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The Functional Conservation of Frazzled in Insects

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The Functional Conservation of Frazzled in Insects

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Abstract

Axons in the developing embryo receive and react to signals that direct their growth to reach target tissues at specified locations. The signal pathways that direct midline crossing of axons during embryonic development have been comprehensively examined in the past years using the *Drosophila* ventral nerve cord or the spinal cord as a model system. A number of these signaling mechanisms are conserved, however disparities have been found between species in general strategy or the molecular signals controlling the response of axons to guidance cues.

The Netrin-Frazzled pathway has been shown to aid in midline crossing of axons in the embryonic ventral nerve cord of *Drosophila*. However, It is uncertain if this function of Frazzled is conserved in other insects. The goals of this research are to gain insight into the evolutionary conservation of axon guidance by the Netrin receptor Frazzled (Fra) and to expand our understanding of how Frazzled affects midline crossing in the flour beetle *Tribolium castaneum*.

The *Frazzled* ortholog in Tribolim is sufficient for replacing loss of function in *Drosophila Fra*. We also expect to see similar expression and function of Frazzled in beetles to those observed in *Drosophila*. These studies expand our knowledge of axon guidance of midline crossing in a species that does not share some of *Drosophila's* derived guidance characters. Using *Tribolium* as an insect model for comparative studies of axon guidance may allow us to see a more ancestral guidance scheme.

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CHAPTER 1:

Introduction

During animal development, the nervous system is patterned in the embryo by integrating many signals and receptors. Neural processes (axons and dendrites) extend from the cell body and build networks that receive and respond to stimuli. The axons extend from the distal end using a dynamic structure called the growth cone. Receptors are differentially expressed on the membrane of the growth cone that relay attractive or repulsive information to the cytoskeleton. This provides the spatial information to determine the direction of each axon to extend toward the proper target. In bilaterally symmetrical animals most axons growing to form the central nervous system are presented with a common guidance decision: whether to cross or not cross the midline. Ipsilateral axons remain on the same side of the body while commissural axons cross the midline (Evans and Bashaw 2010). Failure of axons to properly innervate the appropriate side of the body results in severe neurological defects (Engle 2010).

The midline plays a key role as the organizing center for midline crossing during embryonic axon guidance. Both the attractive and repulsive signaling cues which promote or inhibit midline crossing of axons are produced by midline glia, specialized glial cells located at the midline (Keleman et al. 2002, Mitchell et al. 1996). To be sure that once the axon enters the midline continues across and exits, it never re-crosses, commissural axons control their responses to various attractive and repulsive cues with a high degree of temporal precision. Meanwhile, ipsilateral axons must either ignore attractive signals or have a mechanism to give preference to repulsive cues, keeping them on the side of the body that they originated (Evans and Bashaw 2010).

The relatively simple nervous system of the fruit fly, *Drosophila melanogaster,* embryo requires this patterning mechanism to allow the newly hatched larva to search for food and avoid predators. Similar to the adult fly, the embryo has a segmented body plan including a segmented central nervous system. The fly ventral nerve cord (VNC) is analogous to the vertebrate spinal cord. The VNC is patterned by segmentally repeating neuromeres, temporary repeating developmental signals. Each VNC hemisegment of the abdomen contains the same set of approximately 300 neurons. Many axons are individually identifiable and make predictable and reproducible guidance decisions in each segment and from animal to animal (Figure 1.1, Howard et al. 2019) This provides a model where it is possible to examine phenotypic changes in the same identifiable subset of neurons in multiple segments and multiple animals. This greatly increases quantitative examination of axon guidance decisions.

The identifiable axon pathways in the VNC include FasII, Sema2b, EG, EW, and Apterous (*ap*) expressing neurons (Fig 1.1). FasII-positive axons are comprised of ipsilateral and post-crossing commissural axons, and form three discrete pathways on each side of the midline. One subset of commissural neurons is labeled by the *sema2b-TauMyc* reporter. These axons cross in the anterior commissure and project anteriorly in the intermediate longitudinal pathway. Both the EG and EW clusters and their commissural axons are labeled by the *eg-GAL4* reporter. The EG axons extend through the anterior commissure and the EW axons through the posterior commissure to cross the midline in each segment. A subset of ipsilateral neurons whose axons project towards the midline, then turn anteriorly in the medial longitudinal pathway is labeled by the *ap-GAL4* reporter (Howard et al. 2019).

The *Drosophila* VNC has been well characterized down to the genes required for proper patterning and their relationships. The architecture of the VNC consists of longitudinal axon

tracts and two commissures per segment, anterior and posterior. Each corresponding commissure is composed of the same axons in each segment. These axons can be genetically divided into reproducible subsets for detailed study and mechanistic specificity (Seeger et al. 1993).

The decision to grow ipsilaterally or contralaterally is controlled by signals at the midline and their receptors on the axonal growth cone. These receptors are generally defined as either repulsive (away from the midline) or attractive (toward the midline). Repulsive guidance receptors cause the disassembly of the cytoskeleton and turning away from the midline derived signal. This can be seen with the Slit receptor Roundabout (Robo), the primary repulsive cue in *Drosophila* midline guidance (Dickson and Gilestro 2006). Many other cues influence the steering of axons away from the midline and have been recently reviewed by Howard et al. (2019).

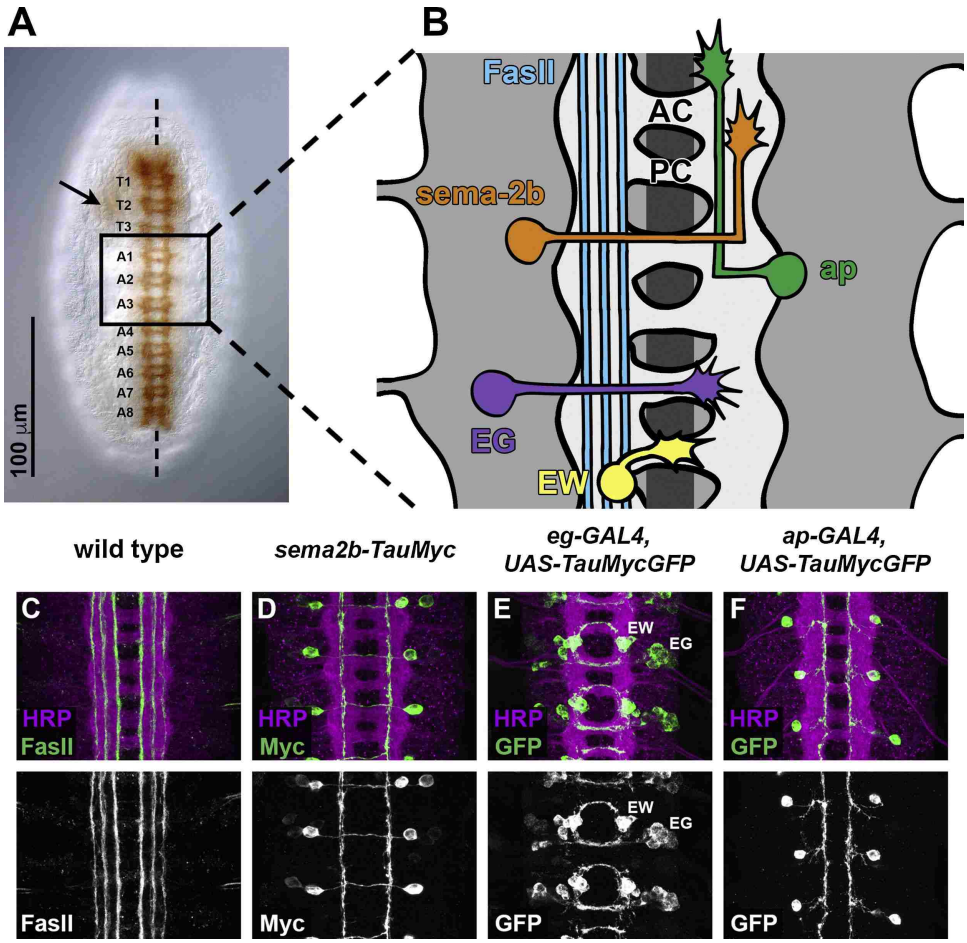

Figure 1.1 The *Drosophila* embryonic CNS. Mature *Drosophila* embryo, all CNS axons labelled with the monoclonal antibody BP102 (**A**). T1-T3 denote thoracic segments; A1-A8 denote abdominal segments. Dashed vertical line designates the midline. Schematic of three abdominal segments of the ventral nerve cord (**B**). Five identifiable neuronal subsets and their axon projection patterns are depicted. The first segment is labeled to designate anterior (AC) and posterior (PC) commissures. Confocal images of wild type (**C**) or transgenic (**D–F**) embryos stained with the indicated antibodies (C–F). Anti-HRP labels all CNS axons (Howard et al 2019)**.**

The opposing signaling mechanism promoting midline crossing of axons in *Drosophila* is also influenced by a number of molecules and signaling pathways also recently reviewed in Howard et al. (2019). The primary attractive pathway is Netrin-Frazzled. This attractive mechanism is evolutionarily conserved across bilaterian animals and promotes midline crossing of axons in a wide variety of animal groups (Evans 2016). The Netrin-Frazzled pathway has been investigated and defined thoroughly in *Drosophila*. In *Drosophila* two functionally redundant Netrin ligands (NetA and NetB) signal through their receptor Frazzled (Fra) to promote midline crossing (Gramates et al. 2017). Frazzled signaling promotes extension of the cytoskeleton in the direction of netrin signaling from the midline. Flies expressing mutations in Fra exhibit reduced or absent commissures, as well as breaks in the longitudinal pathways (Garbe and Bashaw 2007). Net A/B double mutants exhibit a similar reduction of commissures and longitudinals, as the Netrins are functionally redundant no phenotype is seen in a single mutant. Frazzled responds to Netrin in a cell autonomous manner, when a functional Fra is expressed in only a subset of neurons in a Fra mutant embryo, those cells regain their proper axon guidance target (Garbe, O'Donnell and Bashaw 2007).

Frazzled is a transmembrane protein that consists of four immunoglobulin C2 type domains, six fibronectin type III repeats, a transmembrane domain, and a cytoplasmic domain (Kolodziej et al. 1996). Netrin causes dimerization of the Fra protein by binding to two Fra receptor. This binding initiates a signal cascade to promote cytoskeletal rearrangement (Dorsten and VanBerkum 2008). In addition to this canonical role as a Netrin receptor to relay the midline attractive signal, *Drosophila* Fra also functions as a transcription factor to activate expression of *commisureless* (*comm*). Comm is a key modulator of axonal responsiveness at the midline. In response to an as of yet unknown, but Netrin-independent signal, proteolysis of Fra releases its

intracellular domain (ICD). The ICD moves to the nucleus and is sufficient to activate *comm* transcription and inhibit midline repulsion (O' Donnell and Bashaw 2013, Yang, Garbe and Bashaw 2009, Neuhaus-Follini and Bashaw 2015).

A third role of Fra in Drosophila has also been proposed, A non cell-autonomous capture/relocation model in which commissural axons capture Netrin with the Fra receptor (Hiramoto et al., 2000; Hiramoto and Hiromi, 2006). Net is then reposition along the pioneer axons for later extending longitudinal neurons (pCC and dMP2) to follow. For this function, Fra signalling is not required since this function is carried out in the absence of the ICD. As Fra is not expressed in the growing pCC axons, another receptor is thought to respond to Netrin in these neurons. However, lack of Fra in the early pioneer axons causes localization defects in these later axons. The two medial-most fascicles in 1D4 (FasII) stained embryos pioneered by these pCC and dMP2 neurons. The pCC and dMP2 neurons are seen following Net borders during the initial extension of their axons (Hiramoto and Hiromi, 2006).

Conservation of *frazzled*

Much of the work of characterizing axon guidance pathways has focused on *Drosophila*. However, recent attention has turned to investigating whether the mechanisms discovered in *Drosophila* to regulate axon guidance in the embryonic CNS are conserved in other animals. Ligands and receptors of these guidance pathways have been found across insects and other arthropods Huang et al. 2014, Yu et al. 2014, and Behura et al. 2011). Expression patterns and functional analyses support evolutionary conservation of the Net-Fra and Slit-Robo pathways and their roles in midline attraction or repulsion (Evans 2016).

For example in mosquitoes, orthologs of *Drosophila comm2* have been identified. Knockdown in *Aedes aegypti* of *fra* or *comm2* by RNAi produces a commissureless phenotype.

The knockdown of *fra* also results in a loss of *comm2* expression in *A. aegypti*. This suggests that at least within dipterans, Fra's role in activating *comm* transcription is conserved (Sarro et al. 2013, Clemons et al. 2011). Additionally, orthologs of the three *Drosophila comm* genes have not been detected in the genome of the red flour beetle, *Tribolium castaneum*, which suggests this mechanism is not conserved outside of Diptera (Evans and Bashaw 2012, Tribolium 2008). However, orthologs of Fra in vertebrates (DCC and Neogenin) can also regulate transcription via their ICDs. The targets of their transcriptional regulation have not yet been established, and *comm* orthologs have not been identified in vertebrates (Evans 2016). Although, Ndfip has been shown to regulate mammalian Robo1 similar to *comm* (Gorla 2019). All of which could lead to the conservation of the Fra ICD as a transcriptional regulator that has been co-opted for different purposes in various animal groups.

The highly conserved Frazzled/DCC/UNC-40 receptors respond to Net to direct attractive directions in axon guidance in flies, mammals, and worms, respectively. Netrin and Frazzled have coevolved to maintain their function for midline crossing throughout bilaterians incorporating slightly divergent combinations of ligand receptor pairs. The Netrin/Frazzled pairing is seen most basally in planaria and have grown in complexity to include four copies of Netrin and two copies of Frazzled in vertebrates (Huang et al. 2014).

Frazzled is also present in the genomes of insects and other arthropods including *Tribolium*. The Fra gene in beetles shares a significant portion of sequence similarity with that of flies (Fig. 1.2). However, the expression patterns and functions of Fra in Tribolium and other insects remain unexplored. It is more likely that the Netrin-dependent role of Fra in attractive signalling will be conserved than its role in regulating *comm* transcription.

