this code model has been disproved: “swapping” of each of the *robo* alleles with another one while keeping the temporal and spatial expression of the one that has been replaced showed that expression differences can account alone for lateral pathway selection, rather than differences in the structure of the three Robo receptors (Spitzweck et al. 2010). Structural differences are rather important for midline crossing decisions, but not for lateral pathway selection (Spitzweck et al., 2010).

1.4 Molecular mechanisms of Planar Cell Polarity

Recently, unexpected axon guidance roles have been emerging for molecules previously implicated in other developmental processes, including morphogens belonging to the Wnt, Hedgehog and BMP families (Yam & Charron, 2013) and planar cell polarity (PCP) genes (Tissir & Goffinet, 2013). The latter seems to be a good candidate system for regulating midline guidance in *Drosophila*, especially considering the role of some of these genes in midline crossing in the vertebrate spinal cord and in the brain (Lyuksyutova et al., 2003; Tissir et al. , 2005). In the following sections, molecular functions of these “core” PCP genes and, in particular, of the atypical cadherin Flamingo will be described in detail.

Planar cell polarity (PCP) is the term describing the organization of cell sheets in the tangential plane, which reside on the orientation of cells along the proximal-distal axis (Tissir & Goffinet, 2010). Such a polarity is established through asymmetrical localization of “core“ PCP genes on the proximal and distal parts of contiguous cells, thus ensuring transmission of these polarization along the epithelia (Paul N Adler, 2012). Pioneer analysis of orientation of sensory bristles and cellular hairs in the *Drosophila* wing led to the discovery of “core” components, which include Flamingo (Fmi) (Chae et al., 1999; Usui et al., 1999); Van Gogh/Strabismus (Vang/Stbm) (Taylor et al, 1998; Wolff & Rubin, 1998), Frizzled (Fz) (Adler et al., 1997; Vinson et al.,1989), Prickle (Pk) (Gubb et al., 1999), Dishevelled (Dsh) (Theisen et al., 1994) and Diego (Dgo) (Feiguin et al., 2001). Later on, similar role was shown for the polarization of abdominal and thorax epithelia, as well as for ommatidia organization in the eye (Das et al., 2002). Fmi is proposed to have an instructive role in organizing proximal-distal asymmetric distribution of the other core PCP proteins, in a Fz activity-

dependent manner (Chen et al., 2008; Lawrence et al. 2004; Strutt & Strutt, 2008); this intracellular asymmetry is also fundamental for transmitting planar polarity signaling between neighboring cells (Strutt & Strutt, 2008; Wu & Mlodzik, 2008). Homologous proteins are present in vertebrates, where they are important for establishing stereocilia polarity in the inner ear (Guo et al., 2004), follicle and hair shaft orientation in mouse (Wang et al., 2006) and gastrulation movements of convergence and extension in the frog, *Xenopus Laevis*, and zebrafish, *Dario Rerio* (Heisenberg et al. 2000; Wallingford et al., 2000). Unexpectedly, these proteins have been shown to play important roles during neuronal development, in particular in the context of axonal guidance. In the following paragraphs, these roles will be described in detail, starting from Flamingo, which was the first among these molecules to be implicated in axonal pathfinding and the one being more broadly required.

1.5 Roles of PCP genes in axonal pathfinding

1.5.1 Roles of atypical cadherins Flamingo/Celsr mediated signaling

Flamingo/Celsr (Fmi) not only belongs to the PCP molecules but also to the Cadherin family (Halbleib & Nelson, 2006). The Cadherin family comprises more than 100 members involved in diverse developmental processes. The structural feature defining this protein class is the extracellular cadherin repeats domain (Nollet et al., 2000) that mediates homophilic and heterophilic interactions necessary for cell-cell adhesion and sorting (Halbleib & Nelson, 2006). From this basic role in mechanical cell-cell adhesion, cadherins have evolved to function in other aspects of morphogenesis, like cell recognition and sorting, coordinated cell movements, organization and maintenance of cell and tissue polarity and neural circuits formation (Halbleib & Nelson, 2006). This variety of roles and molecular mechanisms is reflected in the great number of family members, subdivided into three major classes: classical cadherins, protocadherins and atypical cadherins. Atypical cadherins present a unique structure among the cadherins, since they have a seven-pass transmembrane domain instead of a single one.

Flamingo (Fmi), also known as Starry Night (Stan) is a seven-pass transmembrane molecule bearing a big extracellular domain that is composed of nine cadherin domains, six epidermal growth factor-like (EGF-like) domains, two Laminin-G-like domains, one hormone receptor domain (HRM) and, next to the transmembrane domain, a GPS cleavage site characteristic of GPCR-adhesion molecules (Bockaet & Pin, 2000). The peculiar seven-pass transmembrane (7TM) is similar to G-protein-coupled receptors (GPCRs) of the secretin receptor family (Harmar, 2001). These structural features are common to the mammalian homologous of Fmi, Celsr1-3, and suggests that Fmi/Celrs can act as adhesion molecules, but also as signaling receptors (Figure1-7).

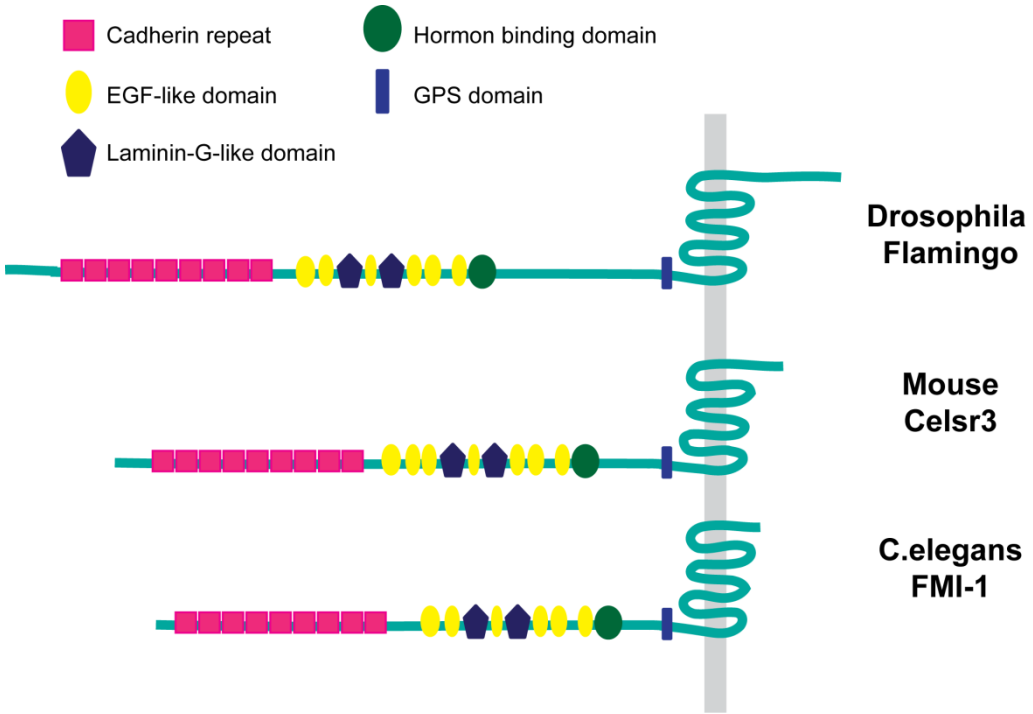


Figure 1-7 Structure of Flamingo and of the homologous Celsr3 and FLAMINGO-1.

Atypical cadherins family members have high conserved structures. The big extracellular domain presents nine cadherin domains, variable number of EGF-like domains, two Laminin-G-like domains, one hormone receptor domain (HRM) and, next to the transmembrane domain, a GPS cleavage site characteristic of GPCR-adhesion molecules. The intracellular domain is short and the 7TM is similar to G-protein-coupled receptors (GPCRs) of the secretin receptor family.

1.5.2 Roles of Flamingo/Celsr in dendritic morphogenesis

In the *Drosophila* peripheral nervous system, Fmi regulates dendritic morphogenesis of sensory neurons in two distinct developmental stages. During late embryogenesis dendrites of dendritic arborisation (da) neurons in the abdominal dorsal body wall start growing toward the midline and pause before reaching it. Later, in early larvae stages, dendrites growth continues until dendrites of contralateral homologous da neurons meet at the midline and repeal each other. This interdendritic avoidance ensures the formation of non-overlapping dendritic fields (Grueber et al., 2002). Loss of *fmi* results in precocious dendrite growth and consequent midline crossing already in embryonic stage (Gao et al., 2000). In *fmi* mutant larvae, dendritic terminals overgrow and invade the contralateral side, creating overlapping dendritic fields with dendrites from homologous contralateral neurons (Kimura et al., 2006). In addition, Fmi represses dendritic extension also in the CNS mushroom body neurons (Reuter et al., 2003), indicating that Fmi is a general negative regulator of dendritic growth in both peripheric and central nervous system in the fly. Celsr2 and Celsr3, in the mammalian nervous system, also regulate dendritic arborization: silencing of *Celsr2* in rat neuronal cultures causes retraction of dendrites, whereas *Celsr3* silencing results in increased neurite growth (Shima et al., 2007; Shima et al., 2004). In both the fly and the mouse dendritic systems, several studies support a signaling role for Fmi/Celsr, rather than a more classical, cadherin-like adhesive function. First, the dendritic overgrowth phenotype of *fmi* mutant embryos can be partially rescued by expression of an Fmi construct lacking the cadherin repeats (indispensable for homophilic adhesion) and the EGF/Laminin-like domains, suggesting that Fmi can transmit a signal independently of homophilic cell adhesion. Second, Matsubara and colleagues showed that a LIM domain protein, Espinas, is capable of binding to an intracellular juxtamembrane domain of Flamingo, thus eliciting repulsion between dendritic branches of class IV sensory neurons (Matsubara et al., 2011). Third, Celrs2 and Celsr3 are capable of triggering intracellular Ca^{2+} increase upon binding to their respective cadherin repeats (Shima et al., 2007), similarly to GPCRs family members.

1.5.3 Roles of Flamingo/Celsr in axon guidance

Flamingo's role in regulation of axonal growth and targeting was first uncovered in the fly visual system. Here, Fmi contributes to axon guidance and synaptic partner selection directing axon-axon and axon-target interactions in different developmental stages. In larval visual system, Fmi is required for maintaining proper distance between pioneer R8 photoreceptor axons, allowing the formation of a continuous topographic map (Lee et al., 2003; Senti et al., 2003). Later, during pupae stages, Fmi ensures proper target selection in both lamina, where it acts in a non-cell autonomous way (Chen et al., 2008) and medulla, where it is required in both photoreceptor axons and their medulla targets, acting via a homophilic axon-target mechanism (Hakeda-Suzuki et al., 2011) (Figure 1-8A and B). Flamingo is widely implicated in axon guidance in *Drosophila*: *fmi* mutants show an axon stalling phenotype in sensory neurons, perturbing the normal interaction between sensory neuron growth cones and their intermediate target cells (Steinel et al., 2009) (Figure 1-8C). Additionally, axon targeting and bifucartion of mushroom body neurons are impaired when Fmi is absent in these neurons (Shimizu et al., 2011) .

Recently, it was shown that loss of *fmi* causes strong axon navigation defects in pioneer and follower axons in the ventral nerve cord of *C. elegans,* likely due to mis-regulating of both axon pathfinding and axon fasciculation (Steimel et al., 2010) (Figure1-8D). *Celsr3* mutant mice also display severe axon guidance defects in many axonal tracts, including the AC and the internal capsule (Tissir et al., 2005); in the latter, Celsr3 seems to be required in neurons but also in cells located in their trajectory, probably acting in both cell types via homophilic interaction (Zhou et al., 2008). Additional defects are observed in the spinal cord, where *Celsr3* mutant commissural axons extend randomly along the anterior-posterior axons instead of proceeding anteriorly (Price et al., 2006) (Figure 1-8E). Similarly, anterior-posterior pathfinding errors are present in serotoninergic and dopaminergic tracts projections in the brainstem (Fenstermaker et al., 2010) (Figure1-8F).

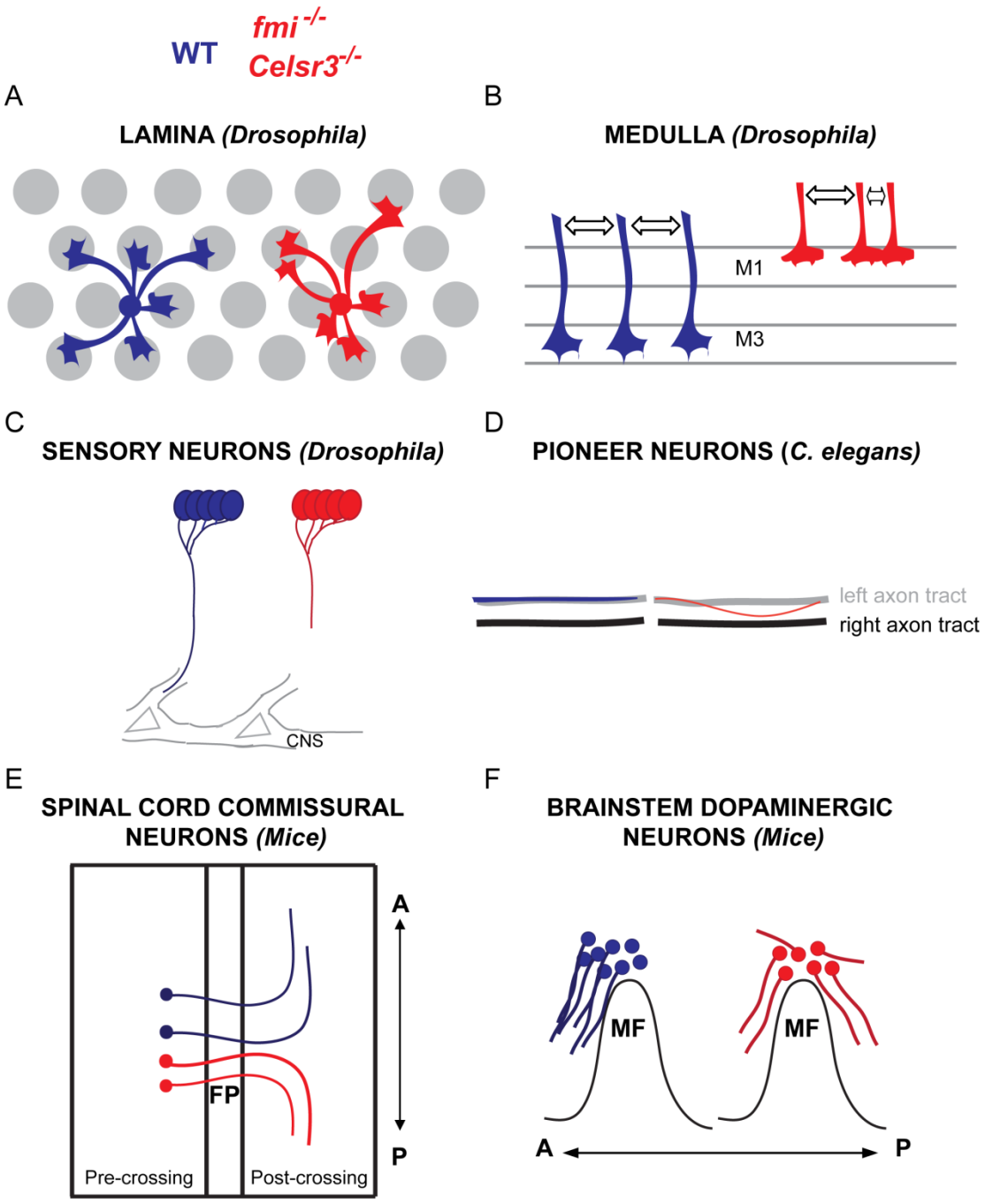


Figure 1-8 Schematic of the major axon guidance phenotypes in *fmi/Celsr3* mutants.

In *Drosophila,* loss of *fmi* causes aberrant synaptic partner selection of R1-R6 photoreceptor axons in the lamina (A) and of R8 in the medulla (B); in the latter, it also causes loss of spacing between R8 axons. Sensory neurons of *fmi*$^{-/-}$ embryos show axon stalling phenotype, thus failing to reach their targets in the CNS (C). In *C. elegans,* pioneer neurons of *FMI-1*$^{-/-}$ animals show irregular midline crossing (D). In *Celsr-3*$^{-/-}$ mice, commissural axons fail to turn anteriorly after crossing the midline (E), whereas dopaminergic neurons fail to project anteriorly in the brainstem and project randomly, including posteriorly (F). WT animals or neurons are depicted in blue, *fmi*

or *Celsr-3* mutants are depicted in red. M1/M3: medulla layers 1 and 3; CNS: central nervous system; FP: floor plate; MF: mesencephalic flexure; A-P: anterior-posterior.

Contrary to what has been observed in dendritic morphogenesis, Fmi can act in axon pathfinding as adhesion molecules as well as receptor. For example, in the fly visual system, Flamingo, being expressed in both pre- and post-synaptic partners, allows recognition and adhesion of photoreceptor axons and their target cells in the medulla in a homophilic manner (Hakeda-Suzuki et al., 2011). Here, the Fmi intracellular domain is not required in photoreceptor axons, arguing against the requirement of an Fmi-mediated downstream signaling. *Celsr3* seems to play a role in synaptic partners recognition in the internal capsule in mammals, being required in both navigating axons and guidepost cells (Zhou et al., 2008). Another compelling evidence of Flamingo acting as an adhesive factor in axon targeting comes from *fmi-1* role in the VNC of *C. elegans*. Here follower axons, which exclusively depend on pioneer axons for correct navigation, show a defasciculation phenotype in *fmi-1* mutant animals.

Interestingly, this phenotype can be rescued by expressing versions of FMI-1 lacking either the intracellular or the seven-pass transmembrane domain (Steimel et al., 2010), arguing for a pure adhesive function in follower axons. Additionally, the worm model offers a good example of Fmi receptor-like activity: opposite to follower axons, FMI-1 intracellular domain is crucial for guidance of pioneer axons, suggesting that axonal pathfinding depends in this case on interaction with intracellular components (Steimel et al., 2010). Thus, FMI-1 acts as cell-type dependent axon guidance factor, exerting its function in different neurons through different structural domains. This axonal cadherin independent activity of Fmi is conserved in *Drosophila* embryonic sensory neurons, where an Fmi construct lacking cadherin repeats and EGF/Laminin domains can partially rescue the stalling phenotype (Steinel & Whitington, 2009).

1.5.4 Involvement of other PCP genes in axon guidance.

Given the fundamental role that Flamingo plays in PCP, a question that many researchers have tried to answer is whether these Fmi/Celsr neuronal functions are PCP-dependent or not. The easiest way to address this point is to analyze whether the other core PCP proteins are required in the same context. Again, a complex and ambiguous mechanism of action has been found, given multiple evidence for both PCP-dependent and –independent pathways. In mice, *Fzd3* (mouse homologous of *Fz*) have similar expression patterns and similar mutant phenotypes to *Celsr3*, showing defects in the anterior commissure and the internal capsule (Tissir et al., 2005; Wang et al., 2002; Zhou et al., 2008). Additional common defects have been shown in post-crossing commissural axons in the spinal cord, where *Celrs3*, *Fzd3* and *Vangl2* (homologous of the *Drosophila Vang Gogh*) mutants display aberrant anterior-posterior projections, in a Wnt4-dependent manner (Lyuksyutova et al., 2003; Shafer et al., 2011). The same genes are also required for anterior-posterior guidance of brainstem serotoninergic and dopaminergic axons, probably responding to a Wnt5 gradient (Fenstermaker et al., 2010; Shafer et al., 2011). Interestingly, a similar cooperative function of the PCP genes *fz*, *dsh*, *vang* and *wnt5* was demonstrated for targeting and branching of *Drosophila* mushroom body neurons (Shimizu et al., 2011). *Fmi* knock-down in these neurons showed a similar phenotype, arguing for a function of core PCP genes in neuronal polarization. However in *Drosophila* and *C. elegans,* Fmi PCP-independent axon guidance pathways have been reported. Mutants for *fz*, *vang*, *dsh* and *pk* show normal photoreceptor targeting in the medulla (Hakeda-Suzuki et al., 2011); similarly, *fz*, *vang* and *dsh* mutants do not display a stalling phenotype in sensory neurons (Steinel & Whitington, 2009). Finally, no defects were detected in *C. elegans* for core PCP mutants (Steimel et al., 2010). An exception is *lin-17/frizzled* mutants, which display phenotypes in pioneer and follower axons. However, double mutants for *frizzled* and *flamingo* show enhanced phenotypes, arguing that they act in a parallel rather than identical pathway (Steimel et al., 2010).

Taken together, these data suggest that genes regulating polarization of cells within epithelial sheets can also act in more dynamic systems, for instance directing growth cone orientation and guidance. Currently, it is not clear whether

the molecular repertoire and the interactions utilized in the two contexts overlap or differ. In other words, are PCP genes activating similar downstream cascades in the two systems? Is asymmetrical localization required for axonal pathfinding? Are the genes acting cooperatively or in a redundant manner with respect to each other? Another open question regards how this novel group of guidance molecules relates with the cardinal axon guidance systems described in paragraph 1.1.

2 AIM OF THIS THESIS

As reviewed above, studies on the nerve cord midline have been particularly meaningful in revealing molecular and cellular pathways used by developing axons to navigate and connect to their targets. Several important regulators of axonal pathfinding have been identified up to now; however, it is important to keep in mind that most of those genes and their molecular pathways have only a partial phenotype, affecting a specific class of neurons and, even within a group of neurons, affecting only some of them, while leaving others unaffected. Thus, a full understanding of axonal guidance mechanisms requires additional studies in order to identify how this redundancy is achieved. In particular in *Drosophila*, loss of the midline attractant Netrin and its receptor Frazzled affects only part of the neuronal population, leaving open the question of how midline crossing of most of the commissural neurons is regulated.

The aim of this study was to explore how different guidance systems cooperatively elicit axonal pathfinding by investigating the role of the PCP molecules and in particular, of the atypical cadherin Flamingo in the fly embryonic CNS. Specifically, this study addressed the following questions:

1) Are PCP genes and in particular Fmi required for guiding neurons at the *Drosophila* midline?
2) How does Fmi cooperate with known axon guidance systems such as Netrin/Frazzled?
3) Where is Fmi required in this system?
4) Is Fmi acting as adhesive factor or as signaling receptor?
5) What is the molecular mechanism acting upstream and downstream of Fmi in this system?

3 RESULTS

3.1 Analysis of PCP genes roles in midline guidance.

"Core" PCP molecules, such as Flamingo (Fmi) (Chae et al., 1999; Usui et al., 1999), Van Gogh/Strabismus (Vang/Stbm) (Taylor et al, 1998; Wolff & Rubin, 1998), Frizzled (Fz) (Adler et al., 1997; Vinson et al.,1989), Prickle (Pk) (Gubb et al., 1999), Dishevelled (Dsh) (Theisen et al., 1994) and Diego (Dgo) (Feiguin et al., 2001), have been recently implicated in important neurodevelopmental processes. For example, Celsr3, Frizzled3 and Vangl2 have been shown to direct anterior-posterior guidance of spinal cord commissural axons and of brainstem serotoninergic and dopaminergic axons (Lyuksyutova et al., 2003; Shafer et al., 2011). To address whether PCP genes play a similar role in the *Drosophila* ventral nerve cord (VNC), loss of function alleles for *fmi, dsh, vang* and *fz* were analyzed in order to identify possible defects in the formation of commissural or longitudinal axon bundles (Figure 3-1). To this aim, stage 16-17 embryos were stained with HRP and FasII antibodies and filleted. HRP labels four axon bundles per segment: two midline crossing bundles, called anterior commissure (AC) and posterior commissure (PC) and two longitudinal tracts. FasII labels three axon bundles within the longitudinal tracts. Both HRP and FasII stainings in $fmi^{E59-/-}$, $dsh^{1-/-}$, $vang^{153-/-}$ and $fz^{KD4-/-}$ embryos failed to reveal any kind of mutant phenotype: HRP positive midline crossing bundles thickness was comparable to WT embryos (Figure 3-1B, magenta), and FasII positive longitudinal axons (Figure 3-1C, green) bundled together forming three nicely separated fascicles on each side of the ventral nerve cord (VNC), as observed in wild-type (WT) embryos.

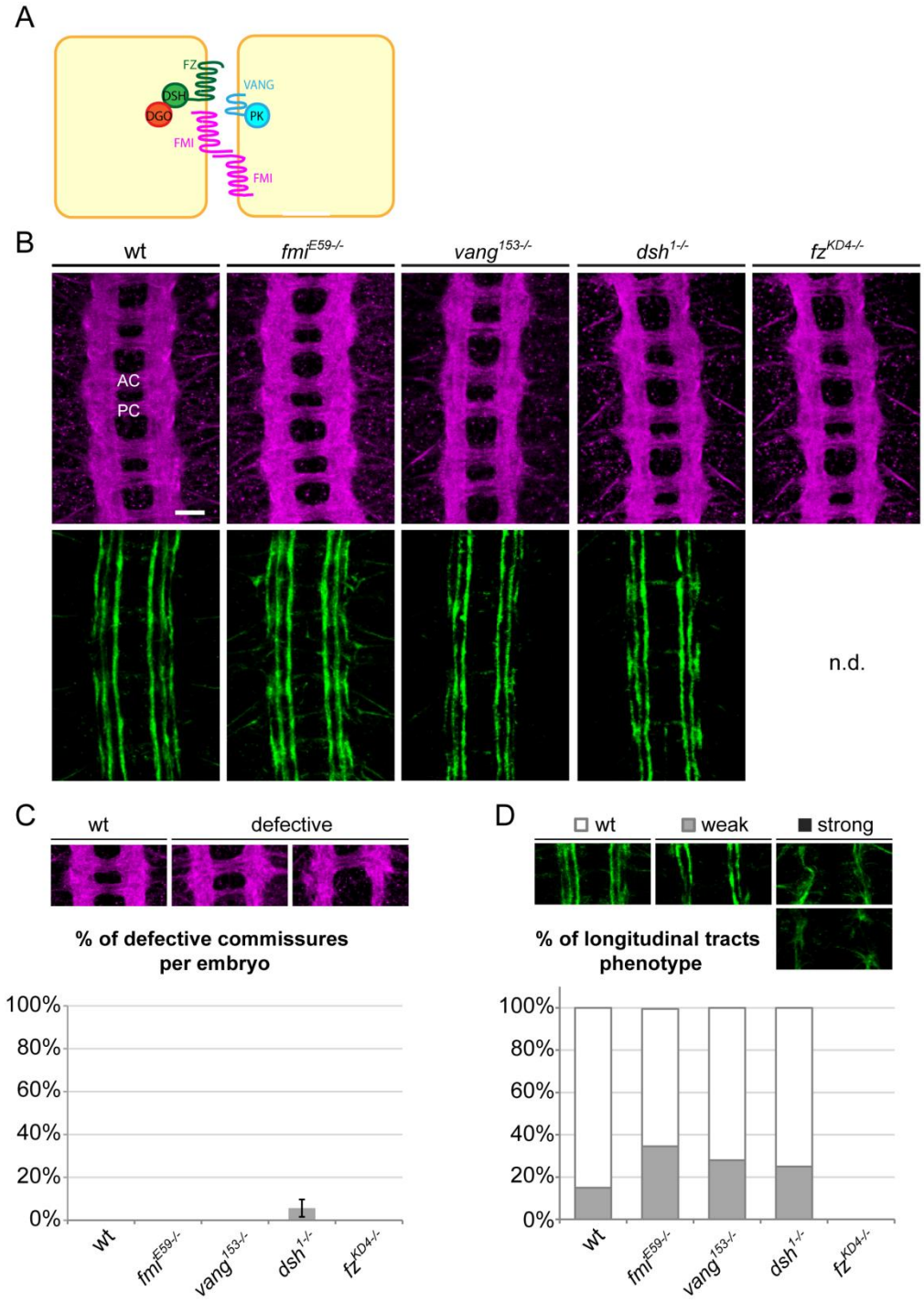


Figure 3-1 "Core" PCP genes and their phenotypes in the embryonic CNS

A. Schematic of the "core" PCP genes, including their cellular localizations and their respective interactions. **B**. Representative stage 16 embryos of indicated genotypes stained with anti-HRP (magenta) to visualize the axon scaffold, or with anti–FasII (green) to label ipsilateral neurons. Anterior is up. N.d.: not determined**.** In wild-type embryos CNS axons are organized in two commissures (AC and PC) per segment and two longitudinal tracts that run along the anterior-posterior axis of the embryo. Mutants for *fmi, vang*, *dsh* and *fz* do not show any relevant phenotype in commissures or longitudinal tracts **C-D**. Quantification of PC defects (C) and

longitudinal tracts (D) defects in *fmi*$^{E59-/-}$, *vang*$^{153-/-}$, *dsh*$^{1-/-}$, and *fz* $^{KD4-/-}$ embryos. 10-20 embryos per genotype were analyzed. Values displayed in C are percentages of defective posterior commissure, and typical defective commissures are shown in the pictures with anti-HRP staining. Error bars indicate s.e.m. Values displayed in D are percentages of defective longitudinal tracts. Weak phenotype refers to loss of the lateral tract, strong phenotype refers to segments where one or none of the fascicles are formed.

