NETRIN-INDEPENDENT FRAZZLED FUNCTIONS IN OOGENESIS AND AXON GUIDANCE

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ABSTRACT

Netrin-independent Frazzled functions in oogenesis and axon guidance

Samantha A. Russell

Greg J. Bashaw

Frazzled (Fra) is a highly conserved receptor expressed on the cell membrane, and it is important for several morphological processes, including cell migration, axon guidance, and adhesion. In addition to these functions, the vertebrate homologue of Fra, Dcc, also functions as a tumor suppressor that has a pro-apoptotic effect in the absence of its canonical ligand Netrin. In both invertebrates and vertebrates, the axon guidance cue Netrin interacts with the extracellular domain of Fra/Dcc to recruit cytoplasmic proteins and affect local cytoskeletal changes to promote axon growth across the midline. In addition, Fra functions independently of Netrin as a transcription factor. Fra is cleaved by gamma-secretase, which allows the Fra intracellular domain to enter the nucleus and activate transcription. Fra activates transcription of Commissureless, and endosome cycling receptor that is required to promote axon growth across the midline. However, whether this signaling mode of Fra is required in other tissue contexts outside of the nervous system is unknown. In Chapter 1, I introduce a subset of axon guidance genes and how they regulate gene expression in the nervous system as well as their known roles in reproductive tissues. In Chapter 2, I show that Fra is required in the fly ovary for egg chambers to progress through mid-oogenesis independently of Netrin, and this requires the transcriptional activation domain within the Fra intracellular domain. Interestingly, in contrast to the pro-apoptotic role that Dcc has in vertebrates, Fra has an anti-

apoptotic role in the germline. In Chapter 3, I use a yeast-two hybrid screen to identify proteins that interact with the Fra intracellular domain, and test the DNA-binding proteins identified for a role in axon guidance and in the female germline. Finally, in Chapter 4 I discuss the implications of this work and potential future directions that would be exciting to explore.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1: Introduction

From Russell S.A., and Bashaw G.J., 2018. Axon guidance pathways and the control of gene expression. Developmental Dynamics 247, 571-580.

Axon guidance molecules regulate gene expression

Abstract

Axons need to be properly guided to their targets to form synaptic connections, and this requires interactions between highly conserved extracellular and transmembrane ligands and their cell surface receptors. The majority of studies on axon guidance signaling pathways have focused on the role of these pathways in rearranging the local cytoskeleton and plasma membrane in growth cones and axons. However, a smaller body of work has demonstrated that axon guidance signaling pathways also control gene expression via local translation and transcription. Recent studies on axon guidance ligands and receptors have begun to uncover the requirements for these alternative mechanisms in processes required for neural circuit formation: axon guidance, synaptogenesis and cell migration. Understanding the mechanisms by which axon guidance signaling regulates local translation and transcription will create a more complete picture of neural circuit formation, and may be applied more broadly to other tissues where axon guidance ligands and receptors are required for morphogenesis.

Introduction

The precise establishment of neural circuits during development is essential for coordinated animal behavior. Cell migration, axon guidance, and synaptogenesis are all processes required for proper neural circuit formation, and axon guidance ligands and receptors regulate these processes. At the tip of the axon is the highly motile growth cone, which encounters a variety of diverse cues, mainly attractants and repellants, as it navigates through its environment. Extracellular cues interact with receptors expressed on growth cones to mediate axon outgrowth, growth cone collapse, and turning. The following axon guidance cues and receptors will be the focus of this review: 1) semaphorins and their neuropilin and plexin receptors, 2) slits and their roundabout (Robo) receptors, 3) netrins and their deleted in colorectal cancer (Dcc), Frazzled (Fra, in *Drosophila*), Unc40 (in *C. elegans*), neogenin (Neo) and Unc5 receptors, and 4) ephrins and their eph receptors (Hou et al., 2008). Sonic hedgehog (Shh), wnt, bone morphogenetic protein (Bmp) and other signaling pathways (Yam and Charron, 2013) have also been shown to play roles in axon guidance, and we refer the reader to previous reviews that discuss these pathways (Bovolenta, 2005; Charron and Tessier-Lavigne, 2007; Sanchez-Camacho and Bovolenta, 2009). We will not cover these pathways in this review, as their involvement in gene regulation is already well studied and reviewed.

Most axon guidance receptors impinge on cytoplasmic proteins to regulate rho family small GTPases, which in turn modulate cytoskeletal and membrane dynamics through diverse downstream effectors. Thus, rho family GTPases can integrate signals from multiple cues to direct growth cone dynamics (Luo, 2002; O'Donnell et al., 2009). Recent reports implicate the Scar/WAVE complex in axon guidance, and suggest that Scar/WAVE may interact directly with axon guidance receptors through the conserved WIRS motif to regulate Arp2/3-dependent actin polymerization (Zallen et al., 2002; Lin et al., 2009; Bernadskaya et al., 2012; Chen et al., 2014). For comprehensive reviews on actin and microtubule dynamics in navigating growth cones and axons, we refer the reader to reviews that explore this topic (Krause et al., 2003; Lowery and Van Vactor, 2009; Dent et al., 2011; Vitriol and Zheng, 2012; Gomez and Letourneau, 2014; Spillane and Gallo, 2014; Stankiewicz and LinSeman, 2014).

The majority of studies on axon guidance receptor signaling have been focused on how axon guidance receptors signal locally to regulate the cytoskeleton and growth cone plasma membrane. In contrast, a smaller body of work has demonstrated that axon guidance cues and receptors also act non-canonically to control cell proliferation, cell migration, and axon guidance by regulating gene expression through translational or transcriptional mechanisms. In this review we aim to synthesize the studies that investigate these mechanisms in an attempt to demonstrate that axon guidance ligands and receptors broadly function to regulate gene expression across a range of neuron subtypes, developmental processes, and organisms.

Part 1: Local translation

Local translation is required for axon guidance *in vitro*

Axons continue to grow and respond to guidance cues even after being severed from their cell bodies (Harris et al., 1987), indicating that all of the required signaling components to mediate these responses are present in growth cones. The observation that growth cones also contain messenger RNAs (mRNAs), translation machinery, and molecules involved in protein degradation (Tennyson, 1970; Bassell et al., 1998; Campbell and Holt, 2001), led to the suggestion that protein synthesis and degradation may occur locally in growth cones. Indeed, vertebrate neurons translate proteins in their growth cones and dendrites (Davis et al., 1992; Crino and Eberwine, 1996). *In vitro,* specific axon guidance cues can rapidly induce local protein synthesis in growth cones and axons to affect axon turning and collapse, and preventing protein synthesis blocks these responses (Farrar and Spencer, 2008; Lin and Holt, 2008). Thus, local translation in growth cones and axons is clearly necessary in order for some axon guidance cues to modulate growth cone behavior. For example, the axon guidance cues Sema3A, slit2, and netrin can all induce local protein translation, and this is required to steer axons in both intact neurons and severed axons *in vitro* (Campbell and Holt, 2001; Wu et al., 2005; Leung et al., 2006; Piper et al., 2006; Lin and Holt, 2007).

The Requirement for Local Translation Depends on Cell Type and the Concentration of Guidance Cues

Despite the fact that several independent studies demonstrated a role for local translation in guidance responses *in vitro*, the limited *in vivo* evidence and conflicting results from *in vitro* experiments caused significant skepticism in the field as to the importance of local translation in axon guidance. The majority of experiments were initially done with *Xenopus laevis* retinal ganglion cell (RGC) axons, but later reports tested the requirement for local translation in axon guidance in other organisms and neuronal subtypes.

In one report, which contrasted substantially from earlier work, Letourneau and colleagues (2009) found that Sema3A-mediated growth cone collapse in cultured chick dorsal root ganglion (DRG) neurons could still occur in the presence of protein translation inhibitors, strongly suggesting that growth cone responses to semaphorin do not strictly depend on protein synthesis. To account for the differences seen in the requirement for local translation in axon guidance, the authors speculated that different neuronal populations might respond differently to guidance cues, as a result of both their intrinsic properties as well as the extrinsic cues the neurons encounter (Roche et al., 2009).

More recently, this apparent conflict has been revisited, leading to the discovery that the concentration of a ligand that growth cones encounter can result in significant differences in the requirement for local translation (Manns et al., 2012; Nedelec et al., 2012). In chick DRG neurons and mouse and human

embryonic stem cell-derived spinal motor neurons (ES-MNs), growth cone collapse in response to treatment with low Sema3A concentrations (<100 ng/ml) requires local protein synthesis (Manns et al., 2012; Nedelec et al., 2012). In contrast, when neurons are treated with high Sema3A concentrations (>625 ng/ml), growth cone collapse still occurs even when protein synthesis is blocked (Manns et al., 2012; Nedelec et al., 2012). Human ES-MNs and mouse brachiothoracic motor neurons show the same bimodal concentration-dependent responses to both Sema3A and Sema3F, suggesting that multiple semaphorins induce local translation. Strikingly, one of the neuronal subtypes analyzed, cervical ES-MNs, lacks the local protein synthesis-dependent response to low Sema3A concentrations. This is thought to be due to lack of local protein synthesis machinery in the growth cones of these neurons (Nedelec et al., 2012).

A better understanding of the Sema3A signaling pathway may provide insight into these concentration-dependent responses. Sema3A treatment leads to the activation of glycogen synthase kinase (Gsk)-3beta, which appears to act downstream of Sema3A regardless of the concentration, and Gsk-3beta activation is necessary for Sema3A-mediated growth cone collapse (Manns et al., 2012). At low concentrations, Sema3A also signals through the mammalian target of rapamycin (Mtor), to activate local protein synthesis of rhoA (Wu et al., 2005; Manns et al., 2012). Inhibiting Gsk-3beta activation results in an increase in protein synthesis, as demonstrated by the increased fluorescence of phosphorylated 4EBP1, a marker for translation. This observation suggests that activated Gsk-3beta may antagonize Mtor. Therefore, high concentrations of

Sema3A may lead to a significant increase in Gsk-3beta activity, which can overcome the need for local protein synthesis in Sema3A-mediated growth cone collapse by inhibiting Mtor and thus protein synthesis. It is unclear how these guidance cue concentrations might relate to the *in vivo* concentrations of cues encountered by growth cones, but it is likely that differential concentration-dependent signaling outputs may serve to diversify axonal responses to a limited set of cues.

Local translation of specific proteins are induced by guidance cues

It is clear that diverse guidance cues can induce local translation and that this activity is important to affect downstream signaling and axon responsiveness. We turn now to the consideration of the proteins that are specifically translated in response to different cues, and, how in turn these proteins contribute to distinct axon guidance responses. In recent studies of cue-induced local translation, a number of distinct mechanisms that control how specific mRNAs are translated locally have begun to emerge (Table 1).

Sema3A Induces the Local Translation of *RhoA* and *NF-protocadherin*

Sema3A has been reported to induce the local translation of two specific proteins, rhoA and NF-protocadherin (Nfpc). In DRGs, Sema3A-mediated growth cone collapse depends on the rhoA effector Rock (rho-associated protein kinase), which acts downstream of axon guidance receptors to regulate cytoskeletal dynamics (Dontchev and Letourneau, 2002). Unsurprisingly then,

rhoA activation is required for Sema3A-mediated growth cone collapse (Wu et al., 2005). Interestingly, *RhoA* transcripts are found in axons at higher levels than other transcripts and are localized in puncta throughout the axon (Wu et al., 2005). Sema3A treatment increases the fluorescence intensity of rhoA protein, while growth associated protein 43 (Gap43), which is expressed at high levels in neurons during development, is not affected, suggesting that Sema3A specifically induces local translation of *RhoA*. In addition, a translation reporter for rhoA reveals that *RhoA* mRNA is translated in growth cones following Sema3A treatment, and translation inhibitors block this effect (Wu et al., 2005). These experiments indicate that Sema3A induces local translation of rhoA in DRG axons and growth cones.

In *X. laevis* RGCs, Sema3A also induces the local translation of the cell adhesion molecule Nfpc *in vitro* (Leung et al., 2013). Nfpc is necessary in RGC axons to maintain the correct levels of adhesion with the optic tract and helps guide RGC axons to their targets. *In vivo* imaging demonstrates the Sema3A-dependent local translation of an *Nfpc* translational reporter in the growth cone, and the observation that a function-blocking antibody for the neuropilin 1 (Nrp1) receptor prevents this effect, reveals a partial requirement for Nrp1 in this process (Leung et al., 2013). In summary, Sema3A induces the local translation of specific mRNAs, *RhoA* and *Nfpc, in vitro* and *in vivo* imaging data strongly supports the conclusion that this regulated translation is likely to contribute to axon guidance.

Table 1: The targets and mechanisms for cue-dependent local translation

Axon Guidance Cue	Translation Target	Candidate Receptor	Mechanism
Semaphorin3	RhoA NFPC	N/a Neuropilin1	N/a
Slit2	Cofilin-1	Robo2/3	Indirect- Slit2 signaling antagonizes the microRNA miR-182, resulting in the release of *cofilin-1* mRNA
Netrin	Beta-actin DSCAM Sensorin N/a	N/a N/a DCC DCC	N/a N/a N/a Direct- interaction between DCC and translational machinery

Table 1. 1 The targets and mechanisms for cue-dependent local translation

Sema3, Slit2, and Netrin all induce local translation of specific mRNAs. Thus far, two mechanisms have begun to be elucidated: Slit2 indirectly induces local translation of cofilin-1 by antagonizing miR-182, and Netrin causes Dcc to directly release translation machinery, allowing local translation to occur.

SLIT2 Induces the Local Translation of *Cofilin1*

In X. laevis RGC axons, there is considerable evidence that slit2 can induce the translation of *Cofilin1*, which destabilizes F-actin and may act downstream of slit2-Robo signaling to cause axon retraction and collapse (Figure 1.1A). *Cofilin1* mRNA interacts with Vg1RBP, an RNA-binding protein implicated in the localization of specific mRNAs to growth cones. Inhibitors of protein synthesis block slit2-induced *Cofilin1* translation, and prevent growth cone collapse (Piper et al., 2006). In addition, a cofilin1 translation reporter, where the 3' UTR of *Cofilin1* mRNA is fused to a photo-convertible kaede protein (Leung and Holt, 2008) is translated in response to slit2 (Bellon et al., 2017). Thus, slit2 treatment induces local translation of *Cofilin1* in RGC growth cones *in vitro*. One method for controlling the specificity of mRNAs translated in response to axon guidance cues could be a relationship between miRNAs with specific targets and axon guidance pathways. miR-182 is the most highly expressed miRNA in *X. laevis* RGC axons. In *Slit* morphants, *X. laevis* RGC axons exhibit targeting defects *in vivo*, where RGC axons target a wider area than in wild-type animals, and the loss of miR-182 in RGCs results in defects that resemble *Slit* morphant phenotypes (Bellon et al., 2017). An algorithm to identify potential targets of miR-182 found *Cofilin1* mRNA as a top target (Zivraj et al., 2010), suggesting a link between miR-182 and slit-cofilin1 growth cone collapse. The loss of miR-182 causes an increase in cofilin1 immunostaining intensity in RGC axons similarly to the fluorescence intensity visualized in control RGC axons treated with slit2,

suggesting miR-182 can block *Cofilin1* translation (Bellon et al., 2017). Unexpectedly, despite having increased cofilin1 present in the *miR-182* morphant RGCs, their axons fail to turn away from slit2 (Bellon et al., 2017). Perhaps a tighter regulation of where *Cofilin1* is translated is required for slit2-mediated growth cone repulsion, and this is lost when miR-182 is knocked down throughout the entire growth cone. While these observations suggest that the effect of slit2 on local translation is important *in vivo*, it is important to point out that the effects observed upon miR-182 manipulation cannot be directly attributed to a role in slit-dependent local translation. Nevertheless, these findings are among the strongest evidence for the *in vivo* importance for local translation in axon guidance. The ability of slit2 to regulate miRNAs provides an intriguing mechanism to explain how specific mRNAs are selected for local translation.