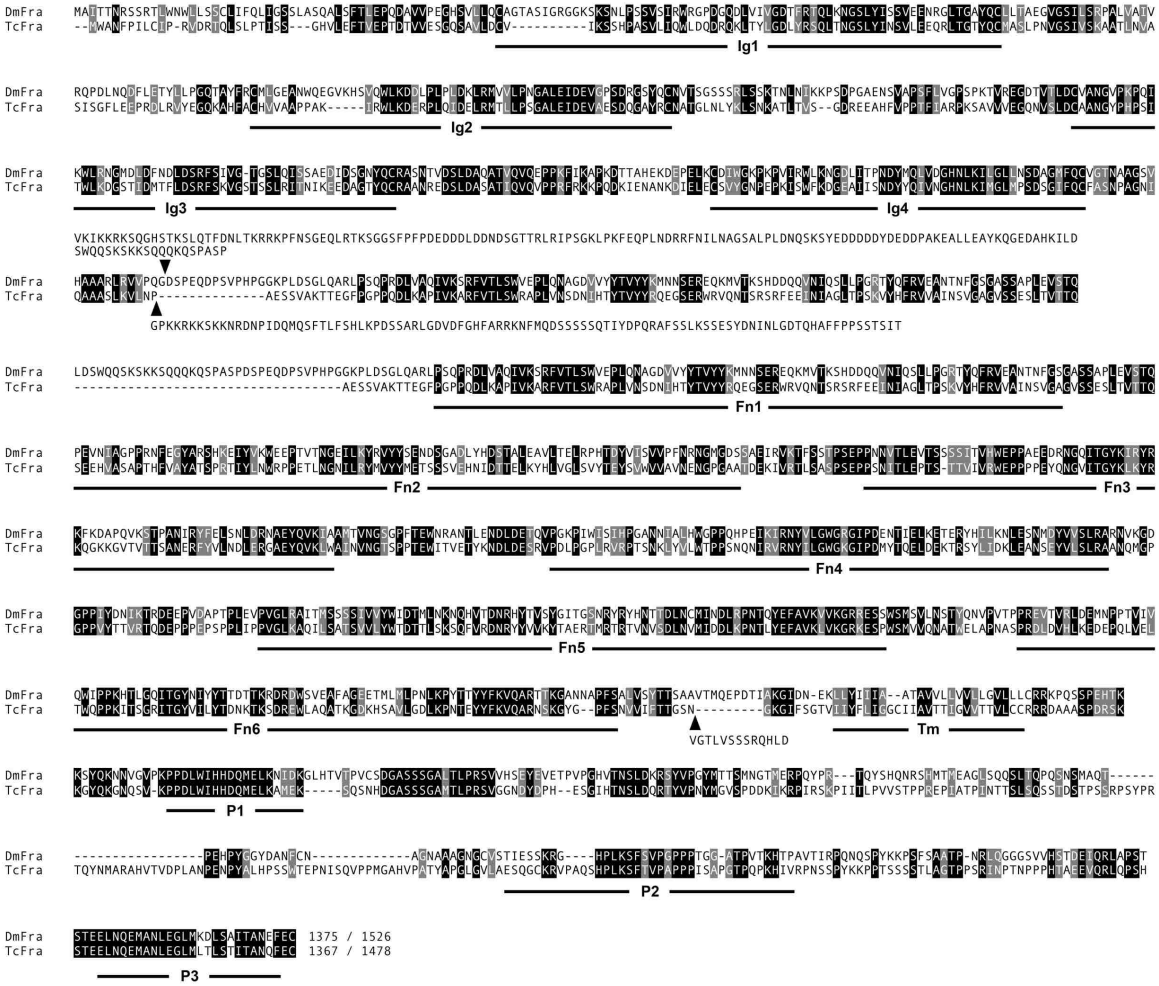

Figure 1.2 Frazzled protein sequence alignment. *Tribolium castaneum* Frazzled (TcFra) protein sequence aligned to *Drosophila melanogaster* Frazzled (DmFra). Domain annotations from DmFra sequence represented with bars below alignment. Black arrows indicate long isoform location and sequence.

A new insect model

The embryonic development of the red flour beetle, *Tribolium castaneum*, is more representative of development in other insects than Drosophila (Tautz, Friedrich, and Schröder 1994). For example, Tribolium develop via short-germ embryogenesis in which segments are added sequentially by a posterior growth zone. This method of adding segments resembles what is seen in basal arthropods and vertebrates but differs from the Drosophila model. Additionally, unlike Drosophila, beetle larvae have fully formed heads with eyes and three thoracic segments with pairs of legs (Tautz 2004, Tribolium 2008).

Beetles exhibit a simplified map of axon guidance effectors when compared to what is known from flies (Fig 1.3). A number of gene duplications and the addition of *comm*, provide additional complexity to Dipteran axon guidance. Most insects including *Tribolium* use only two Robo family members to guide axons at the midline while *Drosophila* employs three Robos. This suggests that *Tribolium* may more closely represent the majority of insect species. As stated, *Tribolium* also lacks an ortholog of *Drosophila* Comm, which regulates Robo localization to the growth-cone. In addition, Tribolium only code for one Netrin (TcNet), as opposed to the two in Drosophila. This suggests that the network of molecular mechanisms controlling VNC development in flies, that is currently used as a template for many studies, may contrast what is occurring in other insects including beetles (Evans and Bashaw 2012). A sizeable gap exists in the known mechanisms of axon guidance during animal development. Developing Tribolium as a model for insect development and central nervous system development will extend our understanding of the conserved features required for building an animal nervous system.

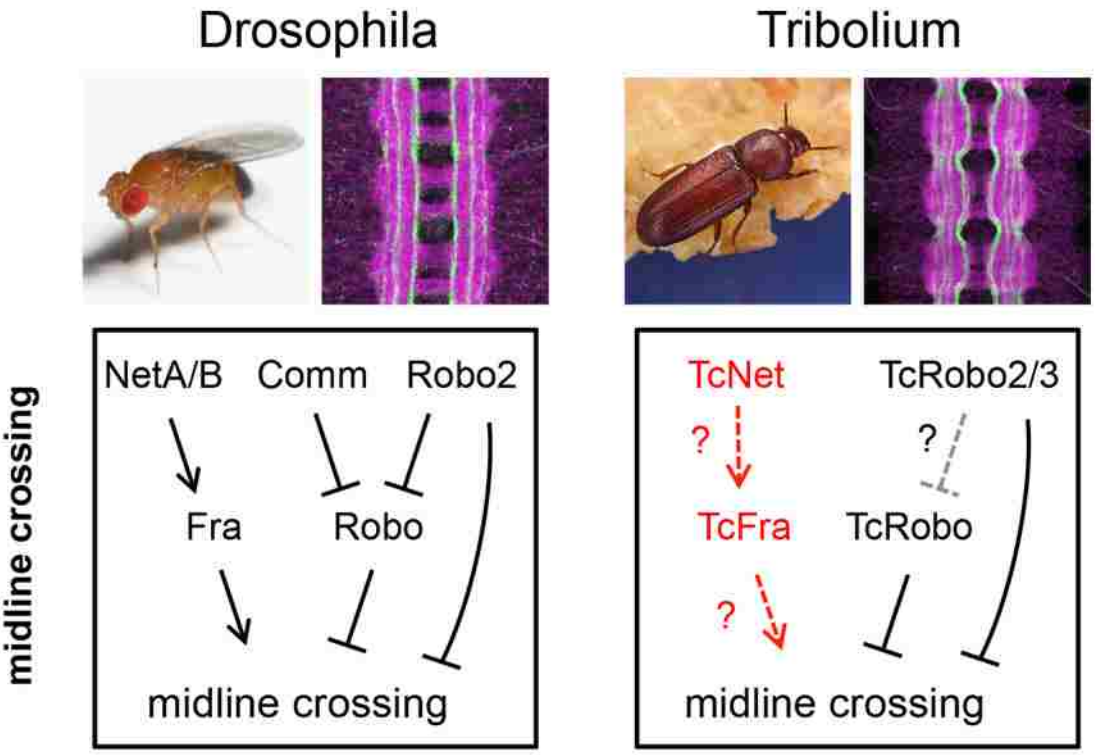

Figure 1.3 Signaling pathways regulating midline crossing of axons in Drosophila and Tribolium. The current *Drosophila* model shows that complexes such as the Slit/Robo, Net/Fra, Comm/Robo synergistically dictate midline crossing decisions. We lack understanding of how midline crossing occurs in animals outside of traditional model systems.

Conservation of other axon guidance genes

There are three Robo paralogs (Robo1, Robo2, and Robo3) in *Drosophila*. These genes have been shown to fill specialized roles in controlling axon guidance in the embryonic ventral nerve cord. The Robo family of guidance molecules have roles in midline crossing and the formation of longitudinal pathways. Based on gene sequences there is one less *robo* family member in other insects. The roles of these Robos from *Tribolium* have been compared to the *Drosophila* Robos (Evans and Bashaw 2012). Knockdown experiments indicate that TcRobo is only involved in midline repulsion similar to its ortholog Robo in *Drosophila*. Knockdown of TcRobo2/3 suggests control of longitudinal pathway formation and its contribution to midline repulsion is similar to a combined *Drosophila robo2+robo3* double mutant. However, in contrast to *Drosophila robo2* mutants, there is no ectopic midline crossing in *TcRobo2/3* knockdown embryos (Fig. 1.4, Evans and Bashaw 2012). However, this RNAi-knockdown approach limits the ability to directly compare the axon guidance functions of the Robo receptors in *Drosophila* and *Tribolium*.

A CRISPR/Cas9 approach to replace *Drosophila robo3* with *Tribolium robo2/3* has to shown the evolutionary conservation of the Robo receptors between *Drosophila* and *Tribolium* (Evans 2017). In the absence of *robo3*, TcRobo2/3 can guide two distinct subsets of developing longitudinal axons axons to their normal positions in the intermediate FasII pathway. This reveals that Robo3 dependent axon guidance decisions can be rescued by TcRobo2/3 in developing Drosophila neurons (Evans 2017). Suggesting that the mechanism by which longitudinal pathway formation is directed by *Drosophila* Robo3 is evolutionarily conserved in TcRobo2/3. This CRISPR/Cas9 gene replacement approach allows for the direct comparison of other Drosophila genes like *frazzled* and their orthologs in other species. This technique also

allows for the development of transgenic lines *Tribolium* to further advance this model of axon guidance.

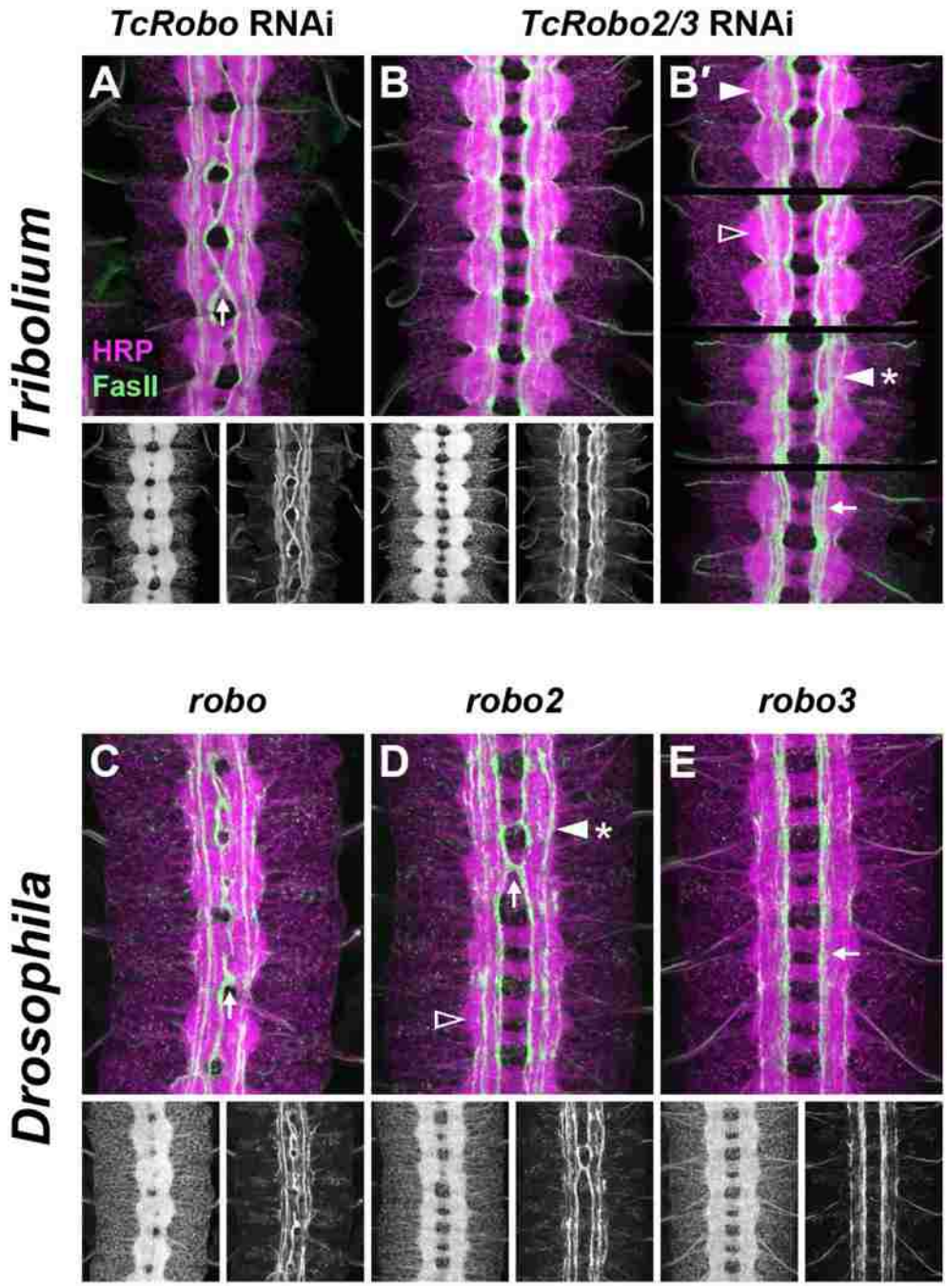

Figure 1.4 Knockdown of Robo receptor function. *Tribolium* (**A,B**) and *Drosophila* (**C-E**) embryos. *TcRobo* knockdown embryo (**A**). *TcRobo2/3* knockdown embryo (**B**). Segments from four different *TcRobo2/3* knockdown embryos showing FasII longitudinal pathway defects (**B'**). *robo* mutant *Drosophila* embryo (**C**). *robo2* mutant *Drosophila* embryo (**D**). *robo3* mutant *Drosophila* embryo (**E**) (Evans and Bashaw 2012)**.**

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CHAPTER 2: Conservation of Frazzled Function

Summary

Axons crossing the midline in developing *Drosophila* require Frazzled along with other guidance cues such as the Robo family of receptors. Frazzled plays a critical role in patterning axonal connections in the developing nervous system by regulating the midline crossing decision. It is uncertain if this function of Frazzled is conserved to the same degree in other animals and even within insects. Directly comparing the function of Fra with the *Drosophila* system will allow us to determine if the controlling mechanisms of axon guidance are equivalent within insects. I used CRISPR gene replacement to directly compare the function of *TcFra* in *Drosophila*, and its ability to rescue axon crossing in *Drosophila*. Axon commissures in fra^{Tcfra} embryos are present and appropriately sized when compared to wild-type embryos. This result suggests TcFra can appropriately signal in *Drosophila* embryos to promote midline crossing and *comm* transcription.

Introduction

Defects seen in midline crossing of *fra* mutants can be rescued by restoring Fra expression in all neurons, or in desired subsets of commissural neurons. (Garbe, O'Donnell and Bashaw). Additionally, preliminary data indicates that under gain of function conditions TcFra can stimulate midline attraction when expressed in *Drosophila* neurons (Fig. 2.1). Thus, I hypothesize that TcFra would rescue reduced midline crossing resulting from a loss of functional Fra. Here I use the CRISPR/Cas9 system to incorporate *Tcfra* into the genome of *Drosophila* replacing the endogenous *fra* protein coding region. If TcFra has the capacity to replace Fra for Netrin binding and *comm* transcription in axon guidance we will see a rescue and proper crossing of commissural axons.