Next, these mutant alleles were analyzed again in a sensitized *fra*$^{-/-}$ background, because functional Net/Fra signaling could mask or compensate for the loss of a pathway playing an analogous guidance role. The analysis included double mutants *fra, fmi* , *fra, vang* , *dsh*, *fra* and *fra, fz*. The mutant alleles *Vang*^{153} and *dsh*^{1} did not enhance *fra* mutant phenotype, whereas both *fmi*^{E59} and *fz*^{KD4} enhanced *fra*$^{-/-}$ phenotype (Figure 3-2). In both of the cases, strong axon pathfinding phenotypes were observed: commissures were significantly much thinner than in single mutants, and the number of segments affected in each embryo also increased (Figure 3-2). In particular, posterior commissures (PC) were almost entirely lost in *fra, fmi* and in *fra, fz* double mutants, whereas in *fra* mutants still 60% of posterior commissures were formed properly and the defective ones were usually thinner, but rarely totally missing. Analysis of FasII positive longitudinal bundles also revealed a highly disrupted axonal pattern (Figure 3-2A and C). In double mutant embryos, axons aberrantly bundle, losing proper separation and often forming only a single fascicle (Figure 3-2A, arrowheads, and 2C, strong phenotype) or stopping prematurely without connecting to the bundle in the next segment (Figure 3-2 asterisks). On the contrary, in *fra* mutants strong defects are only occasionally observed (8%), and mild defects, like presence of only two bundles (Figure 3-2A and C, weak phenotype), are confined to 30% of the segments analyzed.

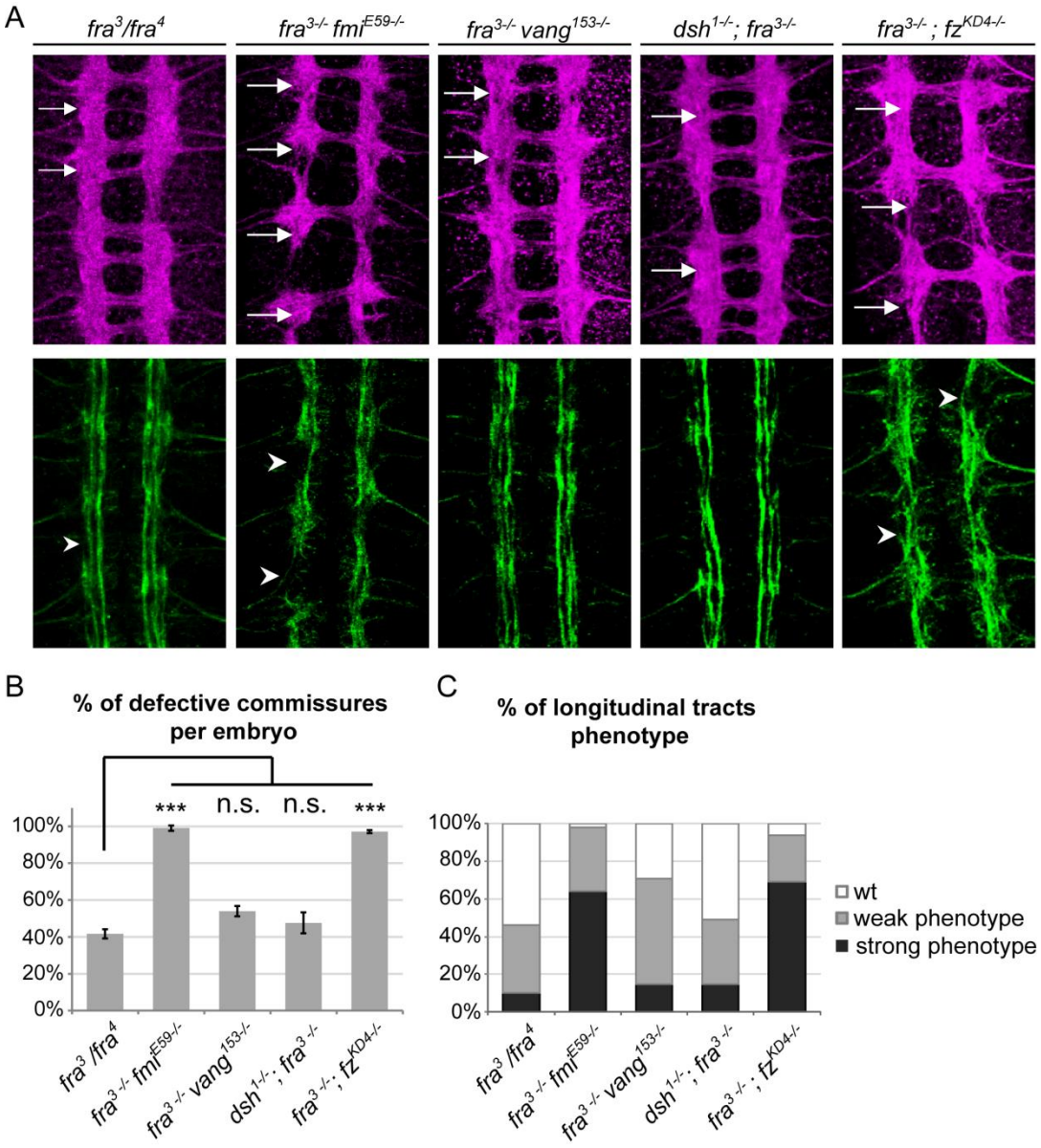


Figure 3-2 Loss of *flamingo* or *frizzled* strongly enhances axonal defects in *frazzled* or *Netrin* mutants.

A-C. Analysis of PCP core genes in axon targeting. **A**. *Vang* and *Dsh* mutations do not significantly enhance the fra^{3}/fra^{4} single mutant phenotype, neither in commissures (anti-HRP, magenta, upper row) nor in longitudinal tracts (anti-FasII, green, bottom row), whereas in $fra^{3-/-}$ $fmi^{E59-/-}$ and $fra^{3-/-}$ $fz^{KD4-/-}$ strong phenotypes are observed: most of the PC are lost (arrows), and longitudinal fascicles are also disrupted, with loss of two or all the tracts in most of the segments **B-C**. Quantification of PC defects (B) and longitudinal tracts (C) defects in $fra^{3-/-}$ $vang^{153-/-}$, $Dsh^{1-/-}$ $fra^{3-/-}$ and $fra^{3-/-}$ $fz^{KD4-/-}$ double mutants. 15-20 embryos per genotype were analyzed. Values displayed in B are percentages of defective posterior commissure. Error bars indicate s.e.m. ***P<0.001. Arrows indicate strong commissure phenotype, arrowheads strong phenotype in

longitudinal tracts. n.s.: not significant. Values displayed in C are percentages of defective longitudinal tracts. Mild phenotype/strong phenotype categories are same as in figure 3-1B.

Although, as mentioned before, loss of *fz* and *fmi* caused in different contexts overlapping mutant phenotypes and the two proteins could directly interact *in vivo* and *in vitro*, in order to transmit an instructive PCP signal among neighboring cells (Chen et al., 2008), the following analysis mainly focused on Fmi, because Fz is known to play a fundamental role in mediating Wnt morphogenic signaling (Charron & Tessier-Lavigne, 2005). Therefore, the phenotype observed upon loss of *fra* and *fz* might be related to loss of such a signaling, rather than being consequent to loss of a Fz-dependent axon guidance pathway.

3.2 Flamingo enhances *Netrin/frazzled* mutant phenotype in midline

Fra is known to mediate Netrin-attractive signaling (Harris et al., 1996; Mitchell et al., 1996); however, Fra was reported to be required for midline crossing in both ligand-dependent (Kolodziej et al., 1996) and – independent (Yang et al., 2009) ways. Thus, in order to clarify which of these pathways was enhanced by loss of *fmi*, embryos lacking *fmi* and both *NetrinA* and *NetrinB*, the two genes encoding for fra ligand in *Drosophila* (Harris et al., 1996), were analyzed (Figure 3-3). Male embryos hemizygous for *NetABΔ* and homozygous for fmi^{E59} also showed strong phenotypes in both commissural and longitudinal bundles, qualitatively and quantitatively similar to the ones observed in $fra^{-/-}$ $fmi^{-/-}$ embryos (Figure 3-1).

In conclusion, loss of *fmi* strongly enhanced axonal pathfinding defects caused by loss of Netrin-dependent Frazzled signaling, leading to the loss of most of commissures and severe disruption of longitudinal projections.

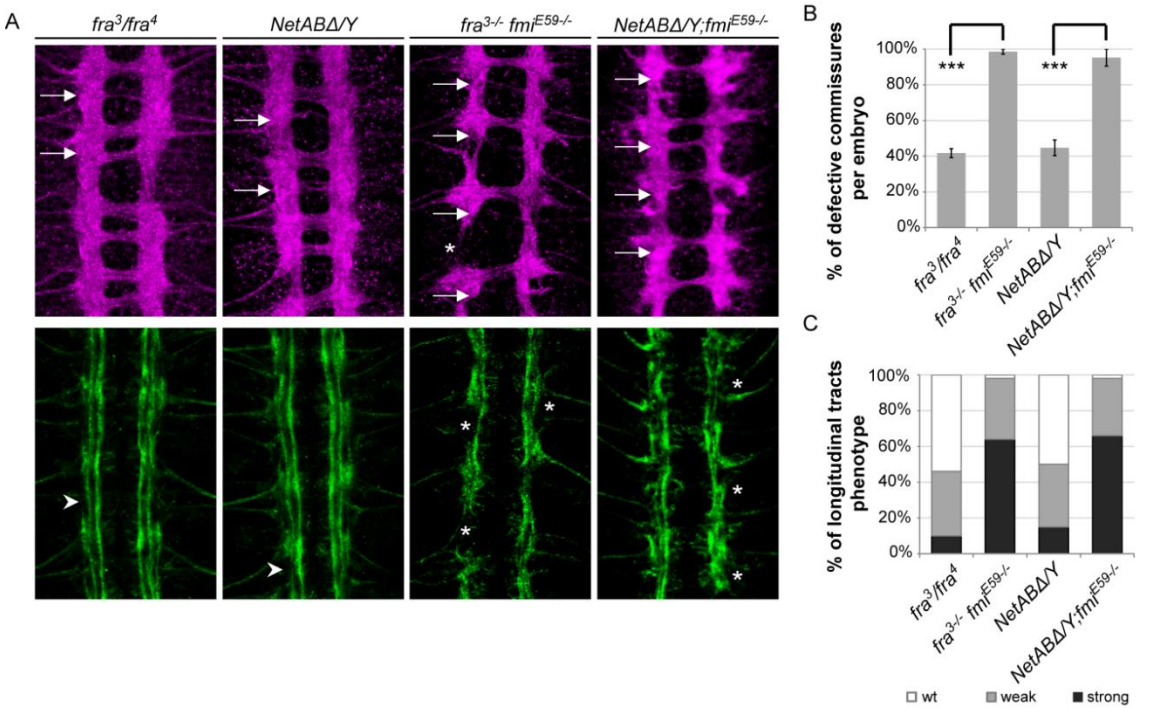


Figure 3-3 Loss of *flamingo* strongly enhances axonal defects in *frazzled* or *Netrin* mutants.

A. Embryos mutants for *fra* or *Net-A* and *Net-B* exhibit defects in commissure formation (anti-HRP, magenta), with some of the PC thinner or missing (arrow).$fra^{3-/-}$ $fmi^{E59-/-}$ and $NetAB\Delta^{-/-}$ $fmi^{E59-/-}$ double mutants show strong enhancement of the commissural phenotype compared to single mutants, with loss of most of the PC. Additionally, breaks in the longitudinal tracts can be observed in double mutants (asterisk). In $fra^{3-/-}$ or $NetAB\Delta^{-/-}$ mutants, occasional breaks or loss of the most lateral longitudinal tract occur (arrowheads) (anti-FasII, green). $fra^{3-/-}$ $fmi^{E59-/-}$ and $NetAB\Delta^{-/-}$ $fmi^{E59-/-}$ embryos show severe disruption of the fascicles, with loss of two or all the tracts in most of the segments (asterisks). Scale bar, 20 µm. **B-C.** Quantification of defective posterior commissures and defective longitudinal tracts in embryos of the indicated genotypes. 15-20 embryos per genotype were analyzed. Values displayed in B are percentages of defective posterior commissure calculated in each embryo. Error bars indicate standard error and three asterisks (***) indicate statistical significance (P<0.001). Values displayed in C are percentages of defective longitudinal tracts calculated in each embryo. Mild phenotype/strong phenotype categories are same as in figure 3-1B.

3.3 Flamingo and Frazzled have overlapping expression patterns.

Flamingo was already shown to be expressed broadly in epithelia and nervous system in both embryos and imaginal tissues (Usui et al., 1999); however, detailed data about the precise temporal and spatial embryonic expression, as

well as a comparison with *fra* expression is not reported yet. Given the availability of a good monoclonal antibody against Fmi, analysis of its localization at the embryonic midline was performed (Figure 3-4). In WT embryos, *fmi* is expressed in virtually all the developing neurons, as revealed by co-staining with anti-Fmi and anti-HRP antibodies (Figure 3-4A). *Fra* was also shown to be expressed in the majority of commissural and longitudinal axons at the CNS (Kolodziej et al., 1996); thus, co-staining with anti-Fra and anti-Fmi antibodies confirmed that indeed the two transmembrane proteins have largely overlapping distribution throughout embryonic axonogenesis, and that it takes place from stage 11-12 to stage 16 (Figure 3-4B, representative images of stage 13 and 16 WT embryos are shown).

In conclusion, analysis of *fmi* expression revealed that *fmi* and *fra* are expressed in the same cells, thus suggesting they might act cooperatively during neuronal development.

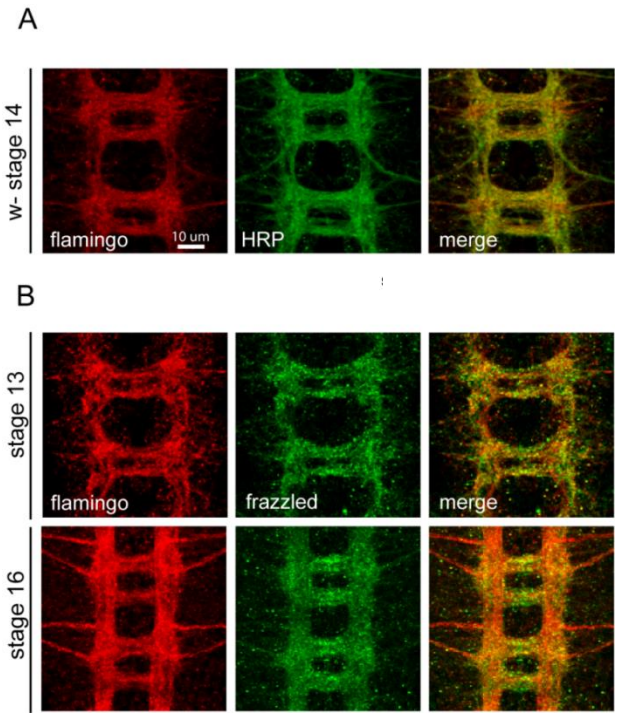


Figure 3-4 F**lamingo and Frazzled have overlapping expression patterns.**

A. Flamingo (anti-fmi, red) is expressed in most of the axons (anti-HRP, green) in wild-type embryos. **B**. In wild-type embryos, anti-flamingo (red) and anti-frazzled (green) antibody staining reveals similar expression pattern for Frazzled and Flamingo from the beginning of axonal extension (stage13) to the end of embryonic development (stage 16). Scale bar, 10 µm.

3.4 Pan-neuronal expression of Flamingo rescues axonal defects of both commissural and longitudinal projecting neurons.

During axonal pathfinding, developing neurons receive and interpret signals coming from surrounding cells, such as other neurons or glia cells. Therefore, abnormal axon pathfinding can arise from aberrant neuron-neuron interactions, as observed in the case of loss of FasciclinII or N-cadherin (Iwai et al., 1997; M. Lin et al., 1994), from absence of midline secreted axon guidance cues, as for loss of Slit or Netrins (Harris et al., 1996; T Kidd et al., 1999; Mitchell et al., 1996; Rothberg et al., 1988), or from irregular glia-neuron interaction, as resulting from loss of Wrapper in midline glia (Noordermeer et al., 1998) or from removal of longitudinal glia during longitudinal pioneer neurons extension (Hidalgo & Booth, 2000). Therefore, the phenotype observed in *fmi* mutants could be due to loss of *fmi* function in either one of the modalities described above. Thus, rescue experiments were conducted in order to determine in which cells Fmi was contributing to axonal outgrowth. To address this question, *fmi* was selectively re-expressed in specific subset of cells in $fra^{-/-}$ $fmi^{-/-}$ embryos, taking advantage of the *Gal4-UAS* system (Brand & Perrimon, 1993). Two different pan-neuronal drivers, *1407-Gal4* (Luo et al., 1994) and *Elav-Gal4* (Hekmat-Scafe et al., 2005), one midline cells driver, *Sim-Gal4* (Scholz et al., 1997) and two different glia cells drivers, *Repo-Gal4* (Sepp et al., 2001) and *Gcm-Gal4* (Sepp & Auld, 1999) were used.

Expression of *UAS-fmi* construct in midline (*with Sim-Gal4*) or glia cells (Gcm-Gal4) still resulted in a disrupted axonal scaffold (Figure 3-5A and B), where both phenotypes in PC and longitudinal tracts were quantitatively similar to control (*fra, fmi* double mutants with the *Gal4* construct) embryos. However, neuronal expression of Fmi significantly reduced the number of defective commissures (Figure 3-5C), as well as number and severity of defective FasII positive bundles (Figure 3-5D). Interestingly, pan-neuronal re-expression of Fmi in *fra, fmi* mutant embryos reduced commissural defects at the extent of *fra* single mutant phenotype, thus rescuing all *fmi* contribution to the phenotype.

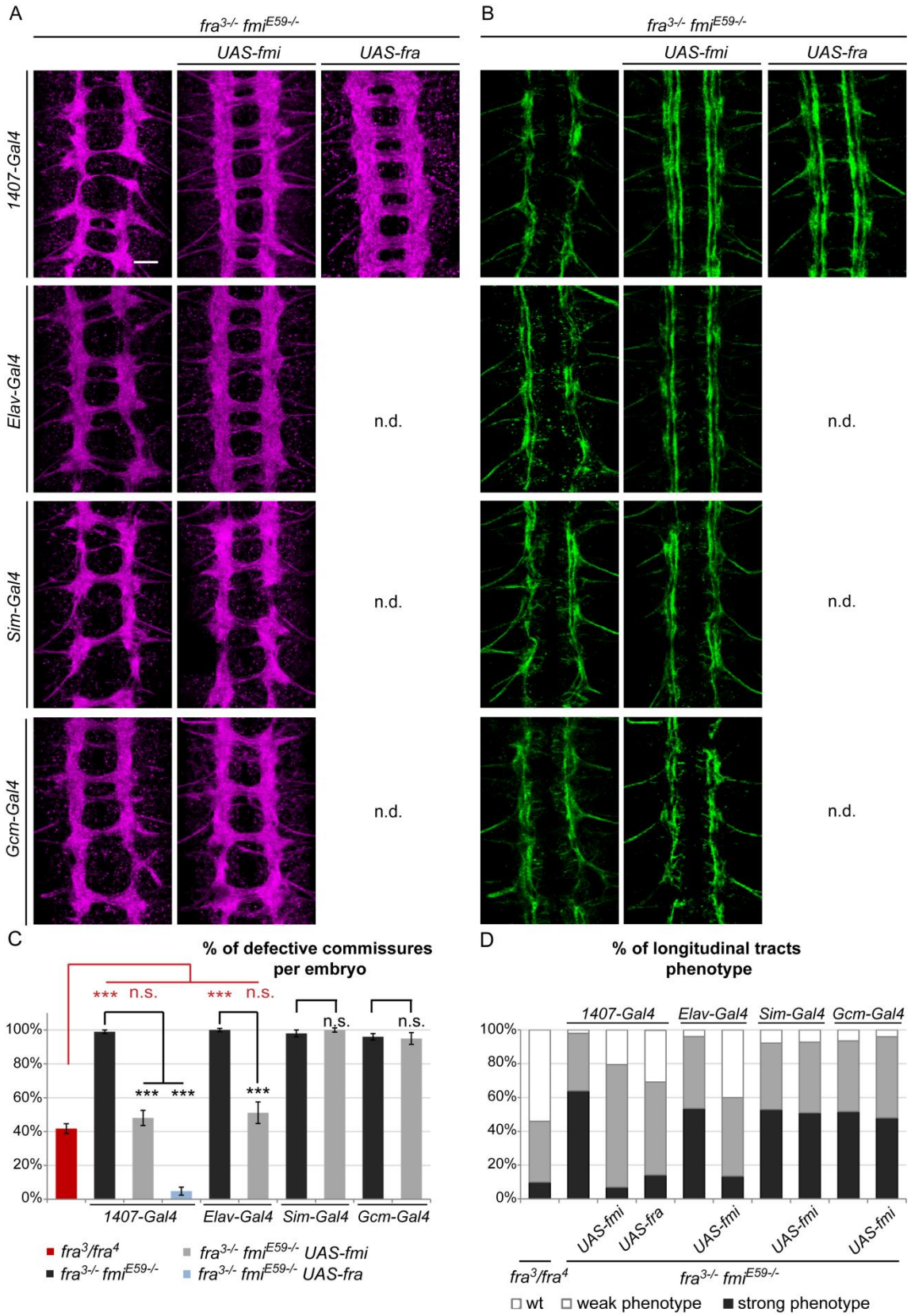


Figure 3-5 Flamingo is required in neurons, but not in midline or longitudinal glia cells, for proper axonal targeting of CNS axons.

A. Commissural defects in *fra*$^{3-/-}$ *fmi*$^{E59-/-}$ mutants are rescued at the level of *fra*$^{-/-}$ single mutants when a wild-type *fmi* construct (*UAS-fmi*) is re-expressed in neurons with *1407-Gal4* or *Elav-Gal4.* Re-expression of Fmi in midline cells with *Sim-Gal4* or in longitudinal glia with *Gcm-Gal4* leads to similar defects as observed in *fra, fmi* double mutants. Re-expression of a wild-type *fra* construct (*UAS-fra*) with *1407-Gal4* fully rescues the commissural mistargeting. Magenta is anti-HRP staining. Scale bar, 20 µm. **B**. Severity of the longitudinal tracts phenotype in *fra*$^{3-/-}$ *fmi*$^{E59-/-}$ mutants is reduced when Fmi is re-expressed in neurons with *1407-Gal4* or *Elav-Gal4*, but not when re-expressed in midline cells (*Sim-Gal4*) or longitudinal glia (*Gcm-Gal4*). Similar effects are observed with pan-neuronal expression of Fra. Green is anti-FasII staining. **C-D.** Quantification for the rescue of the PC defects (C) and of the longitudinal tracts (D) defects in *fra, fmi* double mutants. 15-20 embryos per genotype were analyzed. Values displayed in C are percentages of defective posterior commissure. Error bars indicate s.e.m. ***P<0.001. Values displayed in D are percentages of defective longitudinal tracts. Mild phenotype/strong phenotype categories are same as in figure 3-1D.

Pan-neuronal expression of *UAS-fra* efficiently restored PC bundles (Figure 3-5C), and additionally reduced both the severity and the number of defects in longitudinal bundles, although in this case at a comparable effectiveness of *UAS-fmi* (Figure 3-5D).

These data, together with the previously described neuronal expression pattern (Figure 3-4), suggest that Fmi, similar to Fra, contributes to normal axonal development in longitudinal and commissural neurons.

3.4 Role of apoptosis in *fra, fmi* double mutants.

Recent reports suggested an anti-apoptotic role for Netrins. In mouse or chick primary embryonic neuronal culture, Netrin-1 inhibits the pro-apoptotic activity of DCC in developing commissural neurons, thus supporting the idea that Netrin-1 acts in these neurons not only as guidance cue but also as pro-survival factor (Furne et al., 2008). In *Drosophila* such an anti-apoptotic function has been proposed for *NetB*. Connectivity defects observed in *Net* mutant embryos can be rescued by pan-neuronal expression of either *NetB* (but not *NetA*) or apoptosis inhibitor *p35*, suggesting that *NetB* does not only act as positional cue but also as anti-apoptotic factor. This hypothesis was further validated by the reduced natural cell death observed at the VNC when *NetB* was overexpressed in neurons (Newquist et al., 2013). Interestingly, apoptosis was found to be increased in $Net^{-/-}$ but not in $fra^{-/-}$ embryos, so that the neurotrophic activity of NetB can be mediated by an unidentified receptor (Newquist et al., 2013). All together those results suggested that defects observed in *fra, fmi* or *Net, fmi* double mutants could be a consequence of increased neuronal apoptosis rather than to pathfinding defects. Therefore, a similar approach was taken to evaluate the contribution of cell death to the strong *fra, f*mi double mutant phenotype. The caspase inhibitor p35 was pan-neuronally expressed in $fra^{-/-}$ $fmi^{-/-}$ embryos, but no rescue of the neuronal phenotype was observed (Figure 3-6). Although no direct analysis of neuronal cell death was performed, it is possible to conclude that Fmi does not exhibit anti-apoptotic activity, and that the phenotypes resulting from loss of *fra* and *fmi* are not due to death of the neuronal cells, but rather to defects in axonal outgrowth.