To determine the receptor that slit2 signals through, truncated Robo2/3 receptors that lack their cytoplasmic domains were expressed in RGC growth cones, causing elevated activity of miR-182. This observation suggests that slit2 may require the Robo2/3 receptors in this process (Bellon et al., 2017). However, the use of this 'dominant negative' does raise the question of whether Robo2/3 are acting cell-autonomously in this context, as well as whether the dominant negative receptors are sequestering slits away from other receptors or specifically blocking Robo2/3 activity. The use of morpholinos or RNAi to knockdown *Robo2* and *Robo3* in *X. laevis* RGCs, would be useful to further confirm that Robo2/3 are the receptors involved in slit2-dependent *Cofilin1*

translation.

Netrin Induces the Local Translation of beta-actin and DSCAM

Similar to Sema3A and slit2, netrin1 has also been found to induce the local translation of proteins already implicated in axon guidance, beta-actin and the cell adhesion molecule DSCAM. Beta-actin protein is highly expressed in growth cones and filopodia, and *Beta-actin* mRNA co-localizes with translational machinery in granules detected in neurites, axons, and growth cones (Bassell et al., 1998). The 3' UTR of *Beta-actin* mRNA contains a short sequence, called a zipcode, that is required for the localization of *Beta-actin* mRNA to the plasma membrane (Condeelis and Singer, 2005), and two members of the VICKZ (Vg1 RBP/Vera, IMP-1,2,3, CRD-BP, KOC, ZBP-1) family of RNA-binding proteins, Vg1RBP and ZBP1, interact with *Beta-actin* mRNA via the zipcode sequence to regulate its localization (Zhang et al., 2001; Yisraeli, 2005; Leung et al., 2006; Yao et al., 2006; Welshhans and Bassell, 2011). In *X. laevis* RGC growth cones treated with netrin *in vitro,* granules containing the RNA trafficking protein Vg1RBP move into filopodia that are closer to the source of netrin1, and *Beta-actin* mRNA is asymmetrically translated, with higher levels of Beta-actin protein present on the side of the growth cone encountering higher levels of netrin1 (Leung et al., 2006). Netrin1 can induce the local translation of *beta-actin* protein *in vitro* in both *X. laevis* RGCs, and mammalian cortical neurons (Leung et al., 2006; Welshhans and Bassell, 2011). In mammalian cortical neurons cultured from mice lacking the RNA-binding protein ZBP1, netrin1 no longer induces axon

attraction in a turning assay and does not increase local translation of a beta-actin translational reporter to the levels seen in wild-type neurons (Welshhans and Bassell, 2011). These observations indicate that ZBP1 is required for netrin1-mediated local translation of *Beta-actin* mRNA in mammalian cortical neurons *in vitro.*

Recently, Strohl et al. (2017) developed an imaging technique to visualize translation of single molecules in an *in vitro* culture system. Using this system, the authors determined that *Beta-actin* mRNA is locally translated at multiple sites within growth cones treated with netrin1, and that, remarkably, translation of *Beta-actin* mRNA is induced within 20 seconds of applying netrin1 to neurons in culture (Strohl et al., 2017). It would be interesting to determine whether sites of rapidly induced actin translation co-localize with the Dcc receptor. In addition to inducing the translation of *Beta-actin* mRNA, there is also some evidence that suggests netrin1 can induce the local translation of DSCAM. *Dscam* mRNA is detected throughout the soma, axon, and growth cone of mouse hippocampal neurons, and blocking translation prevents an increase in the expression of the cell adhesion protein DSCAM in response to netrin1 (Jain and Welshhans, 2016).

Several salient points have risen from studies on local translation in axon guidance, including: the local translation of specific mRNAs by guidance cues, and asymmetric translation of certain mRNAs, which are both often required for downstream receptor signaling to regulate axon guidance. Still, several aspects of how guidance cues regulate translation at the growth cone are still unknown. In particular, our current understanding of how receptors interact with and signal

to translational machinery is limited. Indeed, the only axon guidance receptor currently known to directly interact with translational machinery is Dcc (Tcherkezian et al., 2010) (Figure 1.1B).

Dcc directly associates with translational machinery

In the previous mechanisms discussed here, axon guidance receptors might regulate local translation by recruiting cytoplasmic signaling proteins, or receptors could directly interact with translation machinery to regulate local translation. Indeed, the axon guidance receptor Dcc has been shown *in vitro* to directly interact with translation machinery, including eukaryotic initiation factors, ribosomal proteins, small and large ribosomal subunits, and monosomes. Both electron microscopy and immunofluorescence analysis show that Dcc co-localizes with both translation machinery and with newly synthesized protein in axons and dendrites (Tcherkezian et al., 2010). The interaction between Dcc and translation machinery is dependent on netrin1, which causes Dcc to release ribosomal subunits and monosomes, allowing for polysomes to form and translation to occur (Tcherkezian et al., 2010). Removal of the extracellular domain of Dcc inhibits translation in response to netrin1 (Tcherkezian et al., 2010). The conserved P1 motif within the cytoplasmic domain of Dcc is required for Dcc to interact with translation machinery (Figure 1.1B). While the *in vitro* biochemical links between Dcc and translation machinery is quite compelling, the *in vivo* significance of these observations for axon guidance is less clear. *In vivo* evidence linking Dcc-dependent translational regulation to axon guidance is

limited to a single experiment where a Dcc receptor lacking the P1 motif (DccΔP1) is mis-expressed in chick commissural neurons in the developing spinal cord. Neurons expressing DccΔP1 are less likely to extend their axons to the midline in comparison to wild type axons (Tcherkezian et al., 2010). However, the axon guidance defects resulting from over-expressing a dominant negative DccΔP1 cannot be solely attributed to a loss of interaction between Dcc and translational machinery without further analysis. A homolog of Dcc has not been found in the chick, although a homolog of neogenin, a closely related family member that can substitute for Dcc, contains the conserved P1 motif (Phan et al., 2011). Still, the defects resulting from the expression of DccΔP1 could result from blocking netrin1 interactions with neogenin, or alternatively they could be due to an unknown factor that binds to the P1 motif of Dcc. In the *Drosophila* embryo, rescue experiments show that FraΔP1, where Fra is the invertebrate orthologue of Dcc, is able to rescue the midline crossing of a subset of commissural axons in *fra* mutants comparably to the full-length Fra receptor, suggesting the P1 motif is not required for commissural axon guidance (Garbe et al., 2007). However, these experiments were performed with receptors that were expressed at higher than endogenous expression levels, potentially overcoming a requirement for the P1 motif. A more precise analysis to elucidate the function of the P1 motif in axon guidance is necessary. Dcc directly interacting with translational machinery is an exciting finding, and future studies should determine if this interaction is required for Dcc-mediated axon guidance, both *in vitro* and *in vivo*. For example, it would be interesting to determine if the netrin1-induced local translation of *Beta-actin*

mRNA requires Dcc, and if Dcc interacts directly with translational machinery to mediate local translation of either *Beta-actin* or *Dscam.* An interesting alternative possibility is that Dcc control of local translation is important for other neuronal functions of Dcc, such as the regulation of synapse formation or function.

Netrin-mediated Local Translation at the synapse

In addition to its role in axon guidance, netrin is also required for synaptogenesis in *C. elegans* and mammals (Colon-Ramos et al., 2007; Park et al., 2011; Stavoe and Colon-Ramos, 2012; Stavoe et al., 2012; Goldman et al., 2013). For example, *C. elegans* netrin (Unc6) induces synaptogenesis through the Dcc (Unc40) receptor (Colon-Ramos et al., 2007), and this requires Unc40 to interact with CED5/dock180 (a rac GEF) and activate CED10/rac1 to mediate local cytoskeletal rearrangements (Stavoe and Colon-Ramos, 2012). In mammalian cortical neurons, netrin1 also promotes synaptogenesis (Goldman et al., 2013), but the requirement for Dcc as the receptor in this context has not been tested. In *Aplysia* sensory and motor neuron co-cultures *in vitro*, bath application of netrin1 stimulates local translation of the sensory neuron-specific neuropeptide *sensorin* at synapses. In response to netrin1 application, a translation-dependent increase in Sensorin protein is observed in sensory neurons (Kim and Martin, 2015). Notably, while treatment with netrin1 does not convert non-synaptic sites to synaptic sites, it does result in an increase in amplitude of the excitatory post-synaptic potential (EPSP) in sensory neurons, as well as an increase in sites of synaptic connections, suggesting netrin1 increases

synaptic strength between *Aplysia* sensory neurons and motor neurons (Kim and Martin, 2015). The over-expression of *Aplysia* netrin in motor neurons is sufficient to induce increases in sensorin protein in the sensory neurons with which they are co-cultured (Kim and Martin, 2015), suggesting that netrin can act in trans to induce local translation in the sensory neurons. The authors demonstrate that Dcc is required for netrin-mediated induction of *Sensorin* translation by using a function-blocking antibody against Dcc. These experiments imply that Dcc is the receptor that netrin interacts with to increase synaptic strength, and that this is controlled by netrin-Dcc induction of local protein translation. However, the ability of netrin to increase synaptic strength has not been tested in a Dcc-deficient or local translation-blocking assay, which would more definitively demonstrate that Dcc and/or local translation, respectively, are required. Additionally, it remains to be seen whether netrin induction of local translation is required *in vivo* for synaptogenesis or synaptic plasticity.

The control of local translation in axons and growth cones by extracellular cues provides an enticing model for how axon guidance and synaptogenesis can be precisely tuned. The specific expression of proteins in certain compartments may increase the spatial and temporal control provided by axon guidance cues. Still, further investigation of the *in vivo* role for local translation in axon guidance and synaptogenesis is needed to fill in the gaps in our fragmentary knowledge of how receptors signal to translation machinery, and how specific mRNAs are selected for translation.

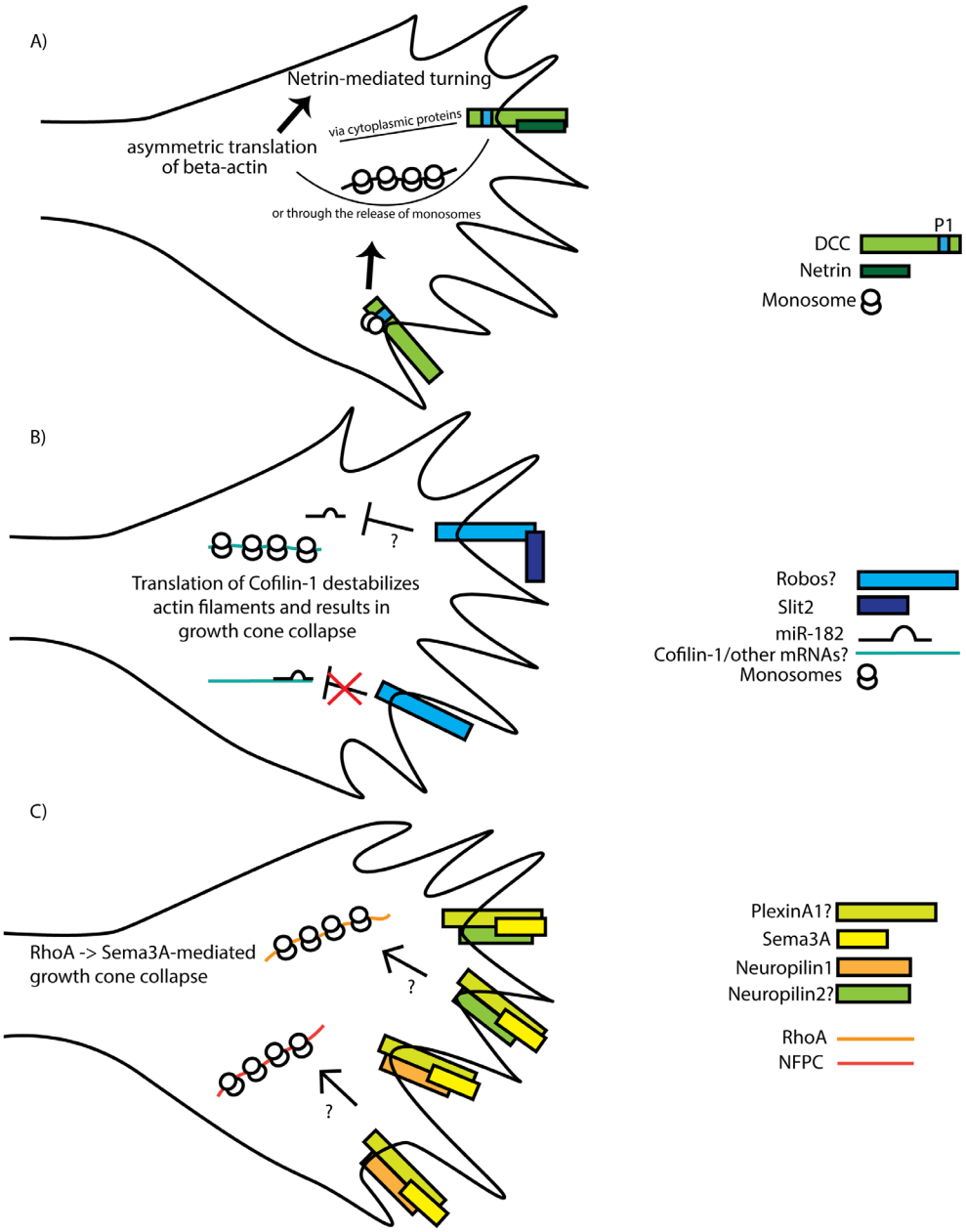

Figure 1. 1**: Netrin, Slit2, and Sema3A induce local translation in axons and growth cones.**

A) A model for indirect regulation of local translation by the axon guidance cue Slit2. Slit2 causes the miRNA miR-182 to release cofilin-1 mRNA, potentiating cofilin-1 local translation and resulting in growth cone collapse. B) A model for direct regulation of local

translation by Netrin-Dcc signaling. Dcc interacts with translational machinery through the conserved P1 motif indicated. Netrin-Dcc interaction induces the release of monosomes from Dcc, allowing them to form polysomes and translate mRNAs locally. Netrin mediates the asymmetric translation of beta-actin, resulting in attractive turning. The induction of beta-actin translation by Netrin could be due to the direct release of translational machinery from Dcc, or through an alternative mechanism via cytoplasmic proteins that link Netrin signaling with translational machinery.