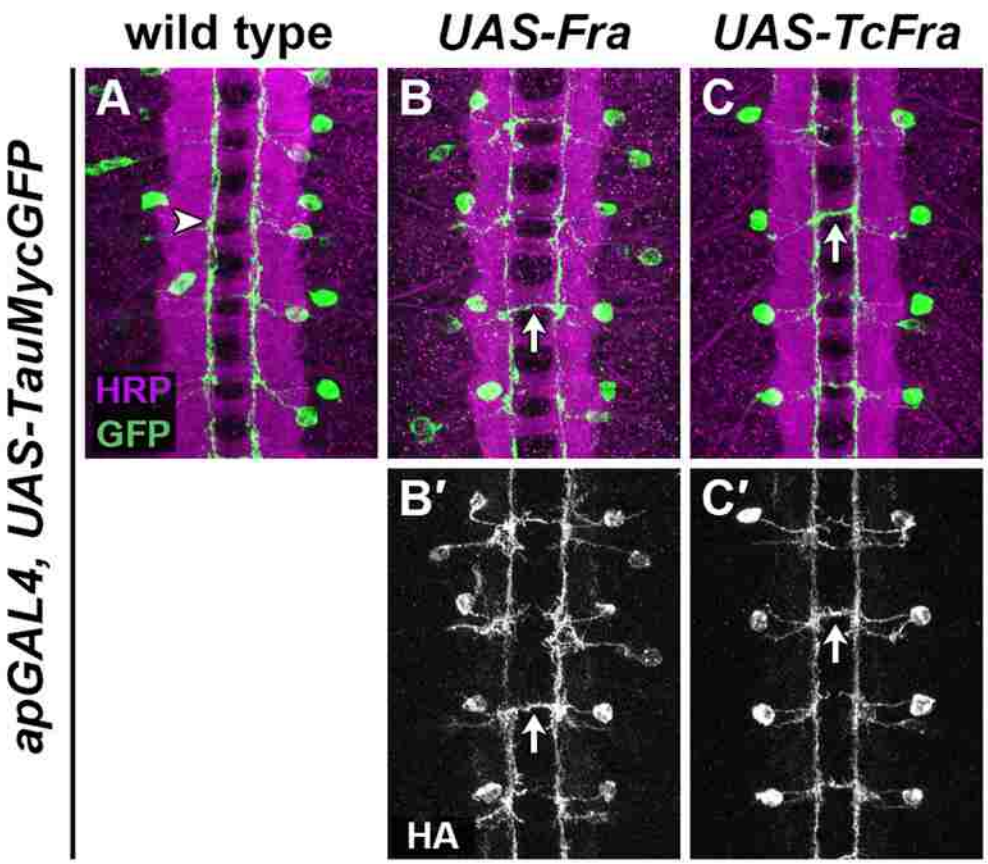


Fig 2.1 Apterous expression of Frazzled and TcFrazzled. Apterous neurons in wild type embryos extend axons in the intermediate longitudinal pathway and do not cross the midline because they do not express Fra (A). Forced expression of UAS-Fra using the apGAL4 driver causes ectopic crossing of axons (B,B'). Using the apGAL4 driver to force expression of TcFra also causes axons to ectopically cross the midline (C,C'). apGAL4 also driving expression of UAS-TMG, apterous axons stained with GFP (A-C). This result suggests that TcFra can respond to *Drosophila* Netrins and signal midline attractive response in fly neurons (Logan Terry unpublished).

CRISPR mediated transgenesis and mutagenesis

The bacterial immune mechanism to detect viral DNA, CRISPR (clustered regularly interspaced short palindromic repeats) can be used to facilitate efficient genome engineering in eukaryotic cells (Fig. 2.2). The RNA-guided Cas9 nuclease from the *Streptococcus pyogenes* has become a powerful tool for mediating genome modification with high precision. Target specification is achieved by simple complementation of a 20-nucleotide sequence that is accompanied by a protospacer adjacent motif (PAM). The 20-nucleotide targeting sequence is then included in the guide RNA (gRNA). Cas9-mediated genome editing triggers DNA repair through either non-homologous end joining (NHEJ, Chapter 3) or homologous recombination (HR) (Ran et al. 2013, Basset and Lui 2014).

With the CRISPR/Cas9 method, the cut genomic DNA can use HR to incorporate a provided tagged transgene (Fig. 2.2). Using two gRNAs with this method also allows for the removal of the entire endogenous *fra* coding region. This can be paired with a homologous piece of DNA carrying the HA tagged *Tcfra* cDNA. Using HR, the repair machinery can use the provided template to replace the excised region with the transgenic sequence (Fig. 2.3). In addition, with this method there is a possibility of completely deleting the *fra* coding region or creating deleterious mutations.

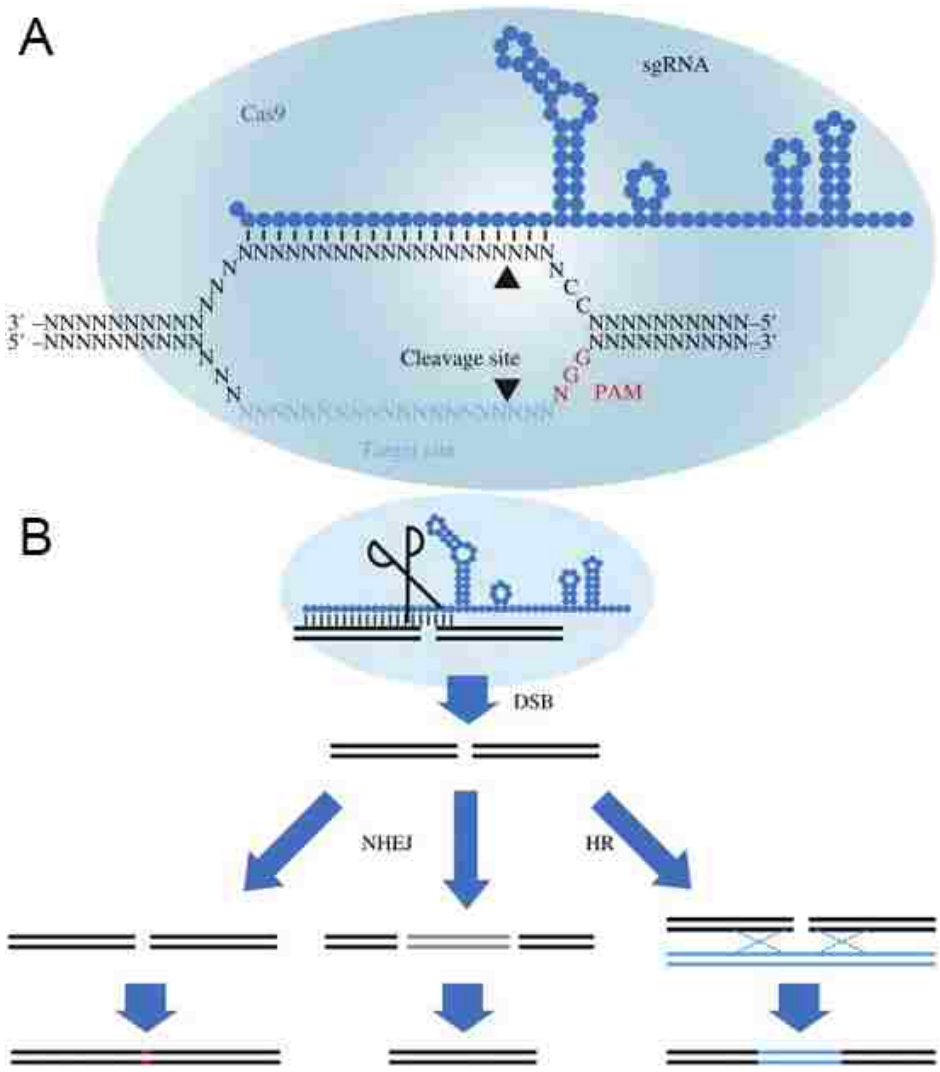


Figure 2.2 CRISPR/Cas9 schematic. Cas9 nuclease is localized by the single guide RNA (sgRNA) to the complimentary target sequence bordering the protospacer adjacent motif (PAM (NGG), red) (**A**). Cleavage occurs 3 nucleotides from the PAM site (black triangles). Double strand breaks (DSB) resulting from Cas9 cleavage are repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ can result in short deletions or insertions (left) or long deletions or inversions (when two cuts are made, middle). HDR can incorporate large insertions from a template strand (right) (adapted from Basset and Lui 2014).

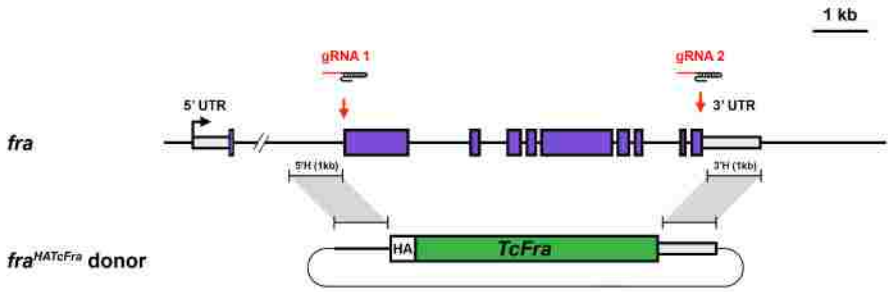


Figure 2.3 Frazzled CRISPR replacement. Replacement schematic of *Drosophila fra* coding region by homology directed repair. Guide RNA (gRNA) cut sites indicated (red arrows). Template with *Tcfra* cDNA and epitope tag flanked by 1kb homology arms.

Commissural axons defects

To quantitatively analyze midline crossing in *Drosophila* embryos, I fluorescently labelled a stereotyped set of commissural neurons, the *egl* neurons. One subset of the *egl* neurons, EG neurons, containing 10-12 cells extend their axons across the midline through the anterior commissure. Four cells make up the second more medial cluster of EW neurons. Three of these neurons send axons through the posterior commissure of the adjacent segment to cross the midline. In wild-type embryos both the EG and EW clusters send their axons across the midline in all segments. In approximately three-quarters of the segments in *fra* mutants the EW axons fail to cross the midline (Fig. 2.4) (Garbe, O'Donnell, and Bashaw 2007).

This subset of commissural axons allows for the quantification of the degree of rescue by scoring the percentage of segments with midline crossing defects. These embryos can be compared to *fra* mutant embryos and embryos in which wild-type *Drosophila fra* is expressed. I expect that TcFra will rescue the midline crossing in *fra* mutant embryos due to Frazzled/DCC function to promote midline crossing of commissural axons being conserved in invertebrates and vertebrates, and thus TcFra is likely to share this role.

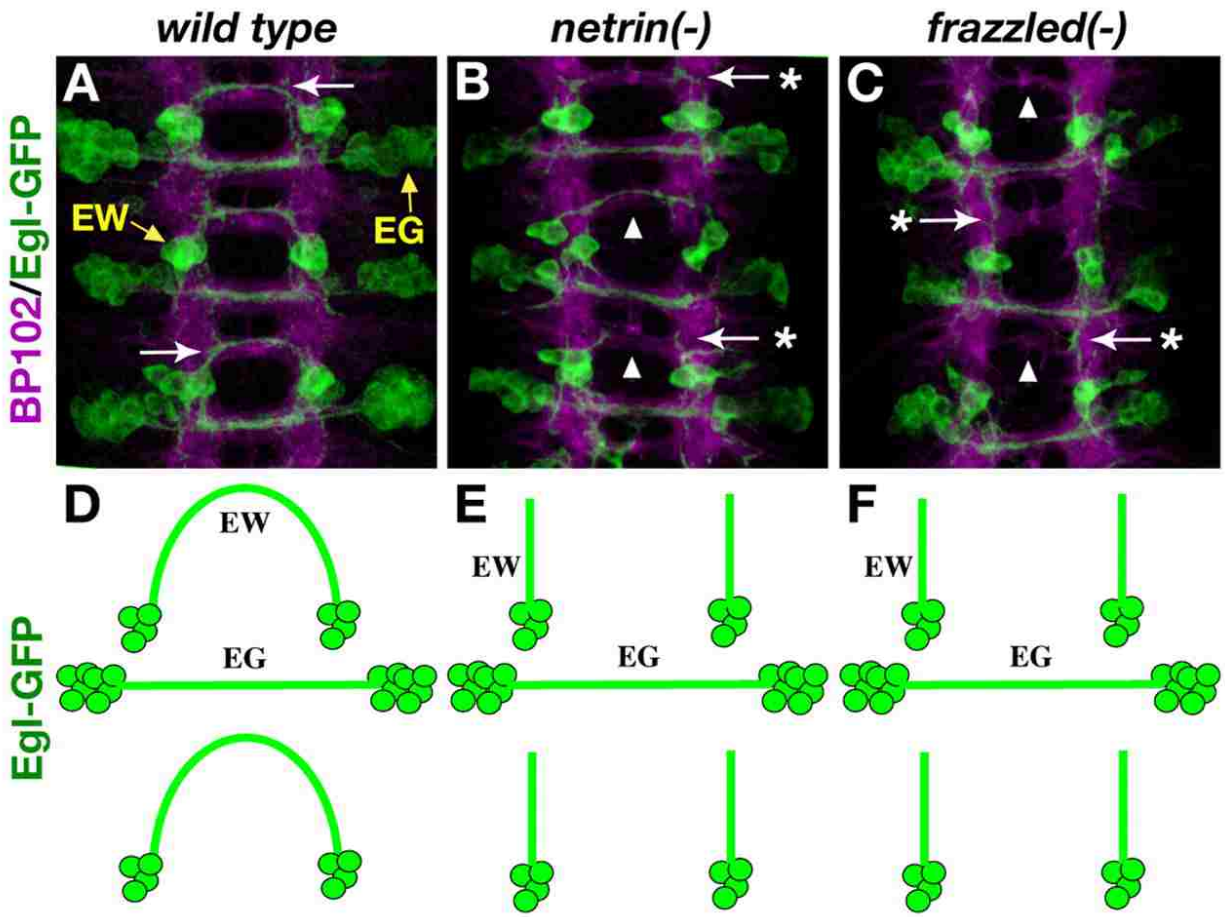


Figure 2.4 Midline crossing defects in EW neurons of *netrin* and *fra* mutants. Neurons labelled with Egl-GFP cross the midline in every segment in wild-type embryos (**A, D**). The EW subset of axons normally crossing in the posterior commissure of each segment frequently fail to cross in *netrin* mutants (**B, E**). Similarly, the EW axons do not cross the midline in most segments of *fra* mutant embryos (**C, F**). Stage 16 embryos stained to display all axons (BP102) and a subset of commissural neurons, Egl neurons (Egl-GFP). Representation of the paths of the EW and EG neurons in each of the specified genotypes (**D-F**) (Garbe, O'Donnell and Bashaw 2007).