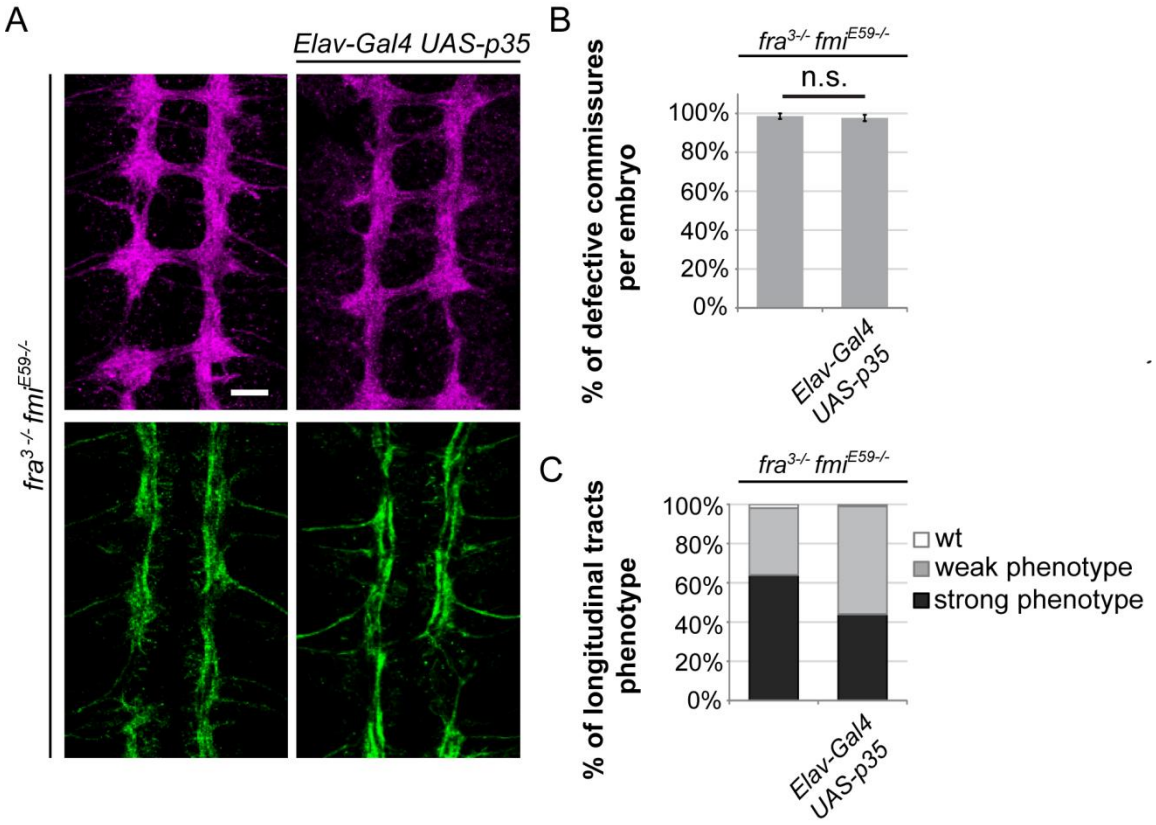


Figure 3-6 Block of apoptosis does not rescue *fra, fmi* double mutant neuronal phenotype.

A Pan-neuronal expression of the anti-apoptotic factor p35 does not ameliorate the *fra*$^{3-/-}$ *fmi*$^{E59-/-}$ neuronal phenotype, neither in commissures (anti-HRP, magenta, upper row) nor in longitudinal tracts (anti-FasII, green, bottom row), Scale bar, 20 µm. **B-C**. Quantification of PC defects (B) and longitudinal tracts (C) defects in *fra*$^{3-/-}$ *fmi*$^{E59-/-}$ double mutants. 15-20 embryos per genotype were analyzed. Values displayed in B are percentages of defective posterior commissure. Error bars indicate s.e.m. n.s.: not significant. Values displayed in C are percentages of defective longitudinal tracts. Mild phenotype/strong phenotype categories are same as in figure 3-1B.

3.5 Flamingo can mediate midline crossing

The analysis presented above showed that Flamingo collaborates with Net/Fra pathway to mediate axon targeting in developing axons; however the molecular and cellular mechanisms of Fmi function are still poorly understood, given that the analysis was performed with general neuronal markers and Gal4 lines. In particular, the poor temporal-spatial resolution of markers such as HRP and FasII could not give further information regarding the precise function of Fmi in different subtypes of neurons. One important question is whether Fmi can specifically mediate midline crossing, one of the prominent functions of Net/Fra signaling pathway (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996).

To address this question, Gal4 lines expressed in smaller subsets of neurons were used. The first one is the *Eagle-Gal4* driver, which label a specific group of commissural neurons, the eagle (Egl) neurons (Dittrichet al., 1997; Higashijima et al., 1996). In WT embryos, expression of a membrane bound GFP (*UAS-mcd8-GFP*) under the *eagle* promoter labels two clusters of neurons: one cluster (EG neurons) of 10-12 cells that have cell bodies located laterally to the longitudinal bundles, extending the axons in the AC, and a medial cluster (EW neurons) of 3-4 cells that extend their axons across the midline in the PC of the adjacent segments (Figure 3-7A).

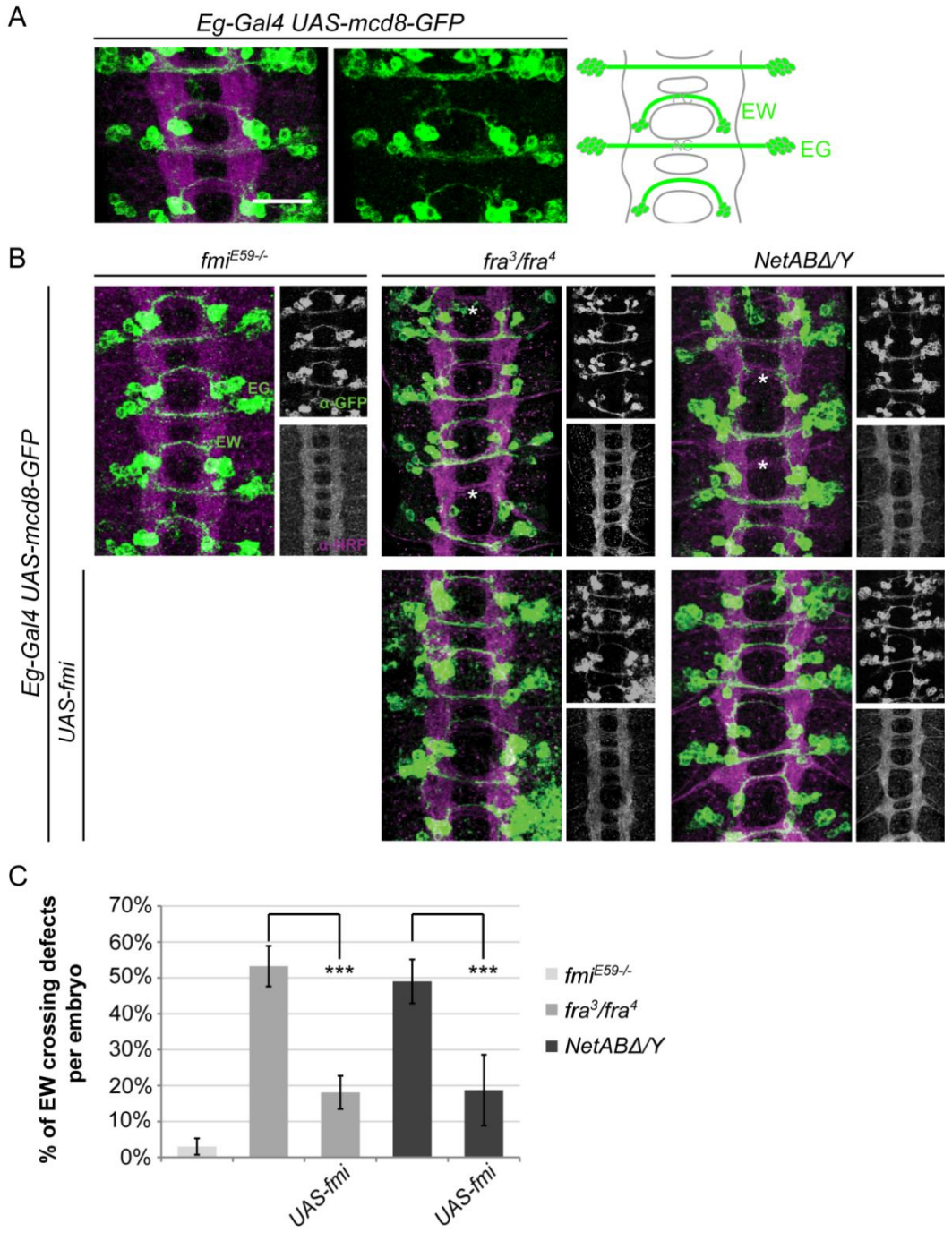


Figure 3-7 Flamingo can elicit midline crossing.

Analysis of commissural axon pathfinding in stage 16 embryos expressing *UAS-mCD8-GFP* in Eagle-positive commissural neurons (with *Eg-Gal4*). **A**. In WT embryos eg-positive commissural axons properly cross the midline in two distinct fascicles: the EG cluster crossing in the AC and the EW cluster crossing in the PC. **B** In *fmi*$^{E59-/-}$ mutants no crossing defects are observed. In *fra*^{3}*/fra*^{4} mutants and in *NetABΔ/Y* mutants some of the EW neurons fail to cross the midline (asterisks), but selective overexpression of Fmi (*UAS-fmi*) in eg-positive neurons specifically rescues the EW pathfinding errors in *fra* and *Net* mutant embryos. anti-GFP staining (green)

labels eg-positive neurons, anti-HRP staining (magenta) labels the axon scaffold. Scale bar, 20 µm. **C**. Quantification of EW crossing defects. 15-20 embryos per genotype were analyzed. Values displayed are percentages of EW crossing defects. Error bars indicate s.e.m. ****P*<0.001.

Analysis of *eagle*-positive axons in embryos lacking Fmi revealed a few defects in the EW neurons, with around 4% of those not crossing the midline. This data is in line with the analysis performed with the HRP antibody, where virtually all the PC seemed to be properly formed. Mutants lacking *fra* or *Net* showed strong phenotypes in this subpopulation of neurons, with 45 and 48% of EW clusters not crossing the midline. Aberrant crossing was already shown in these mutants (Brankatschk & Dickson, 2006; Garbe et al. 2007); and selective expression of *UAS-fra* was sufficient to restore normal crossing behavior (Garbe et al., 2007), arguing a cell autonomous function of *fra* in these neurons. To test whether Fmi can also promote midline crossing, Fmi was expressed specifically in *eagle*-positive neurons in otherwise *fra* mutant background. In these embryos, the EW neurons crossing behavior was significantly restored, with only 18% of EW axons failing to cross the midline. To further confirm that the capability of *fra*-mutant and Fmi overexpressing neurons to cross the midline was Netrin independent, a similar experiment was performed in embryos lacking both *NetA* and *NetB*: also in this case, *UAS-fmi* could rescue the phenotype when selectively expressed in eagle-positive neurons. The ability of Fmi to restore axonal targeting of a subgroup of controlateral projecting neurons in *Net/fra* mutant embryos strongly suggest a role in mediating midline crossing, suggesting that particularly in these neurons Fmi might exploit the same molecular pathway activated by Net/Fra ligand-receptor complex.

The role of Fz, the other PCP gene capable of enhancing *fra* mutant axonal phenotype was also analyzed for its ability of compensate for loss of *fra* in eagle-positive neurons. However, no rescue of the commissural defects was observed upon Fz overexpression (Figure 3-8), suggesting that Fz is not mediating midline crossing and thus that Fmi acts independently of Fz in this context.

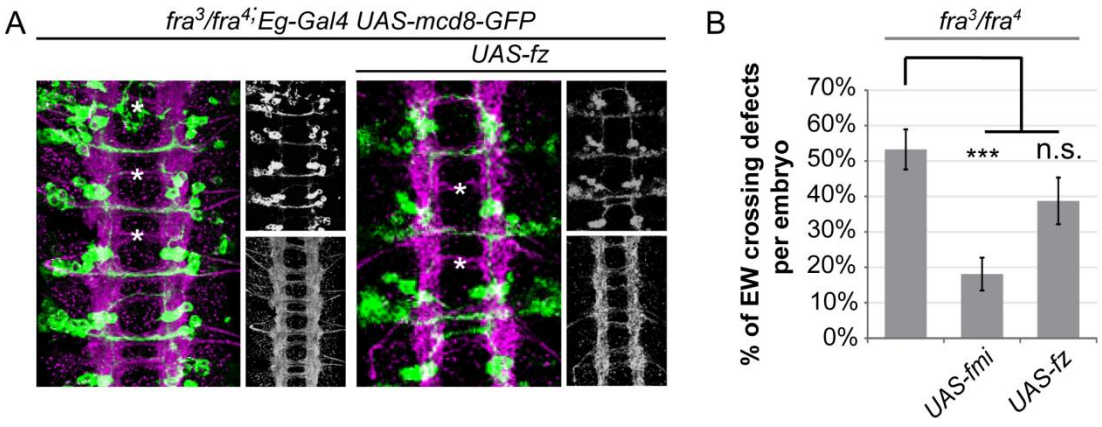


Figure 3-8 Role of Fz in commissural neurons.

Analysis of commissural axon pathfinding in stage 16 embryos expressing *UAS-mCD8-GFP* in Eagle-positive commissural neurons (with *Eg-Gal4*). **A** In fra^3/fra^4 mutants some of the EW neurons fail to cross the midline (asterisks). Selective overexpression of Fz in eg-positive neurons does not ameliorate the EW pathfinding errors in *fra* mutant embryos, whereas overexpression of Fmi does. anti-GFP staining (green) labels eg-positive neurons, anti-HRP staining (magenta) labels the axon scaffold. Scale bar, 20 µm. **B**. Quantification of EW crossing defects. 15-20 embryos per genotype were analyzed. Values displayed are percentages of EW crossing defects. Error bars indicate s.e.m. ****P*<0.001; n.s.:not significant.

To further confirm that Fmi could mediate midline crossing, Fmi was overexpressed in neurons that normally do not cross the midline. For this purpose, the Ftz_{ng}*-Gal4* driver was employed, which is expressed in longitudinal projecting axons that usually project ipsilaterally (Lin et al., 1994) and the behavior of those longitudinal axons was analyzed with the anti-FasII staining. For instance, overexpression of the Ig CAM Dscam in these neurons caused Netrin-independent ectopic crossing (Andrews et al., 2008). Overexpression of one copy of *UAS-fmi* was not sufficient to mediate ectopic crossing, similarly to what observed for *UAS-fra* (Dorsten et al., 2007; Kim et al., 2002). However, increasing the protein amount by adding another copy of either *UAS-fmi* or *UAS-fra* caused some ipsilaterally axons to cross the midline (Figure 3-9).

The ability to mediate ectopic midline crossing when overexpressed in ipsilaterally projecting neurons and to suppress midline crossing defects in *fra* mutant embryos strongly argue for a specific role of Fmi in promoting midline crossing.

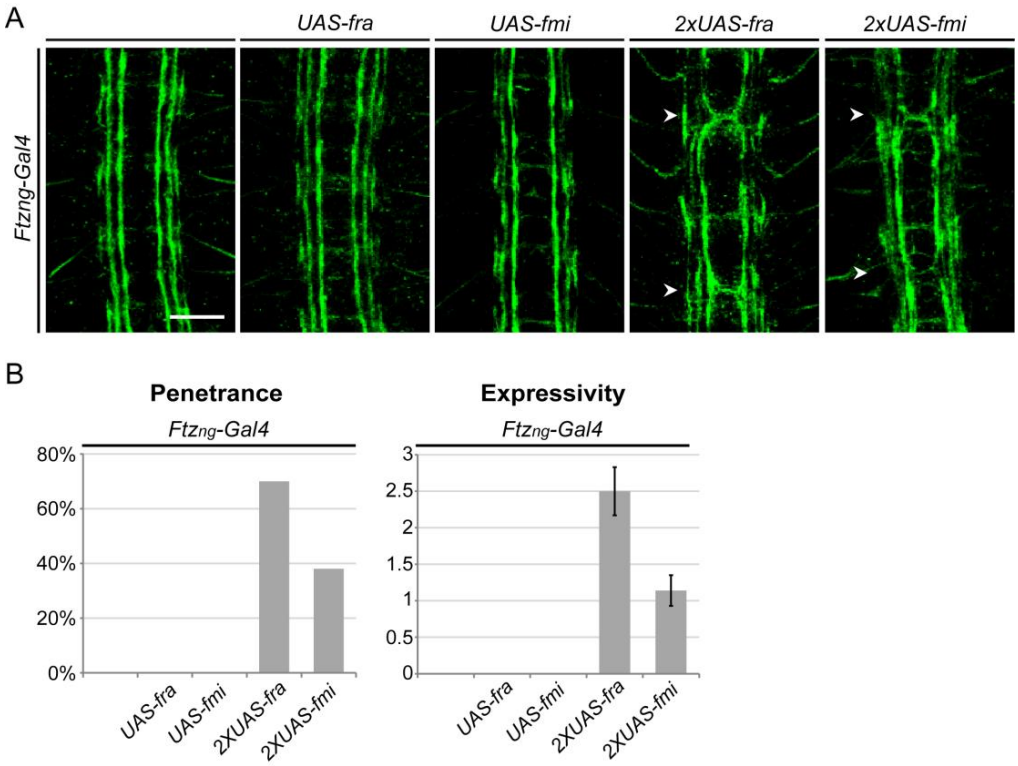


Figure 3-9 Fmi can cause ectopic midline crossing.

Effect of overexpression of Fmi or Fra in ipsilateral neurons. **A.** Stage 17 embryos overexpressing either Fmi (*UAS-fmi*) or Fra (*UAS-fra*) in a small population of *Ftz$_{ng}$-Gal4* positive ipsilateral neurons exhibit ectopic midline crossing (arrowheads). Anti-FasII labels ipsilateral tracts (green). Scale bar, 20 µm. **D.** Quantification of ectopic midline crossing expressed as penetrance (percentage of embryos that display the phenotype) and as expressivity (average number of ectopic crossing per embryos that exhibit the phenotype).

3.6 Flamingo does not act cell-autonomously in *eagle* neurons

It was shown that selective re-expression of Fra in the small eagle-positive population of commissural neurons in an otherwise *fra* mutant background was sufficient to restore normal crossing behavior, arguing for a cell-autonomous function of Fra in these neurons (Garbe et al., 2007). Similarly, selective overexpression of Fmi in eagle neurons in *fra* mutant background can also increase midline crossing (Section 3.5). These findings raise the question of whether Fmi could mediate midline crossing in a cell-autonomous way.

Considering that loss of *fmi* does not cause appreciable defects in eagle neurons, the question was addressed by re-expressing Fmi or Fra in $fra^{3-/-}$ $fmi^{E59-/-}$ embryos using the *Eg-Gal4* driver. In *fra, fmi* double mutants, nearly all the EW axons fail to cross the midline in the PC (Figure 3-10A), but upon re-expression of Fra, only 24% of the EW neurons mis-projected. The rescue was particularly significant, considering that the number of EG bundles non-crossing was lower than the defects observed in *fra* single mutants. Conversely, re-expression of Fmi did not significantly rescue the midline crossing defects. This result was surprising, considering the rescue capability of Fmi when pan-neuronal re-expressed in double mutants, as well as when compared to the effect seen in EW neurons of *fra* single mutant embryos. However, it is important to notice some differences between these experiments. In $fra^{-/-}$ embryos PCs, where EW axons project are usually thinner, but completely lost in very few cases, whereas in $fra^{3-/-}$ $fmi^{E59-/-}$ embryos PC are absent in the majority of segments. This could suggest that Fmi, in order to mediate crossing, needs contact with other axons. The possible mechanism underlying Fmi function in commissural neurons will be described in the discussion part; however, is possible to conclude that Fmi does not act cell-autonomously at least in this subset of commissural neurons.

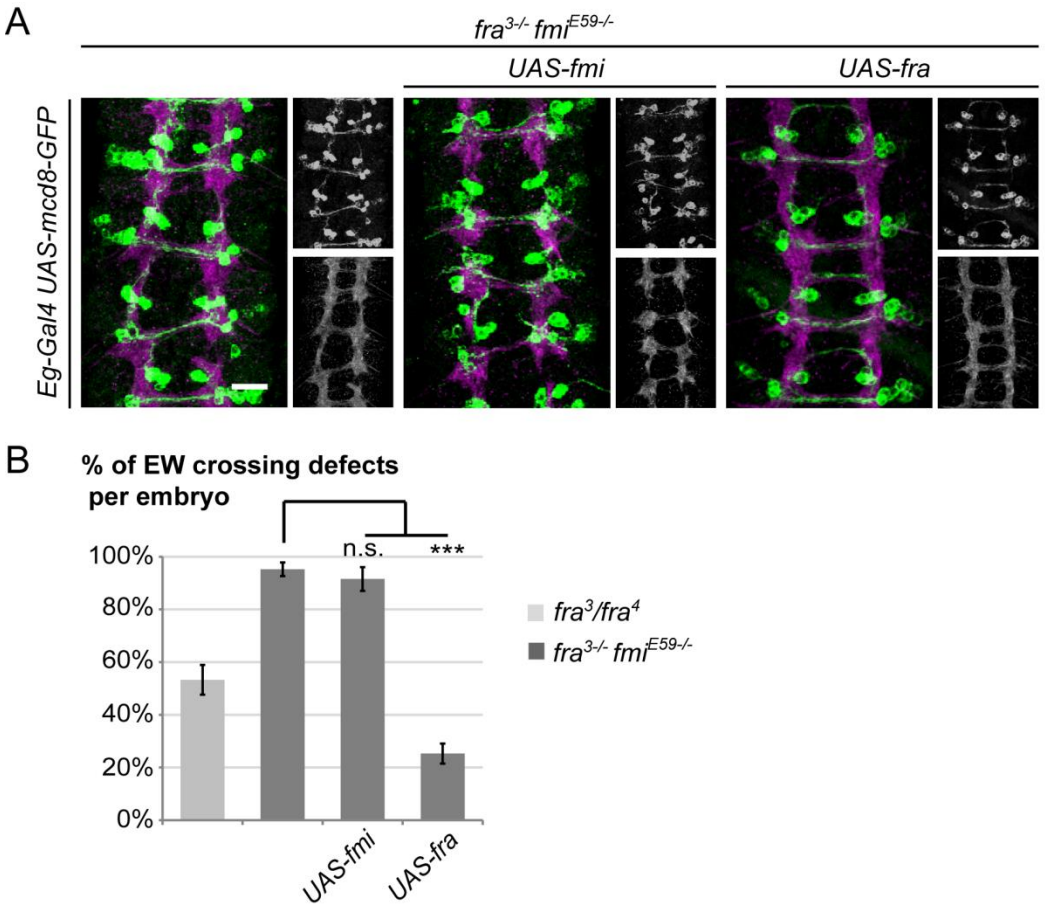


Figure 3-10 Effect of selective Fra or Fmi overexpression in midline crossing neurons.

A.In *fra, fmi* mutants, most of the EW axons fail to cross the midline in the PC. Specific re-expression of Fmi in *eagle*-positive neurons does not ameliorate the crossing defects, whereas selective re-expression of *fra* significantly rescues the defects, reducing the number of non-crossing bundles to less than what is observed in *fra* single mutants. Anti-GFP labels eagle neurons (green), anti-HRP (magenta) labels the axon scaffold. Scale bar, 20 µm. **B**. Quantification of EW crossing defects. 10-20 embryos per genotype were analyzed. Values displayed are percentages of EW crossing defects. Error bars indicate s.e.m. ***P<0.001, n.s.: not significant.

3.7 Role of Flamingo in longitudinal tracts

The mutant analysis (Section 3.1), and the pan-neuronal rescue experiments (Section 3.4) showed that Fmi plays a role in both commissural and longitudinal projecting neurons. Given that this two macro-population of neurons have been frequently described as having a separated and independent development, as well as a different molecular requirement, a more detailed analysis in ipsilateral and, in general, longitudinal projecting neurons is required in order to clarify whether Fmi plays a common role in all the neurons, or its mechanism of action is more dynamic among different classes of neurons.

To address this question, the behavior of some groups of neurons was analyzed, using specific genetic markers, such as *Sema2b-Tmyc, 15J2-Gal4* and *C544-Gal4.*

3.7.1 Analysis of controlateral neurons.

The phenotype resulting from loss of both *fmi* and *fra* appeared to apply to the majority of the neurons in the VNC; however, from the HRP and FasII staining it is really hard to distinguish which neurons are really affected. Controlateral neurons, for example, are a very heterogeneous class, since some of those neurons are interneurons, only crossing the midline and stopping immediately, and others extend their axons further along the anterior-posterior axis, reaching targets that sometimes can be located in the neighboring segments. To analyze in more detail axonal outgrowth, genetic markers that specifically labeled a small subset of neurons were employed. *Sema2b-Tmyc* was used to label a fraction of contralateral neurons (Rajagopalan et al., 2000). This marker labels cell bodies and axons of two to three neurons in each of the A4-A8 segments, as shown in figure 3-11A. The cell bodies are located close to the HRP/FasII positive neuropil, and the respective axons extend along the anterior commissure (AC). After crossing the midline, the axons further extend anteriorly, along the most lateral edge of the intermediate FasII positive fascicle. In $fmi^{-/-}$, no defects are observed in the pathfinding of those neurons (Figure 3-11A). In *fra* mutants, very few defects were observed: around 10% of the neurons were crossing the midline properly, but then they failed to extend anteriorly, or they extended but stop prematurely. In $fra^{-/-}$ $fmi^{-/-}$ embryos, only 30% of the neurons displayed

normal extension. Most of them were not extending (29%) or stopping (24%) after crossing the midline. Some of the neurons were displaying very severe phenotypes, such as thinner commissural bundles (Figure 3-11 A-B, indicated by a (+)), presumably resulting from some of the axons not crossing the midline, and axons crossing the midline, but then extending into the wrong direction, usually posteriorly instead of anteriorly (Figure 3-11 A-B, indicated by an asterisk (*)). These severe phenotypes were observed in 18% of the axons analyzed in *fra*$^{-/-}$ *fmi*$^{-/-}$ embryos, but only in 2% of the *fra*$^{-/-}$ embryos. In some of the *fra*$^{-/-}$ *fmi*$^{-/-}$ neurons (30%) cell bodies were mis-localized in a medial position, closer to the midline, but still the number of cells seemed to be unaffected, and the majority of this mispositioned neurons extended their axons (only 9% did not cross the midline), suggesting that loss of *fmi* is affecting axonal outgrowth and not other neuronal properties. It would have been interesting to investigate whether those phenotypes could be rescued by re-expression of *fmi* in these neurons; unfortunately no *Sema2b-Gal4* line is available, thus not enabling any further experiment.