Part 2: Transcriptional Regulation

The ability of axon guidance signaling pathways to control protein synthesis presents an intriguing mechanism to regulate protein expression in specific areas of the cell. In a similar vein, axon guidance receptors and their ligands have also been implicated in controlling gene expression at the level of transcription in several contexts. There had been hints that axon guidance receptors might regulate transcription similarly to the way that notch controls transcription. However, the evidence was primarily from *in vitro* systems, or only demonstrated a correlational relationship between axon guidance receptors and altered expression of specific genes. In this section of the review, we will discuss recent findings that indicate that guidance receptors can signal to regulate gene transcription, in some cases in surprisingly direct ways.

Axon guidance receptors are transcriptional activators

It is now clear that Dcc, neo, and Fra are able to function as transcriptional activators (Figure 1.2) (Taniguchi et al., 2003; Goldschneider et al., 2008; Neuhaus-Follini and Bashaw, 2015). While early work suggests Dcc and neo could act as transcriptional activators in *in vitro* assays, recent reports demonstrate an *in vivo* role for Fra as a transcriptional activator. Preliminary evidence in vertebrates demonstrated that Dcc is cleaved by gamma-secretase, a protease that cleaves single-pass transmembrane proteins in their transmembrane domain, to release the intracellular domain (ICD) of the protein. Cleavage of Dcc by gamma-secretase is necessary for Dcc to activate a

transcriptional reporter in cell culture (Taniguchi et al., 2003).

Neo is cleaved by a metalloprotease, potentially TACE/ADAM17 (Okamura et al., 2011), which is followed by gamma-secretase cleavage, and the neo intracellular domain (ICD) can subsequently enter the nucleus (Goldschneider et al., 2008). In the nucleus, the neo ICD activates transcription of a reporter in cells, and ChIP on cells reveals several different loci where the neo ICD interacts with chromatin near specific genes (Goldschneider et al., 2008). Several proteins that were found to interact with the N-terminal domain of the neo ICD in a yeast two-hybrid screen are implicated in transcriptional regulation, including the histone acetyltransferase tip60. *In vitro*, neo also interacts with the lim domain only 4 (LMO4) transcription factor in human neurons and in embryonic rat cortical neurons. Neo may also regulate gene expression indirectly, as neo releases LMO4 in response to repulsive guidance molecule A (RGMa), which allows LMO4 to translocate to the nucleus (Goldschneider et al., 2008; Schaffar et al., 2008). Chick RGCs cultured *in vitro* on RGMa have short axons, but a miRNA designed to target LMO4 causes these RGC axons to appear longer, indicating that LMO4 has a role in neo-mediated growth cone repulsion (Banerjee et al., 2016). Interestingly, in chick RGC explant cultures, overexpression of the neo ICD inhibits outgrowth of neurites, yet the neo ICD with its nuclear localization signal removed only partially inhibits neurite outgrowth (Banerjee et al., 2016). This observation suggests that the neo ICD has a nuclear function that can affect neurite outgrowth inhibition *in vitro*. Additional experiments are required to examine whether and how the neo ICD

regulates transcription *in vivo,* and to determine what the transcriptional targets are that the neo ICD regulates. The cleavage of Neo by TACE/ADAM17, as well as the ability of the neo ICD to interact with chromatin is dependent on RGMa *in vitro* (Goldschneider et al., 2008; Okamura et al., 2011). However, RGMa does not interact with Dcc, which leaves open the question of what regulates the transcriptional function of Dcc.

In *Drosophila,* transcriptional activation by Fra is independent of its canonical ligand Netrin (Yang et al., 2009). Fra has also been shown to be cleaved by gamma-secretase (Neuhaus-Follini and Bashaw, 2015), and this cleavage is necessary *in vivo* for Fra to activate transcription of *commissureless (comm)*, whose protein product antagonizes repulsive Slit-Robo1 signaling in *Drosophila* (Neuhaus-Follini and Bashaw, 2015). Both *in vitro* and *in vivo* experiments show that the Fra ICD moves in and out of the nucleus, and the conserved P3 motif is the activation domain required for the Fra ICD to activate transcription (Neuhaus-Follini and Bashaw, 2015). The *in vivo* requirement for the Fra ICD to activate transcription was demonstrated in rescue experiments in *fra* null mutants. A Fra full-length receptor with a point mutation, which abolishes transcriptional activity while leaving other known Fra signaling activities intact, fails to rescue *comm* expression *in vivo*. However, the same receptor with a VP16 activation domain fused to the c-terminus is able to rescue*,* demonstrating that Fra needs an intact activation domain to regulate *comm* expression (Neuhaus-Follini and Bashaw, 2015).

Fra regulates the transcription of one known gene in *Drosophila*, the neo

ICD interacts with several promoters in cells, and there are likely more genes that Dcc, neo, and Fra regulate to control axon guidance or other Dcc-, neo- and Fra-dependent processes. The sole gene currently known to be regulated by Fra is the endosomal sorting protein Comm (Yang et al., 2009; Neuhaus-Follini and Bashaw, 2015), which does not have an orthologue in vertebrates. It is also unclear whether the Fra ICD activates transcription of *comm* directly by binding to the comm promoter, or indirectly by regulating the transcription of other genes.

Control of progenitor dynamics: axon guidance receptors controlling transcription?

Unlike Dcc, neo, and Fra, the Robo receptors have not been implicated in regulating transcription directly. Still, both *Drosophila* and Human Robo1 receptors are cleaved by the metalloprotease Kuzbanian/ADAM10, and this cleavage is necessary for slit-Robo1 signaling (Coleman et al., 2010). In addition, human Robo1 has been shown to undergo a subsequent cleavage by gamma-secretase, which allows the Robo1 ICD to enter the nucleus in cancer cell lines (Seki et al., 2010). These observations suggest Robo1 has the potential to enter the nucleus and act as a transcription factor; however, there is no *in vivo* evidence supporting this idea.

Slit-Robo signaling is required in cortical neurogenesis, and some evidence suggests Robo receptors may regulate transcription in this context; however, whether Robo receptors regulate transcription directly or indirectly is unclear. Furthermore, reports in the field have often produced conflicting results

that complicate our understanding of how Robo receptors might regulate cortical neurogenesis. In the developing mammalian cortex, progenitor cells must strike a balance between dividing for self-renewal, and generating post-mitotic neurons, such as excitatory pyramidal neurons (Noctor et al., 2007). Apical (radial glial cells) and basal (intermediate progenitors) progenitor populations can divide to produce pyramidal neurons. Radial glia typically divide symmetrically to self-renew, and asymmetrically to give rise to either pyramidal neurons, or, more likely, intermediate progenitors (Noctor et al., 2004). Intermediate progenitors always divide symmetrically, either to self-renew or to produce two pyramidal neurons (Miyata et al., 2004; Noctor et al., 2004).

Robo receptors had already been implicated in the regulation of cortical interneuron proliferation (Andrews et al 2006, Hernandez-Miranda 2011), and the expression of Robo1, Robo2, and slit in the ventricular and subventricular zones (VZ and SVZ) of the cortex suggested slit-Robo signaling may also have a role in proliferation of pyramidal neurons (Borrell et al., 2012; Yeh et al., 2014). Here we focus on two recent reports that provide some evidence for Robo receptors regulating transcription, yet they directly contradict each other in several key aspects (Borrell et al., 2012; Yeh et al., 2014). Despite some conflicting observations, both studies support the idea that slit-Robo signaling plays important roles in regulating progenitor dynamics in the developing mammalian cortex (Borrell et al., 2012; Yeh et al., 2014).

Borrell and colleagues show that although Robo1 and Robo2 are both detected in the VZ of the cortex, Robo2 appears to be much more highly

expressed (Borrell et al., 2012). Accordingly, while both *Robo1* and *Robo2* single mutants have an increase in basal progenitors (albeit less severe than the double mutant), *Robo2* mutants have a more severe phenotype than the *Robo1* mutants, suggesting Robo2 has a larger role in regulating progenitor populations in the developing cortex. Similarly, single mutants of *Slit1* and *Slit2* had no significant effect on the progenitor populations in the cortex, yet the *Slit1/2* double mutant resulted in an increase in basal progenitors (Borrell et al., 2012). In direct contrast to these observations, Yeh and colleagues show that Robo1 is expressed in the proliferative zones of the cortex, while Robo2 is undetectable (Yeh et al., 2014). Furthermore, *Robo2* single mutants did not have any defects in the progenitor populations in the cortex, while *Robo1* mutants resulted in an increase in both the apical and basal progenitor populations (Yeh et al., 2014). While the role for Robo receptors reported by the two groups are clearly at odds, there is agreement that slit1 and 2 are necessary for proper regulation of progenitor populations in the cortex. Notably, the two groups used different mutants for *Robo1* and *Robo2* single mutants, raising the possibility that differences in genetic background may explain some of the phenotypic differences that were reported; however, in both cases the mutants used are null mutants, and both groups used the same *Robo1/2* double mutants. While both reports find that slit-Robo signaling is involved in controlling progenitor dynamics, the mechanism each proposes differs greatly. Borrell and colleagues report that there is no difference in apoptosis, and the cell cycle of the basal progenitors is found to be disrupted in *Robo1/2* mutants: basal progenitors divide less

frequently, their cell cycle length is significantly longer, and progenitors fail to separate from the ventricular surface (Borrell et al., 2012). Progenitors that stay attached to the ventricular surface are known to have decreased proliferation (Cappello et al., 2006), suggesting this may be a cause for the slow and less frequent divisions of basal progenitors. Yeh and colleagues, however, find that fewer progenitors undergo apoptosis, progenitors are proliferative for an increased amount of time, but their cell cycle appears otherwise normal, and the *Robo1* mutants have a small but significant decrease in microglia (Yeh et al., 2014), which are reported to cause an increase in progenitor pools (Cunningham et al., 2013). Analyzing conditional knockouts for *Robo1/2* single and double mutants may help to clarify the discrepancies observed in regards to the Robo receptor required for proper cortical neurogenesis, and the mechanism required for proper progenitor dynamics.

Robo receptor signaling and the control of neural progenitor dynamics

How might Robo receptors signal downstream to regulate progenitor dynamics? Interestingly, expression of the notch effector *Hes1* is significantly reduced in the cortex in *Robo1/2* double mutants, and over-expression of hes1 in *Robo1/2* double mutants recues the progenitor defect (Borrell et al., 2012). In addition, RNAi knock down of *Hes1* leads to a reciprocal effect and increases the number of progenitors. These observations suggest that Robo receptors may regulate *Hes1* expression to mediate progenitor dynamics in the developing

cortex. The effect of Robo2 on *Hes1* expression was further tested using an in vitro primary culture system, where a myristolated Robo2 construct was found to activate the hes1 reporter (Hes-luc). Robo2 was able to activate transcription of the hes-luc reporter independently of notch, although co-expression of notch and Robo2 led to a synergistic effect on reporter expression (Borrell et al., 2012). These findings suggest Robo2 may regulate progenitor dynamics in the cortex through the regulation of transcription. Additional evidence pointing to a potential role for Robo receptors in regulating transcription comes from microarray analysis on tissue from the developing cortex, where it was found that over 300 genes are either up- or down-regulated in Robo1 mutants compared to wildtype controls (Yeh et al., 2014). Thus, in the context of progenitor proliferation in the developing mammalian cortex, the Robo receptors may regulate the transcription of genes involved in neurogenesis.

Robo receptors and progenitor dynamics in intestinal stem cells

In the adult *Drosophila* midgut epithelium Robo2 plays a role in maintaining progenitor dynamics. In the midgut, intestinal stem cells (ISCs) give rise to both enteroblast progenitor cells and secretory enteroendocrine (EE) cells (Zeng and Hou, 2015). Robo2 RNAi and *Robo2* homozygous clones generated using MARCM (Lee and Luo, 2001), and the specific knockdown of Robo2 in only ISCs, all result in an increase in EE cells. These observations suggest that Robo2 normally functions in ISCs to control progenitor dynamics and restrict the differentiation of EEs (Biteau and Jasper, 2014). The transcription factor

Prospero (Pros) is necessary but not sufficient to specify EE cell fate (Zeng and Hou, 2015), and genetic interactions with Robo2 suggest Robo2 and Pros might act in the same process (Biteau and Jasper, 2014). While the relationship between Pros and Robo2 in the *Drosophila* midgut remains unclear, one intriguing idea is that Robo2 may regulate transcription of Pros in this system.

Ephrin-Eph signaling and the regulation of neurogenesis

In contrast to the uncertainty over whether Robo receptors can control transcriptional regulation to mediate progenitor dynamics, there is stronger evidence that eph-ephrin signaling regulates transcription during neurogenesis, as reviewed in Laussu et al. (2014). The transmembrane ephrinBs are cleaved by gamma-secretase (Georgakopoulos et al., 2006; Tomita et al., 2006), and the ephrinB1 ICD can interact with zinc finger and homeodomain protein 2 (ZHX2), a transcriptional repressor that is expressed in cortical neural progenitors and inhibits neuronal differentiation (Wu et al., 2009). One transcriptional target of ephrinB1 signaling in neural progenitors is the pro-neurogenic miRNA miR-124 (Arvanitis et al., 2010). EphrinB1 mutant neural progenitor cells have an increase in miR-124 RNA, and cortical sections from *ephrinB1* mutant mice have increased levels of miR-124 RNA (Arvanitis et al., 2010). Interestingly, miR-124 in turn represses expression of ephrinB1 along with other genes (Arvanitis et al., 2010). While ephrinB1 signaling is implicated in repressing transcription, the evidence that ephrinB1 regulates transcription directly is weak. While there are reports that ephrinBs are cleaved by gamma-secretase, it has not been shown

that gamma-secretase cleavage or the translocation of ephrinB1 ICD are required for ephrinB1 to repress transcription of miR-124. Indeed, ephrinB1 ICD interacts with transcriptional coactivator with PDZ-binding motif (TAZ), and phosphorylation of the ephrinB1 ICD results in translocation of TAZ to the nucleus in bone marrow stromal cells (Xing et al., 2010). However, whether transcriptional regulation also requires ephrinB1 to translocate to the nucleus remains unknown.