Methods

Drosophila strains

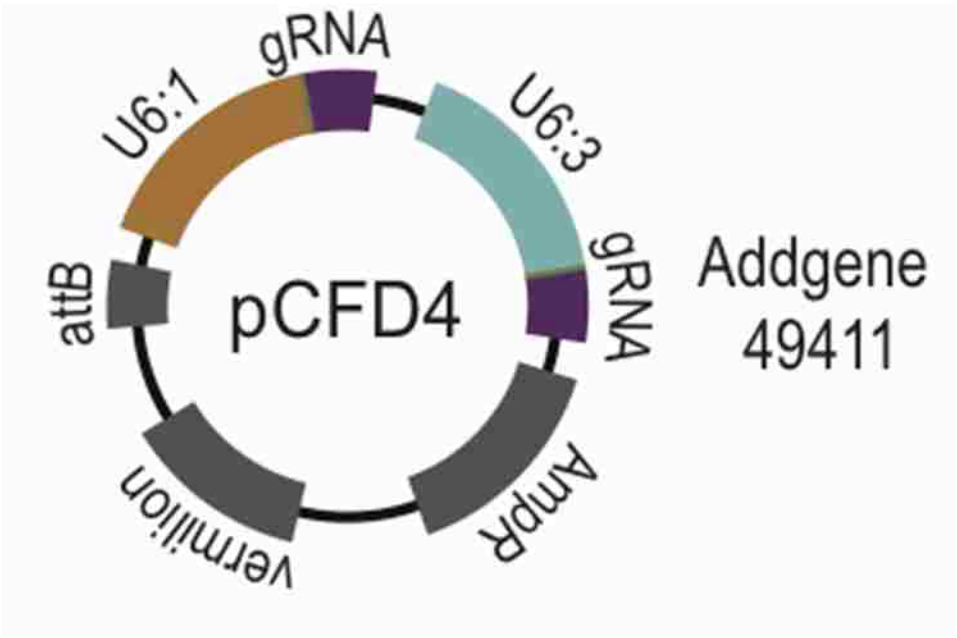
The following stocks were used: *fra*^{3} [UAS-TMG]^{III}, *fra*^{4} [eg-GAL4]^{III}, *Sco*/*CyOwg*, *Sco*/*CyOwg* [UAS-TMG]^{III}, *fra*^{4}, *Robo2*^{robo2}/*CyOwg* [eg-GAL4]^{III}, BDSC# 54591: nos-Cas9.P. The following stocks were generated: *fra*^{Tcfra}/*CyOwg*, *fra*^{Tcfra}/*CyOwg* [UAS-TMG]^{III}, *fra*^{Tcfra}/*CyOwg* [eg-GAL4]^{III}

Creation of CRISPR Plasmids

The guide RNA vector was constructed using the pCFD4 plasmid (Addgene plasmid # 49411; http://n2t.net/addgene:49411 ; RRID:Addgene_49411). This allowed for both upstream and downstream primers to be delivered on the same vector under the control of ubiquitous promoters (Fig 2.5). Primers 617 and 618 were designed with 19-20 base pair regions complementary to target sequences adjacent to a PAM site flanking the coding region (Appendix Table 1). The vector was created using traditional cloning methods in competent *E. coli* cells. The finalized plasmid was cloned, purified, and confirmed by sequencing using the Addgene suggested 5' primers: insertion 1 sequencing primer (Add1) and insertion 2 sequencing primer (Add2) (Appendix Table 1; Addgene (Watertown, MA)).

The Frazzled homology vector was constructed in the pIDT backbone. This was done by PCR of the upstream and downstream homology arms from a single nos-Cas9 (BDSC#54591) fly DNA extraction, with primers 607 – 610 (Appendix Table 1). This is subsequently followed by assembly of the backbone and homology regions by Gibson Assembly (Gibson et al. 2009) The resulting plasmid was cloned using traditional methods in competent cells, purified using the QIAquick Gel Extraction Kit (Qiagen (Hilden, DE)) and digested with Nhe1. Following digestion, a 4x HA tag (primers 619 and 620) was ligated into the site, cloned, and purified. This

plasmid was subsequently digested with Nhe1 and the *Tcfra* cDNA (p10UAST *HATcfra*, primers 745 – 746) was inserted in the restriction enzyme site that was retained at the 3' end of the epitope tag by Gibson Assembly (Figure 2.6) The finalized plasmid was cloned, purified, and confirmed by sequencing with primers 292, 293, 295, 360, 361, 362, and 364 (Appendix Table 1).



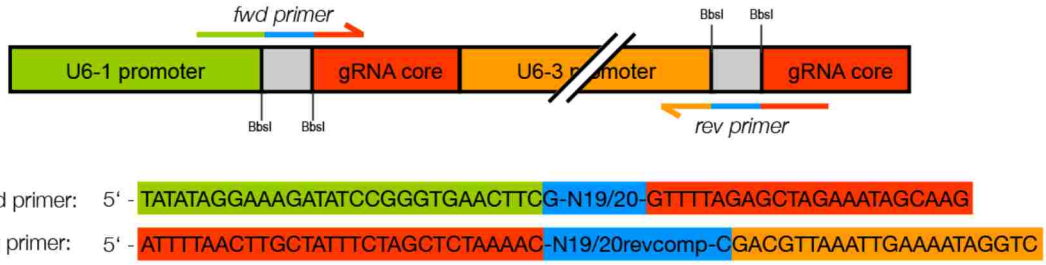


Figure 2.5 pCFD4 guide RNA construction schematic. The pCFD4 plasmid was used to deliver the 5' and 3' gRNAs. Primer 617 was used for the *fwd primer* and 618 for the *rev primer*. gRNAs are under the control of U6:1 and U6:3 promoters (Addgene plasmid 49411).

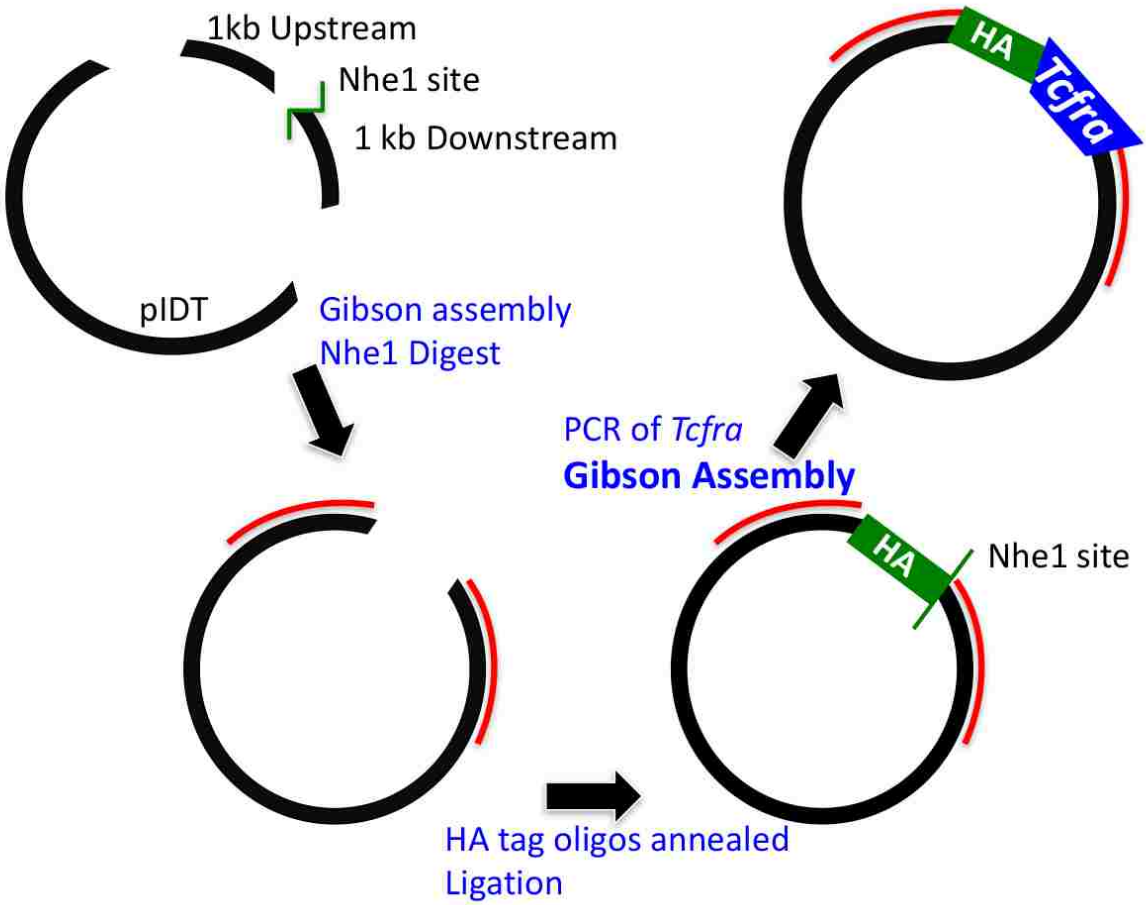


Figure 2.6 pIDT *fra*^{HATcfra} homology vector. The pIDT plasmid backbone and 1kb *Drosophila fra* homology regions combined by Gibson assembly. Nhe1 (green line) site digested between homology arms (red). Epitope tag ligated in, reforming Nhe1 site at 3' end. Nhe1 site digested, *Tcfra* cDNA (blue) inserted by Gibson Assembly.

Analysis and recovery of transgenic lines

Flies received after injection (G0) by Bestgene (Chino Hills, CA) were crossed 1:3 with Sco/CyOwg and all F1 progeny were collected according to sex. DNA from pools of 3 Female F1 progeny were screened by PCR with primer pair 290 and 295, which produce a 1.3-kb product when the *Tcfra* sequence is present. to identify potential replacements. Screening for a complete or partial deletion of Fra was carried out using primers outside of the region to be edited, 767 and 768. Sibling males of PCR positive females were crossed to Sco/CyOwg for 3 days. Males were then removed and screened by PCR with primer pairs 767-768, 290-291, 636-293, and 362-610 to identify founders with the intended mutations. Progeny of PCR positive males were collected to establish a stock (Fig. 2.7). Once the stock was established, 15 flies were collected and DNA extracted using the DNeasy Blood and Tissue Kit from Qiagen (Hilden, DE). Further screening of potential founders and confirmation of the transgenic stock used the primers 767 and 768 listed earlier to reveal the size of the entire inserted fragment. In parallel the Tcfra insertion specific primer pairs 362-610 and 636-293 were used for verification of the presence of the upstream and downstream junctions respectively. The primer pair 290-291 was included to cover the cDNA region and overlap with the previously listed regions for complete sequencing (Fig. 2.8) All of the generated PCR products were cloned with the CloneJET PCR Cloning Kit, ThermoFisher Scientific (Waltham, MA) and sent to Eurofins (LU) for sequencing with primers 358, 360, 361, 362, 364, and 787 (Appendix Table 1).

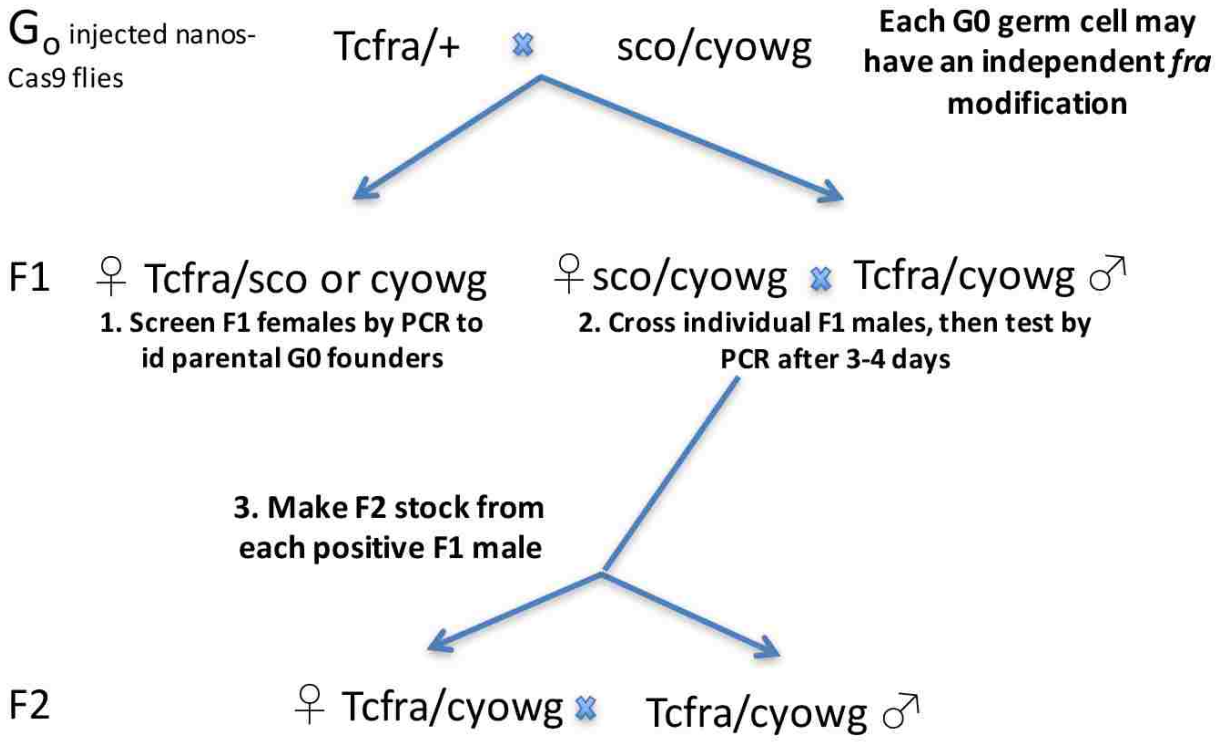


Figure 2.7 Fly recovery crossing scheme. G0 flies were crossed to *sco*/*cyowg*. Female progeny were pooled in sets of 3 and screened by PCR. Male siblings of PCR positive females were crossed to *sco*/*cyowg*, then tested to identify potential founders. Offspring of PCR positive males were collected to create F2 stock.

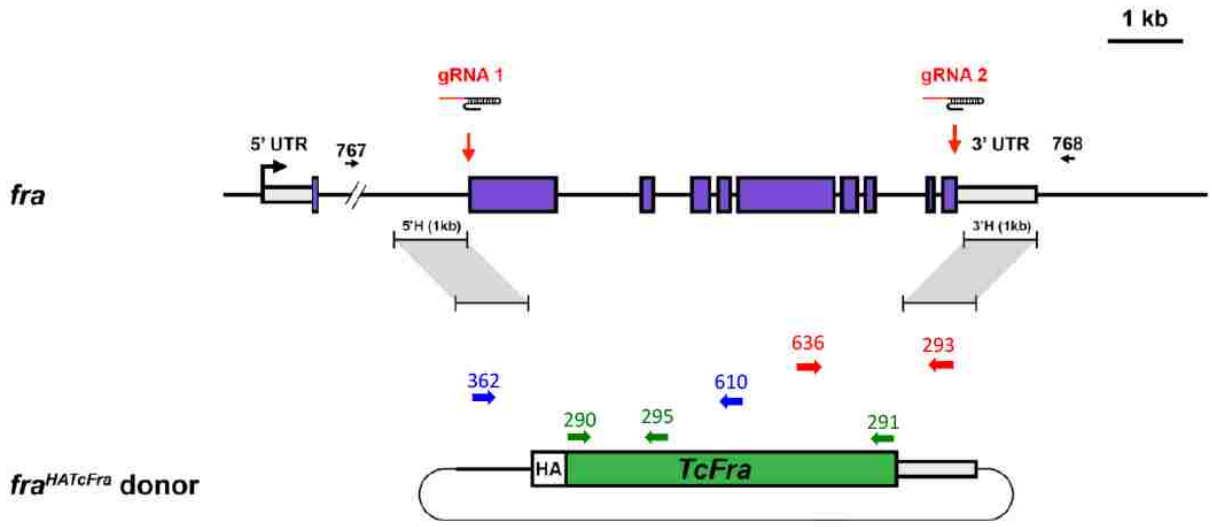


Figure 2.8 PCR screening map. F1 progeny were screened with *Tcfra* specific primers 290 – 295 for insertion of the transgene. F2 stock was screened with Primer sets 767 – 768, 362 – 610, 290 – 291, and 636 – 293 to cover the entire insertion area.

Immunohistochemistry

Standard methods were used to collect, fix, and stain embryos (Patel 1994). The antibodies: FITC-conjugated goat anti-HRP (Jackson ImmunoResearch #123-095-021, 1:100), mouse anti-Fasciclin II (Developmental Studies Hybridoma Bank [DSHB] #1D4, 1:100), mouse anti-βgal (DSHB #40-1a, 1:150), mouse anti-HA (Covance #MMS-101P-500, 1:1000), rabbit anti-GFP (Invitrogen #A11122, 1:1000), Cy3-conjugated goat anti-mouse (Jackson #115-165-003, 1:1000), Alexa 488-conjugated goat anti-rabbit (Jackson #111-545-003, 1:500) were used (Evans 2017). Embryos were genotyped using balancer chromosomes carrying lacZ markers. Ventral nerve cords from embryos of the desired genotype and developmental stage were dissected and mounted in 70% glycerol/PBS. Fluorescent confocal stacks were collected using a Leica SP5 confocal microscope and processed by Fiji/ImageJ (Schindelin et al. 2012) and Adobe Photoshop software.