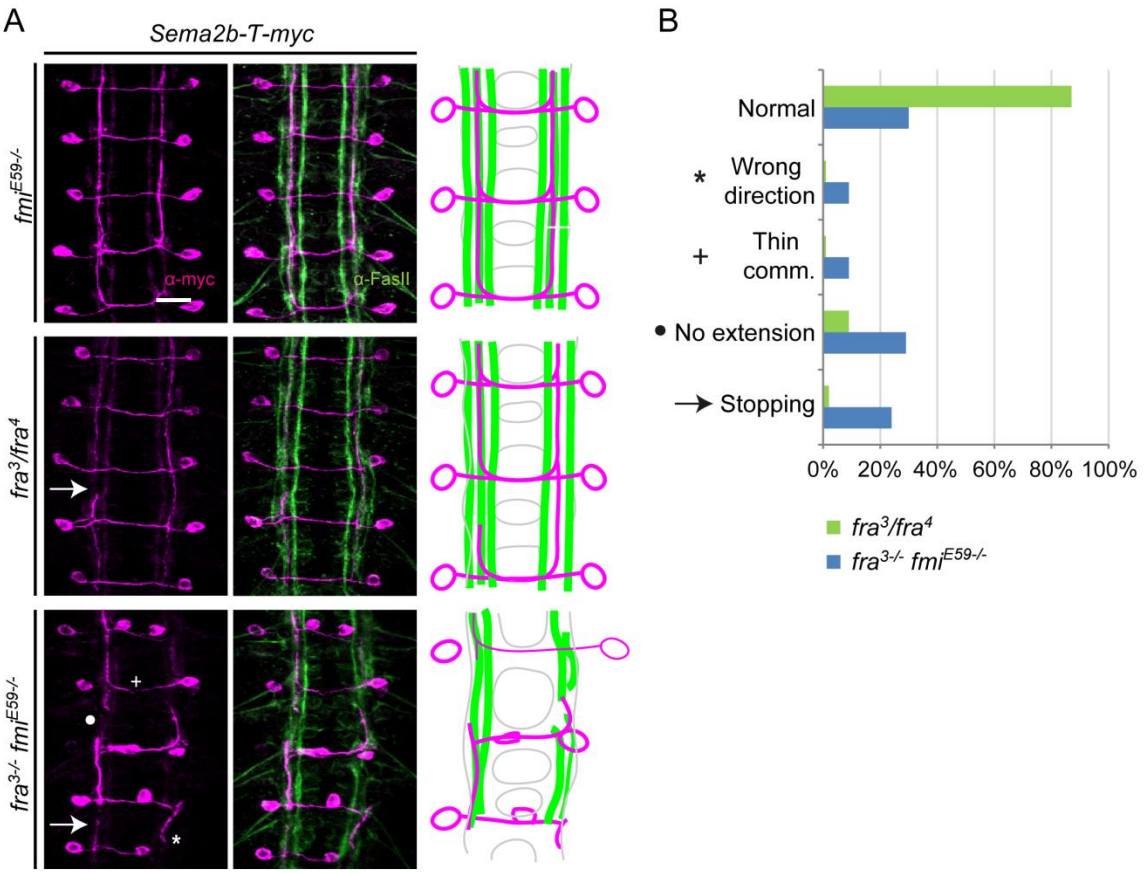


Figure 3-11 Analysis of *Sema2b- Tmyc* contralateral neurons.

A. In *fmi* mutants, no defects are observed, whereas in *fra* mutants 10% of axons do not extend after crossing the midline. In *fra, fmi* double mutants, most of the Sema2b-neurons show phenotypes, such as no crossing, no extension, extension in the wrong direction or premature stopping. Anti-myc labels *Sema2b-Tmyc* neurons (magenta), anti-FasII (green) labels longitudinal tracts. Scale bar, 20 µm. Arrows indicate axons that extend longitudinally but stop prematurely; dots indicate axons that cross the midline but do not extend anteriorly; plus (+) indicate axons that do not cross the midline; asterisks indicate axons that extend in the wrong direction after crossing the midline **B**. Quantification of *Sema2b-Tmyc* path finding defects. 15-20 embryos per genotype were analyzed. Values displayed are percentages of *Sema2b-Tmyc* neurons showing the indicated phenotype; percentages were calculated on the total amount of axons per genotype, not the average defects per embryos.

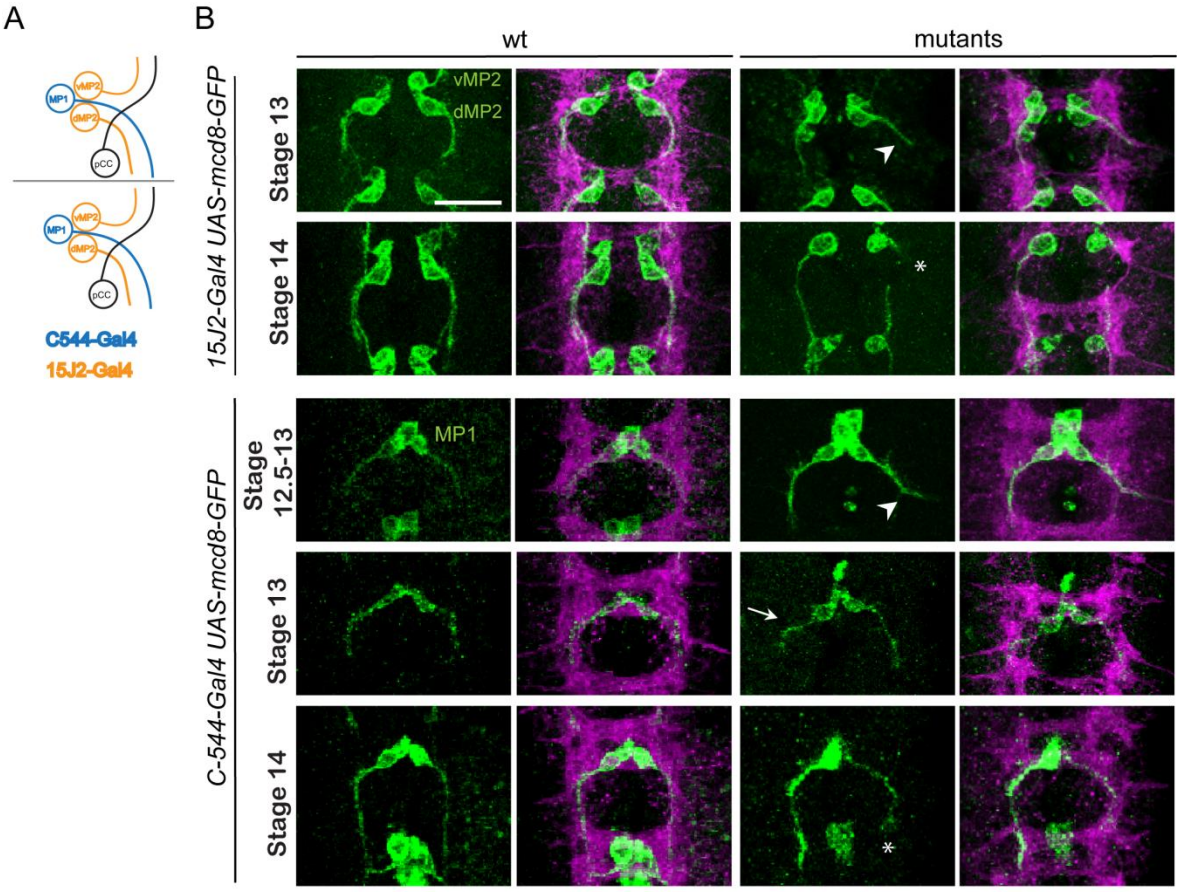
3.7.2 Analysis of pioneer neurons

Axonal outgrowth and pathfinding of longitudinal tracts seem to be particularly dependent on pioneer-follower interactions. In fact, ablation experiments revealed that removal of pioneer neurons impair the normal development of longitudinal tracts (Hidalgo & Brand, 1997). Thus, analysis at a cellular level might be helpful to get insights into the mechanism by which Fmi influences axon outgrowth. Two Gal4 lines are known to be selectively expressed in pioneer neurons: *15J2-Gal4* is expressed in both vMP2 and dMP2 neurons and *c544-Gal4* in MP1 neurons. dMP2 and MP1 neurons extend their axons posteriorly, whereas vMP2 project anteriorly, as represented in the schematic in figure 3-12A. All of them are ipsilateral neurons, and shown to be important for subsequent axonal extension of neurons that form the most medial FasII-positive bundle (dMP2 and vMP2) and the intermediate fascicle (MP1) (Hidalgo & Brand, 1997). *15J2-Gal4* was already used to analyze the role of *fra* in dMP2 pioneer neurons (Hiramoto et al., 2000). Here, *fra* plays a non-cell autonomous role for dMP2 axons, "presenting" Netrin to the extending neurons. In fact, selective re-expression of Fra in dMP2 with *15J2-Gal4* in a $fra^{-/-}$ background is not sufficient to rescue defects in dMP2 neurons (Hiramoto et al., 2000). *15J2-Gal4* was therefore used to label dMP2 neurons in *fmi*, *fra* and *fra fmi* mutants at early developmental stage, such as stage 12.5-13 and 14, when axon extension takes place. In these mutants, two different phenotypes were observed. At early stages, some of the growth cones failed to track the boundary of HRP positive neurons, taking an external trajectory and "exiting" the midline (arrowhead in figure 3-12B); at later stages, axons failed to extend (asterisk in figure 3-12B). Those phenotypes were overall very mild in *fra* or *fmi* mutants and not significantly different from WT embryos (Figure 3-12C). In *fra, fmi* double mutants 16% of axons in stage 13 embryos showed a phenotype, and only 12% in stage 14. Given the weakness of the phenotype, the analysis with *15J2-Gal4* was discontinued.

C544-Gal4 labels mainly MP1 neurons and sometimes midline glia cell bodies. The line is expressed only in few segments in each embryo, causing a variable number of axons labeled across different animals. Thus, analysis was performed in a big number of embryos but only 30 to 50 neurons per genotype per

developmental stage were analyzed. The phenotypes observed in MP1 neurons are similar to the ones seen in dMP2 neurons, with axons exiting the midline (arrowheads), not extending (asterisks) or growing without tracing the HRP positive boundary (arrow) (Figure 3-12B). In *fra* or *fmi* mutants, those phenotypes appeared in 8 to 15% of the axons, whereas in double mutants the phenotypes were strongly enhanced, affecting the 37 to 40% of the axons. Selective re-expression in $fra^{-/-}$ $fmi^{-/-}$ background of either Fra or Fmi partially reduced the phenotypes, although in none of the cases the percentages of axons affected was reduced to the WT situation (Quantification in figure 3-12C). The strong phenotype present in double mutants as well as the partial rescues observed upon re-expression of either of the genes suggested that in MP1 neurons *fra* and *fmi* are both required for axonal growth. The phenotype observed in *fra* and *fmi* mutants were very similar, arguing for a redundant function in these neurons. However, the not fully penetrant expression of *c544-Gal4* limited the possibility to strengthen the conclusions by further analysis, such as for example double re-expression of *fra* and *fmi* in double mutants.

All together, the analysis in longitudinal neurons supported the idea that loss of Fmi affects axonal pathfinding rather than defects in axonal outgrowth, and that Fmi can mediate extension of ipsilateral pioneer neurons in a partially autonomous way.



C

	DEFECTS IN dMP2 NEURONS (15J2Gal4)		DEFECTS IN MP1 NEURONS (c544-Gal4)	
	stage 12-13	**stage 13.5-14**	**stage 12-13**	**stage 13.5-14**
wt	7.5%	0%	2.5%	5%
fmi$^{E59-/-}$	6%	0%	8.3%	8.6%
fra$^{3-/-}$	4.3%	6.8%	15.4%	8.3%
fra$^{3-/-}$ *fmi*$^{E59-/-}$	16%	12.0%	37%	40.7%
fra$^{3-/-}$ *fmi*$^{E59-/-}$ *UAS-fmi*	n.d.	n.d.	33%	31%
fra$^{3-/-}$ *fmi*$^{E59-/-}$ *UAS-fra*	n.d.	n.d.	20.8%	25%

Figure 3-12 Analysis of longitudinal pioneer neurons.

A.Schematic depicting the pioneer neurons of the longitudinal tracts and the Gal4 lines used to selectively label them. *15J2-Gal4* labels dMP2 and vMP2, which extend posteriorly and anteriorly, respectively; *c544-Gal4* labels MP1 neurons, which extend their axon posteriorly. **B**. Examples of wild-type (left) and mutants (right) phenotypes observed in pioneer neurons at early stages of axonogenesis when visualized with *15J2-Gal4* or *c544-Gal4* driving the expression of *UAS-mCD8-GFP* (anti-GFP, green). Anti-HRP (magenta) labels the axon scaffold**.** Scale bar, 20 µm**.** Arrowheads indicate axons extending laterally, asterisk indicates premature stop or not extension. **C**. Quantification of defects of MP1 or dMP2 pathfinding. 10-15 embryos per genotype were analyzed in case of *15J2-Gal4*, whereas given the variable expression of *c544-Gal4*, 30-50 axons per stage per genotype were analyzed. Values are percentages of axons showing the phenotype. n.d.: not determined.

3.8 Flamingo intracellular domain is required for its action.

As non-classical cadherins, Fmi and its homologues contain domains giving them the capability of acting as cell-adhesion molecules (through the cadherin repeats), as well as receptors. Interestingly, both molecular mechanisms appear to be used. Fmi was found to mediate adhesion between photoreceptor axons and their targets in the medulla within the *Drosophila* visual system (Hakeda-Suzuki et al., 2011), as well as adhesion between pioneer and follower axons in the VNC of nematodes (Steimel et al., 2010). Receptor-like functions were found in mammals, where Celrs2 and Celrs3 were proved to trigger intracellular calcium increase upon binding to their respective Cadherin repeats (Shima et al., 2007) and in *Drosophila*, where Fmi extracellular domain was shown to be partially dispensable to mediate repulsion between dendritic branches of peripheral sensory neurons in larvae (Gao et al., 2000), whereas the intracellular domain was required, allowing interaction with the LIM domain protein Espinas (Esn) (Matsubara et al., 2011). In order to assess which domains and consequently, which molecular mechanisms are required in this neuronal context, a series of transgenic fly lines carrying versions of Fmi lacking either the extracellular of the intracellular domains were used (Kimura et al., 2006; H. Strutt & Strutt, 2008). These Fmi variants were specifically expressed in neurons of embryos mutants for both *fra* and *fmi* making it possible to analyze their ability of restoring normal axonal projections. Expression of *UAS-fmiΔIntra*, a version lacking the intracellular tail (schematic in Figure 3-13A), could not rescue the mutant phenotype in neither commissurally nor longitudinally projecting neurons (Figure 3-13B). Instead, expression of *UAS-fmiΔN,* lacking most of the extracellular domain (Cadherin repeats, EGF-like domains and laminin-G-like domains) could partially suppress both phenotypes (Figure 3-13B). In particular, compared to *fra, fmi* mutants, 20% more commissures were formed when the Fmi version with the intracellular domain was expressed. Moreover, a less severe phenotype was observed in longitudinal axons (Figure 3-13C and D).

These rescue experiments showed that the short intracellular tail is required for Fmi activity. The same domain requirement was confirmed for Fmi function in midline crossing by expression of *UAS-fmiΔN* or *UAS-fmiΔIntra* with *EG-Gal4* in *fra*$^{3-/-}$ embryos. *UAS-fmiΔIntra* failed to restore midline crossing, whereas Fmi*ΔN* could reduce defects in *eg-Gal4* neurons (Figure 3-13E-F)

Therefore, Fmi seems to act in this context as a signaling molecule rather than as a pure adhesion molecule.

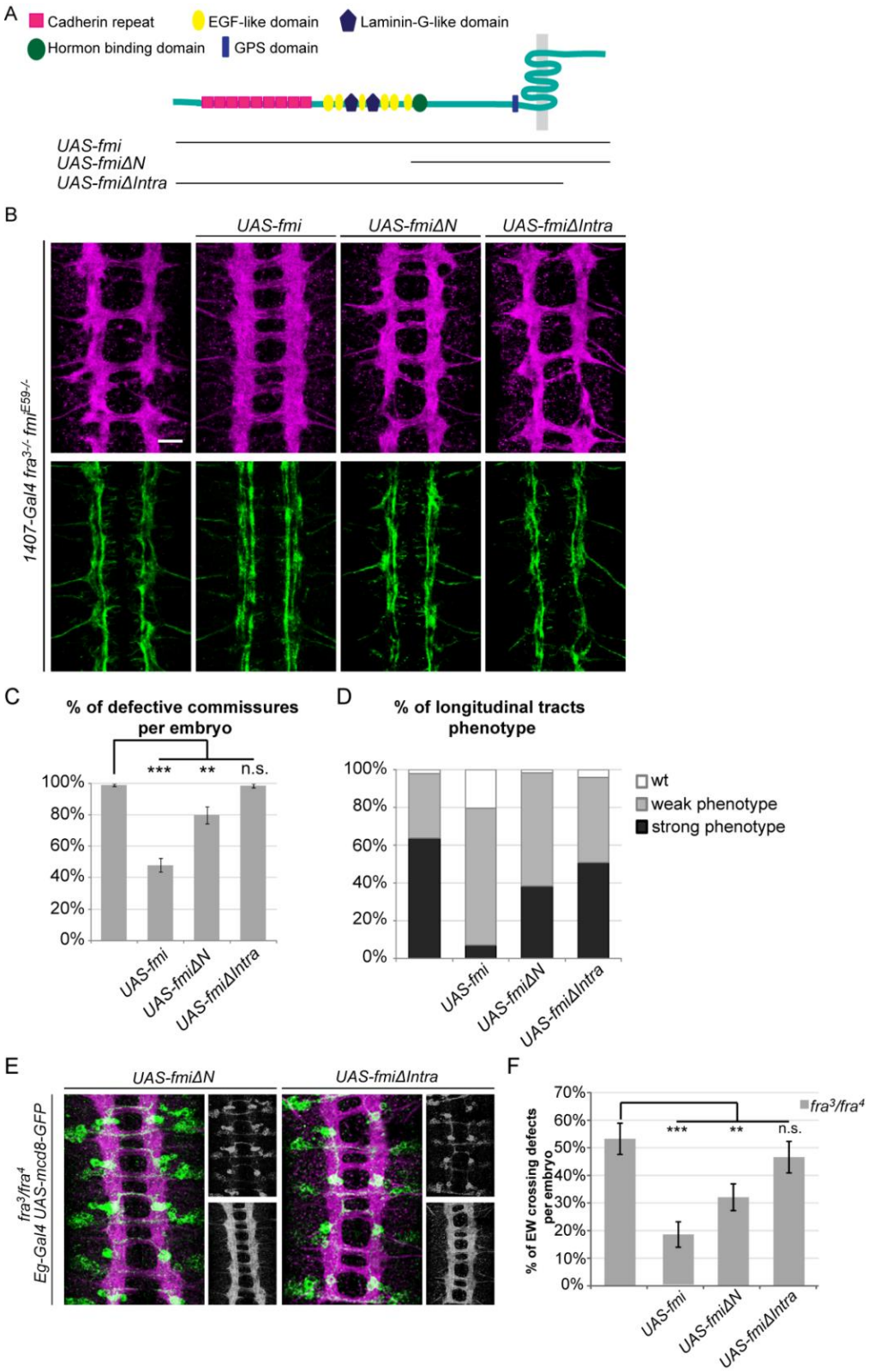


Figure 3-13 Flamingo requires the intracellular C-terminal domain, but not cadherin repeats.

A-F. Analysis of Fmi domain requirement. **A**. Schematic of Fmi structural domains with domains retained in different *UAS* constructs. *UAS-fmiΔN* lacks cadherin repeats, EGF-like domains and

LaminiG-like domains, but retains hormone binding domain and GPS domain, as well as the whole 7-TM and intracellular domain. *UAS-fmiΔIntra* lacks the intracellular domain, except the first 30 amino acids after the last transmembrane domain. In both cases, black line indicates the parts included in the construct. **B**. Neuronal expression (*1407-Gal4*) of *UAS-fmiΔN* in $fra^{3-/-}$ $fmi^{E59-/-}$ mutants partially rescues commissural axon defects (anti-HRP staining, upper row) and the severity of the longitudinal tracts defects (anti-FasII staining in green, bottom row), whereas expression of *UAS-fmiΔIntra* in $fra^{3-/-}$ $fmi^{E59-/-}$ mutants still resembles *fra, fmi* double mutants. Scale bar, 20 µm. **C-D**. Quantification for the rescue of the PC defects (C) and of the longitudinal tracts defects (D) in *fra, fmi* double mutants. 15-20 embryos per genotype were analyzed. Values displayed in C are percentages of defective posterior commissure. Error bars indicate s.e.m. ***P<0.001; **P<0.01, n.s.: not significant. Values displayed in D are percentages of defective longitudinal tracts. Mild phenotype/strong phenotype categories are same as in figure 3-1. **E**. Additionally, selective overexpression of *UAS-fmiΔN* in eg-positive neurons specifically rescues the EW pathfinding errors in *fra* mutant embryos, whereas overexpression of *UAS-fmiΔIntra* fails to reduce crossing defects. Anti-GFP staining (green) labels eg-positive neurons, anti-HRP staining (magenta) labels the axon scaffold. Scale bar, 20 µm. **F**. Quantification of EW crossing defects. 15-20 embryos per genotype were analyzed. Values displayed are percentages of EW crossing defects. Error bars indicate s.e.m. ***P<0.001; **P<0.01, n.s.:not significant.

3.9 Analysis of potential downstream effectors of Fmi signaling.

3.9.1 Role of Rho GTPases in Fmi-mediated midline crossing.

Guidance cues and receptors direct growth cone steering by activating signaling pathways that ultimately change actin cytoskeleton dynamics. Among the molecules eliciting cytoskeleton rearrengements, Rho GTPases play fundamental roles at all stages of axonogenesis (Hall & Lalli, 2010). Among the RhoGTPases members, Rac, Cdc42 and RhoA have been shown to act downstream of many guidance receptors. Usually, Rac and Cdc42 associate with attractive signaling pathway, stimulating growth cone extension, whereas RhoA acts antagonistically downstream of repulsive cues, promoting growth cone collapse. This is not true for all the signaling, since many exceptions have been observed (Vikis et al., 2000; Vikis et al., 2002). In processes involving Netrin mediated attraction, it has been shown that Rac and Cdc42 but not RhoA are required for neurite outgrowth of DCC-positive neurons (Li et al., 2002). In the context of the fly embryonic CNS, positive genetic interaction was shown between Fra and Cdc42 and Rac, whereas negative interaction was reported for

RhoA. In fact, overexpression of dominant active (DA) versions of either Cdc42 (*UAS-cdc42V12*) or Rac (*UAS-rac1V12*) in ipsilateral neurons with *Ftz$_{ng}$-Gal4* causes ectopic midline crossing of ipsilateral axons, but heterozygous loss of *fra* reduces the strength of this phenotype, suggesting that this ectopic crossing might be at least partially depending on Fra signaling (Dorsten et al., 2007). Conversely, overexpression of a constitutively active version of RhoA caused very few midline errors, and those were unaffected by partial loss of *fra* (Dorsten et al., 2007). Considering that a similar phenotype was observed in this study upon overexpression of Fmi (Section 3.5), it is possible that also Fmi and Rac or Cdc42 interact in the context of midline crossing. To test this hypothesis, constitutively active versions of Rac1 and Cdc42 were overexpressed in ipsilateral neurons and one copy of *fmi* was removed in order to see whether it has any effect on the number of ectopic midline crossing observed. Heterozygous loss of *fmi* affects the number of ectopic crossing caused by overexpression of *UAS-rac1V12* by reducing both the number of embryos showing the phenotype and the number of midline crossings (Figure 3-14A-C). Conversely, partial removal of *fmi* did not affect the phenotype observed upon overexpression of Cdc42V12 (Figure 3-14 A-C), suggesting that in the context of midline crossing Fmi acts through a signaling pathway that activates Rac1 but not Cdc42. In order to confirm that Rac can cause midline crossing of commissural neurons, a WT version of it was overexpressed in Eagle neurons in *fra$^{-/-}$* embryos. Additionally, WT versions of Cdc42 and RhoA were overexpressed, in order to clarify their involvement in midline crossing (Figure 3-14 D). Only Rac was capable of increasing the number of EW neurons properly crossing the midline (Figure 3-14 E), suggesting that indeed Rac plays an important role for midline crossing, and that it can be activated from a signaling mechanism independent of Fra. Taken together, those results suggest that at least in the context of midline crossing, Fmi interacts with the GTPase Rac to promote growth cone extension across the midline.

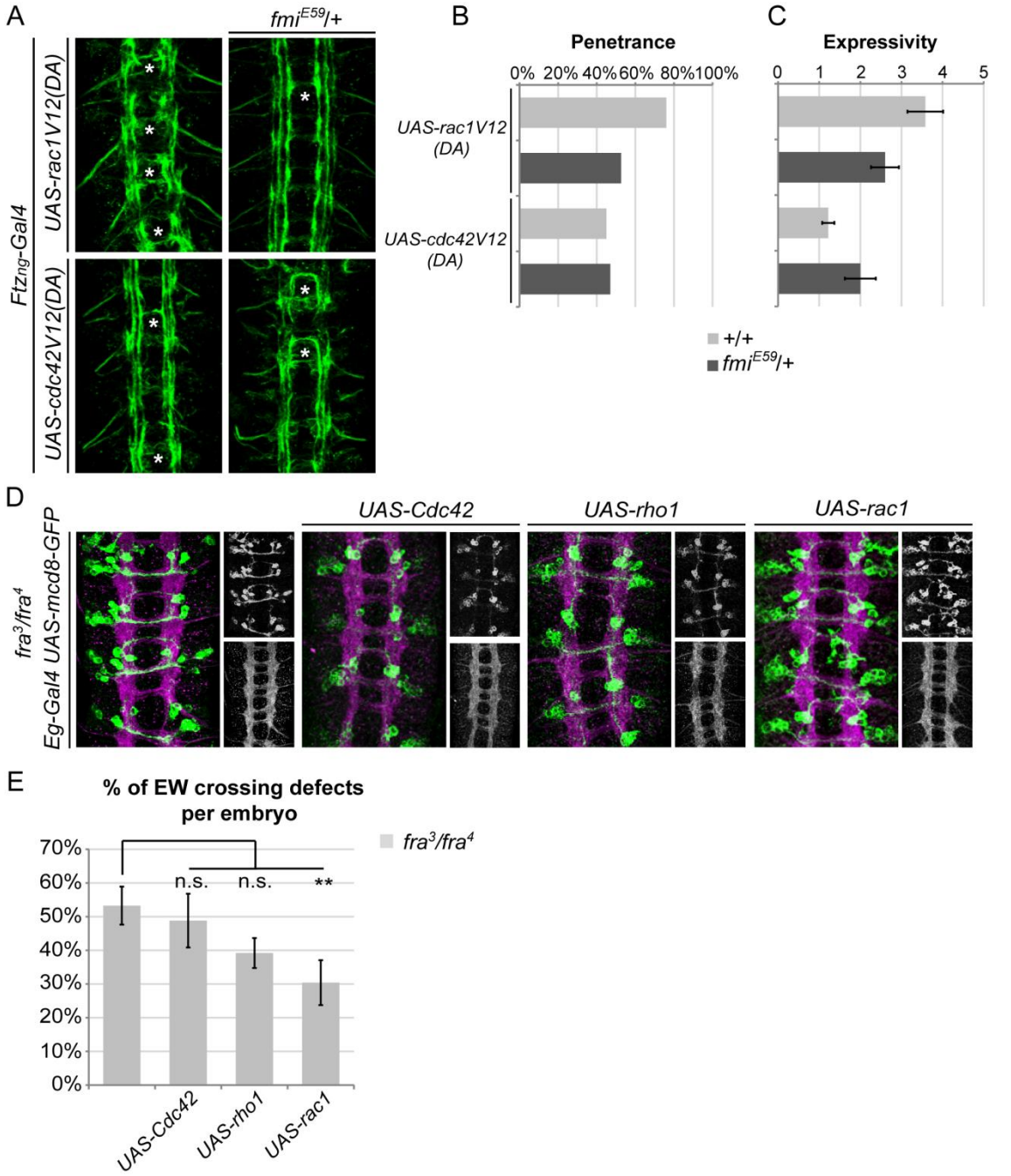


Figure 3-14 Roles of Rho GTPases in Fmi-mediated midline crossing.