Discussion and Future Directions

Axon guidance pathways regulate axon guidance, synaptogenesis, progenitor dynamics, and cell migration using a variety of mechanisms. Originally found to control local cytoskeletal rearrangements, axon guidance pathways also regulate gene expression to control these complex developmental processes. Mounting evidence demonstrates that axon guidance ligands have the ability to induce local translation, and that this is often a requirement for growth cones to respond to axon guidance cues *in vitro*. Axon guidance cues also induce the local translation of specific proteins that are required for the growth cone to respond to the cue. This presents an interesting model where guidance cues induce translation of specific proteins at local sites in the growth cone to mediate growth cone steering, axon branching, and synaptogenesis. However, further research is necessary to demonstrate that local protein synthesis is required *in vivo* for specific axon guidance pathways. In addition, it is not clear how the axon

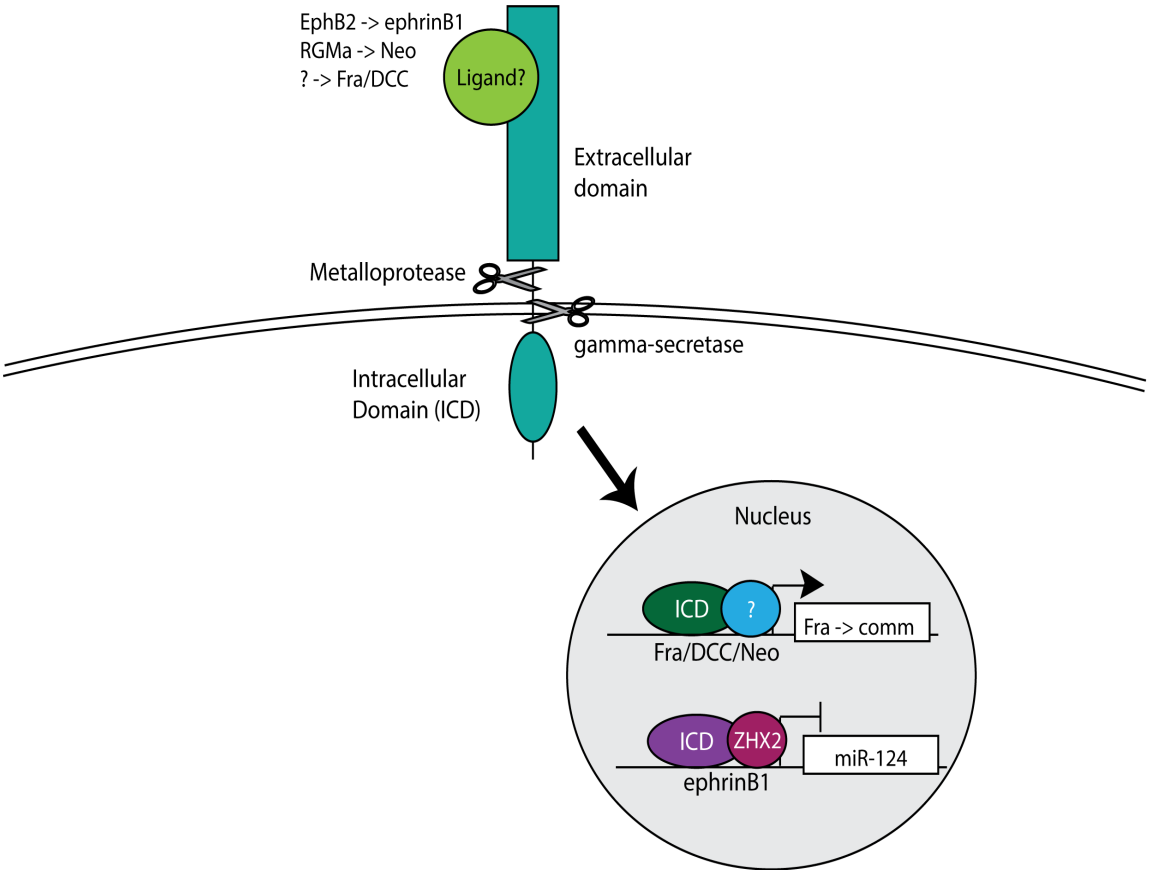

Figure 1. 2**: Axon guidance receptors are cleaved and enter the nucleus to regulate transcription.**

A schematic depicting a general mechanism for axon guidance factors to enter the nucleus and regulate transcription. On the left, a ligand interacts with the extracellular domain of the receptor, triggering ectodomain-shedding by a metalloprotease, and subsequent cleavage in the transmembrane domain by the single-pass transmembrane protease gamma-secretase. The resulting intracellular domain product then enters the nucleus and interacts with nuclear proteins to regulate transcription. It should be noted that while gamma-secretase cleavage and transcriptional activation has been demonstrated to be required for Fra functions in vivo, and in vitro experiments with Dcc and Neo also support this model, the experiments linking the transcriptional regulation

downstream of EphB2-ephrinB1 signaling to this model is substantially weaker. EphB2-EphrinB1 signaling does repress the expression of miR-124, and this is mediated by the transcriptional repressor ZHX2.

guidance receptors required for local translation signal to translation machinery. Thus far, the only receptor shown to directly interact with translational machinery is Dcc, and this interaction has yet to be shown to be required for netrin1-Dcc *in vivo* functional outputs. A more thorough understanding of the receptor signaling mechanisms that converge on translational machinery might allow for the design of more specific receptor manipulations that would directly test their *in vivo* requirement in local translation. Since it is clear that multiple guidance cues regulate translation, at least *in vitro*, how broad of a role does local translation play *in vivo* in axon guidance? A recent report describing the transcripts linked with ribosomes in the axons from both embryonic mice as well as postnatal mice shows an enrichment for transcripts with axon-specific functions (Shigeoka et al., 2016), suggesting that local translation of these mRNAs may play a role in axon guidance and synaptogenesis.

The axon guidance receptors Fra, neo, and Dcc can act as transcription factors, and ephrin and Robo receptors have the potential to at least interact with transcription factors to regulate transcription indirectly. Fra, neo, and Dcc activate transcription *in vitro,* and Fra also has one characterized transcriptional target *in vivo* in *Drosophila*. It remains to be determined whether Fra activates transcription of *comm* directly, or through the transcriptional regulation of other genes. Fra, neo, and Dcc are all sequentially cleaved, and their ICDs can enter the nucleus. Future studies should determine the mechanism through which they activate transcription, and whether they have multiple different targets. Axon

guidance receptors are also expressed in other tissues besides the nervous system, and determining whether they function as transcription factors in other tissues will provide insight into general non-canonical mechanisms, and a better understanding of developmental processes. The evidence that ephrinB1 acts as a transcription factor is promising, but definitive evidence that ephrinB1 has a nuclear function is still lacking. The Robo receptors have a clear role in progenitor dynamics, and they have been tied to alterations in gene expression in mammalian neurogenesis and the *Drosophila* midgut. Whether Robo receptors can directly regulate transcription in these tissues to control progenitor dynamics remains to be determined.

Continuing research into the mechanisms by which axon guidance signaling pathways regulate transcription and local translation will provide a more thorough understanding of axon guidance, synaptogenesis, and ultimately neural circuit formation. Clearly, precise regulation of axon guidance requires more than cytoskeletal rearrangements, and a better understanding of how axon guidance cues and receptors regulate gene expression will be informative for elucidating these processes. Axon guidance cues and receptors are also expressed in tissues outside of the nervous system in normal development, and in cancer cells. Understanding how axon guidance pathways signal to control gene expression will also more broadly provide insight into developmental processes, disease states, and may suggest new therapeutic strategies.

Dcc is a pro-apoptotic tumor suppressor

A second major non-canonical signaling activity of Dcc is as a pro-apoptotic tumor suppressor. Dcc expression is reduced in several cancerous tumors, most notably colorectal cancer (Chen et al., 1999; Forcet et al., 2001; Mehlen et al., 1998). Transgenic re-expression of Dcc in both *Dcc* heterozygous mice and in tumor cells that have lost *Dcc* expression leads to a halt in growth, followed by apoptosis (Mehlen et al 1998, Chen et al 1999). In the presence of Netrin, anti-apoptotic factors are upregulated in Dcc-expressing cells, but when Netrin is absent, Dcc activates cell death via caspase signaling. In the absence of Netrin, caspase 3 cleaves the exposed intracellular domain of Dcc (Mehlen and Mazelin 2003, Goldschneider et al 2010). This allows Dcc-interacting protein 13-alpha (Dip13 alpha) and caspase 9 to interact with the cleaved form of Dcc, which leads to the cleavage and activation of caspase 9. Following this, caspase 9 cleaves and activates caspase 3, forming a positive feedback loop to activate caspase-mediated cell death (Mehlen et al 1998, Forcet et al 2001). In contrast, when Netrin is present, Dip13 alpha instead activates AKT, which results in the inhibition of apoptotic factors such as cytochrome c. Simultaneously, Dip13 alpha increases expression of anti-apoptotic genes. There are conflicting reports as to whether Dcc functions as a dependence receptor broadly in the nervous system, or in select neuronal populations. In addition, it is unclear whether this function of Dcc is required in other non-pathogenic tissues. In the nervous system, increased cell death in the developing brain stem, cerebellum, and spinal cord in *Netrin1*-/- mutant mice have been reported (Llambi et al 2001, Furne et al 2008). However, in the absence of Netrin, oligodendrocyte precursor cells in the embryonic spinal cord do not have an increase in cell death (Jarjour et al 2003). These data support the model that Netrin is required to

prevent apoptosis in some cells in the nervous system. Whether this function of Dcc in controlling cell survival is also conserved in other species has not been determined.

Axon guidance molecules and reproductive tissues

In addition to their well-characterized expression in the developing nervous system, axon guidance molecules are also expressed in several other tissue contexts, including the gut, heart, lungs, and reproductive tissues (Lai Wing Sun et al. 2011, Macabenta et al. 2013, Pert et al. 2015). In these tissues, axon guidance molecules are critical for several morphological processes, including cell migration, tissue morphogenesis, and cell adhesion (Lai Wing Sun et al. 2011, Macabenta et al. 2013, Pert et al. 2015). Still, in many of these contexts the molecular mechanisms underlying the functions of these proteins are unclear. In addition, in some cases axon guidance molecules appear to act in the nervous system to impact the function of other tissues (Trent et al., 1983; Asakura et al., 2007; Ziel et al., 2009; Newquist et al., 2013)

Expression in reproductive tissues

Axon guidance molecules are expressed in reproductive tissues and have been implicated in important processes in several different organisms. While there are some hints as to what these axon guidance genes are doing in vertebrate reproductive tissues, the functional significance of this expression data remains predominantly unknown. Dcc is expressed in human ovarian tissues, and is lost in carcinomas of these tissues (Saegusa et al., 2000; Enomoto et al., 1995). The role of Dcc in ovarian tissues, besides as a tumor suppressor, is unknown. In addition, Netrin is present in porcine reproductive tissues and may have a role in inhibiting vascular growth that could be important for follicular function (Basini et al., 2011; Maeda et al., 2008). Slit and Robo, another pair of

axon guidance genes, are expressed in the human corpus luteum (Dickinson et al., 2008) and the endometrium (Shen et al., 2009). Robo1 is also localized to pre-granulosa cells in the sheep fetal ovary (Dickinson et al., 2010), and Robo2 and Slit2 are localized to oocytes (Dickinson et al., 2010). There is some evidence that the upregulation of Slit and Robo protein levels occurs concurrently with a decrease in the number of proliferating oocytes (Andrews et al., 2008), implying that Slit and Robo may have some role in regulating oocyte proliferation.

Neuronal effects on reproductive tissues

In the worm *C. elegans*, Netrin (Unc-6) and Dcc (Unc-40) are required for egg-laying (Trent et al., 1983; Asakura et al., 2007; Ziel et al., 2009). The HSN motor neuron synapses directly onto the vulval muscles (White et al., 1986), and is required for egg-laying (Sulston and Horvitz, 1982; Trent et al., 1983, Desai et al., 1988; Desai and Horvitz, 1989). Multiple mutant alleles of *unc-6* and *unc-40* cause decreased egg-laying (Trent et al., 1983), and since Unc-6 and Unc-40 are important in guiding the HSN motor neuron (Desai et al., 1988), this indicates that the egg-laying defects seen in *unc-6* and *unc-40* mutants are likely due to defects in the HSN innervating the vulval muscles. Indeed, Unc-6 is expressed in the vulval precursor cells, and is necessary in these cells to attract the HSN neurons ventrally during development (Asakura et al., 2007). Unc-6 and Unc-40 are also required for the formation of the vulva (Ziel et al., 2008). The anchor cell is necessary for connecting the uterus and vulva, to allow for eggs to be laid (Newman and Sternberg, 1996). Mutations in either *unc-6* or *unc-40* cause defects in anchor cell invasion (Ziel et al., 2009). Although Unc-6 is expressed from the ventral nerve cord to promote invasion, Unc-40 is presumably expressed in the non-neuronal

anchor cell which induces vulval precursor cells to differentiate and is important for vulval function (Ziel et al., 2009).

Netrin is also required for egg-laying in *Drosophila* (Newquist et al., 2013). While control flies laid almost 50 eggs per day, global *netAB* mutant flies laid an average of 9 eggs per day (Newquist et al., 2013). It is unclear why *netAB* mutants have a decrease in egg-laying, and the hatch rate of the eggs laid was similar to control flies (Newquist et al., 2013). In addition, one copy of NetB-Myc is sufficient to partially rescue the egg-laying phenotype in *netAB* mutants, indicating that this is a Netrin-specific phenotype (Newquist et al., 2013). Overall, innervation of the ovary appeared grossly normal, and attempts to rescue egg-laying defects by expressing NetB in different neuronal populations failed, suggesting that the requirement for Netrin encompasses many cell populations (Newquist et al., 2013). Thus, it is still unclear in which cells Netrin is required to promote egg-laying. Whether Netrin is functioning extrinsically, such as in the nervous system to guide axons to innervate the ovary, or intrinsically, such as affecting a morphological process that affects egg-laying, remains unknown.

Tissue-intrinsic requirement of axon guidance molecules

While egg-laying phenotypes caused by mutations in axon guidance genes can be due to deficits in innervation of the reproductive system, or physiological effects, some axon guidance genes are expressed and required in the formation of reproductive tissues and for their function in the adult. The *Drosophila* testis contains two stem cell populations that reside at the somatic hub within the stem cell niche, the germline stem cells and the somatic cyst stem cells. Cyst stem cells give rise to cyst cells, and germline stem cells give rise to gonialblasts. It is critical that the ratio at the hub be 2:1 cyst to

germline stem cells because two cyst stem cells encyst each germline stem cell. Subsequently, both populations divide to ensure that the cyst cells encyst the gonialblast daughter cell (Lenhart and DiNardo, 2015). The cyst cells are important for gonialblast differentiation (Kiger et al., 2000; Schulz et al., 2002). Stem cells compete for space at the niche, and it is important that this competition is regulated to ensure the 2:1 ratio (Issigonis et al., 2009). The axon guidance receptor Robo2 is expressed in cyst stem cells and is required for cell competition between cyst stem cells (Stine et al., 2014). Reducing *robo2* expression in all cyst stem cells with RNAi knockdown has no effect on cyst stem cells at the hub. However, *robo2* mutant cyst stem cells are lost from the hub while their wild type neighbors are not, indicating that Robo2 is important for cyst stem cell competition (Stine et al., 2014). This is a clear example of an axon guidance protein functioning intrinsically in the testis.

Interestingly the axon guidance cue Netrin also plays an important role in the stem cell niche in the *Drosophila* ovary. NetrinA (NetA) is expressed in the germarium and is important for germline stem cell maintenance (Tu et al., 2020) indicating a role for axon guidance genes in maintaining stem cells at their niche. In the germarium, four subsets of inner germarial sheath cells have been described and these sheath cells are important for the maintenance or differentiation of the germline stem cells or their progeny, respectively (Tu et al., 2020). NetA is expressed in the two subsets of inner germarial sheath cells closest to the germline stem cells (Tu et al., 2020). Knocking down *netA* specifically in adult inner germarial sheath cells causes a reduction in germline stem cells present at the niche (Tue et al., 2020). This indicates that NetA is required in inner germarial sheath cells to maintain germline stem cells at the niche, providing an example of an axon guidance cue that is expressed in reproductive tissues,

and that is required intrinsically for egg production. Thus, axon guidance genes can also play tissue-intrinsic roles in reproductive tissues.