Results

To test whether *Tribolium fra* can substitute for Drosophila *fra* to promote axon guidance outcomes in the *Drosophila* embryonic CNS, I used a CRISPR/ Cas9-based approach to replace the *fra* gene with *Tcfra*. Two guide RNAs gRNAs were combined with a homologous donor plasmid containing 1-kb flanking regions to induce homologous recombination, replacing the *fra* protein coding region with an HA-tagged *Tcfra* cDNA. The plasmid expressing both *fra* gRNAs (pCFD4) was injected along with the fra^{Tcfra} donor plasmid (pIDT) into *Drosophila* embryos expressing Cas9 under the control of the germline-specific *nanos* promoter, and F1 progeny produced from the injected flies were screened by PCR for the presence of *Tcfra* sequences. Additional PCR screening and DNA sequencing were used to identify correctly modified fra^{Tcfra} loci among the lines recovered from the positive F1 flies. Further details are provided in the methods.

Construction of the pCFD4 gRNA vector was confirmed by PCR (Fig. 2.9) and sequencing. Both *fra* recognition sequences are present in in their respective gRNA and oriented correctly. The homology vector was confirmed at each stage of its construction by PCR (Fig. 2.9) and sequencing. The increase in size of the PCR product compared to negative size indicated insertion and ligation of the additional fragment, and sequencing confirmed the complete fragment was incorporated and oriented correctly. Completed vectors were injected by Bestgene. Flies screened by PCR confirmed one replacement allele recovered from injected embryos (Table 2.1 and Fig. 2.10).

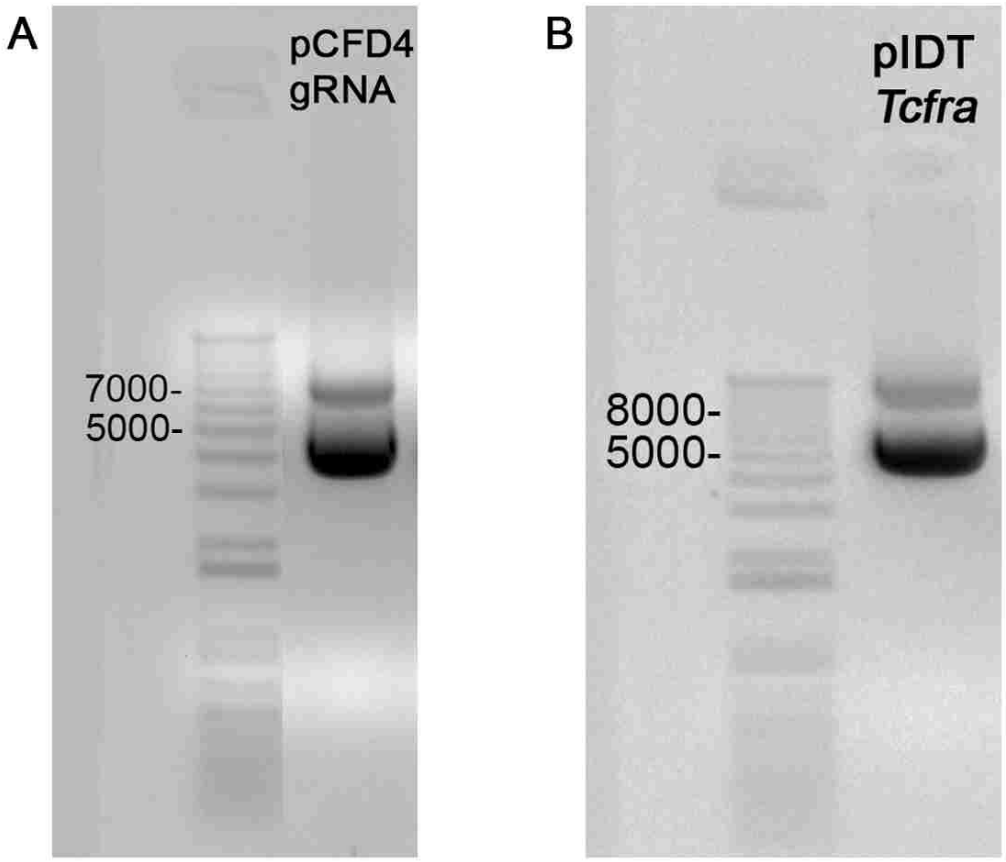


Figure 2.9 gRNA vector and homology vector confirmation. 2µl of mini-prep DNA of gRNAs in pCFD4 vector, 7.1Kb (**A**) and *Tcfra* cDNA in pIDT vector, 8Kb (**B**). Plasmid size compared to 1Kb Plus DNA Ladder from Invitrogen (Carlsbad, CA).

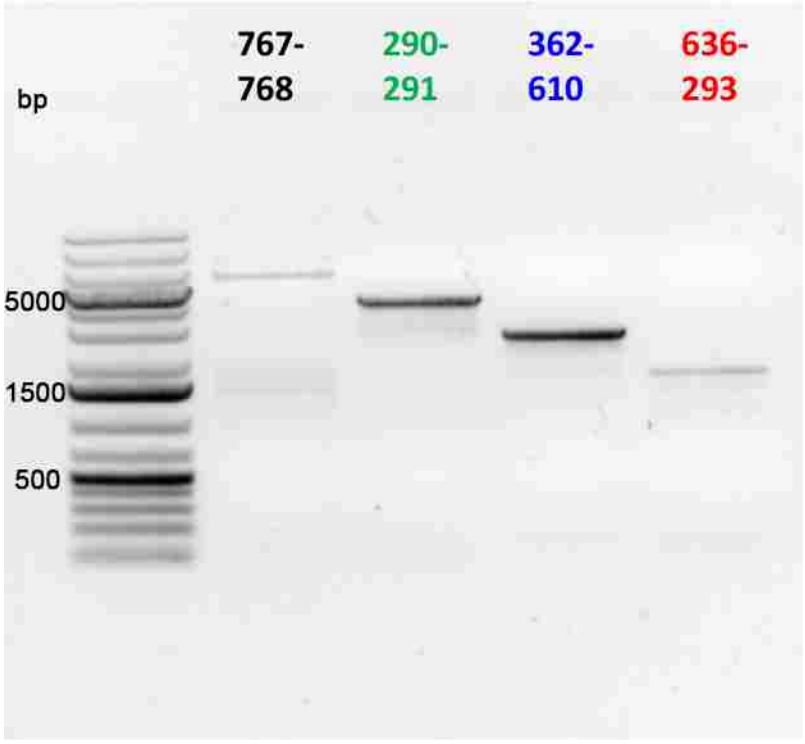


Fig. 2.10 PCR screening. PCR products for indicated primer pairs to screen for insertion of *HATcfra* by homologous recombination. Fragment lengths: 767 – 768 : 6.5-kb, 290 – 291 : 4.0-kb, 362 – 610 : 2.5-kb, and 636 – 293 : 1.5-kb. Gene Ruler 1-kb plus ladder was used from ThermoFisher Scientific (Waltham, MA).

In one founder the tagged *Tcfra* cDNA was successfully incorporated into the *Drosophila* genome, replacing the natural *fra* coding region (Table 2.1). PCR amplification using primer pairs 767 – 768, 290 – 291, 636 – 293, and 362 – 610 (Fig. 2.10) and subsequent sequencing of the PCR products reveal the epitope tag and entire transgene are present and correctly oriented. To characterize the fra^{Tcfca} allele, I first compared expression of HA-tagged TcFra protein from the modified locus to that of the endogenous Fra protein characterized by Kolodziej and colleagues (1996). Fra protein is expressed on developing axons in the embryo. Fra is first detected at stage 13 on the earliest commissural axons. High levels of expression are seen on both commissural and longitudinal axons throughout the development of the CNS. Frazzled is not expressed on tissues that express ligands required for axon pathfinding (muscle, glia, or midline cells) (Kolodziej 1996). In wild-type stage 15 *Drosophila* embryos, Fra is detected on commissural and longitudinal axons and on motor axons projecting from the CNS (Fig. 2.11). Staining of the epitope tag reveals TcFra is present in all commissures and longitudinal pathways of the VNC in fra^{Tcfra} homozygous embryos. The CRISPR introduced *Tcfra* transgene shows proper expression and localization when compared to the wild-type expression seen by Kolodziej and colleagues (1996) using a Fra antibody (Fig. 2.11).

To more closely examine midline crossing in fra^{tcfra} embryos, I used an antibody against the GFP expressing *egl* transgene to label a subset of commissural axons (Fig. 2.12). In wild-type *Drosophila* embryos carrying *eg*GAL4 and UAS-TMG transgenes, the EG and EW subsets of axons cross the midline in the anterior and posterior commissures respectively in each segment. In *Drosophila, fra* is required for proper midline crossing of the EW axons as they fail to cross the midline in 31% of segments in *fra* mutants (Fig. 2.13). Axon commissures in fra^{Tcfra} embryos are present and appear appropriately sized when HRP staining is

compared to wild-type embryos (Fig. 2.12). Staining of *egl* commissural neurons reveals proper crossing of the Fra dependent EW axons in *Tcfra* expressing embryos (Fig. 2.12).

I used an antibody against Fasciclin II (FasII) to label a subset of longitudinal axon pathways. Staining with FasII allows for a closer examination of the function of fra^{tcfra} in non-cell autonomous netrin signaling (Fig. 2.14). In wild-type *Drosophila* embryos, the FasII positive axons form three distinct anterior-posterior axon tracts. Fra is required in *Drosophila* for the formation of these longitudinal tracts. In fra^{Tcfra} expressing flies the FasII longitudinal pathways appear complete and properly formed, similar to wild type (Fig. 2.14). Together these results suggest that *Tcfra* can function equivalently to *Drosophila fra* to properly signal cell autonomously and as a transcription factor to promote midline crossing and non-cell autonomously to promote longitudinal pathway formation.

Table 2.1 *Drosophila* injection table

Injected Line	*# of Injected Embryos*	*% (no.) adult G0 survival*	*% (no.) fertile G0*	*% (no.) G0 founders*
BDSC# 54591: nos-Cas9.P	300	11 (34/300)	35 (12/34)	0 (0/12)
BDSC# 54591: nos-Cas9.P	300	13 (38/300)	37 (14/38)	7 (1/14)

gRNA vector and homology vector were sent to Bestgene for injection into embryos expressing Cas9 in the posterior pole. G0 flies were raised at 25^{o}C.

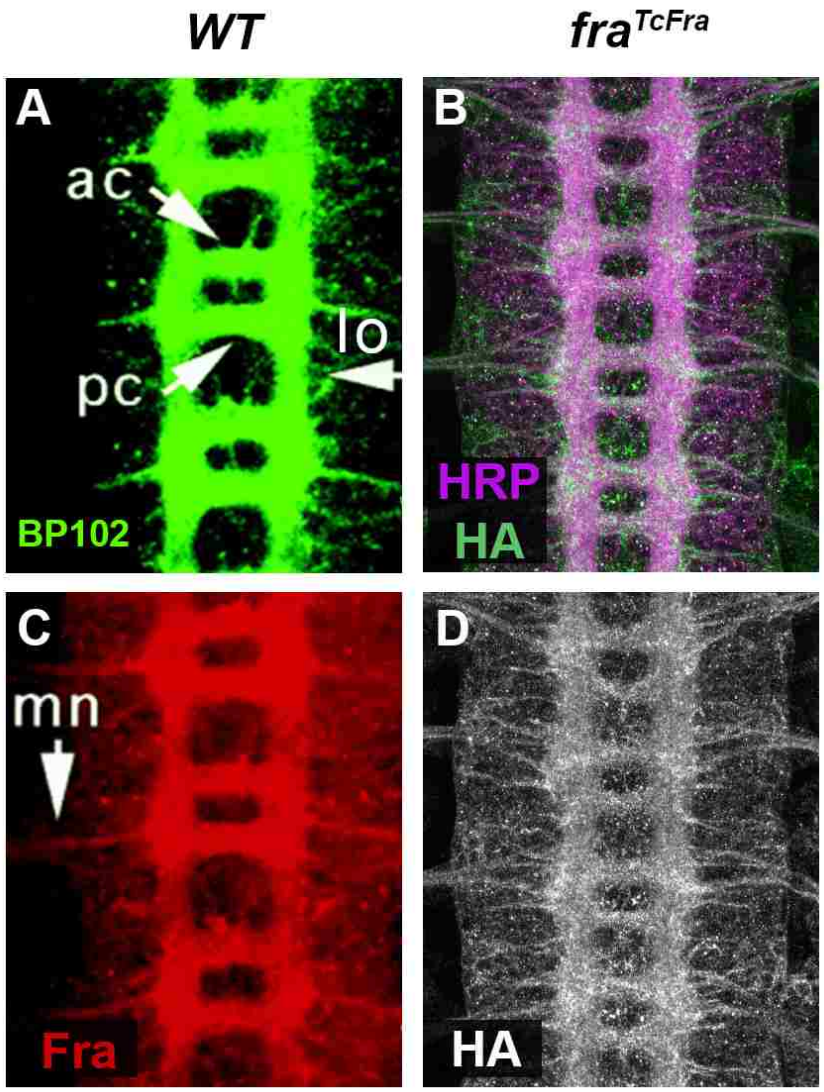


Figure 2.11 Transgene expression pattern. In wild-type stage 15 *Drosophila* embryos, Fra is detected on commissural and longitudinal axons and on motor axons projecting from the CNS (**A,C**). Staining of the HA epitope tag reveals TcFra is present in all commissures and longitudinal pathways of the VNC in fra^{Tcfra} homozygous embryos (**B,D**). The CRISPR introduced *Tcfra* transgene shows proper expression and localization (**D**) when compared to the wild-type expression seen by Kolodziej and colleagues (1996) using a Fra antibody (**C**). ac – anterior commissure, pc – posterior commissure, lo – longitudinal pathway, mn – motor neuron.