A. Stage 17 embryos overexpressing dominant active versions of either Rac1 (*UAS-rac1V12,* upper row) or Cdc42 (*UAS-cdc42V12, bottom row)* in a small population of *Ftz$_{ng}$-Gal4* positive ipsilateral neurons exhibit ectopic midline crossing (asterisks). Removal of one copy of *fmi* reduces the strength of the phenotype of RacV12 overexpression but not of Cdc42V12 overexpression. Anti-FasII labels ipsilateral tracts (green). Scale bar, 20 µm. **B-C.** Quantification of ectopic midline crossing expressed as penetrance (B, percentage of embryos that display the phenotype) and as expressivity (C, average number of ectopic crossing per embryos that exhibit the phenotype).

D. Effect of selective overexpression of either Rac1 Cdc42 or RhoA in eg-positive neurons (labeled with *Eg-Gal4 UAS-mCD8-GFP*) in *fra* mutant embryos. Only overexpression of Rac rescues the EW pathfinding errors. anti-GFP staining (green) labels eg-positive neurons, anti-HRP staining (magenta) labels the axon scaffold. **E**. Quantification of EW crossing defects. 15-20 embryos per genotype were analyzed. Values displayed are percentages of EW crossing defects. Error bars indicate s.e.m. ***P*<0.01. n.s.: not significant.

3.9.2 Espinas mediates Fmi signaling in sensory neuron dendrites but not in midline axons.

The domain analysis reported in chapter 3.8 suggested that Fmi acts as a signaling receptor, mediating an axon guidance signal through its short intracellular domain. Up to now, only one study reported data on potential Fmi binding partners acting as downstream signaling components. Matsubara and colleagues (2011) performed a yeast two-hybrid screening using Fmi C-terminal cytoplasmic tail and found that some members of the *Drosophila* PET-LIM domain family can bind to Fmi: Prickle (Pk), Espinas (Esn) and Testin. Among those, they found that Esn could physiologically bind to the C-terminal tail of Fmi, thus mediating Fmi interaction with intracellular pathways required to transduce a dendritic self-avoidance signal (Matsubara et al., 2011). Some evidence suggests that Fmi-Esn signaling complex could likely play a role in the CNS system. In fact, in both class IV sensory neurons and embryonic CNS neurons Fmi C-terminal intracellular tail is required, suggesting a common activity mechanism. Moreover, Esn is broadly expressed in the embryonic CNS and, as observed for *fmi* mutants, no significant axon guidance phenotype was detected in $esn^{-/-}$embryos (Matsubara et al., 2011). To assess whether Fmi signals via Esn, animals carrying the protein-null mutation esn^{KO6} in combination with fra^{3}, fmi^{E59} or *NetABΔ* mutant alleles were analyzed. None of these double mutant animals showed enhancement of the phenotype compared to their respective single mutant animals (Figure 3-15). Therefore, it was concluded that Esn is not required in this system, and that Fmi exploited a different intracellular signaling pathway such as Rac GTPase mediated signaling to elicit its function during embryonic axonal development.

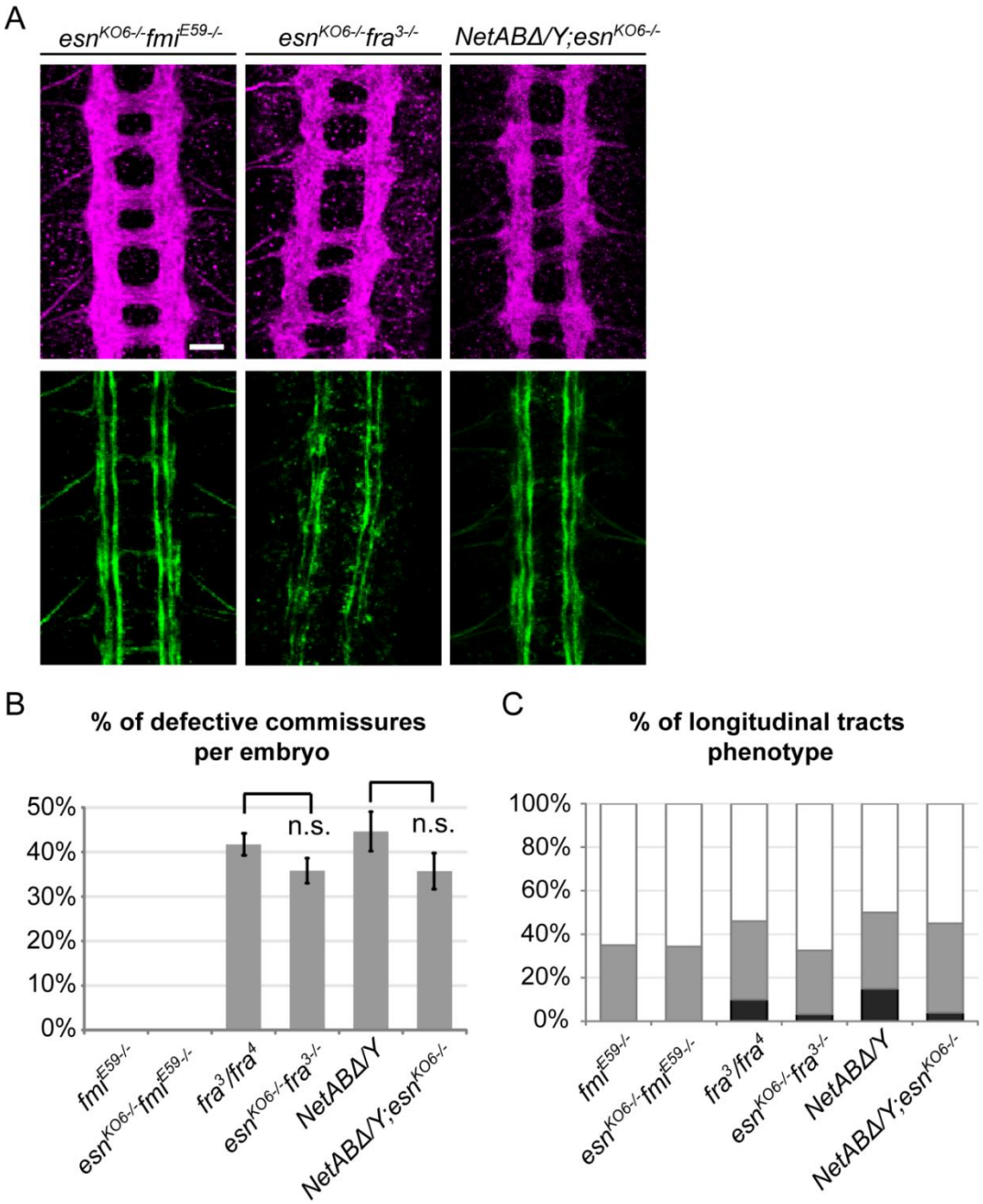


Figure 3-15 Espinas does not mediate Fmi intracellular signaling.

A. Analysis of *espinas* null allele $esn^{KO6-/-}$ in either $fmi^{E59-/-}$, $fra^{3-/-}$ or $NetAB\Delta^{-/-}$ mutant background does not show enhancement of the single mutants phenotypes, neither in midline crossing neurons (magenta, anti-HRP staining) nor in longitudinal projecting neurons (green, anti-FasII staining). Scale bar, 20 µm. **B-C** Quantification of PC defects (B) and longitudinal tracts (C) defects in $esn^{KO6-/-}$ $fmi^{E59-/-}$, $esn^{KO6-/-}$ $fra^{3-/-}$ and $NetAB\Delta^{-/-}esn^{KO6-/-}$double mutants. 15-20 embryos per genotype were analyzed. Values displayed in B are percentages of defective posterior commissure. Error bars indicate s.e.m. n.s.: not significant. Values displayed in C are percentages of defective longitudinal tracts. Mild phenotype/strong phenotype categories are same as in figure 3-1.

3.9.3 Abl mediates Frazzled but not Flamingo dependent midline crossing.

Effective responses to extracellular guidance cues rely on downstream molecules that transduce the signal to the cytoskeleton, ultimately resulting in alterations in growth cone mobility. Among the cytoplasmic proteins that have been implicated in axon guidance, the Abelson tyrosine kinase (Abl) plays a particularly complex role. Analysis of the role of Abl in embryonic CNS development has been particularly challenging because of maternal contribution. In zygotic mutants the strong neuronal phenotype is masked by the maternally produced protein (Grevengoed et al., 2001). In fact removal of both maternal and zygotic Abl resulted in severely disrupted axonal scaffold, with loss of most of the commissural and longitudinal tracts (Grevengoed et al., 2001). In zygotic mutants, few defects were observed (Forsthoefel et al., 2005; Gertler et al., 1989). Additionally, Abl has been implicated in both repulsive and attractive signaling. Abl was shown to antagonize Robo signaling by binding directly to Robo and modulating its phosphorylation state (Bashaw et al., 2000). However another study (Wills et al., 2002) implicated Abl as positive effector of Slit-Robo signaling, arguing that Abl restricts midline crossing. Data seem to be more consistent for AbI as effector of Netrin-midline attractive signaling. It was shown that zygotic mutation of *abl* strongly enhanced *fra* and *Net* mutant phenotypes, suggesting that Abl promotes midline attraction as Net/Fra effector (Forsthoefel et al., 2005). Additionally Fra and Abl were found to physically interact (Forsthoefel et al., 2005). Those physical and genetic interactions have been confirmed in another study, where the function of Abl was analyzed in detail in the context of midline crossing neurons (O'Donnell & Bashaw, 2013). Abl was suggested to act cell-autonomously in commissural neurons, promoting Netrin-dependent midline crossing through its C-terminal F-actin binding domain in a partially kinase-independent fashion (O'Donnell & Bashaw, 2013). The reduction of the crossing defects observed in *fra* mutants when *fmi* was specifically overexpressed in commissural neurons (section 3.5) suggested that Fmi might be activating, in this context, an overlapping molecular repertoire with Fra. Thus, Abl seemed to be a good candidate. Particularly interesting was the *fra, abl* double mutant phenotype, very reminiscent of *fra, fmi* double mutants. In order to

investigate a possible role of Abl as common mediator of Fmi and Fra signaling, several experiments were performed. First, the Abl/Fra physical interaction was validated by testing their ability of binding *in vitro* in co-immunoprecipitation assays. A version of Abl tagged at the C-terminal with 2 copies of c-myc was cloned in a *pUAS*-vector and co-overexpressed in S2 cells together with a version of *GFP* C-terminal tagged Fra (*UAS-Fra-GFP*). The pull-down of Abl using an anti-myc antibody resulted in co-immunoprecipitation of Fra-GFP (Figure 3-16A), confirming the already reported interaction between Abl and Fra. Unfortunately, a similar experiment addressing the interaction between Abl and Fmi failed likely because of the large size of Fmi (data not shown).

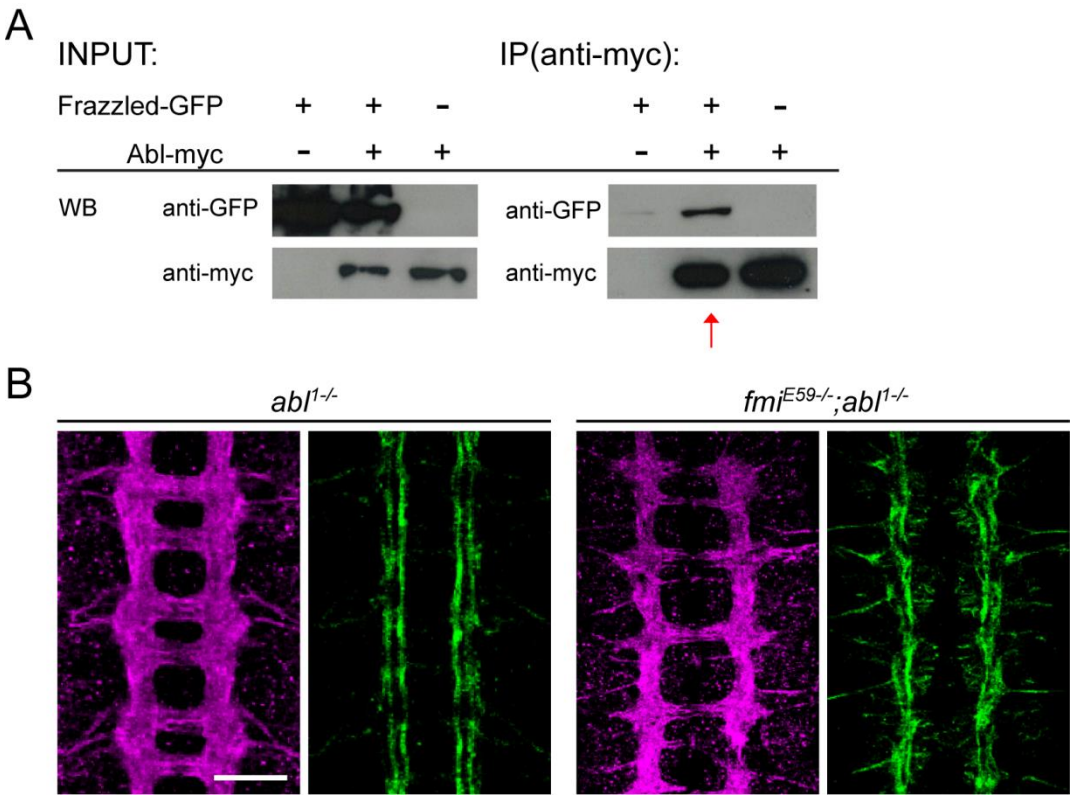


Figure 3-16 Evidences suggesting Abl as common effector of Fra and Fmi signaling.

A.*UAS-frazzled-GFP* and *UAS-abl-myc* were expressed in S2 cells under the control of *Actin-Gal4*. Immunoprecipitation (IP) of Abl with anti-myc antibody also pulled down Fra. **B**. *Abl* zygotic mutant embryos show very little neuronal defects, whereas *fmi, abl* double mutants show disrupted commissures (anti-HRP, magenta) and longitudinal tracts (anti-FasII, green), similar to what is observed in *fra, fmi* double mutants. Scale bar, 20 µm.

This evidence still leaves open the possibility that Abl only works downstream of Fra, and that the *fmi abl* double mutant phenotype results from the loss of two parallel pathways: Fra/Abl in one pathway and Fmi in another. To validate the hypothesis that Abl can be activated independently of Net/Fra, its function in commissural *eagle*-positive neurons was analyzed in a *fra*$^{-/-}$ background. Again, similarly to what had been observed in *fra*$^{-/-}$ embryos overexpressing Fmi, Abl overexpression led to a significant reduction of the midline crossing defects, thus supporting the hypothesis that Abl can be activated independently of Fra in commissural axons (Figure 3-17). Yet, strong evidence for Abl-Fmi interaction in the system was missing. In most of the cases, Abl requires its catalytic activity to function. Yet, some kinase independent functions have been described for Abl (Henkemeyer et al., 1990; Lapetina et al., 2009); additionally, in eg-positive neurons Abl activity was reported to be kinase-independent, although a minimal level of WT Abl was required (O'Donnell & Bashaw, 2013). Thus, given the controversial data on kinase requirement for Abl in commissural neurons, a version of Abl carrying an amino acid mutation causing loss of kinase activity (Abl^{KD}) was assessed for its ability of rescue *fra*$^{-/-}$ commissural phenotype. Abl^{KD} could not restore midline crossing, hinting that tyrosine kinase activity is required for commissural Fra-independent function of Abl (Figure 3-17). In some systems, Abl^{KD} was suggested to act as dominant negative (Hsouna et al., 2003; Sawyers et al., 1994); instead in commissural eg-positive neurons, a version of Abl lacking the C-terminal but retaining the kinase domain was reported to act as a dominant negative, likely competing with endogenous Abl for interaction with Fra (O'Donnell & Bashaw, 2013). Abl^{KD} was anyway tested for its capability of interfering with Fmi-mediated midline crossing. Eagle-specific co-overexpression of Fmi and Abl^{KD} in *fra*$^{-/-}$ background resulted in a rescue of the crossing defects similar to the one resulting from single Fmi overexpression (Figure 3-17B). Although it not possible to rule out the possibility that *Abl*^{KD} is not acting as dominant negative, the experiment suggested that blocking Abl kinase activity does not result in inhibition of Fmi-mediated axonal extension. Therefore, Abl is not a good candidate for a joint molecular mechanism between Fra and Fmi.

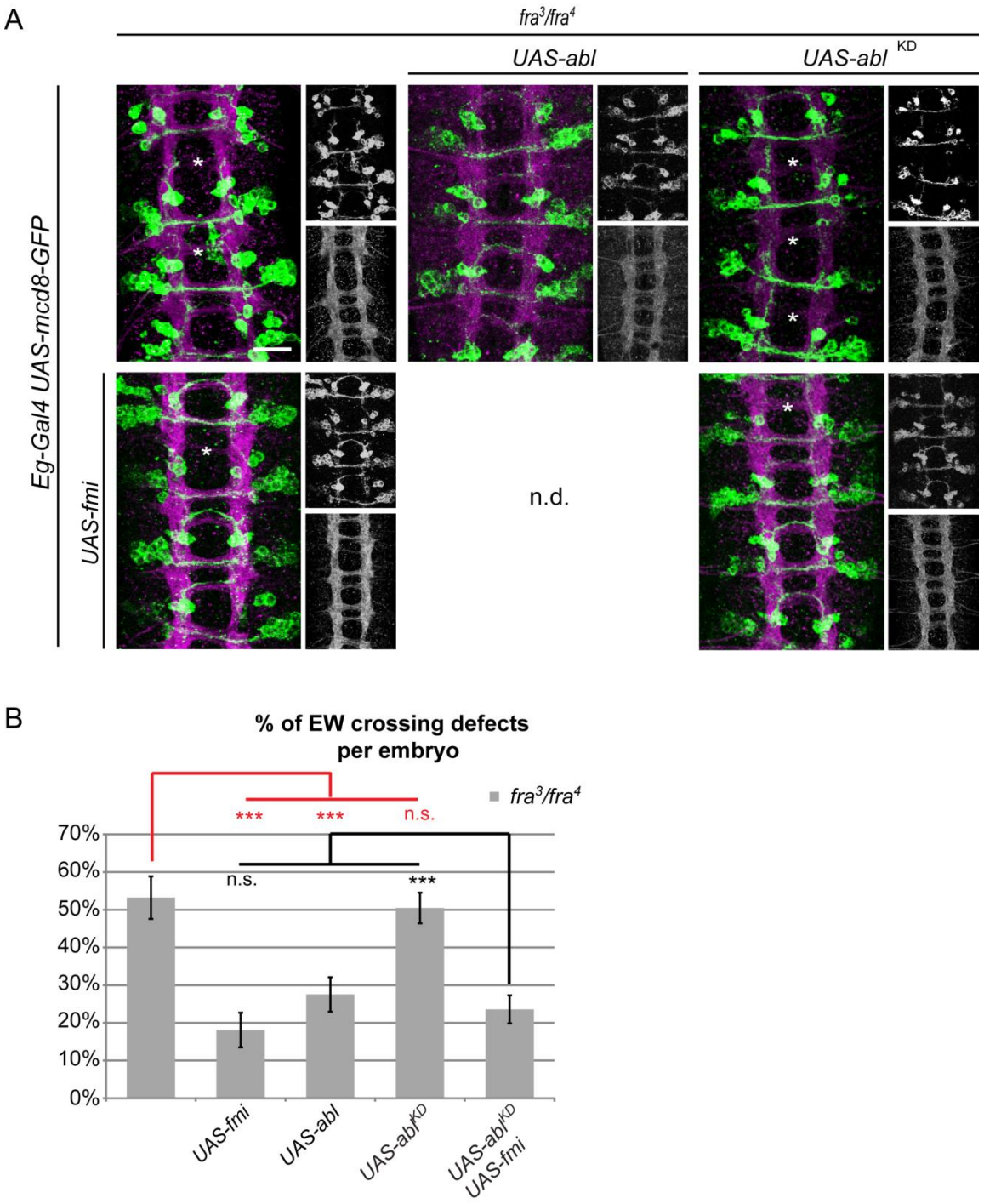


Figure 3-17 Analysis of Fmi/ Abl interaction in commissural axons.

A-B. Effect of selective Abl overexpression in eg-positive commissural neurons in *fra* mutant background. Eg-positive neurons are labeled with *Eg-Gal4 UAS-mCD8-GFP* **A**. Similarly to Fmi, overexpression of Abl (*UAS-Abl*) in a subset of commissural neurons with *Eg-Gal4* rescues the fra^3/fra^4 mutant defects in EW neurons. A kinase dead version of Abl ($UAS\text{-}Abl^{KD}$) does not ameliorate EW pathfinding defects neither interferes with Fmi ability to rescue midline crossing in Eg- neurons when both are co-overexpressed. Anti-GFP labels eagle neurons (green), anti-HRP (magenta) labels the axon scaffold. Scale bar, 20 µm. n.d.: not determined. **B**. Quantification of

EW crossing defects. 15-20 embryos per genotype were analyzed. Values displayed are percentages of EW crossing defects. Error bars indicate s.e.m. ****P*<0.001, n.s.: not significant.

In addition, analysis of Abl, Fra and Fmi overexpression in ipsilateral neurons, with the previously described *Ftz$_{ng}$-Gal4* driver, supported a common pathway between Abl and Fra (Figure 3-9). Overexpression of Abl alone did not cause midline crossing, but co-overexpression with Fra resulted in ectopic crossings in 35% of the embryos analyzed (Figure 3-18), suggesting that ectopic midline crossings observed when Fra levels were increased (with 2 copies of *UAS-fra*, figure 3-9) could be mediated by increase Abl Fra-dependent activity. However, co-overexpression of Fmi and Abl did not result in a phenotype, thus suggesting that the ectopic crossings caused by increased Fmi levels could be Abl-independent. This discrepancy between Fra, Abl and Fmi, Abl overexpressions could as well be due to different levels of expression achieved. However, together with the aforementioned data, these results suggest that Abl, although capable of eliciting midline crossing in a Fra-independent fashion, could as well work independently of Fmi.

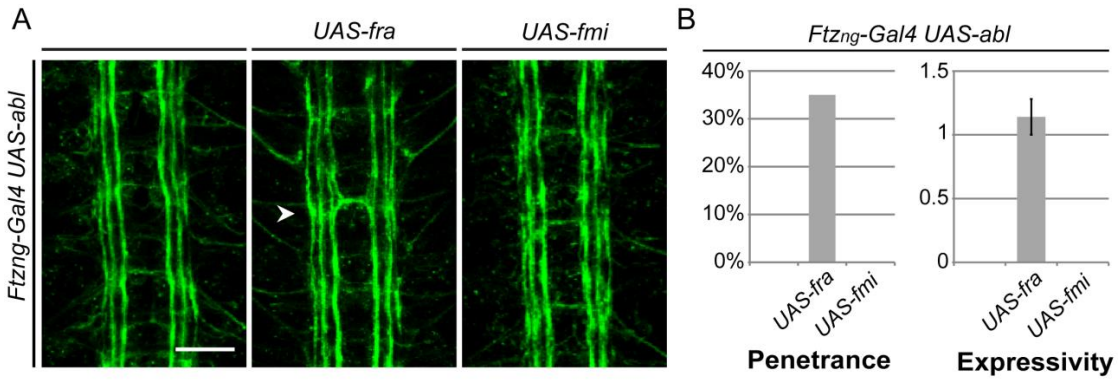


Figure 3-18 Effect of *abl* overexpression in ipsilateral neurons.

A. Stage 17 embryos overexpressing Abl (*UAS-abl*) in *Ftz$_{ng}$-Gal4* positive ipsilateral neurons do not exhibit ectopic midline crossing. When Fra (*UAS-fra*) is co-overexpressed together with Abl, ipsilateral neurons cross the midline (arrowheads), whereas co-overexpression of Abl and Fmi (*UAS-fmi*) does not result in any phenotype. Anti-FasII labels ipsilateral tracts (green). Scale bar, 20 µm. **B.** Quantification of ectopic midline crossing expressed as penetrance (percentage of embryos that display the phenotype) and as expressivity (average number of ectopic crossing per embryos that exhibit the phenotype).

3.10 Analysis of Wnt family members as midline guidance cues.

The data collected till now suggested that Fmi acts as signaling receptor, as it was shown in other biological contexts (Berger-Müller & Suzuki, 2011). However, no candidates acting as ligands, capable of eliciting intracellular signaling have been identified yet. In vertebrates, the Wnt family of morphogens has been shown to act cooperatively with Fz and other PCP proteins in order to direct axonal pathfinding. For example, Wnt4 was shown to be expressed in anterior-high to posterior-low gradient at the vertebrate midline, directing the post-crossing anterior turning of commissural neurons. The effect is mediated by the receptors Fzd3, Celrs3 and Vangl2 expressed on the neurons (Lyuksyutova et al., 2003; Shafer et al., 2011; Tissir et al., 2005). The same molecular repertoire is present in serotoninergic and dopaminergic axons in the hindbrain and midbrain, where *Wnt5* directs their extension along the anterior-posterior axis (Blakely et al., 2011; Fenstermaker et al., 2010). Wnt signaling has been shown to regulate axon guidance also in other system, such as olfactory sensory neurons (Rodriguez-Gil & Greer, 2008) and cortical neurons (L. Li, Hutchins, & Kalil, 2009). Also in *Drosophila*, evidence for Wnt-PCP dependent axon guidance mechanisms emerged. *Wnt5* directs AC selection of commissural axons expressing the receptor Derailed (Drl), repelling them from the PC (Yoshikawa et al., 2003) and directs mushroom body neurons targeting and branching through Fz, Dsh and Vang (Shimizu et al., 2011). It is important to mention that although in vertebrates Wnts family members have been known to regulate PCP establishment (Gao et al., 2011; Vivancos et al., 2009), such a mechanism has been described in *Drosophila* only recently (Wu et al., 2013). Nevertheless, Wnts family members seem to be good candidates as midline attractants. In *Drosophila*, seven family members have been identified till now; however, some of them, namely Wnt6, Wnt10 and Wnt8 are not expressed at the VNC during embryonic development (Janson et al., 2001). Therefore the analysis was focused on Wg, Wnt2, Wnt4 and Wnt5.