The *Drosophila* ovary as a model system

The *Drosophila* ovary is a well-characterized and highly tractable genetic system that has served as a model for studying cell migration, cell adhesion, and cell death. Since axon guidance molecules are involved in these processes in other tissues, the ovary presents an apt tissue to investigate whether these molecules are required for some of these processes, and to determine whether they signal in similar ways to their function in the nervous system. In addition, the ovary contains relatively few distinct cell populations, the cells tend to be quite large, and the process of oogenesis is divided into distinct, well-characterized stages. In addition, techniques like RNAi and mosaic analysis work well in this system, making it a powerful system to investigate signaling mechanisms.

Stages of oogenesis

Each female *Drosophila* contains a pair of ovaries that make up the majority of their abdomen. Each ovary consists of 15-20 strings of developing eggs, called ovarioles. The germarium, which houses the stem cell populations for the germline cells and the somatic cells, resides at the anterior end of the ovariole (Kirilly and Xie, 2007). The germline stem cells, which reside in their niche at the anterior tip of the germarium, divide asymmetrically to self-renew and to give rise to a daughter cystoblast (Spradling, 1993). The cystoblast undergoes mitosis another four times, with incomplete cytokinesis, to form a 16-cell cyst. These dividing cysts are ushered by escort cells through the germarium (Spradling, 1993). Each cyst is made up of one oocyte and 15 nurse cells,

which will become large polyploid cells that make the mRNA and proteins for the oocyte. Midway through the germarium, the germline cyst is passed from escort cells to the somatic follicle cells. These follicle cells divide to encapsulate the germline cyst in a single-cell layer, and then this egg chamber buds from the germarium.

Within the ovariole, there are 14 characterized stages of growth determined by morphological criteria, including the size of the egg chamber, nurse cell polyploidy, oocyte size, and the ploidy and position of follicle cells (King 1970). After the egg chamber buds from the germarium, the egg chambers grow in size. This is due to both the replication of the nurse cell genome without mitosis, called endocycling or endoreplication, and the dividing follicle cells which continue to maintain a single layer encapsulating the germline cyst. At stage six, the follicle cells switch from mitotic cycles to endocycles, where they grow larger to continue surrounding the growing germline cyst. Stage eight marks mid-oogenesis, or vitellogenesis, and the oocyte within the egg chamber begins to take up yolk proteins and grow larger from this stage until a mature egg is formed. At stage nine the majority of the follicle cells migrate towards the oocyte, and a small group of 6-8 cells, called border cells, migrate from the anterior tip of the egg chamber through the nurse cells towards the anterior side of the oocyte. These cells will create the micropyle, which is important for sperm entry into the egg. At stage ten, the nurse cells will dump their mRNA and protein into the oocyte, and then begin to die. By stage 14, the follicle cells have completely surrounded the oocyte and have created the vitelline membrane, and a mature egg is made, ready to be ovulated (McLaughlin and Bratu, 2015).

Diet and degeneration at mid-oogenesis

Drosophila egg production is a highly energy-dependent process, and there are two checkpoints in place during oogenesis to ensure that this energy investment leads to the production of high-quality eggs. The first checkpoint is within the germarium, where cell death occurs in response to poor nutrient conditions (Drummond-Barbosa and Spradling, 2001). The second checkpoint is at mid-oogenesis, where cell death occurs in response to poor nutrient conditions or abnormal egg chambers (Drummond-Barbosa and Spradling, 2001; Beachum et al., 2021; Tanentzapf et al., 2000; Chao and Nagoshi, 1999). Oogenesis is blocked at these checkpoints in response to starvation as well as mutation of components of diet-dependent pathways, such as the insulin signaling pathway (Bohni et al., 1999; Chen et al., 1996; Montagne et al., 1999; Drummond-Barbosa and Spradling, 2001). Insulin, target of rapamycin (Tor), Amp Kinase, and nuclear hormone signaling all function in the ovary to mediate effects from diet (Laws and Drummond-Barbosa, 2017). In Drosophila, insulin-like peptides bind and activate the insulin receptor (InR), a receptor tyrosine kinase (Nassel et al., 2015). InR activation results in PI3k phosphorylation, which leads to the recruitment of the serine/threonine kinase Akt1 to the membrane. This causes Akt1 to be phosphorylated and activated. Activated Akt1 is able to affect several substrates that regulate a variety of cellular processes: Akt1 can inhibit GSK-3beta and the transcription factor FoxO, and can activate mTORC1 (Manning and Toker, 2017; Nassel et al., 2015). The GSC division cycle requires PI3K/FoxO signaling, while growth at later stages requires PI3K/Tor signaling (Hsu et al., 2008; LaFever et al., 2010).

Poor nutrient conditions cause a partial block in ovulation and also result in the retention of eggs (Drummond-Barbosa and Spradling 2001). This block in ovulation is

caused by the death of germline cells in the germarium and at mid-oogenesis (Drummond-Barbosa and Spradling 2001; Mazzalupo and Cooley, 2006; Pritchett et al., 2009; Buszczak et al., 2002; Terashima and Bownes, 2006). Poor nutrient conditions also cause the accumulation of enlarged P bodies and microtubule rearrangements in younger egg chambers, and these egg chambers are thought to be protected from death, as their development considerably slows down and egg chamber degeneration seems to only occur post stage 7 (Drummond-Barbosa and Spradling 2001; Shimada et al., 2011). The effects of poor nutrient conditions on the germline requires functional Insulin signaling in the follicle cells (Burn et al., 2015).

Abnormal egg chambers and degeneration at mid-oogenesis

While diet-dependent pathways and the ovarian diet responses have been well studied at mid-oogenesis, it is less well understood how “abnormal egg chambers” activate the checkpoint at mid-oogenesis. The activation at this checkpoint can be triggered by disrupted polarity (Beachum et al., 2021; Tanentzapf et al., 2000), or follicle cell death (Chao and Nagoshi, 1999). Follicle cell polarity is important to establish patterning in the future embryo. Disruptions to egg chamber polarity can lead to degeneration at mid-oogenesis (Tanentzapf et al., 2000; Beachum et al., 2021).

Activation of the mid-oogenesis checkpoint results in egg chamber degeneration. Typically, the germline cells die first and are engulfed by the surrounding follicle cells, which will die soon after. This degeneration requires the death effector caspase Dcp-1 in the germline (Peterson et al., 2003). Under poor nutrient conditions and in abnormal egg chambers, Dcp-1 is activated after stage eight. Dcp-1 is also known to be required for germline death in response to poor nutrient conditions at this stage (Peterson et al., 2003). Germline survival depends on the suppression of apoptosis, and the egg

chambers from starved flies where Dcp-1 signaling is inactive in the germline have follicle cells that die prior to the germline (Peterson et al., 2003). These egg chambers are called peas without pods (pwops), as the follicle cells all die off, leaving only the germline cyst (Pritchett and McCall, 2012). While much is known about how poor nutrient conditions activate the checkpoint at mid-oogenesis, the molecule(s) that control the checkpoint, as well as the different types of abnormal egg chambers that can activate the checkpoint are still unknown.

The goal of this work is to determine how Fra functions in the ovary, and whether this system can be used to further our knowledge of how Fra activates transcription, and to identify proteins that might be required to interact with Fra to regulate transcription. In Chapter 2, I characterize Fra's function in the ovary, and find that Fra is required for egg chambers to progress through mid-oogenesis independently of Netrin. In contrast to the pro-apoptotic function that Dcc has as a dependence receptor, Fra is anti-apoptotic in the ovary and promotes germline survival. In addition, the transcriptional activation domain within the conserved P3 region of Fra is required for this function, suggesting that Fra acts as a transcription factor in the ovary. In Chapter 3, I conduct a yeast two-hybrid screen to identify proteins that interact with the Fra intracellular domain. In particular, I follow-up on interactors that have DNA-binding domains, Clawless/C15 (Cll) and Pleiohomeotic-like (Phol), as proteins potentially important for nervous system development. Finally, in Chapter 4 I discuss the implications of this work, and future directions.

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CHAPTER 2: Frazzled/Dcc acts independently of Netrin to promote germline survival during *Drosophila* oogenesis

Modified from Russell SA, Laws KM, Bashaw GJ Frazzled/Dcc acts independently of Netrin to promote germline survival during *Drosophila* oogenesis (In revision at Development).

Abstract

The Netrin receptor Frazzled/Dcc (Fra in *Drosophila*) functions in diverse tissue contexts to regulate cell migration, axon guidance and cell survival. Fra signals in response to Netrin to regulate the cytoskeleton and also acts independently of Netrin to directly regulate transcription during axon guidance in *Drosophila*. In other contexts, Dcc acts as a tumor suppressor by directly promoting apoptosis. In this study, we report that Fra is required in the *Drosophila* female germline for the progression of egg chambers through mid-oogenesis. Loss of Fra in the germline, but not the somatic cells of the ovary, results in the degeneration of egg chambers. While a failure in nutrient-sensing and disruptions in egg chamber polarity can result in degeneration at mid-oogenesis, these factors do not appear to be affected in *fra* germline mutants. However, similar to the degeneration that occurs in those contexts, the cell death effector Dcp-1 is activated in *fra* germline mutants. Fra's function in the female germline is independent of Netrin and requires Fra's transcriptional activation domain. In contrast to Dcc's role in promoting cell death, our observations reveal a role for Fra in regulating germline survival by inhibiting apoptosis.

Introduction

Netrin and its receptor Deleted in colorectal cancer (Dcc, Frazzled in *Drosophila*) play critical roles in the development and maintenance of multiple tissue types, including the *Drosophila* heart and gut, as well as the vertebrate pancreas, lung, mammary glands, vascular system, and musculature (Lai Wing Sun et al., 2011; Macabenta et al., 2013; Pert et al., 2015). In the developing nervous systems of invertebrates and vertebrates, Netrin signals through its receptors Frazzled (Fra)/Dcc to promote attractive axon guidance (Boyer and Gupton, 2018). This activity requires receptor interactions with intracellular effector proteins that remodel the growth cone cytoskeleton to steer the navigating axon (Zang et al., 2021).

In *Drosophila* commissural neurons, Fra also acts independently of Netrin to regulate gene expression (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009). In this context, Fra is proteolytically processed to release its intracellular domain (ICD), which can translocate into the nucleus and activate transcription of *commissureless (comm)* (Neuhaus-Follini and Bashaw, 2015). It is unknown whether this mode of signaling is conserved or functions outside of the nervous system. In human embryonic kidney cells, vertebrate orthologs of Fra, Neogenin and Dcc, activate transcription of a luciferase reporter gene, and the Neogenin ICD can bind upstream of open reading frames and regulate their mRNA expression (Goldschneider et al., 2008; Taniguchi et al., 2003). This suggests that Fra's ability to activate transcription is conserved across species. However, it is unclear how the transcriptional activity of Fra is regulated. Nor is it known how Fra interacts with transcriptional machinery and what other target genes it may regulate.

A second non-canonical function of Dcc is to act as a tumor suppressor to promote cell death. In the absence of Netrin, expressing Dcc in human embryonic kidney cells, prostate and colon carcinoma cells, and neuroblastomas results in the cleavage of the ICD of Dcc by caspase 3, leading to the activation of caspase-mediated cell death (Chen et al., 1999; Forcet et al., 2001; Mehlen et al., 1998). These studies have led to the "dependence receptor" hypothesis, which posits that Dcc depends on the presence of Netrin to prevent cell death. Dcc also promotes cell death when Netrin expression is limited in the mouse spinal cord and enteric nervous system, and the chick neural tube (Castets et al., 2012; Furne et al., 2008). Furthermore, in adult mouse and rat brains, *netrin* conditional knockouts lead to Dcc-mediated dopaminergic neuron death (Jasmin et al., 2021). While Dcc may function as a dependence receptor in human tumor cells and in some vertebrate neurons, whether this function is conserved in other species and other tissue contexts remains to be determined.

Netrin-Fra signaling has been predominantly studied in the developing nervous system; however, this signaling pathway plays diverse and essential roles in many tissue contexts (Lai Wing Sun et al., 2011; Macabenta et al., 2013; Pert et al., 2015). Netrin and its receptors may also play a role in reproduction. In *Drosophila, netrinAB* mutant females have decreased fertility (Newquist et al. 2013), although it is unclear whether this reflects tissue-intrinsic or neuronal requirements. While the nervous system profoundly influences organismal physiology, including reproduction (Drummond-Barbosa, 2019), Netrin also affects cell migration and adhesion by acting on its receptors in other tissues. For example, Netrin (Unc-6) is required for the normal innervation of the *C. elegans* reproductive system (Asakura et al., 2007), and Unc-6 secreted from neurons also promotes anchor cell invasion to shape the developing reproductive system (Ziel et al., 2009). In other cases, Netrin and its receptors have clear tissue-

intrinsic roles. For example, a recent study shows that NetrinA is expressed in the *Drosophila* germarium and is required in escort cells for germline stem cell maintenance (Tu et al., 2020). Intriguingly, Netrin and Dcc are expressed in porcine and human adult female reproductive tissues, respectively (Basini et al., 2011; Enomoto et al., 1995; Maeda et al., 2008; Saegusa et al., 2000). While there are some hints that Netrin may be important for blood vessel development in porcine reproductive tissues, the importance of Netrin and Dcc to reproductive tissue development and function remains largely unknown (Basini et al., 2011; Enomoto et al., 1995). Furthermore, the mechanism of Dcc signaling in these tissues has yet to be explored.

To further investigate the diverse signaling mechanisms of Fra/Dcc, we sought to define a novel tissue context that would allow us to directly observe changes in cell morphology and survival; therefore, we turned to the *Drosophila* ovary. The *Drosophila* ovary is an excellent system to address linkages between cell morphology and survival since the process of oogenesis requires coordination of multiple morphogenetic events as egg chambers grow and differentiate. Furthermore, germline survival depends on the suppression of apoptosis (Peterson et al., 2003), allowing us to test whether Fra regulates this process. *Drosophila* ovaries consist of ovarioles, or strings of developing egg chambers. Oocyte development begins in the germarium at the anterior end of the ovariole. At the anterior of the germarium, germline stem cells divide to give rise to daughter cystoblasts, which divide four times with incomplete cytokinesis to create sixteen-cell cysts containing one oocyte and fifteen nurse cells (Spradling, 1993). Nurse cells endoreplicate, producing mRNA and proteins that are eventually transferred to the oocyte and are necessary for its growth (Spradling, 1993). At the midpoint of the germarium, somatic follicle cells encapsulate the cyst in a single layer as it buds off of the germarium to form an egg chamber (Kirilly and Xie, 2007) (Figure. 1A). In the

vitellarium, egg chambers progress through 14 stages of growth that are characterized by well-established morphological criteria (King, 1970). At mid-oogenesis, also known as vitellogenesis, the oocyte grows dramatically as it takes up yolk, and follicle cells migrate to surround the growing oocyte. Shortly thereafter, nurse cells dump their contents into the oocyte, follicle cells create the vitelline membrane of the egg, and the mature egg is ovulated (McLaughlin and Bratu, 2015).

Oogenesis is an energy-intensive process, and it stands to reason that such an investment should be reserved for the production of high-quality eggs (Laws and Drummond-Barbosa, 2017). Poor nutrient conditions can trigger programmed cell death both in the germarium and during mid-oogenesis (Drummond-Barbosa and Spradling, 2001). This checkpoint activation leads to the cleavage and activation of the cell death effector caspase Dcp-1 and egg chamber degeneration (Peterson et al., 2003). Similarly, egg chamber abnormalities such as disrupted polarity (Beachum et al., 2021; Tanentzapf et al., 2000) or follicle cell death (Chao and Nagoshi, 1999) can trigger the mid-oogenesis checkpoint. Little is known about the mechanism of how these developmental events trigger the checkpoint at mid-oogenesis.