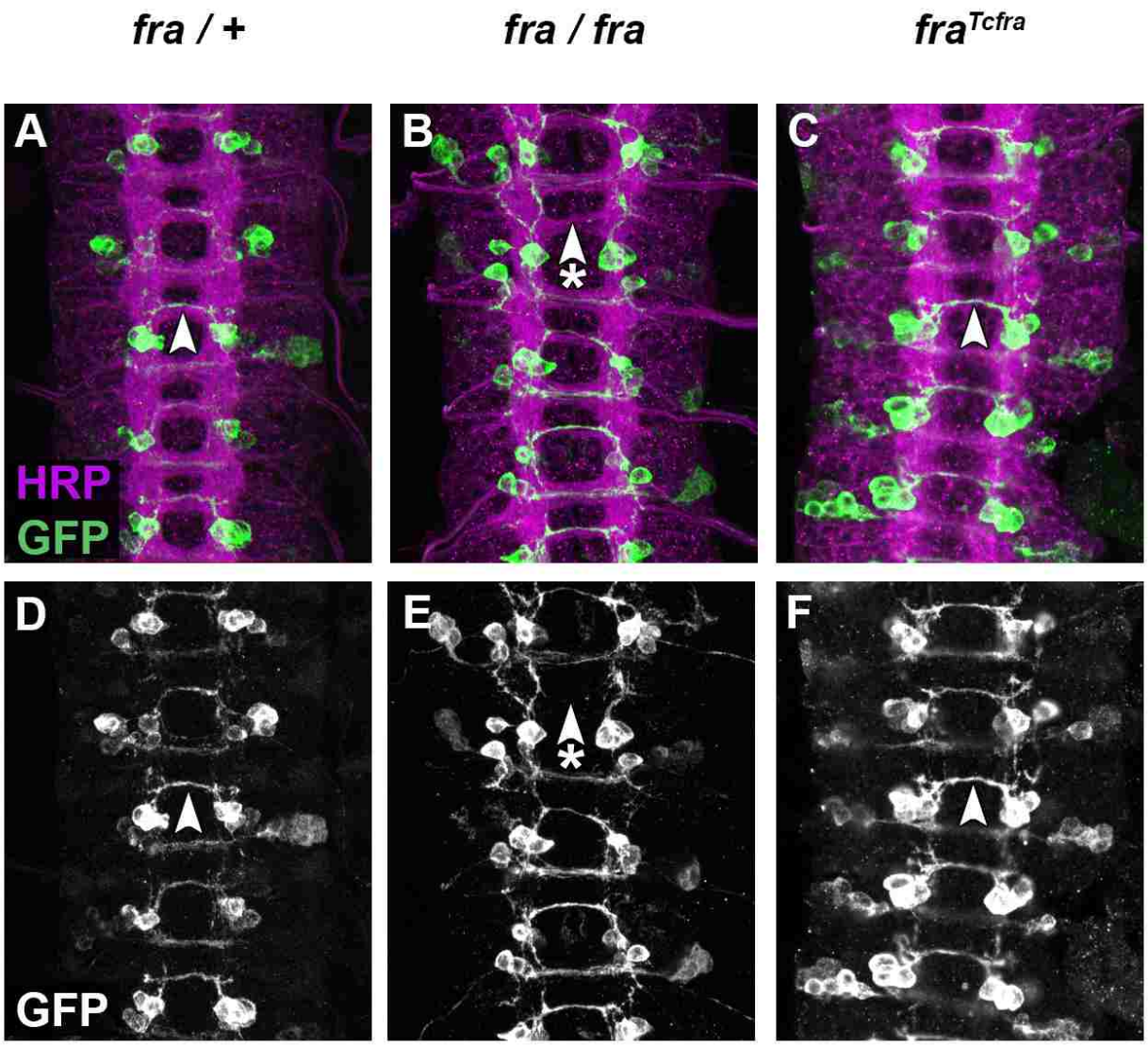


Figure 2.12 Midline crossing defects. Antibody staining of HRP (magenta) labels all axons and GFP (green) expressed by *eg*GAl4 transgene labeling a subset of commissural axons (**A-C**). In *Drosophila, fra* is required for proper midline crossing of the EW axons (arrow **A,D**) as they fail to cross the midline in *fra* mutants (asterisk **B,E**). EW axons in *fra*^{Tcfra} embryos (arrow **C,F**) are present and appear appropriately sized when GFP staining is compared to wild-type embryos (**A,D**).

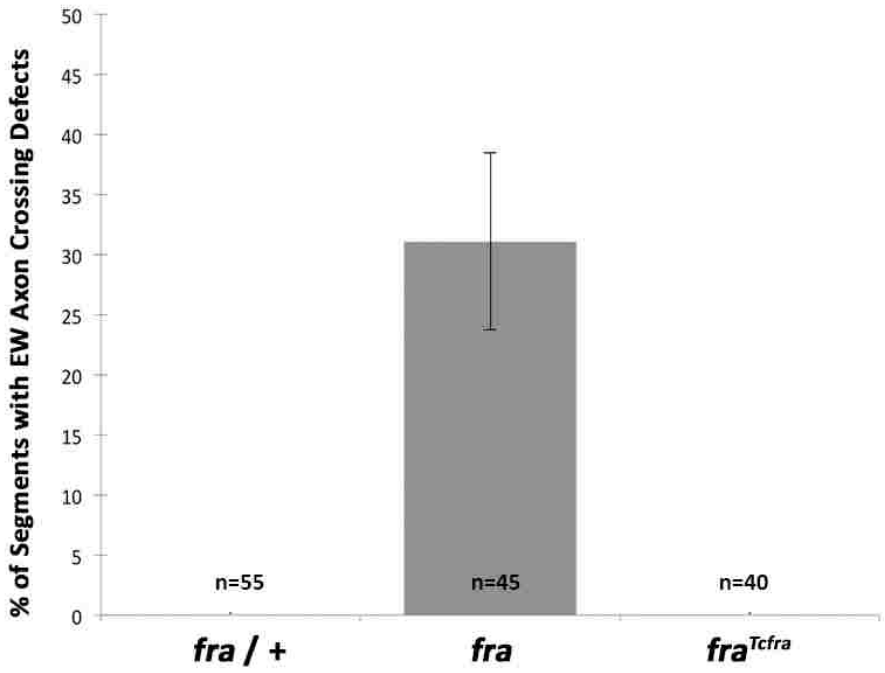


Figure 2.13 Midline crossing defects. Quantification of midline crossing defects of EW axons. Error bar represents standard error, n = total number of segments.

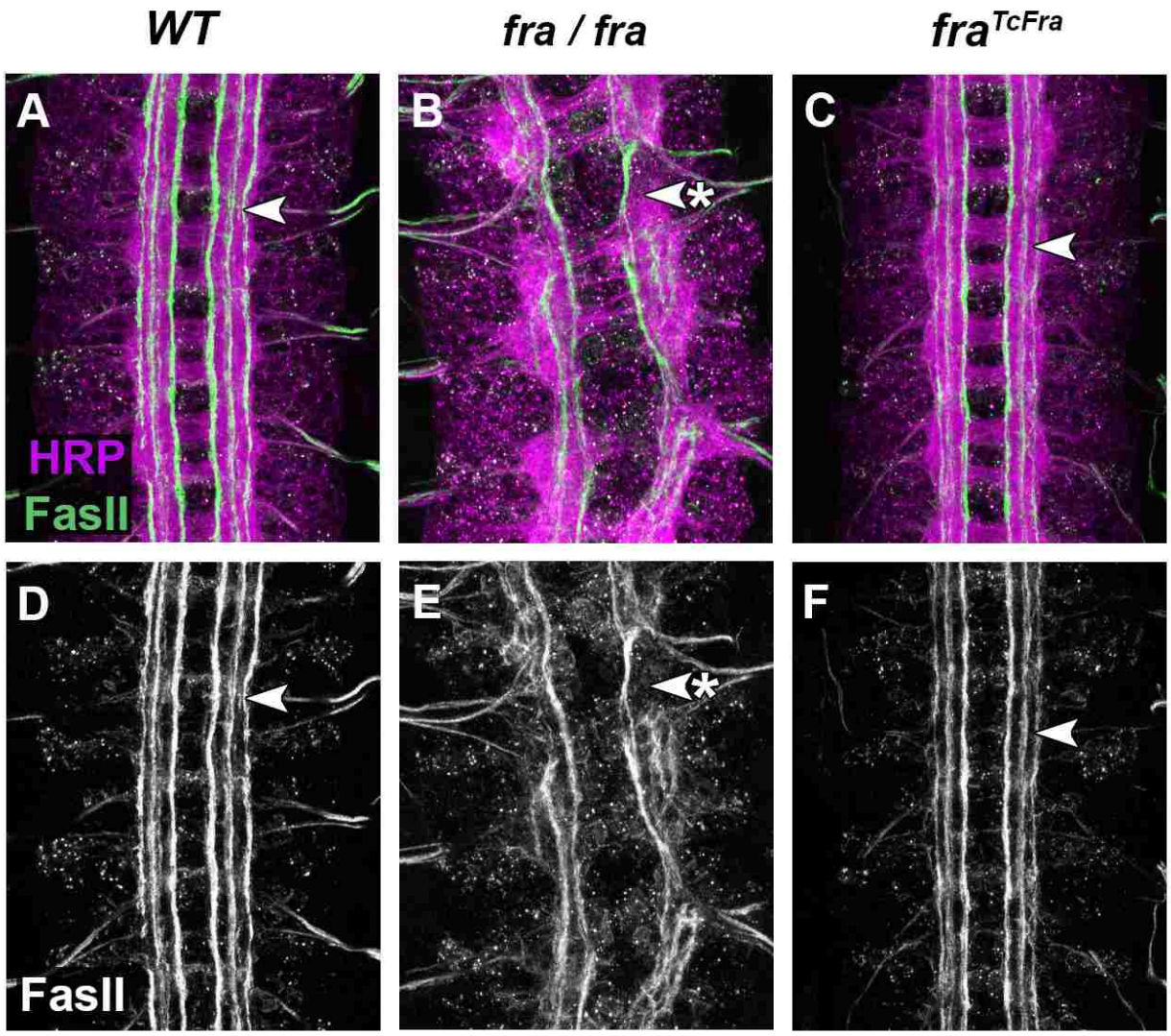


Figure 2.14 Longitudinal pathway formation. Antibody staining of HRP (magenta) and Fasciclin II (green) to label a subset of longitudinal axon pathways (**A-C**). In wild-type *Drosophila* embryos (**A,D**), the FasII positive axons form three distinct anterior-posterior axon tracts (arrow). Longitudinal pathways fail to form in *fra* mutant embryos (asterisk **B,E**). In fra^{Tcfra} expressing flies the FasII longitudinal pathways appear complete and properly formed, similar to wild type (arrow **C,F**).

Discussion

In this chapter I used the CRISPR/Cas9 gene replacement technique to directly examine the function and evolutionary conservation of a crucial gene in axon guidance. I replaced the *Drosophila melanogaster frazzled* gene with its ortholog from *Tribolium castaneum* (*Tcfra*). Proper expression of the tagged TcFra protein reveals that the sequences required for the production and trafficking of Fra in *Drosophila* are present in the *Triboulium fra* sequence. Thus, we can conclude that the necessary localization signals are conserved between flies and beetles. The commissures of *Tcfra* homozygous embryos appear full when compared to wild type. Appropriate crossing of the Fra dependent EW axons is also rescued in transgenic embryos. These results demonstrate that the netrin dependent role of Fra is conserved in these insects and the signaling cascade remains operational. The result also indicates the conserved function of the ICD to translocate to the nucleus and activate transcription of *comm*. The longitudinal pathways also appear to form correctly in the transgenic embryos. This indicates that the third documented role of Fra, to move netrin into the longitudinal axon tracts for the later growing ipsilateral axons to follow. I have demonstrated in this chapter that two of the three functions of *frazzled* that have been described in the *Drosophila* CNS are conserved in the *Tribolium* homolog *Tcfra*.

Acknowledgements

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Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH, and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242.

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CHAPTER 3: Characterization of Frazzled in Tribolium

Summary

Our understanding of the molecular and genetic regulation of axon guidance in the insect CNS has come almost exclusively from studies in *Drosophila*. Developing *Tribolium castaneum* as a model for development and central nervous system patterning will extend our knowledge of the conserved features required for building an animal nervous system. The Frazzled gene in beetles shares a significant portion of sequence similarity with that of flies. However, the function of this gene in *Tribolium* remains unexplored. In this chapter I investigate *fra* function in *Tribolium* midline crossing using CRISPR to create loss of function mutations by Cas9 mediated double strand breaks. Investigating the role of the Netrin-Frazzled pathway in a non-dipteran insect will give insight to the evolutionary connection between axon guidance by Frazzled in insects, DCC in vertebrates and UNC-40 in *C. elegans*.

Introduction

The ladder-like VNC of insects develops in pairs of commissures that repeat in each segment. These connect symmetric longitudinal tracts running along the anterior-posterior axis on each side of the midline. This architecture is conserved in insects and the choice of midline crossing or longitudinal pathway is essential to establishing this precisely organized neural framework. The embryonic midline is an important source of cues to growing axons whether or not to cross the midline (Evans 2016, Howard et al. 2019). Relatively little is known about these axon guidance cues outside of conventional model organisms. Evidence has shown divergence in some axon guidance pathways for example, *commissureless* and *robo2* orthologs are limited to dipterans (flies and mosquitos). Although the *frazzled* pathway is conserved across insects, its functions have not been examined in other insects and little is known about how well these

functions are conserved as other axon guidance molecules evolve. The study of conservation and divergence of these developmental genetic mechanisms will offer understanding into how divergent axon guidance activities contribute to the evolution of nervous systems.

Axon guidance cues are an essential factor in the development of the nervous system. These cues regulate axon guidance decisions during development of the central nervous system. The two principal opposing signaling pathways of are the repulsive Slit-Robo pathway, and the attractive Netrin-Frazzled pathway (Howard et al. 2019). In *Drosophila,* a mechanism of cross-regulation and interplay between the two opposing pathways has emerged. The diversification of ligand-receptor families has also become apparent (Evans 2016). Beetles exhibit a simplified map of these axon guidance cues when compared to what is known from flies. Gene duplications and the addition of *commisureless*, provide additional complexity to Dipteran axon guidance. This suggests that *Tribolium* may more closely represent the majority of insect species.

Midline repulsion is mainly conserved in *Tribolium*, however Robo control of longitudinal pathway formation is different from that in *Drosophila*. Robo2/3 orthologs are not found outside insects, suggesting that duplication and diversification of the Robo family has contributed to the evolution of nervous system development in insects and other animals (Evans 2016). In *Drosophila,* Robo2 and Robo3 divide the control of distinct aspects of longitudinal pathway formation that in *Tribolium,* are both controlled by TcRobo2/3 (Evans and Bashaw 2012). Specifically, TcRobo2/3 determines the intermediate and lateral positioning of axons in longitudinal pathways. The evolution of this system becomes more intriguing given that *Drosophila* Robo3 is unable to substitute for Robo2, while Robo2 could rescue a loss of Robo3. However, Robo2 is not expressed in the appropriate neurons to do so naturally (Evans 2016). The evidence of diversity from the Robo family shows the capacity for functional change in the

development of the nervous system. This brings into question changes in attractive signaling mechanisms.

As stated, *Tribolium* along with all other non-dipteran insects lack an ortholog of *comm*, which regulates Robo localization to the growth-cone. Specifically, no orthologs are detected in the *Tribolium* genome sequence of the three *comm* genes from *Drosophila* (Evans and Bashaw 2012 and Tribolium 2008). This temporal control of Robos repulsive midline signaling allows for precise control of midline crossing in *Drosophila.* A loss of Comm in flies results in, as indicated by its name, a completely commisureless phenotype (Tear et al. 1996). The pathway responsible for the activation of *comm,* also appears to be the attractive Net-Fra pathway, which lends to appropriate attraction/loss of repulsion cross talk at the midline of Drosophila. Within Diptera, genes orthologous to *Drosophila comm2* have been identified in mosquitoes. Subsequent RNAi knockdown of *fra* or *comm2* both produced a commissureless phenotype in *A. aegypti*. Additionally, *fra* knockdown resulted in a loss of *comm2* expression, suggesting that Fra's role as a transcription factor may be conserved within dipterans (Clemons et al. 2011 and Sarro et al. 2013). Still the absence in other insects of such a necessary developmental control gene is interesting.

Netrin, the attractive ligand, is expressed in a number of insect and non-insect arthropods at the embryonic midline. Studies of Netrin have included *Drosophila* (Mitchell et al. 1996 and Harris et al. 1996), *Tribolium*, mosquito, amphipod crustaceans, isopod crustaceans (Simanton et al. 2009), and arachnids. Most of these species are lacking functional studies, however knockdown of netrin using RNAi in spiders has shown midline crossing defects (Linne and Stollewerk 2011). This evidence supports a conserved function for Netrins in midline attraction throughout the arthropod clade. In spite of the conservation, *Tribolium* only code for one Netrin

(TcNet), as opposed to the two in *Drosophila*. This suggests that the network of molecular mechanisms controlling attractive signaling in flies, that is currently used as a template for many studies, may be more complex than what is occurring in other insects including beetles (Evans and Bashaw 2012).