Mutants for any of these genes were never reported to show a phenotype in the embryonic CNS. Given that the phenotype of other genes required in Fmi-signaling was revealed in *fra* or *Net* mutant background, loss of function alleles for the *wg*, *wnt2*, *wnt4* and *wnt5* were analyzed in combination with *Net* or *fra*

mutation. It was already known that *wnt5* mutants have fused commissures, due to the switch of some axons from the AC to the PC when they lose repulsiveness towards the *wnt5* expressing PC (example shown in figure 3-17A). In *wnt5*$^{D7-/-}$ *fra*$^{3-/-}$ embryos the AC defects are not enhanced (Figure 3-19B), but Fra-dependent PC crossing defects observed in *fra* single mutants are partially suppressed (Figure 3-19C). This compensation could be explained by an increased number of axons aberrantly crossing in the PC, thus resulting in a wild-type size bundle. In other double mutant combinations, only *Net/fra* loss of function phenotypes were observed, with no enhancement upon loss of Wnt genes (Figure 3-19C). To ensure that effects on midline crossing were not obscured because of redundancy among the family members, overexpression analysis was performed. In fact, it was shown that midline expression of Wnt5 with the *Sim-Gal4* driver caused loss of AC, because Wnt5 was not restrained anymore to the PC, thus repelling Drl-positive axons from the AC (Yoshikawa et al., 2003). Therefore, functions of other Wnts family members could have also been revealed by ectopic expression.

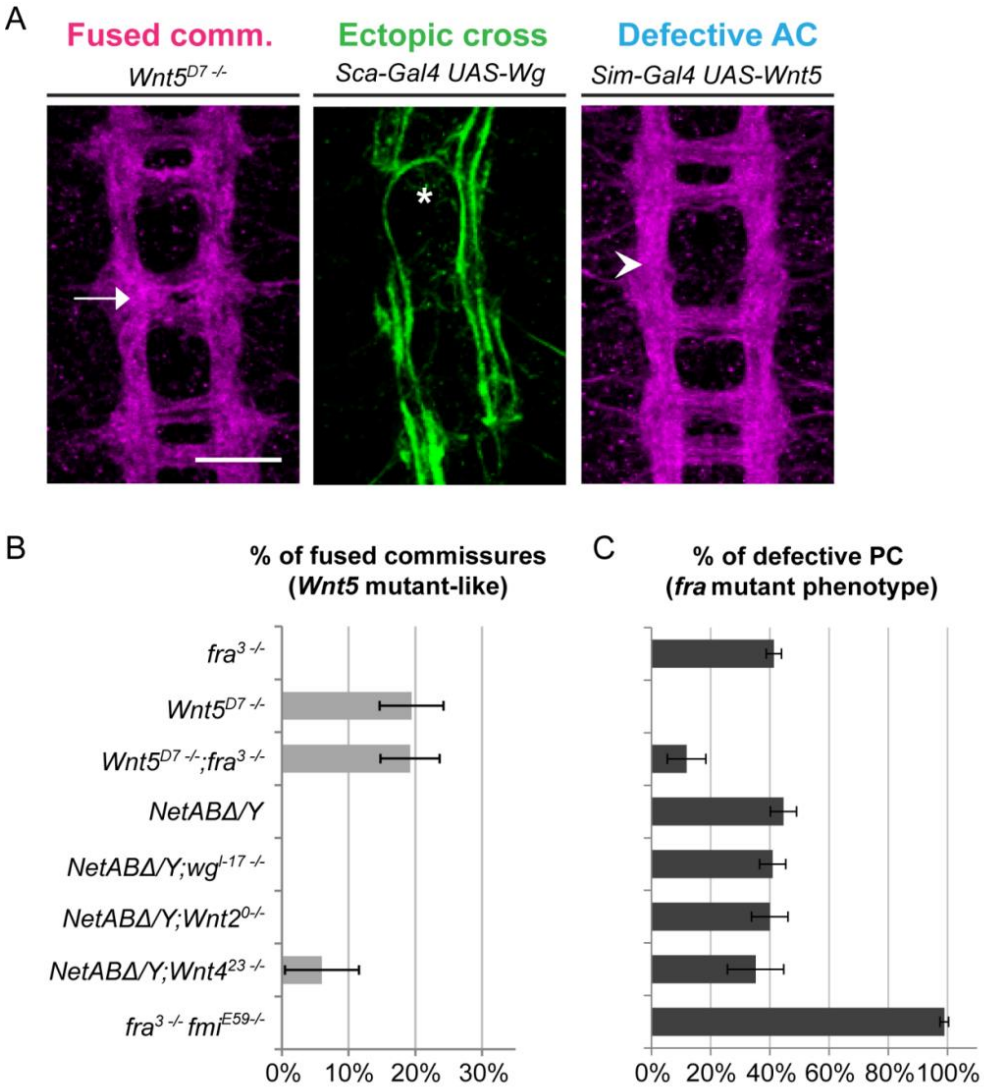


Figure 3-19 Analysis of Wnts family members.

A.Examples of the phenotypes observed in case of loss or overexpression of Wnts family members. Fused commissures results from loss of *Wnt5* or pan-neuronal overexpression of Wg or Wnt5. ectopic midline crossings of ipsilateral axons are present in embryos overexpressing Wg with *Sca-Gal4*, whereas loss or reduction of AC happens when Wnt5 is miserexpressed at the midline with *Sim-Gal4*.Commissures are visualized through HRP staining (Magenta), ipsilateral axons through anti-FasII staining (green). Scale bar, 20 µm. Arrow indicates fused commissures, asterisk indicates ectopic midline crossing and arrowhead indicates missing AC. **B-C**. Quantification of defects observed in embryos lacking *Net/fra* in combination with either *wg, wnt2, wnt4 or wnt5.* **B** is the quantification of the Wnt5 mutants-like defects as shown in A, consisting in axons occupying the area between the two commissures, thus causing fusion of the commissures. **C** is the quantification of the number of PC missing or reduced. Both the quantification are based on HRP staining .

Wg, Wnt2, Wnt4 and Wnt5 were expressed under the control of different promoters: *Ftz$_{ng}$-Gal4* was used for expressing ipsilateral neurons, *Elav-Gal4* for expression in post-mitotic neurons, *Sim-Gal4* for expression at the midline and *Sca-Gal4* for a strong expression in neurons, starting from early neuroblast stages, in epidermal precursor cells and in midline cells precursors (Bossing & Brand, 2006; Mlodzik et al., 1990). Only in few combinations a phenotype was observed, as reported in Table1. Mild loss of AC was observed upon *Sim-Gal4* dependent overexpression of Wnt4, reminiscent of the phenotype caused by midline overexpression of Wnt5. The phenotype is almost negligible, and would support the idea that *wnt4* acts, similarly to *wnt5*, in AC-PC selection and not in midline crossing. *Elav-Gal4* and *Sca-Gal4* overexpression of Wnt5 gave rise to fused commissures. This phenotype could be caused by loss of specific PC localization of Wnt5, thus leading to repulsion of Drl-positive axons from the AC now expressing Wnt5. Overexpression of Wg with *Sca-Gal4* caused fused commissures, but also ectopic crossing of ipsilateral neurons and reduced AC (Figure 3-19A, Table 1). This phenotype was unexpected, especially considering that no effect was observed upon overexpression of Wg in post-mitotic neurons (with *Elav-Gal4*) or at the midline (with *Sim-Gal4*). Wg is required for correct neuroblast specification and formation during embryonic development (Chu-LaGraff & Doe, 1993) and overexpression of Wg with the early onset driver *Sca-Gal4* might interfere with this process. Taken together, these results suggest that Wnt family members do not act as axon guidance cues in this context, although the possibility that they have overlapping phenotypes as it was shown for *wg* and *wnt4* in PCP has not completely been ruled out(Wu et al., 2013). Analysis of triple mutants might reveal the phenotype, but was technically too difficult to perform.

	Fused commissures		Ectopic crossings		Defective AC	
	penetrance	expressivity	penetrance	expressivity	penetrance	expressivity
Sim-Gal4 UAS-Wg						
Sim-Gal4 UAS-Wnt2						
Sim-Gal4 UAS-Wnt4					12.50%	1
Sim-Gal4 UAS-Wnt5					50%	1.5
Sca-Gal4 UAS-wg	42.10%	2.2	36.80%	1.6	26%	1.15
Sca-gal4 UAS-wnt2						
Sca-Gal4 UAS-wnt4						
Sca-Gal4 UAS-Wnt5	14.28%	1				
Elav-Gal4 UAS-Wg						
Elav-Gal4 UAS-Wnt2						
Elav-Gal4 UAS-Wnt4						
Elav-Gal4 UAS-Wnt5	37.00%	1				
Ftzng-Gal4 UAS-Wg						
Ftzng-Gal4 UAS-Wnt2						
Ftzng-Gal4 UAS-Wnt4						
Ftzng-Gal4 UAS-Wnt5						

Table 1 Phenotypes caused by overexpression of Wnts family members.

Examples of the three categories (fused commissures, ectopic crossing and defective AC) are shown in Figure 3-19.

4 DISCUSSION

The processes of axonal navigation and target selection are crucial for building neural circuits and, consequently, for correct functioning of nervous systems. Thus, molecular and cellular mechanisms involved in axon guidance have been of central interest in neuroscience for the last few decades. Studies performed with different model organisms and systems revealed the fundamental role of four conserved families of guidance cues and their receptors: Netrins, Slits, ephrins and Semaphorins (Kolodkin & Tessier-Lavigne, 2011; Tessier-Lavigne & Goodman, 1996). However, soon other studies started to reveal axon guidance functions for molecules implicated in very different developmental processes. For example, members of the Hedgehog, TGF-β/BMP, and Wnt families of morphogens have been shown to direct growth cone turning via alternative pathways to the one employed for cell fate specification (Yam & Charron, 2013). Other molecules that surprisingly emerged for their roles in axonal development are Planar Cell Polarity (PCP) proteins (Tissir & Goffinet, 2013). They were originally identified for their function in organizing and regulating the coordinated orientation of cells along a particular axis within the plane of a tissue in *Drosophila* (Chae et al., 1999; Feiguin et al., 2001; Usui et al., 1999; Vinson et al., 1989; Wolff & Rubin, 1998). Similarly, their homologs in vertebrates have conserved functions for the polarization of the inner ear epithelium (Ezan & Montcouquiol, 2013), convergent extension during gastrulation (Gray et al., 2011) and patterning of hair follicles (Chang & Nathans, 2013; Devenport & Fuchs, 2008). However, growing evidence supports roles for “core” PCP genes in neuronal development, especially in axon guidance and dendritic growth, but also in polarization of ependymal cells or neural tube closure (Tissir & Goffinet, 2010).

In this study, the role of the PCP protein and atypical cadherin Flamingo (Fmi) was analyzed in the context of *Drosophila* embryonic CNS guidance. Here, Fmi was shown to cooperatively act with Net/Fra signaling in guiding both commissural and longitudinal axons. In fact, loss of *fmi* and either *Net* or *fra* results in loss of most of the posterior commissures as well as defects in

ipsilateral axons fasciculation and extension. Consistent with its expression pattern, Fmi is required in neurons; in particular, Fmi is capable of mediating midline crossing of commissural neurons and is important for correct axonal directionality in some ipsilateral pioneer neurons. Interestingly, Fmi acts through a largely adhesion-independent mechanism and signals instead via its intracellular domain, to elicit an in part Rac1-mediated signaling. Together with the analysis of other possible interacting partners presented in this thesis, the present data suggest that Fmi acts in this context through a novel Wnt and PCP independent mechanism.

In conclusion, this study proposes a model where axonal extension towards and along the midline cooperatively require two different signaling pathways, which converge at the level of the Rac1 GTPase to influence the cytoskeleton dynamics: a Fra-dependent signaling, responsive to the midline attractant Net, and a Fmi-dependent signaling, responsive to a yet unidentified, likely membrane bound, guidance cue.

4.1 Roles of Flamingo in different neuronal types.

This study revealed a new role for Fmi in directing axonal pathfinding. Previously, two receptor systems were shown to regulate the formation of the embryonic CNS midline in *Drosophila:* Slit/Robo and Net/Fra. Slit is the major midline repellent, and it is acting through the Robo family of receptors (Kidd et al., 1999; Rothberg et al., 1988; Seeger et al., 1993). They are required for preventing ipsilateral and contralateral neurons from crossing or re-crossing the midline, respectively (Battye et al., 1999; Simpson et al., 2000). Additionally, this signaling is important for lateral pathway selection of ipsilateral axons (Rajagopalan et al., 2000b; Spitzweck et al., 2010) . Netrins and their receptor Fra act instead as midline attractants, guiding commissural axons across the midline (Brankatschk & Dickson, 2006; Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Although Net/Fra signaling was revealed as major attractant signaling at the midline, loss of function phenotypes of receptor or ligand lead to only partially penetrant phenotypes (Harris et al., 1996; Mitchell et al., 1996). By analyzing the *Drosophila* midline, the present work has shown that

Fmi acts together with Fra during the formation of the central nervous system midline. Severe axonal phenotypes were observed in both commissural and longitudinal neurons through analysis of loss of function alleles of *fmi* in the absence of either *fra* or *Net*. Hence, Fmi represents another important attractive mechanism at the embryonic midline that functions redundantly to the Net/Fra system. This finding could thus explain the discrepancy between Fmi broad expression pattern in the embryonic CNS and the absence of a neuronal phenotype in *fmi* mutants (Usui et al., 1999 and figure 3-2).

The mutant phenotype and the expression pattern suggested a neuronal role for Fmi, but rescue experiments were performed in order to determine in which kind of cells Fmi was required. These experiments revealed that in the context of *Drosophila* midline CNS Fmi is required neither at the midline nor in intermediate targets such as longitudinal glia (Hidalgo & Booth, 2000; Learte & Hidalgo, 2007). Instead, pan-neuronal re-expression of Fmi in $fmi^{-/-}$ $fra^{-/-}$ embryos reduced significantly both commissural and longitudinal neuron phenotypes. The cell-autonomous requirement of Fmi in targeting neurons contrasts in part with recent work in mice. Here, it was shown that the Fmi homolog Celsr-3 acts non-cell autonomously during the development of corticothalamic projections. In fact, conditional removal of *Celsr3* in the mouse basal forebrain and diencephalon, but not in the thalamus or the cortex causes loss of the internal capsule by preventing the entry of those tracts into the basal telencephalic corridor (Zhou et al., 2008). However, migration of facial branchiomotor (FBM) neurons in the fish brainstem is regulated by *celsr2* and other PCP genes such as *vangl2*, *fzd3a*, *prickle1a* and *1b* in both cell-autonomous and non-cell autonomous manners (Bingham et al., 2002; Rohrschneider et al., 2007; Wada et al., 2006).

Furthermore, rescue of both commissural and longitudinal phenotypes indicates that Fmi plays a role in most of the neuronal types. In fact, double mutant embryos for *fmi* and *fra* are characterized by almost complete loss of posterior commissures (PC), mild thinning of anterior commissures (data not quantified) and strong defects in ipsilateral neuron pathfinding and fasciculation, as observed with HRP and anti-FasII staining (Figure 3-3). Analysis performed with Gal4 lines labeling subsets of those neurons confirmed these observations. For instance, *Sema2b-Tmyc* positive contralateral neurons of *fra, fmi* mutants exhibit

defects in both midline crossing neurons, with some commissural bundles appearing very thin, and longitudinal projections, with many axons stopping or failing to extend anteriorly after crossing the midline or extending in the wrong direction. This last phenotype, although observed only in 10% of the *Sema2b-Tmyc* axons, is reminiscent of the role of Celsr3 in the mouse in post-crossing commissural axons, were loss of the gene results in mistargeting along the anterior-posterior axis (Tissir et al., 2005; Zhou et al., 2008). Conversely, in *fra* single mutants only a low percentage of the axons shows pathfinding errors, with axons stopping before reaching the next segment. The *fra*$^{-/-}$ phenotype is consistent with observations that Net/Fra signaling affects only the crossing of posterior commissures (Brankatschk & Dickson, 2006; Garbe et al., 2007; Kolodziej et al., 1996; Mitchell et al., 1996). However, it is important to notice that even in *fra, fmi* double mutants, only few of the anterior crossing neurons are affected. In fact, analysis of *Eagle-Gal4* positive commissural interneurons showed that the EG cluster, which extends across the AC, is not affected by loss of both Fmi and Fra signaling, whereas in the same embryos the EW cluster, which extend across the PC, is unable to cross the midline in virtually all the segments.

Why are posterior crossing axons more sensitive than anterior crossing axons to loss of *fmi* and *fra*? Firstly, not all the neurons face the same kind of guidance decision during pathfinding. For instance, some axons may be more sensitive to guidance cues secreted from the midline, thus extending in a cell-autonomous way, whereas some others might rely on contact mediated signaling or being more dependent on pioneer-follower interactions. It is known since long time which neurons extend early across the midline (Jacobs et al., 1989; Klämbt et al., 1991); however, a true pioneer role has yet to be assigned for commissural axons. In the context of posteriorly crossing neurons however, Fra was shown to direct midline crossing in a cell autonomous way (Garbe et al., 2007), whereas this study suggests that Fmi in this context acts in a non-cell autonomous way. Nevertheless, overexpression of Fmi in eagle axons was sufficient to significantly reduce midline crossing defects in *fra* mutants, but not in *fra, fmi* double mutants. Two not mutually exclusive mechanisms can be envisioned. First, a “community effect” might contribute to the growth of individual axons within a bundle.

Considering that in *fra*$^{-/-}$ the PC bundle in which eagle neurons extend is thinner, but not yet missing as observed in *fra*$^{-/-}$ *fmi*$^{-/-}$ embryos, Fmi might strengthen axon-axon interactions. Those interactions are lost or weakened either as a cause or as consequence of mistargeting, as observed in double mutants. Second, extending axons might actively signal to each other. For instance, Fmi might require a neuronally localized, contact-mediated binding partner in order to be activated and transmit an intracellular signaling. Given that the cadherin domain is less important, Fmi does not rely on homotypic interactions with Fmi expressed on surrounding posterior commissural neurons. Instead, it might interact with this unidentified ligand mostly through the hormone binding domain or the seven pass-transmembrane domain. This second scenario is particularly intriguing since it might also explain why pan-neuronal expression of Fmi can rescue PC bundle targeting.

In the context of ipsilateral neurons, genetic ablation experiments showed that four cells play a pioneer role (Hidalgo & Brand, 1997). Therefore, analysis of pioneer axon pathfinding has been performed by taking advantage of two Gal4 lines, *15J2-Gal4* and *c544-Gal4* (figure 3-12). Analysis of *c544-Gal4* positive MP1 neurons, although partially compromised by the low amount of neurons labeled in each embryo, revealed a fully cooperative effect of Fmi and Fra. Here, absence of either of the proteins caused guidance defects in a low number of the neurons, whereas in double mutants those defects appeared significantly enhanced (40%), arguing for a cooperative role of the two signaling pathways. Selective re-expression of either one of the two genes reduced the number of defective axons only partially, suggesting that both are equally required at least in this class of pioneer neurons. The fact that mild defects are observed in *fmi* mutants MP1 neurons at this early stages of axonogenesis, whereas no significant defects are observed in follower axons at stage 16 support the finding that removal of only one of the pioneer neurons is not sufficient to cause mis-targeting of follower neurons (Hidalgo & Brand, 1997).

Taken together, a model can be envisioned in which Fmi and Fra cooperatively contribute to axonal targeting of both commissural and ipsilateral neurons. However, whereas Fra seems to be acting cell-autonomously in both neuronal types, Fmi displays two different molecular mechanisms: in pioneer ipsilateral

neurons it acts cell-autonomously, whereas in commissural axons it acts non-cell autonomously, probably mediating an important axon-axon signaling.

4.2 Does Flamingo have a real axon guidance phenotype?

Given the recently discovered role of Netrin-B as anti-apoptotic factor for *Drosophila* embryonic neurons (Newquist et al., 2013), it is possible that the strong axonal phenotype observed in *Net*$^{-/-}$ *fmi*$^{-/-}$ embryos results from increased apoptosis rather than from abnormalities in axonal pathfinding. In fact, HRP and FasII staining only label axonal projections, but fail to mark cell bodies. Therefore, the evaluation of the number of neurons present in the different kinds of mutants is difficult. However, at least three lines of evidence suggest that Fmi does not increase or cause apoptosis. Firstly, in animals mutant for *fmi* no difference on the thickness of axonal bundles was observed, suggesting that the number of axons and thus the number of neuronal cells are unchanged. Second, pan-neuronal expression of p35, a potent caspase inhibitor, was not sufficient to rescue the defects observed in *fra*$^{-/-}$ *fmi*$^{-/-}$ embryos, whereas it was shown to be sufficient for rescuing defects in *Netrin* mutants. Lastly, labeling subsets of neurons such as pioneer neurons (with *15J2-Gal4* or *c544-Gal4,* figure 3-12), contralateral neurons (with *Sema2b-Tmyc,* figure 3-11) or interneurons (with *Eagle-Gal4,* figure 3-10) in *fmi* single mutants or *fra, fmi* double mutants, failed to show any difference in the number of cell bodies. Taken together, these results argue against a function of Fmi in neuronal survival, and support the idea that the defects observed in commissural and longitudinal neurons are due to disturbed axonal growth and/or guidance. However, the present data shows that axonal growth is also not impaired by loss of *fmi*. While in *fra, fmi* mutant embryos EW neurons fail in almost every segment to cross the midline, they are capable of extending an axon. Nevertheless, these axons then fail to cross the midline and either stop prematurely or extend anteriorly toward the next segment. Similar effects are observed in longitudinal pioneer neurons, where the phenotypes observed in MP1 neurons depend on axonal pathfinding, with axons growing laterally exiting the CNS instead of following a more intermediate path (Figure 3-12). Thus in *fra, fmi* mutant embryos neuronal cells are initially capable

of growing an axon, but then the axon is misguided toward a wrong target/direction. These results are in line with data in vertebrates, where loss of *Celsr3* does not affect axonal growth per se but alters guidance, resulting in stalling at intermediate targets or misrouting of axons (Tissir et al., 2005).

4.3 Atypical cadherins: adhesive molecules or signaling receptors?

It has been known since a long time that cadherins play a role in neuronal development. In particular, their capability of mediating homophilic and heterophilic cell-cell interactions is fundamental in many processes such as axonal fasciculation or synapse formation (Halbleib & Nelson, 2006). For example, N-cadherin mediates axon-axon interactions in ipsilateral neurons in the *Drosophila* embryonic CNS (Iwai et al., 1997), whereas Cadherin-13 inhibits axon extension of spinal motor neurons (Fredette et al., 1996). Notably, most of the known neuronal functions of cadherins depend on their capability of mediating adhesion through homophilic cell-cell interactions. However, atypical cadherins have been reported to be both structurally and functionally different from the rest of the cadherin family members (Berger-Müller & Suzuki, 2011; Bockaet & Pin, 2000). In fact, they possess a peculiar structure, in which a seven-pass transmembrane domain is present, thus hinting that they could act either as a classical cadherin-like adhesion factor or as a signaling receptor, potentially through the G-protein coupled receptors (GPCRs) like transmembrane domain (Harmar, 2001). In neuronal contexts, Fmi-mediated homophilic cell-cell interactions have been reported to be important for i) correct internal capsule formation, where Celsr3 plays a role in both neurons and in cells located on their trajectory, probably directing axonal pathfinding in a homophilic manner (Zhou et al., 2008), ii) photoreceptor targeting at the medulla in the *Drosophila* visual system, where Fmi mediates axon-target recognition through its extracellular domain (Hakeda-Suzuki et al., 2011) and iii) pioneer-follower interaction in the *C.elegans* VNC, where expression of a version of *fmi-1* lacking either the intracellular or the seven-pass transmembrane domain in follower neurons is sufficient to rescue the defasciculation phenotype (Steimel et al., 2010). In the context of embryonic CNS, rescue experiments performed with

truncated versions of Fmi demonstrated that the extracellular domain is partially dispensable for mediating Fmi function, thus arguing for a non-adhesive, cadherin-independent activity. In fact, FmiΔN, a version of Fmi lacking Cadherin repeats, EGF-like domains and laminin-G-like domains, was capable of partially restoring crossing defects of commissural axons as well as reducing the severity of the longitudinal axons phenotype. In particular in the context of ipsilateral neurons, the strong fasciculation phenotype observed upon loss of *fra* and *fmi*, could be easily related to loss of adhesion among axons belonging to the same bundle. However, the result that cadherin domain is partially not required for Fmi activity supports the idea that axon-axon interactions in longitudinal tracts are regulated not only by purely adhesive factors. Similar cadherin-independent functions have been reported for Fmi in other contexts, such as dendritic arborization field formation in *Drosophila* larvae (Kimura et al., 2006), advance of *Drosophila* embryonic sensory axons (Steinel & Whitington, 2009) and guidance of pioneer neurons in the *C.elegans* VNC (Steimel et al., 2010).

The confirmation that Fmi can act in the CNS context as signaling receptor by activating an intracellular signaling pathway came from the rescue experiment performed with a version of Fmi lacking a very short intracellular tail. Pan-neuronal expression of this construct completely failed to restore Fmi-dependent axonal defects observed in *fra, fmi* double mutants embryos. Notably, the intracellular tail of Fmi was shown to be important for its interaction with proximal polarity proteins (Strutt & Strutt, 2008), although some capability of transmitting intercellular PCP signaling seems to be retained by FmiΔIntra (Chen et al., 2008; Strutt & Strutt, 2008).

Two important aspects emerged from this domain study in the context of CNS axon guidance: first, Fmi does not bind to itself, but to a yet unidentified ligand and, second, Fmi mediates intracellular signaling. Thus, two important questions are opened: 1) what's the intracellular signaling activated in this context? And 2) what is the possible ligand/ receptor complex utilized?

4.3.1 Signaling pathways activated by Fmi.

Little is known about how Fmi mediates intracellular signaling. In fact, only one study tried to address this question up to now: Matsubara and colleagues performed a yeast-two hybrid screening and found that some members of the *Drosophila* PET-LIM domain family can bind to Fmi: Prickle (Pk), Espinas (Esn) and Testin. They confirmed that Esn-Fmi interaction is required for transducing a dendritic self-avoidance signal (Matsubara et al., 2011). The LIM domain is recognized as a modular protein-binding domain (Kadrmas & Beckerle, 2004), therefore Esn might as well work downstream of Fmi for axonal targeting, especially considering its expression in the embryonic VNC (Matsubara et al., 2011). However, double mutants for *esn* and *fra* or *esn* and *Net* looked similar to single *fra* or *Net* mutants, thus suggesting that Esn does not mediate Fmi signaling in the context of axonal pathfinding. It is likely that, given the role of Esn in mediating a repulsive signal among dendrites, a positive/attractive neuronal signal is transduced by a completely separate pathway, starting from the direct cytoplasmic binding partner for Fmi. This idea, together with the redundancy of Fmi when Fra is present, led to the hypothesis that Fmi, at least in the context of midline crossing, might exploit a common pathway activated by Net/Fra-dependent signaling. This is also supported by the finding that Fmi can mediate midline crossing in absence of either *fra* or *Net.* The best candidate for such a role was the Abelson Tyrosine Kinase (Abl), because it was already shown to bind to Fra and to mediate midline crossing through its C-terminal F-actin binding domain in a cell-autonomous way (O'Donnell & Bashaw, 2013). The analysis performed in this study revealed unclear results regarding a possible role of Abl downstream of Fmi. On the one hand, *fmi, abl* double mutants display neuronal phenotypes very similar to *fra, abl* and *fra, fmi* double mutants (figure 3-16b). Additionally, selective overexpression of Abl in $fra^{-/-}$ embryos rescued midline crossing defects, suggesting that Abl can be activated independently of Fra. However, overexpression of a kinase-dead Abl did not abolish the capability of Fmi to rescue crossing defects in $fra^{-/-}$ embryos (figure 3-17) nor did it interfere with the gain-of-function phenotype caused by Fmi overexpression in ipsilateral neurons (figure 3-18). This suggests that Abl is not essential downstream of Fmi-mediated midline crossing. However, the kinase-

dead version of Abl was shown to act as a dominant negative in some contexts (Hsouna et al., 2003; Sawyers et al., 1994) but not in commissural axons, where instead the loss of the C-terminal domain interferes dominantly with midline crossing (O'Donnell & Bashaw, 2013). Even though there is a possibility that Abl kinase dead does not act as dominant negative, the presented evidence does not serve as a strong support for a genetic interaction between Abl and Fmi. Moreover, a direct physical interaction is also unlikely to be found, given the difficulties reported in other studies on performing co-immunoprecipitation experiments with Fmi (Hakeda-Suzuki et al., 2011; Matsubara et al., 2011).