Here, we find that while Fra is expressed in both germline and somatic cells in the *Drosophila* ovary, it is required specifically in the germline for progression through mid-oogenesis. The starvation response in *fra* mutant germline cysts is unaffected, indicating that *fra* is unlikely to regulate the ovarian response to diet. Furthermore, both germline and follicle cell polarity appear to be intact in egg chambers with *fra* mutant germlines. Nevertheless, ovarioles containing these mutant egg chambers express activated Dcp-1 and initiate apoptosis. Thus, in contrast to vertebrate systems where Dcc promotes apoptosis in some contexts, our results indicate that Fra can play the opposite role to promote germline survival by negatively regulating apoptosis. Global

netrin mutants have morphologically normal ovaries, suggesting that Fra acts independently of Netrin in this context. Intriguingly, the transcriptional activation domain of Fra is required for egg chambers to progress through mid-oogenesis, providing *in vivo* evidence that Fra may act as a transcription factor outside of the nervous system. Together, this work reveals a critical Netrin-independent role for Fra in allowing progression through mid-oogenesis by preventing apoptosis and establishes the ovary as a system to investigate Fra signaling.

Results

Fra is expressed in the ovarian germline and the soma

To determine if and where Fra is expressed in the ovary, we took advantage of the *fra-MiMIC* allele from the MiMIC protein trap collection (Nagarkar-Jaiswal et al., 2015), which produces a GFP-tagged Fra from its endogenous locus. GFP-Fra is expressed throughout the ovariole, with higher expression in egg chambers that have bud from the germarium (Figure. 1B,B'). In the vitellarium, GFP-Fra is present on the membrane of somatic follicle cells, where it is enriched at the apical domain (Figure. 1B', arrowhead). We also detect GFP-Fra on both nurse cell (arrow) and oocyte membranes in the germline. In addition, GFP-Fra is present on F-actin enriched ring canals (asterisk), the intracellular bridges between syncytial germ cells. A similar expression pattern is seen with a c-terminal Fra antibody (Figure. 1C,C') (Kolodziej et al., 1996). To test the specificity of this antibody in the ovary, we generated genetic mosaic females and compared Fra expression in homozygous null clones to neighboring cells still expressing

Fra (Figure. 1E,F,G). As expected, GFP-negative cells, which are mutant for *fra*, are depleted of Fra (Figure. 1F).

Fra is required for oogenesis

We generated *fra* mosaic flies using the Flp-dominant female sterile technique to determine if there is an ovary-intrinsic role for Fra (Chou and Perrimon, 1996). Ovo is a transcription factor involved in female germline differentiation, and the ovo^{D} allele produces a dominant negative protein that causes germline degeneration early in oogenesis (Vazquez-Pianzola et al., 2011). We used a heat shock inducible flippase to induce recombination at FRT sites on chromosome 2R, where one chromosome carried the ovo^{D} allele, and the other carried either a wild-type or mutant *fra* allele. Since germline cells carrying ovo^{D} die early in oogenesis, we were able to compare control ovarioles with germlines that are nearly completely mutant for *fra*. We generated *fra* mutant germlines using three different alleles: two null alleles, fra^{3} and fra^{4}, and a hypomorphic allele, fra^{6} (Kolodziej et al., 1996; Yang et al., 2009). In control ovarioles, egg chambers bud from the germarium, grow progressively larger, and rarely degenerate (McLaughlin and Bratu, 2015) (Figure. 2A). In *fra* germline mutants, egg chambers appear morphologically normal prior to mid-oogenesis; however, a striking number of ovarioles contain degenerating egg chambers at the onset of mid-oogenesis (Figure. 2B-D). This degeneration is easily recognized by the presence of pyknotic nurse cell nuclei (Figure. 2B') and is accompanied by the apparent enlargement of some follicle cells (Figure. 2B''), suggesting they could be engulfing nurse cell debris (Etchegaray et al., 2012). Consistent with differences in Fra protein function in these

alleles, only 42% of *fra*^{6} ovarioles contain degenerating egg chambers, while *fra*^{3} and *fra*^{4} ovarioles exhibit 60.22% and 85.71% degeneration, respectively (Figure. 2C,D). Since both *fra*^{3} and *fra*^{4} are protein null alleles, the increased degeneration seen in *fra*^{4} is most likely due to a linked background mutation. These observations suggest that Fra is required in the germline for egg chambers to progress through mid-oogenesis.

Fra is cell-autonomously required in the germline for egg chamber survival

While *ovo*^{D} clones generate germlines almost entirely mutant for *fra*, this approach also creates undetectable follicle cell clones, albeit less frequently. Since Fra is expressed in both the soma and the germline, we investigated where Fra is required for egg chambers to progress through mid-oogenesis. Since all three *fra* alleles lead to degeneration with the *ovo*^{D} system, we selected one allele, *fra*^{3}, to continue our analysis. To determine whether Fra is required for oogenesis in the germline, follicle cells, or both, we generated negatively-marked homozygous *fra* clones, which we identified by the absence of GFP (Figure. 3A,C). We identified ovarioles containing GFP-negative clones (*fra* mutants) in either follicle cells or the germline and determined whether these ovarioles also contained degenerating egg chambers (Figure. 3A,C). We compared the rate of degeneration to control mosaic ovarioles, where all cells are wild-type at the *fra* locus. Since cell death leads to membrane perforation and the leaking out of cytoplasmic GFP, we could not definitively determine the GFP status of degenerating egg chambers. Therefore, we restricted our analysis to ovarioles that had germline or follicle cell clones in non-degenerating egg chambers. Consistent with our results using the *ovo*^{D} system, ovarioles with at least one GFP-negative *fra* mutant germline cyst contain more degenerating egg chambers than ovarioles with control cysts (Figure. 3A,B).

Furthermore, egg chamber degeneration in *fra* mutant mosaic germlines primarily occurs at mid-oogenesis (Table 1). To evaluate the contribution of follicle cells to this phenotype, we quantified degeneration in ovarioles with large follicle cell clones (>50% of each egg chamber). Similar to control ovarioles, fra^3 mosaic ovarioles with large follicle cell clones rarely contain degenerating egg chambers (Figure. 3C,D), suggesting that Fra is dispensable in follicle cells for progression through mid-oogenesis. To confirm that the degeneration in germline *fra* mutants is due to the loss of Fra, we used the germline-specific driver *nanos-GAL4* to express a full-length Fra transgene in *fra* mutant mosaic flies (Figure. 3E). As expected, germline expression of the full-length Fra receptor rescues the *fra* mutant degeneration phenotype (Figure. 3F). Thus, Fra is required specifically in the germline to promote egg chamber progression through mid-oogenesis.

Germline *fra* is not required for nutrient sensing and does not appear to impact polarity

In *fra* germline clones, degeneration occurs at mid-oogenesis. While a low level of egg chamber degeneration occurs stochastically at this checkpoint, flies subjected to specific stressors, including starvation and disruptions to egg chamber polarity, experience higher levels of degeneration (Beachum et al., 2021; Drummond-Barbosa and Spradling, 2001; Tanentzapf et al., 2000). Given their morphological similarities, we reasoned that Fra could be impinging on the ovarian response to diet. Alongside egg chamber degeneration at mid-oogenesis, starved flies exhibit a dramatic shift in the localization of the Insulin-responsive transcription factor Forkhead Box O (FoxO). Under well-fed conditions, *Drosophila* insulin-like peptides signal through the Insulin receptor,

leading to FoxO phosphorylation and sequestration in the cytoplasm (Manning and Toker, 2017; Nässel et al., 2015). When insulin signaling is low, FoxO is not phosphorylated and is transported into the nucleus, where it activates target genes (Nässel et al., 2015). Insulin signaling is required by the germline for egg chamber progression through mid-oogenesis (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005), and while FoxO is not an effector of insulin signaling in this context (LaFever et al., 2010), its re-localization is insulin-dependent. We tested whether the absence of *fra* could shift FoxO from the cytoplasm into the nucleus. However, we find that FoxO localization is unchanged in GFP-negative *fra* mutant germline cysts (Figure. 4A,B,C), indicating that insulin signaling is not compromised in these cells. To explore whether Fra controls a different aspect of the ovarian response to diet, we tested whether starvation could further increase degeneration in ovarioles with *fra* mutant germline cysts. When flies are starved or fed a protein-poor diet, egg chamber degeneration at mid-oogenesis increases (Drummond-Barbosa and Spradling, 2001; Shimada et al., 2011). If *fra* mutant germline cysts degenerate due to a failure to sense the nutrient environment, then starving these flies would not dramatically increase egg chamber degeneration. However, upon starvation, flies with *fra* mutant germlines have a drastic increase in degeneration, closely mirroring the response of flies with wild type germlines (Figure. 4D). Taken together, these results indicate that germline Fra is unlikely to be involved in nutrient sensing during oogenesis.

Polarity of both follicle cells and germline cysts determines the embryonic body plan (Merkle et al., 2020), and disruptions in the polarity of either follicle cells or germ cells can lead to an increase in egg chamber degeneration during oogenesis (Beachum et al., 2021; Tanentzapf et al., 2000). We evaluated *fra* germline clones for defects in germline and somatic polarity. Shortly after the formation of the sixteen cell cyst, Orb

accumulates in the oocyte, where its expression is maintained throughout oogenesis (Lantz et al., 1994). In the vitellarium, the oocyte is positioned at the posterior end of the egg chamber (Figure. 4E) (King, 1970). To evaluate germline cyst polarity, we monitored Orb and oocyte localization in GFP-negative cysts. Orb accumulates normally in *fra* germline cysts, and oocytes in mutant cysts are appropriately oriented at the posterior end of the egg chamber (Figure. 4F,G). Thus, Fra does not appear to control germline polarity preceding the checkpoint at mid-oogenesis.

While Fra is expressed robustly in follicle cells, it is not intrinsically required in the soma for germline cyst survival (Figure. 3C,D). We tested the possibility that Fra non-autonomously regulates apicobasal and lateral follicle cell polarity. Armadillo (Arm, Beta-catenin) is localized to the cell membrane of both follicle cells and germline cells and is enriched at the apical domain of follicle cells (Figure. 4E,H). Arm localization in follicle cells adjacent to *fra* germline cysts is indistinguishable from its localization in wild type ovarioles (Figure. 4I), suggesting that Fra does not regulate apicobasal follicle cell polarity non-autonomously. Similarly, Discs large (Dlg), which localizes to lateral domains of follicle cells (Goode and Perrimon, 1997), has an unchanged localization pattern in egg chambers with *fra* mutant germline cysts (4J). As expected, localization of both Arm and Dlg is unperturbed in *fra* mutant follicle cells (Figure. S1) While we cannot exclude the possibility that Fra controls other aspects of egg chamber polarity, the grossly normal morphology of *fra* germline mutants prior to degeneration suggests that any effects Fra has on polarity are subtle. Overall, the degeneration in *fra* mutants does not appear to be due to an activation of known triggers of the mid-oogenesis checkpoint.

Fra prevents apoptosis to promote progression through the mid-oogenesis checkpoint

How does *fra* germline degeneration compare to degeneration induced by poor nutrition and abnormal egg chambers? In nutrient-dependent egg chamber degeneration, follicle cells upregulate Draper and engulf the germline following nurse cell nuclei condensation and fragmentation (Etchegaray et al., 2012). Similarly, the follicle cells in degenerating egg chambers from ovarioles with *fra* germline cysts upregulate Draper (Figure. S2A,B), indicating that engulfment signaling in the follicle cells is active in degenerating egg chambers. Thus, while known upstream triggers of the mid-oogenesis checkpoint appear unaffected in *fra* mutants, degeneration is similar to that caused by the checkpoint's known triggers.

In contrast to Dcc, which promotes cell death in the absence of Netrin (Chen et al., 1999; Forcet et al., 2001; Mehlen et al., 1998), loss of *fra* from the germline results in egg chamber degeneration, suggesting that Fra has a pro-survival function. The cell death effector caspase Dcp-1 is required at mid-oogenesis for germline cell death in response to checkpoint activation (Peterson et al., 2003). We hypothesized that loss of germline Fra results in activated Dcp-1 expression and lead to apoptosis. While control cysts rarely express activated Dcp-1, we often detect it in late-stage *fra* germline mutant egg chambers and degenerating egg chambers in ovarioles with *fra* germline cysts (Figure. 5A,B). Furthermore, germline-specific expression of the baculovirus caspase inhibitor p35 (*nanos-Gal4>UASp-p35*) rescues the degeneration phenotype in ovarioles with *fra* germline clones (Figure. 5C,D), creating egg chambers with a persistent germline and missing follicle cells ("balding" egg chambers, Figure. 5C,E). Based on Dcp-1 staining and pyknotic nuclei, the follicle cells appear to be dying (not shown). This

is consistent with previous reports describing the effect of inhibiting caspases in the germline of starved flies (Mazzalupo and Cooley, 2006). Thus, in contrast to Dcc's role as a "dependence receptor," Fra has an anti-apoptotic role in the *Drosophila* female germline.

Because Fra prevents germline apoptosis, we explored the possibility that it regulates cell death more directly. Dcp-1 is inhibited by *Drosophila* Inhibitor of apoptotic protein 1 (Diap1) (Hawkins et al., 1999). Diap1 protein and mRNA are detected in egg chambers prior to mid-oogenesis; its expression decreases at mid-oogenesis, then increases again after stage eight (Baum et al., 2007; Foley and Cooley, 1998) (Figure. 5F). Germline overexpression of Diap1 suppresses Dcp-1-induced germline cell death at mid-oogenesis (Peterson et al., 2003). Similarly, over-expressing Diap1 in the germline is sufficient to prevent degeneration caused by starvation (Baum et al., 2007; Mazzalupo and Cooley, 2006). We hypothesized that if Fra were preventing Dcp-1 activation through its negative regulator Diap1, then *fra* germline cysts in younger egg chambers might have reduced Diap1 levels, causing increased degeneration at mid-oogenesis. We compared Diap1 expression in GFP-negative *fra* germline cysts to neighboring GFP-positive control germline cysts and detected no differences in Diap1 levels (Figure. 5G,H). Thus, if Fra interacts with cell death machinery, it does not do so by regulating Diap1 levels.

Fra acts independently of Netrin in the ovary to promote germline survival

In the *Drosophila* nervous system, Fra signals through both Netrin-independent and -dependent mechanisms (Boyer and Gupton, 2018; Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009). Since germline Fra is required during oogenesis, we asked

whether Netrin is also present in the ovary. In the developing nervous system, the two *Drosophila netrin* genes, *netrinA* and *netrinB,* have overlapping expression domains and can function interchangeably to control axon guidance (Harris et al., 1996; Mitchell et al., 1996). We first evaluated Netrin expression with the *netrinA-MiMIC* allele from the MiMIC protein trap collection (Nagarkar-Jaiswal et al., 2015), which produces NetrinA-GFP from the endogenous locus. Consistent with a recent report (Tu et al., 2020), we detect NetrinA-GFP in a subset of escort cells, somatic cells that support germline cyst development in the germarium (Figure. 6A). We do not detect NetrinA outside of the germarium. In contrast, in flies expressing NetrinB-Myc from its endogenous locus (Brankatschk and Dickson, 2006), we detect Myc signal throughout the ovariole (Figure. 6B,B'). Thus, while NetrinA is unlikely to signal through Fra in the vitellarium, the NetrinB expression pattern is consistent with such a role at mid-oogenesis.