Frazzled orthologs are likewise present across insects and arthropods. Outside *Drosophila* few studies have examined the expression and function of these orthologs. The conservation of the multiple roles of Fra is not clear. Fra's dual roles in promoting Netrin-dependent attraction and Netrin-independent activation of comm transcription might be conserved across arthropod species. However, it would be more likely that the canonical Netrin-dependent role of Fra will be heavily conserved than the role as a *comm* transcription factor, as discussed previous *comm* is not conserved within insects. The severity of the *fra* loss of function phenotype is indicative of the overlapping and redundant attractive guidance pathways in *Drosophila*, discussed by Howard et al. (2019). Comparing the severity of the loss of function phenotype in another model system would be indicative of the evolution of the complexity of these models.

The embryonic development of *Tribolium* is more representative of development in other insects than *Drosophila* (Tautz, Friedrich, and Schröder 1994). For example, *Tribolium* develop via short-germ embryogenesis in which segments are added sequentially by a posterior growth zone. This method of adding segments resembles what is seen in basal arthropods and vertebrates but differs from the Drosophila model. Additionally, unlike Drosophila, beetle larvae have fully formed heads with eyes and three thoracic segments with pairs of legs (Tautz 2004 and Tribolium 2008). Developing *Tribolium* as a model for insect development and central

nervous system development will extend our understanding of the conserved features required for building an animal nervous system.

In this chapter methods and reagents were developed using CRISPR/Cas9 for the creation of mutations by non-homologous end joining in *Tribolium*. This can introduce small insertions or deletions at the site of repair, within the *Tcfra* gene. Cas9-mediated genome editing triggers DNA repair through either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (Ran et al. 2013). After Cas9 cuts the target sequence the NHEJ repair process can often exclude nucleotides creating mutations at the cut site. Mutations resulting from NHEJ can be useful by producing non-functional alleles for knockout analysis. CRISPR/Cas9 has proven to be an effective and efficient method for mutagenesis in multiple insects including *Tribolium* (Gilles et al. 2015).

Methods

Stocks

Beetle stocks are raised at 25^{o}C in a mixture of 500 grams of flour and 25 grams of active dry yeast. Stocks are flipped by moving 5ml of adult beetles to fresh flour/yeast mixture every month. Wild type beetles were used for injection and surviving adult beetles were numbered according to round of injection. Lines carrying potential mutations in *Tcfra* were named based on the injected founder and F1.

Creation of CRISPR Plasmids

Guide RNA plasmids were constructed using the pU6b plasmid (Addgene plasmid # 64247; http://n2t.net/addgene:64247 ; RRID:Addgene_64247). Primers were designed with 20-30 base pair regions complementary to a target sequence adjacent to a selected PAM site (Fig. 3.1). Primers 551 and 552 were used to create gRNA E, 553 and 554 for gRNA F. Additional gRNAs (G and H) were constructed using primers 780-783 (Appendix Table 1). Forward and Reverse primers were used for PCR and products ligated into the Bsa1 site of the pU6b plasmid. The resulting plasmid was cloned into chemically receptive *E. coli* cells.

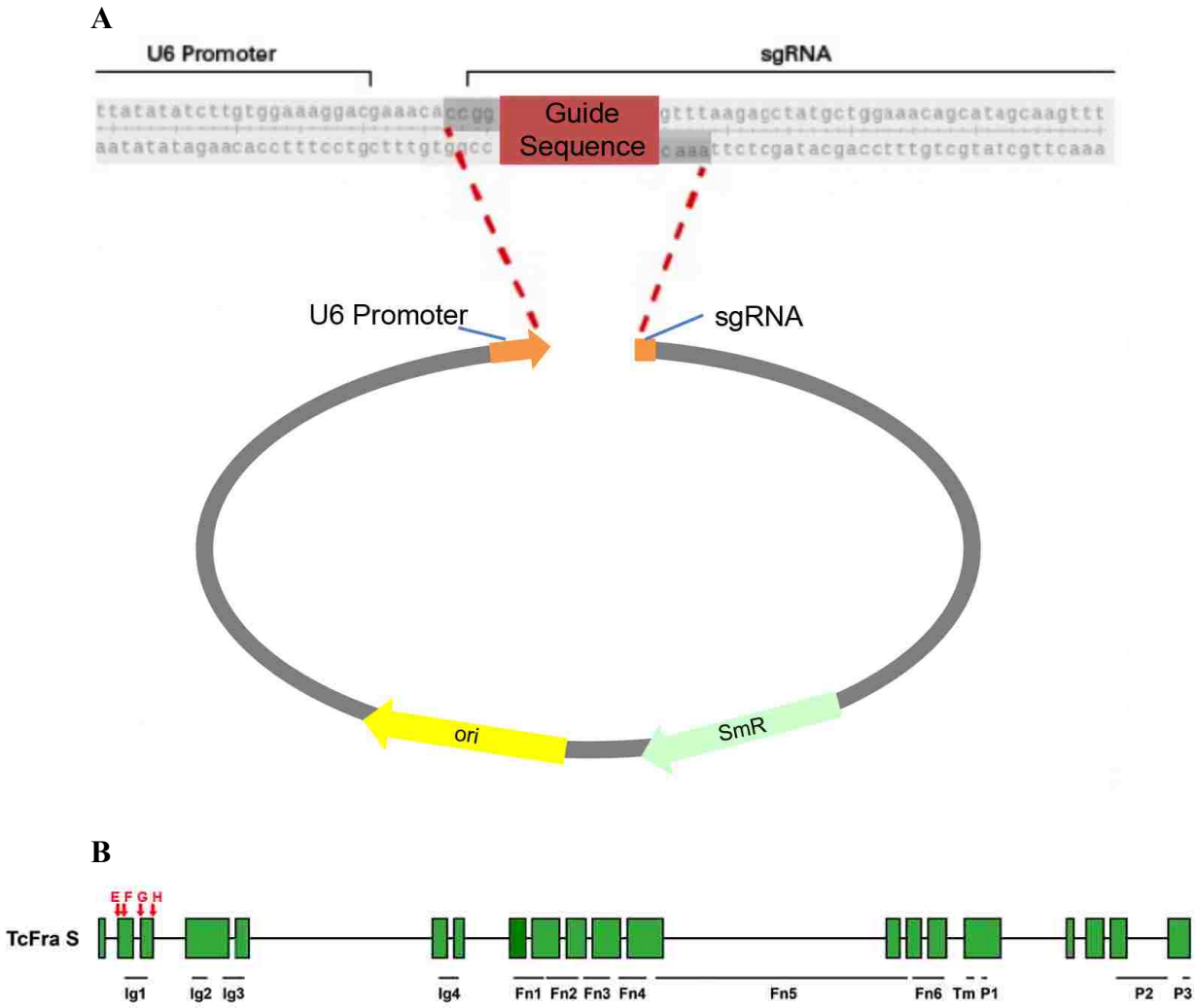

Figure 3.1 pU6b gRNA design. (A) Guide RNA vector plasmid design (Addgene plasmid # 64247; http://n2t.net/addgene:64247 ; RRID:Addgene_64247). Primers overlapping Bsa1 restriction enzyme site were ordered for each desired gRNA. **(B)** Schematic of intended gRNA target sites located within the coding region for the first immunoglobulin domain of *Tcfra*.

Injection of Embryos

A mixture of Cas9 encoding DNA and two gRNA encoding plasmids was used for injection. For each injection mixture, the Cas9 encoding DNA was held at 500ng/µl. The remainder of the injection mix was comprised of gRNA plasmids at equal concentrations (Table 3.1) to a total volume of 20µl. 2µl of food dye, diluted 1:100 in distilled water, was added to the final concentration of injection mixture. Injection mixes were stored at -20ºC between uses. Needles used for injection were pulled on a P-1000 Flaming/Brown Micropipette Puller (Sutter Instruments. Novato, CA). The capillary tubes used were 100mm length, 1.0mm outside diameter, 0.56mm inside diameter borosilicate glass capillaries with filament (#1B100F-4 World Precision Instruments Inc. Sarasota, FL). The program used for creating the needles was as follows: Heat 501 (Ramp -5), Pull 120, Vel. 60, Time 175, and Pressure 200. Needles were then opened with micro-dissection scissors. Open needles were filled with 2µl of injection mix, attached to a syringe, and mounted in a micromanipulator (Fig. 3.2).

One to three-hour old beetle embryos were collected for injection. This was done by adding 5ml of wild type beetles in a 5% mixture of active dry yeast in 100g white flour. The adults were left to lay eggs at 25ºC for two hours. Adults were removed by sifting with an 850µm sieve. The eggs remaining in the flour were returned to 25ºC to mature one hour. Embryos were then collected by sifting through a 300µm sieve. Embryos were washed with water and 70- 100 embryos were aligned on a microscope slide with the posterior pole to the right (Fig. 3.2). Injection of embryos was accomplished on a Nikon TS100 microscope. Embryos were injected by moving the stage to insert the needle in the posterior end of the embryo and applying pressure to the syringe until blue food dye was visible in the embryo.

Injected embryos were incubated at 32ºC in a humid chamber for 3 days. G0 hatched larvae were moved to 5% yeast and flour mixture and incubated at 25ºC. 21 days post injection pupae were sexed and placed in individual vials of flour/yeast mix to finish developing and labeled according to date of injection.

PCR analysis of Fra CRISPR mutants

Upon reaching adulthood, Injected G0 beetles were crossed 1:3 to wild type virgins. After one week, adults are removed. After two more weeks, F1 progeny were collected according to sex as pupa and crossed again to wild type. After crossing F1 adults will be screened by PCR with primer set 354 - 355 (Appendix Table 1) to identify potential deletions. Progeny of PCR positive F1s are then collected to establish stock (Fig 3.3).

Table 3.1 *Tribolium* embryo injection

Injection Mix	Cas9 conc. *(ng/µl)*	gRNA 1 conc. *(ng/µl)*	gRNA 2 conc. *(ng/µl)*
E – F	500	450	450
G – H	500	400	440

Mixes were calculated for final concentration based on original concentration of plasmid Midi prep (Qiagen, Hilden, DE).

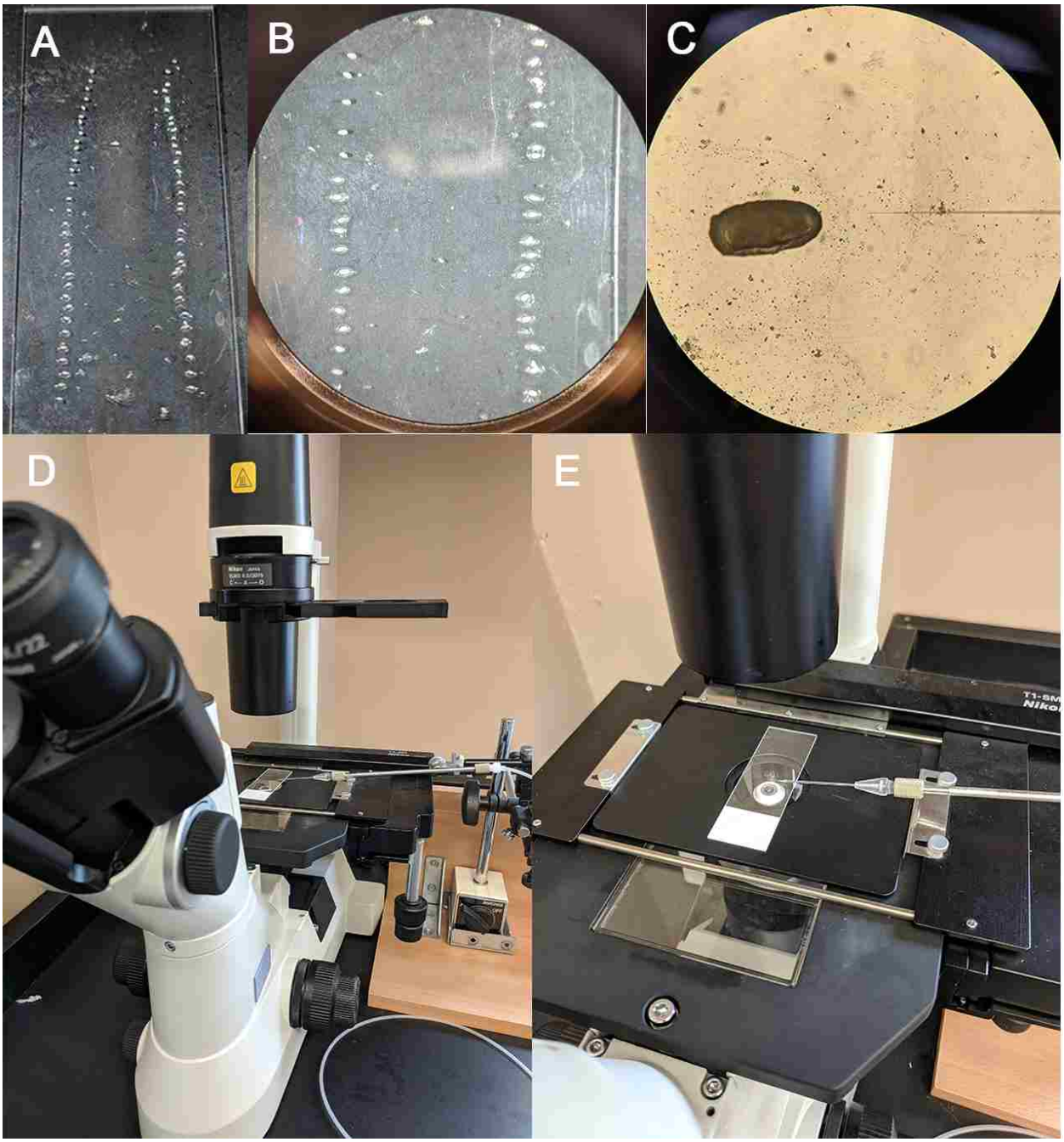

Figure 3.2 Beetle embryo injection setup. Rows of *Tribolium* embryos aligned on microscope slide with posterior ends pointed outward (**A**). Slide from A, viewed at 10x magnification (**B**). Embryo (left) and injection needle (right) before injection, viewed at 100x magnification (used for injection) (**C**). Needle mounted in micromanipulator aligned with slide (**D**, **E**).