In addition to the results obtained from Esn and Abl, analysis of Rho GTPases revealed important insights on how Fmi might act on cytoskeleton dynamics. In fact partial removal of Fmi, similarly to published experiments carried out for Fra (Dorsten et al., 2007), reduced ectopic midline crossings caused by ipsilateral overexpression of a constitutively active version of Rac1. This result can be explained by the fact that a constitutively active version of Rac might be acting by sequestering all the GAPs that would be switching Rac from an active to an inactive state, thus increasing the levels of active Rac. Therefore, Fmi's ability to reduce the phenotype suggests that there is less active endogenous Rac produced. However the same kind of interaction was not observed for the other GTPase implicated in Fra signaling, Cdc42. This suggests that Fmi can only partially compensate for loss of Fra signaling, but not substitute it completely. Conversely, Fra signaling presumably would be able to compensate for loss of Fmi, which is well in line with the fact that single *fmi* mutants do not show guidance phenotypes. Also Rho1 GTPase was analyzed in midline guidance, because in the context of dendrite morphogenesis, genetic interaction was observed between Rho1 and Fmi in class IV neurons (Matsubara et al., 2011). However, Rho1 seems to not play a role in midline crossing (figure 3-14). Thus, this is the first reported interaction between Fmi and Rac (Figure 4-1). An attractive explanation for the differential requirement of GTPases downstream of Fmi would be that in contexts where Fmi mediates a repulsive signal, such as among dendrites, Rho1 is activated, whereas in cases where Fmi mediates axonal extension, Rac is activated. Additional data would be required to prove this model.

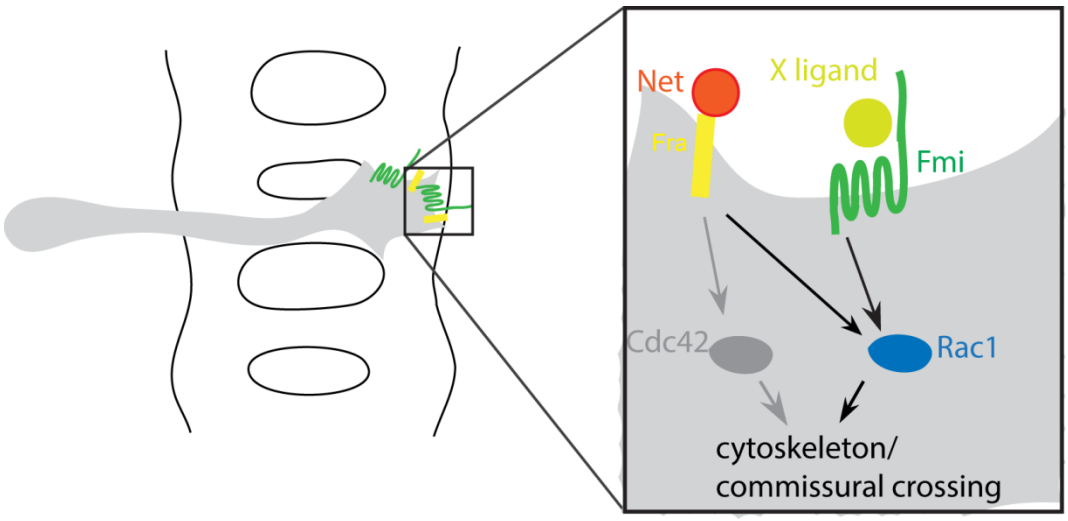


Figure 4-1 Targeting of commissural axons requires Fra and Fmi signaling pathways.

In the *Drosophila* embryonic CNS commissural neuron midline crossing relies on two signaling: Net/Fra signaling, and a novel Fmi-dependent signaling, which likely is activated by a membrane bound unknown ligand. The two signaling act on the cytoskeleton dynamics by activating Rac1 GTPase. Fra further influences actin remodeling by activating Cdc42.

Cytoskeleton dynamics have to be regulated not only in the case of growth cone steering, but also in the context of epithelial cell polarity. For example, actin cytoskeleton activity seems to be fundamental for the correct position, size and number of wing hairs at the distal edge of wing cells. In this context, the signal arising from asymmetrical localization of PCP proteins in cells is transmitted intracellularly through planar cell polarity effector proteins (PPE proteins): inturned (in), fritz (frtz), fuzzy (fy), and the most downstream effector multiple wing hairs (mwh) (Adler, 2012); notably, they also localize asymmetrically. Mwh seems to be directly modulating the actin cytoskeleton. In fact, it contains a G-protein binding domain and it has sequence similarity to the Diaphanous family of formins (Strutt & Warrington, 2008; Yan et al., 2008), which are known to act as actin nucleators by promoting the formation of long actin filaments in a Rho1 dependent manner (Wallar & Alberts, 2003). Genetic interaction and co-immunoprecipitation experiments revealed that Mwh is activated by Rho1 (Yan et al., 2009). However, Mwh lacks the actin nucleator activity, and given its ability

of inhibiting actin filament formation *in vitro*, is thought to have a dominant negative effect on the activity of other actin nucleators to inhibit actin cytoskeleton (Strutt & Warrington, 2008). Several reports support the idea that also distally-enriched proteins such as Dsh and Fz are responsible for actin cytoskeleton regulation, although no evidence has been found for a putative candidate. It is known however, that Dsh functions in a PPE independent manner when controlling the number of hairs (Lee & Adler, 2002). This suggests the existence of a yet unidentified molecule linking to actin regulation, maybe a formin or another cytoskeleton regulators. Additionally, in the context of ommatidia polarity Fz and Dsh genetically interacts with Rho1 (Strutt et al., 1997), suggesting that tight regulation of RhoA GTPase activity is important to achieve correct epithelia polarity.

In conclusion, the data collected in this thesis, together with previous work, support the idea that Rho GTPases activity is required for processes involving polarity genes in both epithelia and neuronal cells, although the molecular requirement to elicit cytoskeletal regulation in the two systems is likely to diverge at the level of GTPases and probably at the level of effectors as well.

4.3.2 Roles of Wnt/PCP molecules in Fmi-mediated guidance/ Concept of planar cell polarity in axon guidance.

To identify the molecular mechanism underlying Fmi function in CNS neurons, analysis of other “core” PCP molecules has been performed. In fact, in some neuronal contexts more than one PCP gene was implicated, although is not clear whether the genes act in a cooperative manner. For example, guidance of post-commissural axons along the anterior-posterior axis in the spinal cord is affected by loss of either *Celsr3, Fzd3,* or *Vangl2* (Fenstermaker et al., 2010; Tissir et al., 2005; Wang et al., 2002; Zhou et al., 2008). Additionally, axonal branching and extension of mushroom body neurons are defective in *fz*, *dsh,* or *vang* mutants (Shimizu et al., 2011). Moreover, a possible role of other PCP genes in CNS neurons was justified by the finding that, similarly to *fmi,* loss of function alleles for *fz*, *dsh* or *vang* did not display phenotypes in the embryonic CNS (Figure 3-1). However, analysis of double mutants for *fra, vang* and *dsh, fra* displayed a phenotype identical to *fra* single mutants. Combined loss of *fra* and *fz* instead

caused a phenotype indistinguishable from *fra, fmi* double mutants, suggesting that *fz* could cooperate with *fmi*, likely acting in a signaling complex. This hypothesis is supported by the fact that Fmi and Fz can physically interact, as it was shown in the context of epithelial cell polarity (Chen et al., 2008). To further investigate Fz's role in midline crossing and to verify whether it could restore midline crossing at similar extent of Fmi, Fz was selectively expressed in *eagle*-positive commissural neurons in $fra^{-/-}$ animals. No reduction of crossing defects was observed, suggesting that either Fmi and Fz do not act together or, if they do interact, they do it *in trans*, for example mediating glia-neuron interaction or axon-axon interaction. Such a mechanism would be really different from what is observed in epithelial cells, where the two molecules interact *in cis* (Chen et al., 2008; Strutt & Strutt, 2008). To address this point, either rescue experiments in which Fz is re-expressed in different kinds of cells or the analysis of *fmi, fz* double mutants should give important insights. However, these experiments proved difficult to be carried out, because of technical difficulties related to the low number of embryos with the right genotype obtained. Thus, in order to clarify whether the phenotype observed in $fra^{-/-}$ $fz^{-/-}$ embryos is due to interference with a putative fz-fmi signaling or with a Fmi-independent Fz signaling, such as the Wnt morphogenic signaling (Yam & Charron, 2013), analysis of members of the Wnt family has been performed. Axon guidance roles for Wnt morphogens have been reported in different systems, such as spinal cord, brainstem, olfactory system in vertebrates (Blakely et al., 2011; Fenstermaker et al., 2010; Lyuksyutova et al., 2003; Rodriguez-Gil & Greer, 2008) and mushroom body neurons and VNC in *Drosophila* (Shimizu et al., 2011; Yoshikawa et al., 2003). Additionally, PCP signaling seems to be directed by Wnt gradient in some systems (B. Gao et al., 2011; Vivancos et al., 2009; Wu et al., 2013), suggesting that Fmi positive neurons might be responsive to a Wnt gradient at the midline. Loss of function alleles for members of the Wnt family known to be expressed at the midline, namely *wg, wnt2, wnt4,* and *wnt5* were analyzed in either $Net^{-/-}$ or $fra^{-/-}$ backgrounds. Additionally, they were overexpressed in different kinds of cell types, in order to evaluate effects of ectopic expression or overexpression. However, none of these genetic manipulations resulted in a phenotype that could be linked to Fmi activity in these neurons, thus arguing against a role of a single Wnt protein as Fmi-ligand. This analysis however could not rule out the

possibility that some of the Wnt genes have overlapping and redundant functions, as it was shown for *wg* and *wnt4* in PCP (Wu et al., 2013). Analysis of triple mutants might reveal the phenotype, but because of the complexity of the genetics and the weakness of the resulting flies, these experiments were not performed.

Taken together, analysis of PCP genes and Wnts morphogens suggest that Fmi acts in the context of axonal pathfinding across and along the midline through a novel molecular mechanism, which might require an interaction with Fz *in trans*. Moreover, comparison of the role of Fmi between single and groups of neurons suggests that the Fmi ligand is likely to be a localized cue rather than a diffusible, gradient-distributed cue (see Paragraph 4.1).

Although Fmi in the context of the *Drosophila* embryonic midline seems to act largely in a PCP- and Wnt-independent manner, it is interesting that in many other neuronal contexts more than one PCP gene is usually required for correct axon targeting. PCP signaling playing a role in the neuroepithelium is expected, given that the nervous system originates from a epithelial neural plate. However, less predictable was the involvement of PCP molecules in axon guidance, dendrite maturation, neural migration and maturation (Tissir & Goffinet, 2010). Given that the growth cone is a high motile structure and epithelial sheets are a stationary system, it is hard to explain how PCP like mechanisms can affect axonal pathfinding. How can cell polarity signaling be utilized by axons during their targeting? The growth cone is per se a polarized structure, given that microtubules point their (+) growing end toward the tip of the growth cone and actin filaments also possess (+) barbed ends pointing toward the tip of the filopodia. Additionally, endo- and exocytosis are also polarized in the growth cones (Itofusa & Kamiguchi, 2011), suggesting that one of the mechanisms to convey polarity in the growth cone is asymmetric localization of proteins on their membrane. Such model is supported by the observation that in live growth cones, Vangl2 protein is highly enriched on the tips of extending filopodia but not on the shrinking filopodia (Shafer et al., 2011). Moreover, Vangl2 seems to be required for regulating membrane localization of Fzd that in the context of commissural axons senses a Wnt5 gradient along the anterior-posterior axis. This interaction leads to anterior turning of post-commissural axons. Thus,

asymmetrical localization of polarity proteins on filopodia might influence the recruitment or the localization of transmembrane proteins. In turn these proteins, acting as guidance receptors, might locally activate the actin cytoskeleton machinery, causing directional extension of the growth cone. This mechanism can be important not only for polarity genes that act as receptors, but might also be a general mechanism for mediating directional control by many axon guidance molecules. It is possible that Fmi is localized asymmetrically in the growth cone in the embryonic midline. At this point, however, no direct evidence is available.

4.4 Roles of Fmi and Fra signaling during development of the Drosophila and the mouse midline systems.

Consistent with the overlapping expression pattern in the embryonic CNS observed for Fmi and Fra in this study, the mouse Celsr-3 was also shown to be abundant in commissural neurons that express DCC in the spinal cord (Shafer et al., 2011). This finding supports the idea that most of the guidance cues and their signaling mechanisms are conserved from flies to mice (Kolodkin & Tessier-Lavigne, 2011). However, floor plate and invertebrate midline present some functional and structural differences that may account for differential molecular requirements or diverse mechanism of action of the same molecules (Arendt & Nübler-Jung, 1999). For example, structural differences can be observed: in the vertebrate spinal cord, the dorsal spinal cord commissural neurons extend their axons over a long distance, finally reaching the floor plate after growing along a circumferential route and turning medially when reaching the ventral side, where the floor plate resides (Kolodkin & Tessier-Lavigne, 2011). In the fly nerve cord, commissural neuron cell bodies are located laterally to the midline, and project their axons medially, crossing in either one of the two main commissural bundles (Dickson & Zou, 2010). Additionally they are intermingled with other types of neurons, such as ipsilateral interneurons and motor neurons, whereas vertebrate commissural neurons are segregated in the dorsal half of the spinal cord. These structural differences suggest that guidance systems, even though conserved between the two animal species, might slightly differ in their mechanisms of

action. This is true in the case of Netrin: given the long distance that commissural axons have to cover before reaching the floor plate, Netrin-1 acts as long range attractant, distributed in a gradient over long distances (Kennedy et al., 1994). In the fly midline, no such gradient has been observed for NetA or NetB; instead, it has been shown that Netrins act as short range guidance cues, given that replacement of secreted Netrins with a version tethered to the membrane is sufficient to mediate midline crossing (Brankatschk & Dickson, 2006). This local function of Netrin is in line with the fact that commissural neurons have cell bodies located in close proximity to the midline, thus suggesting that a localized signal is sufficient to drive axonal pathfinding. Another big difference between the two systems is the fact that the floor plate provides not only secreted guidance cues, but it is also an important source of morphogens, which in turn specify neuronal cell fates along the dorsal-ventral axis. Additionally, those morphogens are important later in development as axon guidance signals (Sánchez-Camacho et al., 2005; Yam & Charron, 2013). For example, members of the BMP, hedgehog and Wnt families of morphogens have been described to perform such a role (Yam & Charron, 2013). In particular, Wnts play an important axonal guidance role in relationship to PCP proteins, considering that Fzd3, Celsr3, and Vangl2 are responsive to the gradient of some of these morphogens, conferring commissural axons the ability to turn anteriorly after crossing the midline (Lyuksyutova et al., 2003; Shafer et al., 2011). The midline of the fly embryo is not a major source of morphogens, sharing only the axonal pathfinding role with the vertebrate counterpart (Dickson & Zou, 2010). In fact, even though some of the Wnt family members are expressed at the *Drosophila* midline, loss-of-function and gain-of-function experiments conducted in this study failed to reveal any neuronal phenotype, suggesting that Fmi capability of mediating midline crossing is not related to its responsiveness to a Wnt gradient. Interestingly, Fmi seems to be responding to a rather short range, local cue rather than to a global cue. In fact, when overexpressed in eagle-commissural axons, it can elicit midline crossing only when some surrounding axons target correctly, suggesting that contact-mediated interaction is needed.

Thus, Fra and Fmi signaling seem to be short range signaling mechanisms in the *Drosophila* nerve cord, whereas in vertebrates evidence are present for a long distance signal in response to diffusible molecular gradients. These data suggest that the distance travelled by axons in order to reach their targets might also influence the type of guidance cue required.

4.5 Similarities and divergences of Fra and Fmi signaling in other axon guidance systems.

Fmi and Fra signaling act in a cooperatively way to ensure proper midline crossing of commissural axons, and also for correct pathfinding of ipsilateral neurons. These partially redundant mechanisms confer robustness to the system, by ensuring that at least some neurons can target correctly even in absence of one of the molecular pathways. The nerve cord and spinal cord systems are not unique for the cooperative function of Fmi and Fra signaling in axonal targeting. In fact, Fra and Fmi have been shown to play a common role in midline crossing in the vertebrate brain and also in the *Drosophila* visual system. In mice, Net/DCC signaling is important not only for guiding commissural axons towards the midline and direct crossing, but also for the development of other axonal tracts in the brain (Fazeli et al., 1997; Serafini et al., 1996). Indeed, in *Net-1*$^{-/-}$ and *DCC*$^{-/-}$ mice the corpus callosum, which joins the left and right cerebral cortices, and the hippocampal commissure, which joins left and right hippocampi, are completely absent. Additionally the anterior commissure is severely affected, with only a small bundle of crossing axons remaining commissure (Fazeli et al., 1997; Serafini et al., 1996). Notably, loss of Net/DCC signaling does not affect all the commissures, since the habenular and the posterior commissure appear intact. Interestingly, the anterior commissure is absent also in *Celsr-3*$^{-/-}$ mice (Tissir et al., 2005; Zhou et al., 2008). Moreover, loss of either *Celsr-3* or *Net-1* affects guidance of the thalamocortical tracts extending into the internal capsule (Braisted et al., 2000; Finger et al., 2002; Molnár et al., 2012; Powell et al., 2008; Zhou et al., 2008). In the *Drosophila* visual system, both Fmi and Fra are required for the correct targeting of R8 photoreceptor axons at the M3 layer in the medulla. Here, removal of either of

the genes specifically in the eye causes mistargeting of R8 axons in other medulla layers, such as M1-M2 (Lee et al., 2003; Senti et al., 2003; Timofeev et al., 2012). Fmi regulation of axon-target recognition is mediated through interaction with the transmembrane molecule Golden Goal (Gogo), which displays a similar mutant phenotype in R8 (Hakeda-Suzuki et al., 2011; Mann et al., 2012; Tomasi et al., 2008). In particular, Fmi seems to be required in both photoreceptor axons and medulla target cells, increasing axon-target adhesiveness through homotypic interaction (Hakeda-Suzuki et al., 2011). Fra is also required in both photoreceptors and target cells, but target selection is achieved through a different mechanism. In fact, Fra is expressed both at the target region in the medulla and in photoreceptor axons. In the medulla M3 layer Fra acts by capturing and localizing Netrin, in order to present it to the Fra-positive R8 axons seeking for the correct layer in the medulla (Timofeev et al., 2012). The selective expression of Net/Fra at the M3 layer, compared to the broad expression of Fmi in many medulla layers nicely fits the hypothesis that Fmi promotes release of the R8 axons from their temporal M1-M2 layers and, after Net/Fra system has elicited target selection, stabilizes axon-target interaction via homophilic binding between photoreceptor axon and medulla. The roles of Net and Fmi signaling in the visual system support the idea that the two systems are mediating axonal targeting in *Drosophila* by mediating short range interactions; however, Fmi does not elicit intracellular signaling per se as is observed in the nerve cord.

4.6 Conclusive remarks.

The work presented in this dissertation reveals a novel role of the PCP molecule Fmi during axon targeting of commissural and longitudinal axons at the *Drosophila* midline. In this context, Fmi works in a cooperative way with Net/Fra signaling. In particular, Fmi transmits a short-range guidance information, acting together with Fra upstream of the Rac1 GTPase. This new role for Fmi reinforces the idea that molecular pathways utilized to confer polarity in epithelia cells are also involved in directed growth of axons, possibly by regulating regional responsiveness of growth cones. Additionally, this work demonstrates that correct axonal pathfinding does not rely only on classical attractant and repulsive signaling, but also on local signals, that might influence axon-axon interactions and therefore affect axonal targeting.

This work could be further validated and expanded by analyzing in more detail the interaction between Fmi and Rac1 GTPase, for instance by biochemically validating the role of Fmi as activator of Rac. Moreover, it would be interesting to understand the molecular pathway connecting the two proteins, starting with the identification of the GEF upstream of Rac in this context. Given the lack of knowledge regarding signaling pathways elicited by Fmi in most contexts in both invertebrates and mammals, it would be interesting to verify whether the very same molecular pathway is utilized in contexts such as dendritic morphogenesis and PCP in epithelia. Another interesting point would be to verify whether Fmi is indeed preferentially localized on growth cones at filopodia tips and whether its overexpression or loss is sufficient to mediate growth cone extension/ retraction in embryonic primary neuron cultures. This approach could also help in understanding whether Fz is important in the context of Fmi mediated signaling. Lastly, an important contribution would be to find a possible ligand/interacting partner for Fmi, especially screening for molecules capable of interacting with the hormone-binding domain and the 7-pass transmembrane domain that are shown to be required for many cadherin independent roles of Fmi.

5 MATERIALS AND METHODS

5.1 Materials

5.1.1 Chemicals

Table 2 List of chemicals used and their sources.

CHEMICAL	SOURCE
Agarose, high electro endosmosis	Biomol
Agar-Agar danish	Roth
Ethanol absolute	Sigma-Aldrich
Glycerol	Merck
Methanol	Sigma-Aldrich
Isopropanol (2-Propanol)	Sigma Aldrich
Tween 20	Sigma Aldrich
EDTA	Sigma Aldrich
Formaldeyde (10%)	Polyscience
Heptane	Fluka
Phenol-chloroform	Amresco
Triton X-100	Roth
Tris Base	Sigma Aldrich
Sodium Lauryl Sulfate (SDS)	Roth

5.1.2 Consumables and Kits

Table 3 List of consumables and their manufacture companies.

DEVICE	MANIFACTURER
Fly Work and Embryos Collection	
Blue fly food	Fisher Scientific
Apple juice	Aldi
Sodium Hypochlorite solution	Sigma-Aldrich
Cell culture	
Schneider Media	PromoCell GmbH
Fetal Bovine Serum (FBS)	PromoCell GmbH
Penicillin/ Streptomycin 100X	PAA Laboratories
Effectene Transfection Reagent	Qiagen
Molecular biology	
iProof High Fidelity DNA Polymerase	BioRad
Steril Surgical Blades	Bayha
Gel loading dye blue (6X)	New England Biolabs
Ampicillin	
1kb DNA ladder	New England Biolabs
QIAprep Spin Miniprep Kit	Qiagen
Plasmid Maxi Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Immunoblotting	
Transfer Buffer	Pierce
APS	Biorad
Poliacrilamide	Biorad
Milk powder	Roth
Nitrocellulose membrane	Whatman
ECL solution	GE Healthcare
Protein marker	New England Biolabs
Complete Mini Protease Inhibitors	Roche

5.1.3 Buffers and solutions

Phosphate buffered saline (PBS): 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2 HPO_4, 8g NaCl in 1 l H_2O at pH 7.4

PBT (0.1%): 0.1% (v/v) Triton X-100 in PBS

Solution A: 0.1M Tris HCl pH 9.0, 0.1M EDTA pH 8.0, 1% SDS in water.

TAE (50X) (2000 l): 484 g Tris base, 50 mM EDTA (pH 8.0), and 114.20 ml glacial acetic acid (pH 8.5).

Fly water: 8 ml Propionic acid in tap water.

Luria Bertani Medium (LB medium) (1000 ml): 10 g NaCl, 10 g Bacto - tryptone, 5 g yeast extract, 20 g agar (pH 7.5). 1.5% Agar added for making LB plates. Desired antibiotics were added after autoclaving and cooling down the media.

Lysis buffer: 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton, protease inhibitor (Sigma)

TBST: 24.23 g/l Tris base HCl, 80.06 g/l NaCl, pH 7.6 0.1% (v/v) Tween 20

5.1.4 Drosophila media

Standard *Drosophila* medium

For preparing 50 l of Standard *Drosophila* medium, 585 g of agar were dissolved in 30 l of water by heating the mixture to the boiling point. 5 kg corn flour, 925 g yeast, 500 g soy flour, 4 kg molasses were mixed with water and added into the dissolved agar. The volume was filled up to 50 l and cooked at 96°C for 1.5 h. After cooling down the mixture to 60°C, 315 ml of propionic acid, 120 g of methykparaben, 125 g niparsin/methylparaben, 1 l of 20% Ethanol and 500 ml of 10% phosphatidic acid were added to the mixture.

Blue yeast paste

Blue yeast paste, added to weak stocks or crossed, was prepared mixing instant dry yeast (Femipan Inc.), Instant blue *Drosophila* medium (Fisher Scientific) and water.

Apple juice agar plates

500 ml of apple juice were mixed with 14 g of agar and melted in a microwave.

5.1.5 Equipment

Table 4 List of Equipment.

DEVICE	MANIFACTURER
Fly Maintenance	
Incubators	Percival
Microscopy and Immunohistochemistry	
Confocal microscope Olympus FV-1000	Olympus
Fluorescence microscope M205 FA	Leica
Cover glasses for microscopy (18x18mm)	Thermo Scientific
Microscope slides with frosted ends	Menzel-Gläser
Light microscope stemi 2000	Zeiss
Forceps	Inox
Vectashield fluorescence H-1000 mounting media	Vector laboratories Inc.
Type F Immersion Liquid	Leica Microsystems CMS Gmbh
Cell culture	
Incubator FTC90i	Uniequ.
Laminar air flow hoods	Heraeus
Cell culture flasks BD Falcon	BD Biosciences
Cell culture wells, 60 mm	BD Biosciences
Molecular biology	
Spectrophotometer NanoDrop 1000	PeqLab
Thermocycler DNA engine tetrad	MJ Research
Bacterial Incubator	Heraeus
Culture shaker incubator	Unitron
Immunoblotting	
Running	
Blotter	

5.1.6 Antibodies

Table 5 List of primary antibodies for immunohistochemistry and immunoblotting

ANTIGEN	HOST	DILUTION	SUPPLIER
Primary antibodies for immunofluorescence			
Anti-GFP (Living colors)	rabbit	1:1000	Clonetech
Anti-HRP conjugated-Dylight 567	goat	1:250	Jackson
Anti-Fasciclin II (1D4)	mouse	1:100	DSHB
Anti-sex lethal (M18)	mouse	1:100	DSHB
Anti-β-Galactosidase	chicken	1:1000	Abcam
Anti-Futsch (22C10)	mouse	1:100	DSHB
Anti-Flamingo	mouse	1:20	DSHB
Anti-Frazzled	rabbit	1:100	from Y.N.Jan
Anti-myc-TRICT conjugated	mouse	1:200	Santa Cruz
Primary antibodies for western blot			
Anti-myc	mouse	1:2000	Santa Cruz
Anti-GFP	mouse	1:2000	Clonetech

Table 6 List of secondary antibodies for immunohistochemistry and immunoblotting

ANTIGEN	HOST	DILUTION	SUPPLIER
Secondary antibodies for immunofluorescence			
Rabbit IgG Alexa Fluor 488	donkey	1:250	Invitrogen
Rabbit IgG Alexa Flour 568	goat	1:250	Invitrogen
Mouse IgG Cy3)	donkey	1:250	Jackson
Mouse IgG Alexa Flour 488	goat	1:250	Invitrogen
Chicken IgG Alexa Fluor 568	goat	1:250	invitrogen
Secondary antibodies for western blot			
Anti-rabbit HRP conjugated		1:2000	Jackson
Anti-mouse HRP conjugated		1:2000	Jackson

5.1.7 Fly stocks

Table 7 Fly stocks and their origin.