To determine whether Fra function in the ovary is dependent on Netrin, we tested whether Netrin is required in the ovary. Female flies homozygous for a small deletion removing both *netrinA* and *netrinB* (*netrinAB*$^{\Delta GN}$, Brankatschk and Dickson, 2006; Newquist et al., 2013) survive to adulthood at low frequency, and we used these "escapers" to examine the effect of global Netrin removal on oogenesis. A previous study found that *netrinAB*$^{\Delta GN}$ escaper females lay fewer eggs than control flies; however, no defects were observed in ovary morphology (Newquist et al., 2013). Consistent with these results, ovarioles from *netrinAB*$^{\Delta GN}$ escapers appear morphologically indistinguishable from control ovarioles (Figure. 6C). Specifically, egg chambers progress through mid-oogenesis normally, and sibling heterozygote controls and *netrinAB*$^{\Delta GN}$ mutants have similar rates of egg chamber degeneration (Figure. 6D). This demonstrates that Netrin is dispensable for progression of egg chambers through mid-oogenesis and that the role of Fra in this process must be Netrin-independent.

Fra's transcriptional activation domain is required in the germline for egg chamber survival

Given that Netrin is dispensable for egg chamber progression through mid-oogenesis, how is Fra signaling in this context? Fra has a Netrin-independent function in the embryonic nerve cord, where it activates transcription to regulate axon guidance (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009). To activate transcription, Fra must be proteolytically processed by gamma secretase, which releases the Fra ICD from the cell membrane and allows it to enter the nucleus (Neuhaus-Follini and Bashaw, 2015). Once there, the Fra ICD activates transcription of *comm* (Neuhaus-Follini and Bashaw, 2015), whose protein product downregulates the expression of the repulsive guidance receptor Robo1 (Keleman et al., 2005, 2002).

Since Fra functions independently of Netrin in the ovary, we considered the possibility that Fra regulates transcription in this context. Previously, the Fra ICD (*UAS-FraICDMyc*) and a transgene with a point mutation that inactivates Fra's transcriptional activation domain (*UAS-HAFraE1354A*) were used to rescue *fra* mutant phenotypes in the embryonic nervous system (Neuhaus-Follini and Bashaw, 2015). While neuronal expression of *UAS-FraICDMyc* rescued Fra's transcriptional regulation of *comm*, *UAS-HAFraE1354A* did not, demonstrating that Fra's activation domain is required in that context. To test the possibility of a similar mechanism operating in the germline, we cloned the *FraICDMyc* and *HAFraE1354A* constructs into the germline optimized *pUASp* vector (Rørth, 1998). Importantly, all transgenes were inserted at the same location, and when overexpressed using the germline-specific driver *nanos-GAL4* in wild-type flies, none changed the rate of degeneration at mid-oogenesis (Table 2). We then tested the

ability of each Fra variant to rescue degeneration in ovarioles containing *fra* mutant germline cysts and compared the level of rescue to that of the full-length Fra receptor (Figure. 3). While the full-length Fra receptor is able to rescue degeneration in ovarioles with *fra* mutant germline cysts, *UASp-FraE1354A* fails to rescue this degeneration (Figure. 7A,D). Since this E1354A point mutation disrupts the transcriptional activation domain in Fra without disrupting the nuclear export signal or Netrin-dependent Fra signaling (Neuhaus-Follini and Bashaw, 2015), we hypothesized that Fra's transcriptional activation domain is specifically required for its function in the ovary. Indeed, a version of this transgene with an added VP16 activation domain (*UASp-FraE1354A-VP16*; Neuhaus-Follini and Bashaw, 2015) rescues degeneration in ovarioles with *fra* germline clones, consistent with the model that Fra's transcriptional activation domain is necessary for its anti-apoptotic role in the ovary (Figure. 7B,E). Surprisingly, the Fra ICD alone fails to rescue degeneration in *fra* germline clones (Figure. 7C,D), even though it is sufficient to rescue Fra's transcriptional function in the nerve cord (Neuhaus-Follini and Bashaw, 2015). One possibility is that the full-length receptor contains interaction domains that, while dispensable for nervous system function, are necessary for Fra function in the germline. Alternatively, the levels of ICD expression achieved using the *nanos-GAL4* element may not be sufficient to rescue the germline phenotype. Nevertheless, the failure of *UASp-FraE1354A* to rescue degeneration in ovarioles with *fra* mutant germline clones and the rescue provided by *UASp-FraE1354A-VP16*, suggests that the transcriptional activation domain in Fra is required to promote germline survival.

While Fra most likely activates the transcription of multiple genes, *comm* is the only target that is currently known (Neuhaus-Follini and Bashaw, 2015). To determine whether *comm* is expressed in the ovary, we conducted reverse transcription

polymerase chain reaction (RT-PCR) using two different sets of primers directed against *comm* cDNA on mRNA extracted from both *Drosophila* ovaries and embryos. While we detect *comm* mRNA in the ovary with this method, we were unable to determine its expression pattern (Figure. S3A). To identify *comm*-expressing cells, we used small molecule fluorescence *in situ* hybridization (smFISH) (Little and Gregor, 2018). Unexpectedly, we do not detect endogenous *comm* mRNA (Figure. S3B). A positive control, in which we induce transgenic Comm expression in follicle cells using *traffic jam-GAL4*, demonstrated that our probe can detect *comm* mRNA (Figure. S3C), suggesting that *comm* is either not expressed in the ovary, or is expressed at levels below our threshold of detection. Indeed, a recently published RNA-seq study detected *comm* mRNA at very low levels in certain follicle cells in the ovary, with no detection in germline cells (Jevitt et al., 2020). While these observations do not support a germline role for Comm, it remains possible that low-level expression is functionally relevant for oogenesis. To further evaluate potential expression and function of *comm* in the germline, we used two approaches that have revealed functional connections between *fra* and *comm* during axon guidance. First, we tested whether mis-expression of Fra could induce *comm* transcription. Overexpressing Fra in the germline is unable to induce *comm* expression (Figure. S3D). Moreover, when we compared *fra*/+; *comm*/+ female flies to their sibling controls, we did not observe a significant increase in egg chamber degeneration. Taken together, our observations indicate that Fra is unlikely to be regulating *comm* in the ovary. Nevertheless, the clear requirement for the Fra transcriptional activation domain to promote germline survival suggests that Fra is regulating the transcription of key target genes in the germline.

Discussion

In this paper, we explore the role of Fra in the ovary and demonstrate that Fra intrinsically promotes germline survival independently of Netrin. Fra is expressed on the cell membrane of nurse cells, oocytes, and follicle cells. Loss of germline, but not follicle cell, *fra* causes a significant increase in egg chamber degeneration at mid-oogenesis. Degeneration at mid-oogenesis is often caused by starvation or disruptions in egg chamber polarity (Beachum et al., 2021; Drummond-Barbosa and Spradling, 2001; Tanentzapf et al., 2000), and *fra* germline degeneration shares the morphological hallmarks of these pathways. However, *fra* mutants do not alter the starvation-induced degeneration response or FoxO localization, suggesting that Fra is not involved in nutrient sensing. Furthermore, Orb, Armadillo, and Discs large are localized normally in egg chambers with *fra* mutant germline cysts, indicating that loss of *fra* is not likely to affect germline polarity or the apicobasal or lateral polarity of follicle cells. Analysis of apoptotic markers in *fra* mutant germlines reveals a striking elevation of Dcp-1 expression, suggesting that the degeneration observed at mid-oogenesis is triggered by the activation of the Dcp-1 caspase. Accordingly, the expression of a *UASp-p35* transgene, which acts specifically to inhibit apoptosis, results in a robust rescue of the *fra* mutant degeneration phenotype. In the nervous system, Fra functions through both Netrin-dependent and -independent mechanisms. Interestingly, ovarioles from *netrinAB*$^{\Delta GN}$ mutants appear morphologically normal and do not degenerate at mid-oogenesis, indicating that Fra functions independently of its canonical ligand Netrin in this process. Consistent with a Netrin-independent role for Fra in the ovary, rescue experiments demonstrate that Fra's transcriptional activation domain is required for germline cyst survival at mid-oogenesis. Together, our results demonstrate that Fra is

required in the germline, independently of Netrin, to promote egg chamber progression through mid-oogenesis. We have established the ovary as a novel tissue context to further investigate Fra's Netrin-independent activity.

Fra functions independently of known regulators of the mid-oogenesis checkpoint

At mid-oogenesis, both external and internal factors can activate a checkpoint that leads to cell death. Since the late stages of oogenesis require significant energy input, this checkpoint may prevent a costly investment in a low-quality oocyte, which would be detrimental to both the female and her offspring (Drummond-Barbosa and Spradling, 2001; Mazzalupo and Cooley, 2006; Pritchett et al., 2009). Starvation (Buszczak et al., 2002; Drummond-Barbosa and Spradling, 2001; Terashima and Bownes, 2006) and disruption to egg chamber polarity (Beachum et al., 2021; Tanentzapf et al., 2000) trigger the mid-oogenesis checkpoint. When wild-type flies are starved or fed a protein-poor diet, degeneration at mid-oogenesis (also described as a "block to vitellogenesis") increases dramatically (Drummond-Barbosa and Spradling, 2001). While nutrient-dependent degeneration is morphologically similar to the *fra* mutant germline phenotype, FoxO is primarily localized in the cytoplasm in *fra* germline mutants, indicating that insulin signaling is functioning in these cells. In addition, when flies with *fra* mutant germlines are starved, egg chamber degeneration at mid-oogenesis increases compared to well-fed counterparts. This further increase in degeneration suggests that *fra* germline cysts are still competent to respond to dietary signals. Taken together, these observations indicate that Fra is unlikely to be involved in the ovarian response to diet.

Disruptions in egg chamber polarity can also increase degeneration at mid-oogenesis (Beachum et al., 2021; Tanentzapf et al., 2000). However, Orb, Armadillo, and Discs large localization in and adjacent to *fra* mutant clones indicates that neither germline polarity nor apicobasal or lateral follicle cell polarity are controlled by Fra. It remains possible that other aspects of egg chamber polarity are affected in *fra* germline cysts. Based on the absence of diet-related phenotypes and the normal morphology of egg chambers prior to degeneration, it is unclear why *fra* mutant germline cysts undergo apoptosis. A better understanding of downstream Fra signaling in the ovary will give insights into Fra's functions here. Furthermore, Netrin is expressed in the germarium, and appears to be important for germline stem cell maintenance at the niche (Tu et al., 2020). It would be interesting to see if Fra is also required for this process and functions with Netrin in the germarium.

Fra is anti-apoptotic in the ovary

In the developing enteric nervous system, as well as the nervous system across multiple species, Dcc can act as a "dependence receptor" (Castets et al., 2012; Furne et al., 2008; Jasmin et al., 2021). Limiting Netrin, either *in vitro* by its absence in the serum, or *in vivo* through conditional knockouts, prevents Netrin from interacting with Dcc. This ultimately leads to caspase-mediated cell death in many contexts, including the nervous system and in both human embryonic kidney and cancer cell lines (Forcet et al., 2001; Goldschneider and Mehlen, 2010; Mehlen et al., 1998; Mehlen and Mazelin, 2003). Whether this function is only active in select cells, and whether the homolog of Dcc in other organisms can also act in a similar way, is unknown.

In contrast to the pro-apoptotic role of Dcc in some tissues, Fra has an anti-apoptotic role in the *Drosophila* ovarian germline. We find that removing Netrin has no effect on egg chamber degeneration. However, loss of Fra causes an increase in egg chamber degeneration and a concomitant increase in ovarioles with Dcp-1 positive egg chambers. Future studies should address whether Fra is a substrate for caspase cleavage and how Fra/Dcc can have both pro and anti-apoptotic activity. Indeed, it is unclear whether the mechanism through which Fra prevents apoptosis in this context bears any similarity to that in which Dcc engages the caspase signaling pathway to promote cell death in vertebrate systems. Interestingly, although the precise Caspase3 cleavage site in Dcc is not conserved in Fra, the Fra ICD does undergo multiple cleavage events to generate fragments that are similar in size to Dcc ICD fragments (Neuhaus-Follini and Bashaw, 2015; Taniguchi et al., 2003). One intriguing possibility is that the Fra ICD may interact directly with Dcp-1 to prevent its activation.

Netrin-independent Fra transcriptional regulation

Netrin is required for fecundity in *Drosophila*, and global *netrinAB*$^{\Delta GN}$ mutants lay fewer eggs than wild type controls (Newquist et al., 2013). We observe no defects in the morphology of *netrinAB*$^{\Delta GN}$ mutant ovaries, and, in contrast to flies with *fra* germline clones, we do not observe changes in egg chamber survival. Global removal of Netrin is likely to affect multiple tissues in adult flies, including the nervous system, and reproduction is sensitive to organismal physiology (Laws and Drummond-Barbosa, 2017). Nevertheless, the absence of egg chamber degeneration in global *netrinAB*$^{\Delta GN}$ mutants indicates that Fra acts independently of Netrin to promote germline survival.

We have previously shown that in addition to its Netrin-dependent role in axon guidance, Fra signals independently of Netrin in the nerve cord to transcriptionally activate *comm* (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009). Consistent with this Netrin-independent mode of signaling, we find that Fra's transcriptional activation domain is required for the progression of egg chambers through mid-oogenesis. Unlike the embryonic nervous system, where the Fra ICD partially rescues *fra* mutant phenotypes (Neuhaus-Follini and Bashaw, 2015), expression of the Fra ICD in the germline fails to rescue the *fra* mutant oogenesis phenotype. This difference may reflect different requirements for Fra in these two tissue contexts. One possibility is that germline Fra binds a co-activator at the cell membrane, facilitating its transport to the nucleus following gamma-secretase cleavage. Alternatively, the failure to rescue may reflect a technical limitation due to insufficient expression levels of the Fra ICD in these experiments. In the nervous system, gain of function effects of ICD expression are dose dependent, and multiple copies of the transgene are required to generate robust phenotypes. Transcriptional signaling requires the Fra ICD to translocate to the nucleus (Neuhaus-Follini and Bashaw, 2015). While we are unable to detect the Fra ICD in the nucleus, this does not rule out the possibility that it is entering the nucleus to regulate transcription. Indeed, in the nerve cord, nuclear Fra ICD is only detected occasionally when *UAS-FraICDMyc* is overexpressed in neurons, and detected more often when the Fra ICD's nuclear export signal is also removed (Neuhaus-Follini and Bashaw, 2015).

Currently, Fra's only known transcriptional target is *comm* (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009), and *comm* does not appear to be expressed in the germline. Furthermore, germline-specific expression of Fra using *nanos-GAL4* does not induce *comm* mRNA expression, suggesting that *comm* is not a transcriptional target of Fra in these cells. Since the transcriptional activation domain is required for Fra to

promote germline survival, this indicates that Fra has other transcriptional targets that are necessary for preventing apoptosis in the germline. Indeed, the Neogenin ICD binds upstream of several genes and regulates their transcription *in vitro* in human embryonic kidney cells (Goldschneider et al., 2008). Future studies should determine other transcriptional targets of Fra.