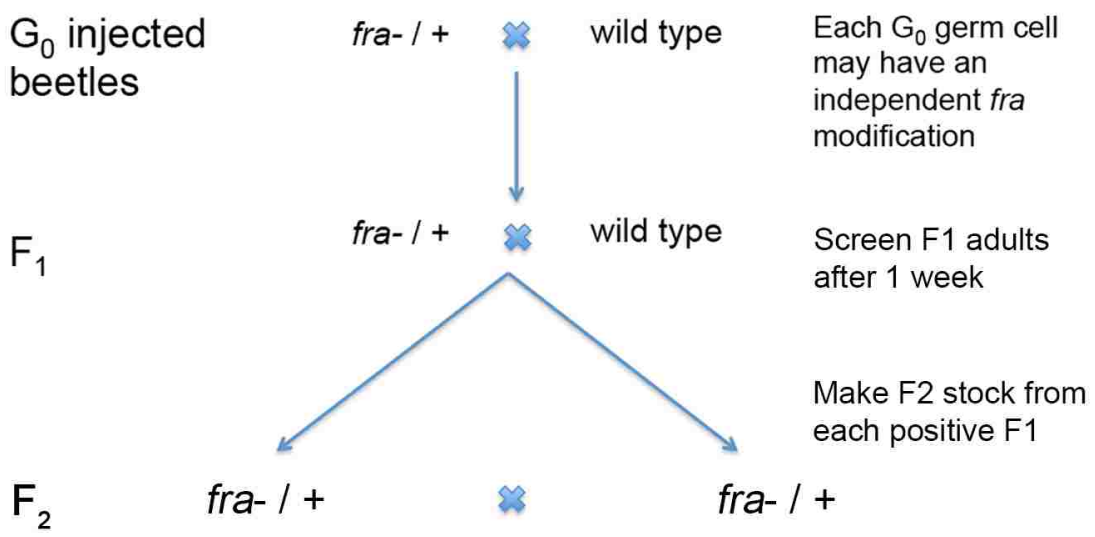

Figure 3.3 *Tribolium* crossing scheme for mutant allele recovery. G0 injected beetles are crossed to 3 virgin wild type beetles. F1 progeny are crossed again to wild type. Adult F1s are screened by PCR post crossing. Progeny of PCR positive adults are collected to create stock of *fra* deficient allele.

Results

In this chapter CRISPR/Cas9 was used to potentially create mutations by non-homologous end joining. Four gRNAs were constructed and confirmed with sequencing (Fig. 3.4). These were subsequently injected into *Tribolium* embryos in pairs. Injected embryos were raised, crossed to wild type, and offspring were collected for screening to identify germ line mutations in *frazzled*. A total of 804 embryos were injected with a combination of gRNAs and Cas9 mRNA, 474 embryos with mix E-F, 330 with mix G-H (Table 3.2). Of the injected embryos, 12 survived to adulthood. After crossing to wild type 9 potential founders produced offspring. Totaling an injection survival rate of 1.4% of injected embryos to adulthood and 75% fertility rate for those adults.

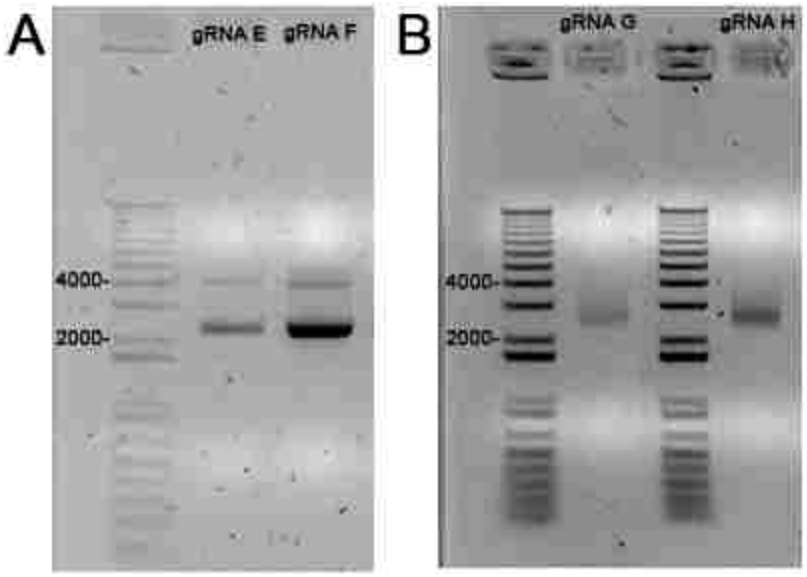

Figure 3.4 Guide RNA PCR confirmation. 2µl of mini-prep DNA of gRNAs E, F, G, and H in pU6b vector, 4.1Kb (**A**, **B**). Plasmid size compared to 1Kb Plus DNA Ladder from Invitrogen (Carlsbad, CA).

Table 3.2 *Tribolium* embryo injections

Injection Mix	*# of Injected Embryos*	*% (no.) adult G0 survival*	*% (no.) fertile G0*	*% (no.) G0 founders*
Tcfra gRNA E-F	111	(2)	0	-
Tcfra gRNA E-F	141	0	-	-
Tcfra gRNA E-F	222	0	-	-
Tcfra gRNA G-H	101	1(1)	100(1)	
Tcfra gRNA G-H	163	2.5(4)	75(3)	
Tcfra gRNA G-H	66	7.5(5)	100(5)	

gRNA vector and Cas9 vector were injected into posterior pole of 1-3-hour old embryos.

Discussion

In this chapter methods and reagents were developed to adapt the CRISPR/Cas9 system to create mutations by non-homologous end joining in *Tribolium*. This can introduce small insertions or deletions at the site of repair, within the *Tribolium frazzled* gene. The low injection survival rate to adulthood severely lowered the efficiency of the producing mutations. With additional trials and improved technique this pitfall should be remedied in future studies.

Further work still needs to be done to identify mutations generated in this study. Mutant phenotypes generated in this study can then be used to compare the role of *fra* in generating the CNS. Investigating the evolution of genes controlling development of the nervous system will give insight into not only the evolution of insects, but also assist in the development of *Tribolium* as a model system.

The CRISPR/Cas9 system can be further used to examine *Fra* expression in *Tribolium* in the embryonic CNS. Future studies aimed at determining *Tcfra* expression in the beetle CNS midline can use CRISPR to knock in an epitope tag to the *Tcfra* locus by homologous recombination. In addition to studying the expression and loss of endogenous *fra* a more direct comparative approach, similar to Chapter 2, can be taken using CRISPR in *Tribolium*.

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Chapter 4:

Conclusion

The Netrin-Frazzled pathway has been shown to aid in midline crossing of axons in the embryonic ventral nerve cord of *Drosophila*. However, It is unclear if this function of Frazzled is conserved across insects. The goals of this research were to gain insight into the evolutionary conservation of axon guidance by the Netrin receptor Frazzled and to expand our understanding of how Frazzled influences midline crossing in the flour beetle *Tribolium castaneum*.

The *frazzled* ortholog from *Tribolium* is shown in chapter 2 to rescue loss of function in *Drosophila* Fra. Appropriate crossing of the Fra dependent EW axons is rescued in transgenic embryos. This rescue demonstrates that the netrin dependent role of Fra is conserved in these insects. The longitudinal pathways also appear to form correctly in the transgenic embryos. This indicates that the role of Fra to move netrin into the longitudinal axon tracts for the later growing ipsilateral axons to follow. However, these results fall short of confirming a conservation of the function of the ICD to translocate to the nucleus and activate transcription of *comm*. Using the transgenic line generated in this study additional work needs to be done to determine if the transcription factor role of Frazzled is also conserved. An *in-situ* hybridization experiment looking at the expression of *comm* under the transcriptional control of endogenous *fra*, a *fra* knockout, and *Tcfra* would clarify the conservation of this role. These results demonstrate that two of the three functions of *frazzled* that have been described in the *Drosophila* CNS are conserved in the *Tribolium* homolog *Tcfra*.

Methods and reagents were developed to adapt the CRISPR/Cas9 system for studying midline crossing in *Tribolium* (Chapter 3). The low injection survival rate to adulthood severely lowered the efficiency of producing mutations. With additional trials and improved technique

this pitfall should be remedied in future studies. We also expect to see similar expression and function of Frazzled in beetles to those observed in *Drosophila*. These studies expand our knowledge of axon guidance of midline crossing in a species that does not share some of *Drosophila's* derived guidance characters. Using *Tribolium* as an insect model for comparative studies of axon guidance may allow us to see a more ancestral guidance scheme. Further work still needs to be done to identify mutations generated in this study. Mutant phenotypes generated in this study can then be used to compare the role of *fra* in generating the insect CNS.

The CRISPR/Cas9 system can be further used to examine *Fra* expression in *Tribolium* in the embryonic CNS. Future studies aimed at determining *Tcfra* expression in the beetle midline can use CRISPR to knock in an epitope tag to the *Tcfra* locus by homologous recombination. In addition to studying the expression and loss of endogenous *fra* a more direct comparative approach, similar to Chapter 2, can be taken using CRISPR in *Tribolium*. These studies can then be extended to additional genes for a more complete comparative picture of axon guidance.

[redacted]

Primer #	Name	Sequence	Target	Application
607	DmFra upstream homology fwd	CCTGAATGATATGCGGCCTCTCAACGTCGAAACTCGATGTCTAATTAAGG	DmFra upstream genomic with 20nt matching pIDT	PCR and vector construction
608	DmFra upstream homology rev	TTATTAAGGCGGCTTCAGTGGCTAGCCGAAAGAGCTGTACAAA	DmFra exon2 junction with NheI and 20nt matching DmFra downstream	PCR and vector construction
609	DmFra downstream homology fwd	TTTTTTGTACAGCTCTTTCGGCTAGCCACTGAAGCCGCCTTAATAATGTCCGGCTG	DmFra downstream homology region with NheI and 20nt matching DmFra upstream	PCR and vector construction
610	DmFra downstream homology rev	AATGCCGTAAGATCACGCGCTTTCACAATTCGTAAATGCCTTTAGTGTGC	DmFra downstream homology region with 20nt matching pIDT	PCR and vector construction
745	TcFraF Gibson	TGTGCCCGATTACGCCGGAGCTCATGTGCTGGAGTTTACGGTGGAGCCGACG	Dm Vector HA , Tcfra upstream ORF	Vector construction
746	TcFraR Gibson	TATTAAGGCGGCTTCAGTGGTCAACATTCGAACTGATTCGCTGTGATTG	Dm Vector HA , Tcfra downstream ORF	Vector construction
617	DmFra upstream gRNA F	TATATAGGAAAGATATCCGGGTGAACTTCGACGGCATCCTGGGGCTCCAGTTTTAGAGCTAGAAATAGCAAG	pCFD4, upstream gRNA target (G added)(protospacer 19nt+G)	gRNA construction
618	DmFra downstream gRNA R	ATTTTAACTTGCTATTTCTAGCTCTAAAACTGATTGCGCTTAGATCCTTCGACGTTAAATTGAAAATAGGTC	pCFD4, downstream gRNA target (rev comp)(protospacer 20nt, includes G)	gRNA construction
292	TcFraexon4T7F	TAATACGACTCACTATAGGGGTATCTCGGGCTTTCTGGAGGAGC	TcFra exon4 with T7 promoter	Sequencing
293	TcFraexon4T7R	TAATACGACTCACTATAGGGGTCATGTCGATGGTCGACCCATC	TcFra exon4 with T7 promoter	Sequencing
295	TcFraexon8T7R	TAATACGACTCACTATAGGGCTTTCGCTGCCCTCCTGCCTGTAG	TcFra exon8 with T7 promoter	Sequencing
358	TcFra1170	GATGCCGGGACTTATCAATGC	TcFra ORF	Sequencing
360	TcFra2170	AGTGCTCCAACACACTTTGTG	TcFra ORF	Sequencing
361	TcFra2670	ACTAGCCCACCAACCGAATGG	TcFra ORF	Sequencing
362	TcFra3170	TACACCGCCGAAAGAACGATG	TcFra ORF	Sequencing
364	TcFra4170	CAATCGAGCACAGACTCGACG	TcFra ORF	Sequencing
787	DmFra seq CRISPR site	ATCATCCAAAGAAATTTGAATTCCCAAGTG	Dmfra sequencing CRISPR site upstream	Sequencing
619	4xHA fwd ultramer 5'XbaI-3'NheI overhang	CTAGATCTTACCCCTACGATGTGCCCGATTACGCCGGATCTTACCCCTACGATGTGCCCGATTACGCCGGATCTTACCCCTACGATGTGCCCGATTACGCCGGATCTTACCCCTACGATGTGCCCGATTACGCCGGAG	Epitope tag vector construction
620	4xHA rev ultramer 5'NheI-3'XbaI overhang	CTAGCTCCGGCGTAATCGGGCACATCGTAGGGGTAAGATCCGGCGTAATCGGGCACATCGTAGGGGTAAGATCCGGCGTAATCGGGCACATCGTAGGGGTAAGATCCGGCGTAATCGGGCACATCGTAGGGGTAAGAT	Epitope tag vector construction
767	DmFra CRISPR upstream	TAAGTGCACAGTGTCCTGCCTGAGTCCTCG	DmFra CRISPR PCR screening upstream fwd, outside homology region	PCR screening and Sequencing
768	DmFra CRISPR downstream	TACAAAGTTTGGAGGGTACAGGTGGCAGTG	DmFra CRISPR PCR screening downstream rev, outside homology region	PCR screening and Sequencing
290	TcFraF	CATGTGCTGGAGTTTACGGTGGAG	TcFra lg1	PCR screening and Sequencing
295	TcFraexon8T7R	TAATACGACTCACTATAGGGCTTTCGCTGCCCTCCTGCCTGTAG	TcFra exon8 with T7 promoter	PCR Screening
291	TcFraR	TCAACATTCGAACTGATTCGCTGTG	TcFra C-term	PCR screening and Sequencing
636	DmFra upstream homology seq F	TCATTGGCAGGTCCTGCCACCCTTACATCC	DmFra CRISPR donor upstream homology region	PCR screening and Sequencing
293	TcFraexon4T7R	TAATACGACTCACTATAGGGGTCATGTCGATGGTCGACCCATC	TcFra exon4 with T7 promoter	PCR screening and Sequencing
362	TcFra3170	TACACCGCCGAAAGAACGATG	TcFra ORF	PCR screening and Sequencing
610	DmFra downstream homology rev	AATGCCGTAAGATCACGCGCTTTCACAATTCGTAAATGCCTTTAGTGTGC	DmFra downstream homology region with 20nt matching pIDT	PCR screening and Sequencing
551	TcFra gRNA E fwd	TTCGATTCCACGACTGTGTCCGT	TcFra gRNA target E with p(U6b) overhang for cloning	Tc gRNA construction
552	TcFra gRNA E rev	AAACACGGACACAGTCGTGGAAT	TcFra gRNA target E with p(U6b) overhang for cloning	Tc gRNA construction
553	TcFra gRNA F fwd	TTCGTTCGTTTCAGGTCATGTGC	TcFra gRNA target F with p(U6b) overhang for cloning	Tc gRNA construction
554	TcFra gRNA F rev	AAACGCACATGACCTGAAACGAA	TcFra gRNA target F with p(U6b) overhang for cloning	Tc gRNA construction
780	Tcfra gRNA G Fwd	TTCGCTACGACTCGAATTACTTCT	TcFra gRNA target G with p(U6b) overhang for cloning	Tc gRNA construction
781	Tcfra gRNA G Rev	AAACAGAAGTAATTCGAGTCGTAG	TcFra gRNA target G with p(U6b) overhang for cloning	Tc gRNA construction
782	Tcfra gRNA H Fwd	TTCGGTGAGTAGAATTCATTTATTC	TcFra gRNA target H with p(U6b) overhang for cloning	Tc gRNA construction
783	Tcfra gRNA H Rev	AAACGAATAAATGAATTCTACTCAC	TcFra gRNA target H with p(U6b) overhang for cloning	Tc gRNA construction
354	TcFra5UTR-F	ACCGATAAGAGCAACTGTTGTCCCACC	TcFra predicted 5'UTR	PCR Screening
355	TcFra3UTR-R	ACTAACTAAAGTCCCCTTTCACGCCAC	TcFra predicted 3'UTR	PCR Screening
Add1	Addgene pCFD4 insertion 1 fwd	CGTTTTTAATAAAATACATTGCATAGATCTGAATTCATTTTCAACGTCCTCGATAGTA	pCFD4, upstream gRNA target	Sequencing
Add2	Addgene pCFD4 insertion 2 fwd	AGGTTAGCTCGCCAAGCAG	pCFD4, downstream gRNA target	Sequencing