STOCK	SOURCE
NetABΔ/FM7, lacZ	Barry Dickson
w; FRTG13 *fra*^{3} /CyO, lacZ	Bloomington Stock Center
w; FRTG13 *fra*^{4} /CyO, lacZ	Bloomington Stock Center
yw; frt42B *fmi*[E59]/CyO.y^{+}	Kirsten Senti
yw;*UAS-fra*	Bloomington Stock Center
yw; *UAS-fmi*	Tadashi Uemura
yw; *UAS-fmiΔN*-EYFP	Tadashi Uemura
UAS-fmiΔIntra-GFP	David Strutt
Elav-Gal4(III)	Bloomigton Stock Center
w;*Mz1407-Gal4*	Bloomigton Stock Center
w;*Sim-Gal4*/CyO y^{+}	Bloomigton Stock Center
w; *Repo-Gal4*/TM3, Sb	Gaia Tavosanis
Gcm-Gal4	Bloomigton Stock Center
Eagle-Gal4(Eg-Gal4)	Bloomigton Stock Center
yw;Pin/Cyo;*UAS-mcd8*-GFP	Bloomigton Stock Center
yw Baz^{4}FRT9-2/FM7a,GMR-nvYFP	Bloomigton Stock Center
w; Sco/CyO, GMR-nvYFP	Bloomigton Stock Center
w; Dr^{1}/TM3, GMR-nvYFP , Sb	Bloomigton Stock Center
fmi[E59] *fra*^{3} / CyO, GMR-nvYFP	This study
UAS-Abl/TM3, Sb	Bloomigton Stock Center
Sema2b-Tau myc	Bloomigton Stock Center
c-544-Gal4	Alicia Hidalgo
Fz ^{KD4}	Takashi Suzuki
UAS-Fz	Takashi Suzuki
vang^{stbm153}/CyO	Bloomigton Stock Center
vang^{stbm6}	Bloomigton Stock Center
dsh^{1}	Bloomigton Stock Center
dsh^{3}FRT19A/FM7a	Bloomigton Stock Center

Wg	Bloomigton Stock Center
Wnt2^{O}/CyO	Bloomigton Stock Center
Wnt4^{EMS23}/CyO	Bloomigton Stock Center
Wn5	Bloomigton Stock Center
Uas-wg	Bloomigton Stock Center
UAS-Wnt2.5-	Bloomigton Stock Center
uas-wnt4	Bloomigton Stock Center
uas-wnt5	Bloomigton Stock Center
UAS-Abl^{K417N}*;Abl*^{1}/TM6B, Tb	Bloomigton Stock Center
*UAS-Rac1.*W	Bloomigton Stock Center
UAS-Rac1N17	Bloomigton Stock Center
UAS-Rac1V12	Bloomigton Stock Center
*UAS-Cdc42.*W	Bloomigton Stock Center
UAS-Cdc42N17	Bloomigton Stock Center
UAS-Cdc42V12	Bloomigton Stock Center
UAS-Rho1	Bloomigton Stock Center
Abl^{1}/TM6B, Tb	Bloomigton Stock Center
Abl^{4}/TM6B, Tb	Bloomigton Stock Center
esn^{KO6}	Tadashi Uemura
w^{1118}	Bloomigton Stock Center
Gal4-Ftz$_{ng}$/TM3, act-LacZ	Bloomigton Stock Center
UAS-p35.H	Bloomigton Stock Center

5.1.8 Plasmids

Table 8 List of Plasmids used for CoIP experiments.

PLASMID	ANTIBIOTIC RESISTANCE
pUAST	Amp
Actin-Gal4	Amp
pUAS-Frazzled-GFP	Amp
pUAS-Abl-myc	Amp
pBluescript Abl cDNA GH09917	Amp

5.1.9 Oligonucleotides

Table 9 Primers for cloning and sequencing

PRIMERS	SEQUENCE
Primer for cloning of Abl cDNA in the pUAS vector and for tagging it	
Fwd 1st PCR ABL	5' CAAATGGGGGCTCAGCAg 3'
Rev 1st PCR ABL	5'CAGATCTTCTTCAGAAATAAGTTTTTGTTC CCTGTTAAGCGCATTGGAGAT 3'
Fwd 2nd PCR ABL NotI	5'CACCGCGGCCGCCAAATGGGGGCTCAG 3'
Rev 2nd PCR ABL XBaI	5'GCGCTCTAGACTACAGATCTTCTTCAGAAA TAAGTTTTTGTTCAGGCAACAGATCTTCTTCAGAA AT 3'
Primer for sequencing	
Abl seq primer_1:	5'GAACTCTGAATAGGGAATTGGGA 3'
Abl seq primer_2:	5'GCGTCGGGAATGAGCTACC 3'
Abl seq primer_3:	5'GAGCACATCGGGCGTGG 3'
Abl seq primer_4:	5'ATGCAGCATTCTCTTCACAGC 3'
Abl seq primer_5:	5'CAGTAGCTCACCCAAGCGGA 3'
Abl seq primer_6:	5'AAAGCTGACCAACGGCAATA 3'
Abl seq primer_7:	5'GGGGGCCATCAATACGGTT 3'
Abl seq primer_8:	5'GTACGAGCAGAAGCCACAGA 3'

5.2 Methods

5.2.1 Molecular cloning

DNA gel electrophoresis

1% agarose gels were prepared in TAE buffer, and 2µl of Ethidium Bromide were added to the melted agarose before puring into the cast. Samples were prepared adding 6X DNA loading buffer to the DNA. TAE was also used as running buffer.

Abl cloning strategy

In order to express Abl in S2 cells, the coding sequence (CDS) was cloned into a pUAS-attB vector. The final construct was designed in order to have a C-terminus 2Xc-myc tag. Abl CDS was amplified through PCR from a cDNA clone (clone GH09917, DGRC). This first PCR also removed the stop codon, adding in frame the c-myc coding sequence. After purification, the PCR product was used as a template for a second PCR, in which a restriction site for NotI was added at the 5', whereas at the 3' a second c-myc coding sequence, a STOP codon and a XbaI restriction site were added.

The purified PCR product and the pUASattB vector were digested with NotI and XbaI. The reaction was:

NotI 1 µl

XbaI 1 µl

Buffer 2(10X) 5 µl

DNA (300 ng-1 µg)

H_2O up tp 50 µl

The reaction was performed at 37ºC for 2 hours.

The digested products were separated on agarose gel. Bands corresponding to the desired DNA fragments were cut out with a sterile razor blade and purified using Qiagen Gel Extraction Kit. The DNA was the eluted in 30 µl of ddH_2O.

The ligation reaction was conducted at 16ºC o/n using T4 DNA ligase. Insert/vector molar ratio was 1:1

T4 DNA ligase buffer (10X):2 µl

T4 DNA ligase: 1 µl

Vector: 50 ng

Insert:25 ng

Water up to 20 µl

Ligation reaction was transformed into bacteria cells (see below)

Polymerase Chain Reaction (PCR)

I-Proof polymerase mix was used for cloning Abl cDNA, and was used according to manufacture specifications; a final volume of 50 µl was prepared for each reaction.

5.2.2 Fly mantainance

Flies were raised and cultured in plastic bottles containing *Drosophila* standard medium. For crosses and weak stocks blue yeast paste was added to the media. Vials (diameter 25mm) were used for normal stock keeping and crosses; bottles (diameter 50mm) were used for expansion and virgins collection. Flies were kept in incubators with controlled temperature and humidity (60-70%). Fly stocks were kept at 18°C, whereas flies kept for expansion, crosses and embryos collection were kept at 25°C unless otherwise stated. For selection and collection, flies were anesthetized with CO2 and observed with stereomicroscopes.

5.2.3 Drosophila genetics

Balancer chromosomes

Embryonic specific marked balancer chromosomes were exploited in order to select embryos with the wanted genotypes after collection, fixation and staining. The balancers used for this purpose were:

yw Baz^{4}FRT9-2/FM7a,GMR-nvYFP
w; Sco/CyO, GMR-nvYFP
w; Dr^{1}/TM3, GMR-nvYFP , Sb
The GMR enhancer is composed of five copies of a *Glass response element* from the *Rh1* gene. It drives expression in all cells behind the morphogenetic furrow (Hay, Wolff, & Rubin, 1994) . Therefore, embryos could be easily selected screening for the YFP fluorescence in the anterior part of the embryo (Le et al., 2006)

Gal4/UAS system

Gal4/UAS system allows spatio-temporal control of gene expression (Brand & Perrimon, 1993), and was used in this study for overexpression of specific gene or for performing rescue experiment. Gal4 encodes in yeast for a transcription activator protein that can bind to the *UAS* (*Upstream Activation Sequence*) promoter and activate gene transcription. The cDNA of the gene of interest is generally fused to a UAS sequence, and the expression can be driven in a spatio-temporal controlled manner by a Gal4 element fused to specific promoter. The result is the expression of the gene of interest only in the promoter specific fashion. In this study, the system was exploited for performing rescue experiments, for overexpression or ectopic expression of genes, or in combination with a reporter gene (usually mcd8-GFP) for selectively label/visualize specific populations of neurons.

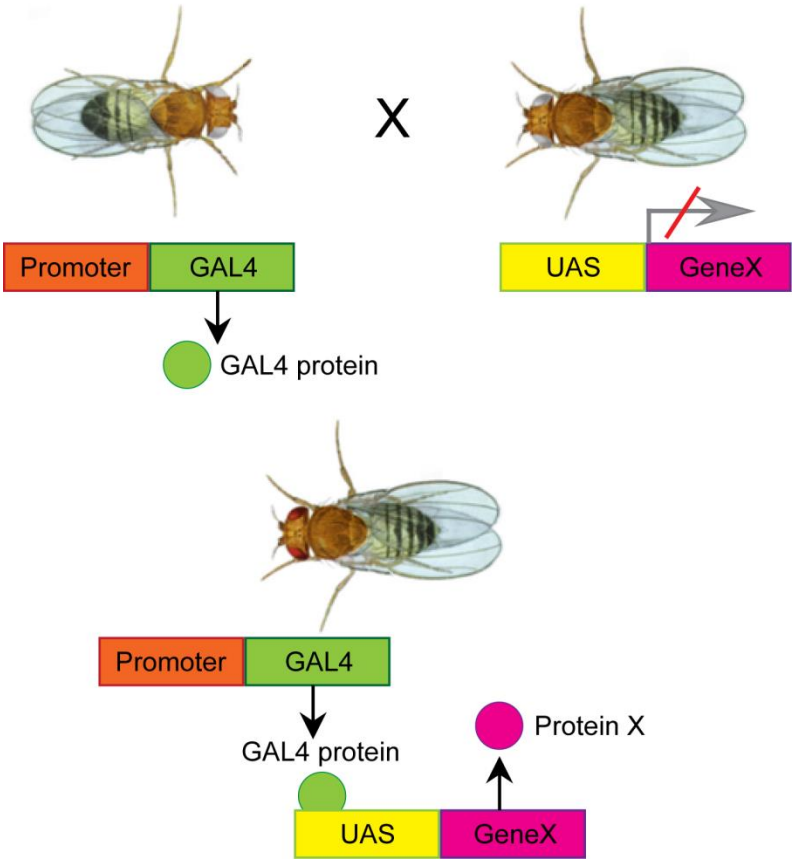


Figure 5-1: Schematic of the Gal4/UAS system in flies.

Spatio-temporal controlled gene expression is achieved in flies crossing transgenic flies carrying the Gal4 transcription activating protein under the control of a specific promoter for transgenic flies carrying the UAS sequence upstream of the gene of interest. The progeny carrying both of the transgenes will be expressing the gene of interest in a specific subset of cells.

5.2.4 Embryo collection

Embryos were collected on apple juice agar plates after the parental cross was kept in vials for at least 3 days. Flies were then transferred in collection cages closed with apple juice agar plates covered with dry yeast (to maximize the egg laying) and let laying eggs at 25°C. The plate was exchanged twice per day, once in the morning and once in the evening. The evening plate was then kept at 18°C to slow down development and embryos were then collected and fixed together with the overnight collection the following morning.

5.2.5 Embryo fixation

Embryos were fixed as described in (REF). Plates were treated with 10% bleach for 3 min at room temperature in order to dechorionate the embryos. The

embryos were then collected in a sieve and rinsed with tab water in order to remove all the bleach. They were subsequently transferred in Eppendorf tubes and fixed under vigorous shaking for 30 min at rt. The fixative used was a 50% Heptane, 45%PBS, 5% Formaldehyde mixture. After fixation, the lower phase of the fixative (PBS/Formaldehyde solution) was replaced with Methanol, the tube vortexed and the bottom phase removed again. This sequence was repeated until the embryos were all sinking at the bottom of the tube, meaning that the vitelline membrane was removed. The whole fixative was then removed and exchanged with Methanol, and the embryos were washed for 10 min on rotatory shaker. Embryos could be then directly stained or stored at -20°C after exchanging methanol with Ethanol in order to avoid bleaching of GFP.

5.2.6 Immunohistochemistry

Embryos were rinsed with 0.1% PBT in order to remove any trace of Methanol or Ethanol, and then washed three times with 0.1% PBT for permeabilization. Blocking solution consisting of 5% of Donkey or Calf serum in 0.1% PBT was then applied for 1 hour. Primary antibodies dilutions were prepared in Blocking solution and applied to embryos overnight at 4°C or at least 4 hours at r.t. Embryos were then washed three times for 10 min with PBT and proper secondary antibodies diluted in 0.1% PBT were applied for at least 2 hours at r.t. Embryos were washed 3 x 10 min with 0.1% PBT and Vectashield Mounting media was applied. Embryos of the desired stage were then selected according to anatomical features (Bownes, 1975; Campos-Ortega & Hartenstein, 1985) and filetted, exposing the ventral midline. Imaging was done using the Olympus FV-1000 confocal microscope; images were processed with Adobe Photoshop Image J and Adobe Illustrator.

5.2.7 Genomic DNA isolation

For genomic DNA isolation 2-3 adult flies were collected in Eppendorf tube and kept on ice. 250 µl of Solution A was added to the flies and they were homogenized using plastic homogenizer. Flies were then incubated for 30 min at 70°C. 28 µl of 8M Potassium Acetate solution was added and samples incubated on ice for 30 min. Samples were centrifuged for 30 minutes at 13.000 rpm and the resulting supernatant was transferred to a new tube and mixed with.

250 µl of Phenol-Chloroform. After 5 min centrifugation at 13.000 rpm the upper fraction was collected and transferred to a new tube, where DNA was precipitated by adding 125 µl of Isopropanol. The DNA was spun down by 15 min centrifugation at 13.000 rpm, and the supernatant removed. After washing the pellet with 125 µl of 70% Ethanol, DNA was again centrifuge and let dry at room temperature in order to get rid of all the Ethanol. DNA was diluted in 30 µl of distilled water and concentration determined with NanoDrop.

5.2.8 Transformation and plasmid preparation

One Shot Top10 chemical competent cells (Invitrogen) were used for transformation of ligation products, due to their high efficiency. Cells were thaw from -80ºC on ice, and 5 µl of the ligation reaction were added; cells were kept for 30 minutes on ice. Bacteria were then heat-shocked at 42ºC for 1 minute ad immediately returned on ice for 2 minutes. 250µl of LB media were added and the cells were incubated at 37ºC for 1h with shaking (300rpm).20-50 µl of the cells were plated on LB plates containing the selective antibiotic and incubated at 37ºC overnight. The following day, single colonies were picked up and grown in LB media containing the selective antibiotic. The plasmids were extracted using Qiagen Kits according to manufacture protocols.

For routine DNA amplification, DH5α Electro competent cells were thaw on ice, inoculated with 2-3 µl of DNA and transferred in electroporation cuvettes. After electroporation, 200 µl of LB media was added and cells were incubated at 37ºC for 1h with shaking (300rpm). 10µl of cells were plated on LB plates and containing the selective antibiotic and incubated at 37ºC overnight. Single colonies were then treated as described before for chemical competent cells.

5.2.9 Cell culture and transfection

Schneider S2 cells were initially derived from a primary culture of late stage *Drosophila Melanogaster* embryos (20 hr) (Schneider, 1972).

Cells were maintained in a 28°C incubator without CO_2 in complete Schneider's medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged every 3-4 days at a 1:2.5 dilution.

S2 were transfected using the Effectene Kit according to manifacture manual (Qiagen). Briefly, cells were seeded the day before transfection in 60mm petri dishes in 4 ml of media containing both serum and antibiotics at the density of 0.5 to 2.0 x 10^6 cells/ml. The day after, 1 µg of DNA was mixed with 150ul of the supplied buffer EC and 8µl of Enhancer, and incubated at r.t. for 2-5 minutes. 25µl of Effectene Reagent was added to the DNA mixture, and incubated for 10 minutes to allow transfection-complex formation. After the incubation, the 1ml of medium was added and the the final mixture added drop-wise to the cells. The cells were incubated 48-72 cells before harvesting them.

5.2.10 Co-immunoprecipitation

S2 cells were harvested 2-3 days after transfection, centrifuged for 5min at 3400 rpm and washed twice with PBS. The cells were resuspended in 300 µl of lysis buffer and lysed using a homogenizer for 1 min at full speed. The pellet was incubated at 4°C for 30 min, then centrifuged at 3400 rpm for 5 min. Supernatants were transferred to a new tube and diluted in lysis buffer. Solutions were incubated with the anti-myc antibody for 2 hours at 4°C. Afterwards 40 µl of beads (slurry 50% (v/v), were added and the mixture incubated for 2 hours at 4°C. The beads were washed with 500 µl of ice-cold lysis buffer, then once with 500 µl of 50 % v/v lysis buffer in PBS, and once with 500 µl of PBS. The beads were removed by centrifugation and the samples were boiled in 6 µl SDS buffer (6x) for 10 minutes and loaded onto a protein gel.

5.2.11 Immunoblotting

Samples (20 µl) were separated by SDS-PAGE on 5 % bis/tris polyacrylamide gels. Gel electrophoresis was performed in 1x SDS running buffer at 140 V and 75 mA for 1 h. Gels were blotted on Whatmann nitrocellulose membrane for 45 min at 25 V and 200 mA with a semi-dry apparatus (Thermo Scientific), after that membrane and filter paper were soaked in Fast Semi-Dry Transfer Buffer (1x) for 15 min. Afterwards the blots were blocked in a TBST solution with 5 % w/v non-fat for 30 min. The membranes were incubated with the primary antibody diluted in blocking solution o/n. The next day, the blots were rinsed and washed 3x with TBST (1x), for 15 min each time and then incubated for 2 h with the secondary antibody diluted in blocking solution. After being washed three times with TBST,

the membranes were developed in the 1:1 mixture of the Amersham ECL Western Blot Detection Reagents from GE Healthcare. Images at different exposure times were taken in a dark room with Amersham Hyperfilm ECL or using the Fusion Fx7 video camera system.

5.2.12 Summary of the experimental genotypes

Table 10 Detailed genotypes of the embryos per figure relative to the results section.

FIGURE	EXPERIMENT	GENOTYPE
3-1	Mutant analysis	*w^-* *fmi^{E59}/fmi^{E59}* *dsh^1/dsh^1* *$vang^{153}/vang^{153}$* *fz^{KD4}/fz^{KD4}*
3-2	Mutant analysis	*$fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$* *$fra^3\ vang^{153}/fra^3\ vang^{153}$* *$dsh^1/dsh^1; fra^3/fra^3$* *$fra^3/fra^3; fz^{KD4}/fz^{KD4}$*
3-3	Mutant analysis	*fra^3/fra^4* *fmi^{E59}/fmi^{E59}* *NetABΔ/Y* *$fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$* *NetABΔ/Y; fmi^{E59}/fmi^{E5}*
3-4	Expression	*$^9w^-$*
3-5	Rescue	*$^9w^-$1407-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$* *1407-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;UAS-fmi/+* *1407-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;UAS-fra/+* *$fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;Elav-Gal4/+* *$fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;Elav-Gal4/UAS-fmi* *Sim-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$* *Sim-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;UAS-fmi/+* *Gcm-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$* *Gcm-Gal4 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;UAS-fmi/+*
3-6	Rescue	*$fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;Elav-Gal4/+* *UAS-p35 $fra^3\ fmi^{E59}/fra^3\ fmi^{E59}$;Elav-Gal4/+*

3-7	Mutant analysis	*Eg-Gal4 UAS-mcd8*-GFP/ *UAS-mcd8*-GFP *fmi^{E59}/fmi^{E59}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP *fra^{3}/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP
	Rescue	*NetABΔ/Y; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP *fra^{3}UAS-mcd8*-GFP/*fra^{4};Eg-Gal4 UAS-mcd8*-GFP/*UAS-fmi* *NetABΔ/Y;Eg-Gal4 UAS-mcd8*-GFP/ *UAS-mcd8*-GFP *UAS-fmi*
3-8	Rescue	*fra^{3}/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP *fra^{3} UAS-mcd8*-GFP */fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/ *UAS-fz*
3-9	Overexpression	*Ftz$_{ng}$-Gal4/*+ *Ftz$_{ng}$-Gal4/UAS-fmi* *Ftz$_{ng}$-Gal4/UAS-fra* *Ftz$_{ng}$-Gal4 UAS-fmi /UAS-fmi* *Ftz$_{ng}$-Gal4 UAS-fra/UAS-fra*
3-10	Rescue	*fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP *fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP *UAS-fmi* *fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-mcd8*-GFP *UAS-fra*
3-11	Mutant analysis	*fmi^{E59}/fmi^{E59};Sema2b-T-myc* *fra^{3}/fra^{4}; Sema2b-T-myc* *fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; Sema2b-T-myc*
3-12	Mutants	*fmi^{E59}/fmi^{E59};15J2-Gal4/ UAS-mcd8*-GFP *fra^{3}/fra^{4}; 15J2-Gal4/ UAS-mcd8*-GFP *fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; 15J2-Gal4/ UAS-mcd8*-GFP *c-544-Gal4 fmi^{E59}/fmi^{E59}; UAS-mcd8*-GFP *c-544-Gal4 fra^{3}/fra^{4}; UAS-mcd8*-GFP *c-544-Gal4 fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; UAS-mcd8*-GFP
	Rescue	*c-544-Gal4 fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; UAS-mcd8*-GFP/*UAS-mcd8*-GFP *UAS-fmi* *c-544-Gal4 fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}; UAS-mcd8*-GFP/*UAS-mcd8*-GFP *UAS-fra*
3-13	Domain Analysis	*1407-Gal4 fra^{3} fmi^{E59}/ fra^{3} fmi^{E59}* *1407-Gal4 fra^{3} fmi^{E59}/ fra^{3} fmi^{E59};UAS-fmiΔN/*+

		1407-Gal4 fra^{3} fmi^{E59}/ fra^{3} fmi^{E59};UAS-fmiΔIntra/+ *fra^{3} UAS-mcd8*-GFP*/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-fmiΔN* *fra^{3} UAS-mcd8*-GFP*/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-fmiΔIntra*
3-14	Overexpression	*Ftz$_{ng}$-Gal4 /UAS-racV12* *fmi^{E59}/+; Ftz$_{ng}$-Gal4 /UAS-racV12* *Ftz$_{ng}$-Gal4 /UAS-Cdc42V12* *fmi^{E59}/+; Ftz$_{ng}$-Gal4 /UAS-Cdc42V12*
	Rescue	*fra^{3} UAS-mcd8*-GFP*/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-rac1* *fra^{3} UAS-mcd8*-GFP*/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-cdc42* *fra^{3} UAS-mcd8*-GFP*/fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/*UAS-Rho1*
3-15	Mutant analysis	*esn^{KO6} fmi^{E59}/ esn^{KO6} fmi^{E59}* *esn^{KO6} fra^{3}/ esn^{KO6} fra^{3}* *NetABΔ/Y; esn^{KO6}*
3-16	Mutant analysis	*Abl^{1}/abl^{1}* *fmi^{E59}/ fmi^{E59}/ abl^{1}/abl^{1}*
3-17	Rescue	*fra^{3} /fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/ *UAS-mcd8*-GFP *fra^{3} UAS-mcd8*-GFP */fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/ *UAS-fmi* *fra^{3} UAS-mcd8*-GFP */fra^{4}; Eg-Gal4 UAS-mcd8*-GFP/ *UAS-abl* *fra^{3} UAS-mcd8*-GFP */fra^{4} UAS-abl^{K417N}; Eg-Gal4 UAS-mcd8*-GFP/+ *fra^{3} UAS-mcd8*-GFP */fra^{4} UAS-abl^{K417N}; Eg-Gal4 UAS-fmi/ UAS-mcd8*-GFP
3-18	Overexpression	*Ftz$_{ng}$-Gal4 /UAS-abl* *Ftz$_{ng}$-Gal4 UAS-fmi /UAS-abl* *Ftz$_{ng}$-Gal4 UAS-fra/UAS-abl*
3-19	Mutants	*wnt5^{D7}/Y* *wnt5^{D7}/Y; fra^{3}/ fra^{3}* *wg^{l-17}/ wg^{l-17}* *NetABΔ/Y; wg^{l-17}/ wg^{l-17}*

		wnt2^{0}/ wnt2^{0}
		NetABΔ/Y; wnt2^{0}/ wnt2^{0}
		wnt4^{23}/ wnt4^{23}
		NetABΔ/Y; wnt4^{23}/ wnt4^{23}
	Overexpression	*Sca-Gal4/UAS-wg*
		Sca-Gal4/UAS-wnt2
		Sca-Gal4/UAS-wnt4
		Sca-Gal4/UAS-wnt5
		Sim-Gal4/UAS-wg
		Sim-Gal4/UAS-wnt2
		Sim-Gal4/UAS-wnt4
		Sim-Gal4/UAS-wnt5
		Elav-Gal4/UAS-wg
		Elav-Gal4/UAS-wnt2
		Elav-Gal4/UAS-wnt4
		Elav-Gal4/UAS-wnt5
		Ftz$_{ng}$-Gal4/UAS-wg
		Ftz$_{ng}$-Gal4/UAS-wnt2
		Ftz$_{ng}$-Gal4/UAS-wnt4
		Ftz$_{ng}$-Gal4/UAS-wnt5

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Publications and Scientific communications

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- CSHL Asia conference, Francis Crick Symposium on Neuroscience-the changing brain: “Atypical cadherin Flamingo collaborates with Netrin/Frazzled signaling during axon targeting in the *Drosophila* embryonic CNS”. Poster presentation.
- CSHL conference, Neurobiology of Drosophila:” The role of Flamingo in the *Drosophila* Ventral Nerve Cord axon guidance”. Poster presentation.