Our results establish the ovary as a second *in vivo* tissue context where Fra regulates transcription. In the nervous system, Fra functions both via cytoskeletal modifications and transcriptional regulation (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009; Zang et al., 2021). However, teasing apart the different functions of Fra is challenging: both occur in the same cells and depend on the conserved P3 motif within the Fra ICD (Garbe et al., 2007; Neuhaus-Follini and Bashaw, 2015). We find that Fra functions independently of Netrin and requires its transcriptional activation domain to promote egg chamber survival through mid-oogenesis. This provides a complementary, tractable system to specifically study how Fra regulates transcription, and to identify the upstream and downstream components involved in this signaling pathway.

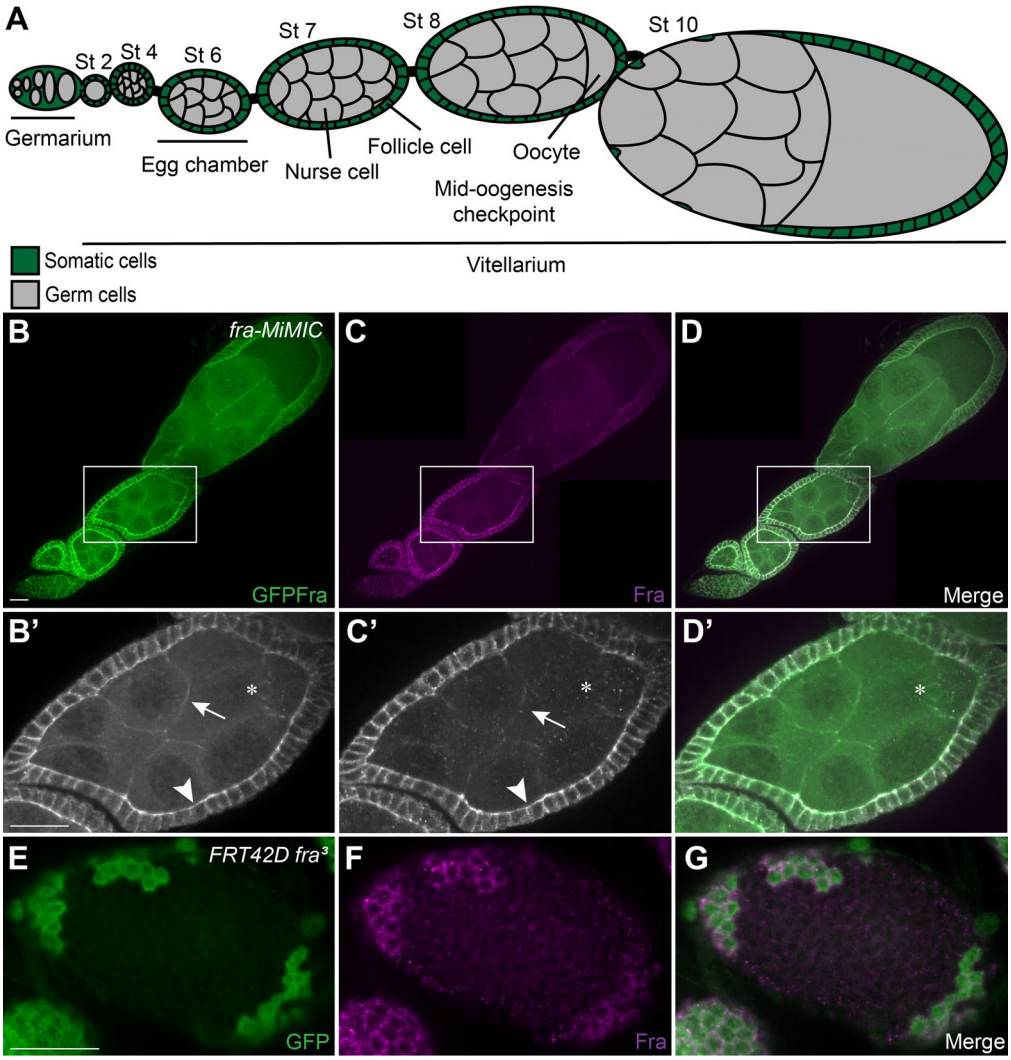

Figure 2. 1. **Fra localizes to the cell membrane of both follicle and germ cells in the Drosophila ovary**.

(A) Schematic of an ovariole with the germarium at the anterior and multiple egg chamber stages, each completely encapsulated by a single layer of follicle cells. Mid-oogenesis starts at stage eight, when the oocyte begins to take up yolk. (B-D) Single channel images of a fra-MiMIC ovariole stained for (B) GFP (GFP-Fra, green), (C) Fra (magenta), along with the merged image (D). (B'-D') Insets of indicated egg chamber from B, C, D, respectively. Arrows indicate Fra on nurse cell membranes, and

arrowheads indicate Fra enrichment on the apical side of follicle cell membranes. Asterisks mark Fra localized to a ring canal. (E-G) Single channel images of an ovariole with fra^3 clones, where the GFP+ cells are wild-type and GFP- cells are mutant for fra, stained for (E) GFP (green), (F) Fra (magenta), along with the merged image (G). Scale bars are 20 microns.

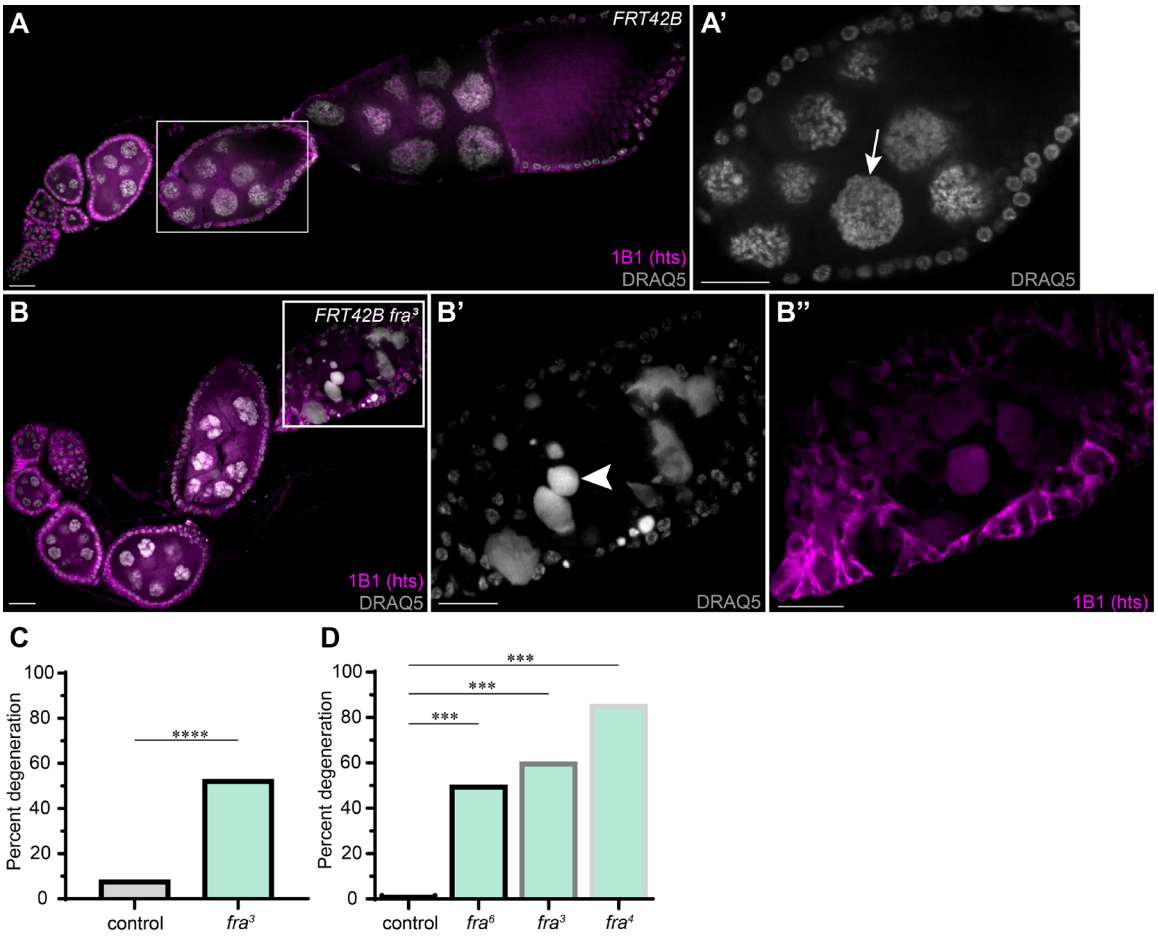

Figure 2. 2. **Fra is required for germline survival in the ovary.**

(A) Wild type ovariole from ovo^D control flies stained for 1B1 (magenta) to mark cell membranes and DRAQ5 (grey) to mark nuclei. (A') DRAQ5 channel of boxed region in

A. Arrow indicates a healthy nurse cell nucleus. (B) Ovariole with a fra^3 mutant germline. (B') DRAQ5 channel of boxed region in B. The arrowhead indicates a pyknotic nurse cell nucleus. (B'') 1B1 channel of boxed region in B illustration morphological changes to follicle cell membrane. (C) Percentage of ovarioles containing a degenerating egg chamber in ovarioles with a wild-type germline versus a fra^3 mutant germline. N=122, 36 ovarioles from one trial. (D) Percentage of ovarioles containing a degenerating egg chamber in ovarioles with a wild-type germline versus fra^3, fra^4, or fra^6 mutant germlines. N=26, 50, 93, 21 ovarioles from one trial. Statistical significance determined by Fisher's exact test and p-value adjusted using Bonferroni-Dunn for multiple comparisons, ***= p-value <0.0003. Scale bars are 20 microns.

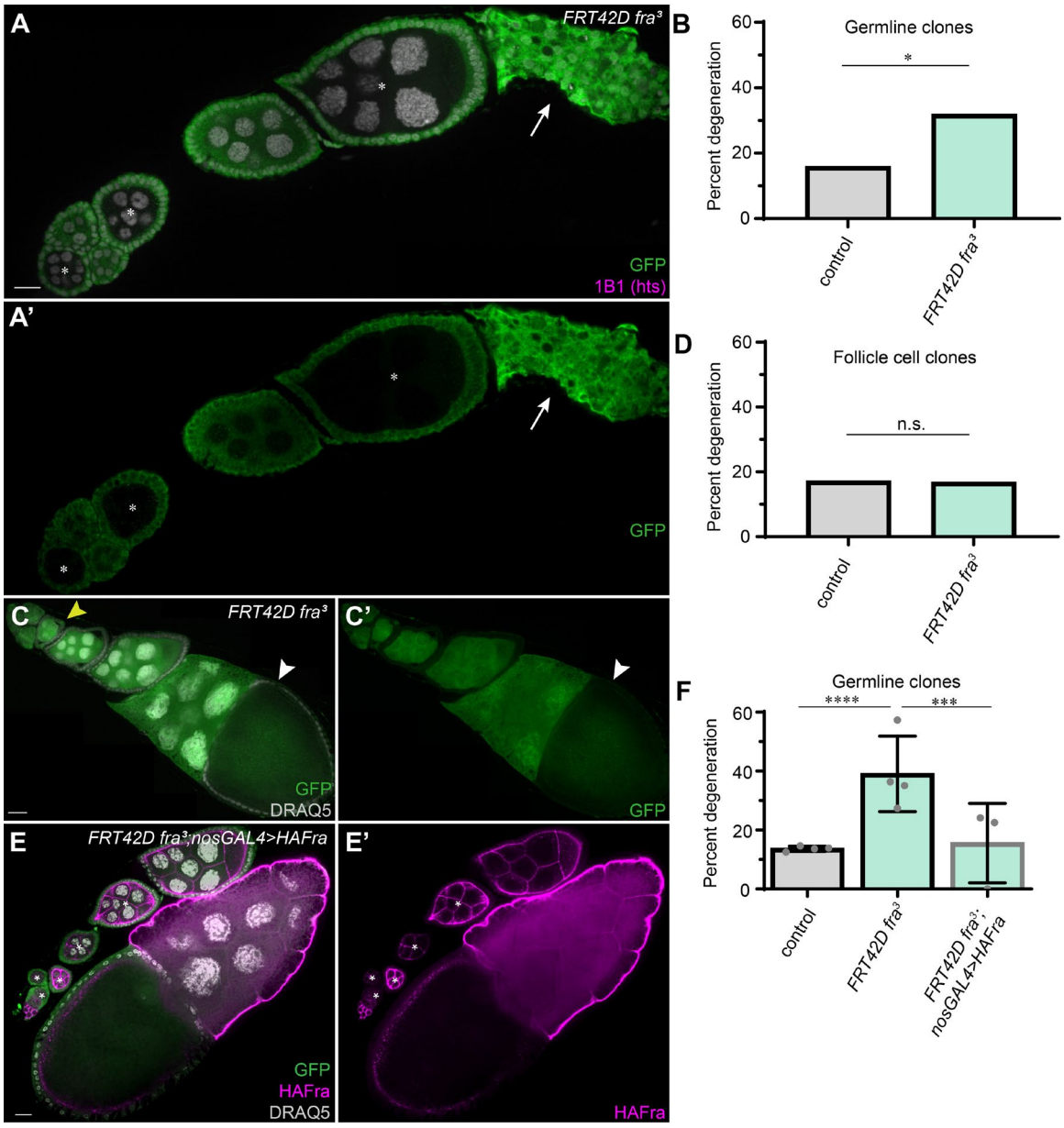

Figure 2. 3. **Fra is required in the germline for egg chambers to progress through mid-oogenesis**.

(A) Ovariole stained for GFP (green) and 1B1 (cell membranes; magenta) with fra^3 germline clones (GFP-, white asterisks). A degenerating egg chamber is at the posterior end of the ovariole (white arrow). (A') GFP channel from A. (B) Percentage of ovarioles containing a degenerating egg chamber out of all ovarioles with at least one GFP-

germline cyst. N=82, 63 ovarioles from one trial. (C) Ovariole stained for GFP (green) and 1B1 (cell membranes; magenta) with fra^3 mutant follicle cells (GFP-, white arrowhead) and few wild-type follicle cells (GFP+, yellow arrowhead). (C') GFP channel from C. (D) Percentage of ovarioles containing a degenerating egg chamber in ovarioles with large GFP- follicle cell clones (more than 50% GFP- follicle cells in all egg chambers). N=44, 15 ovarioles from two independent trials. (E) Ovariole with fra^3 germline clones (GFP-, white asterisks) expressing full-length Fra tagged with HA (magenta) in the germline. DRAQ5 marks nuclei (grey). (E') HA channel from E. (F) Percentage of ovarioles containing a degenerating egg chamber. This graph also appears in Fig 7C with additional genotypes that were tested simultaneously using the same controls. N=208, 260, 116 ovarioles from at least three independent trials. Statistical significance determined by Fisher's exact test and p-values were adjusted using Bonferroni-Dunn for multiple comparisons, *= p-value <0.05, ***= p-value= 0.0003, ****= p-value <0.0001. Error bars represent s.d. Scale bars are 20 microns.

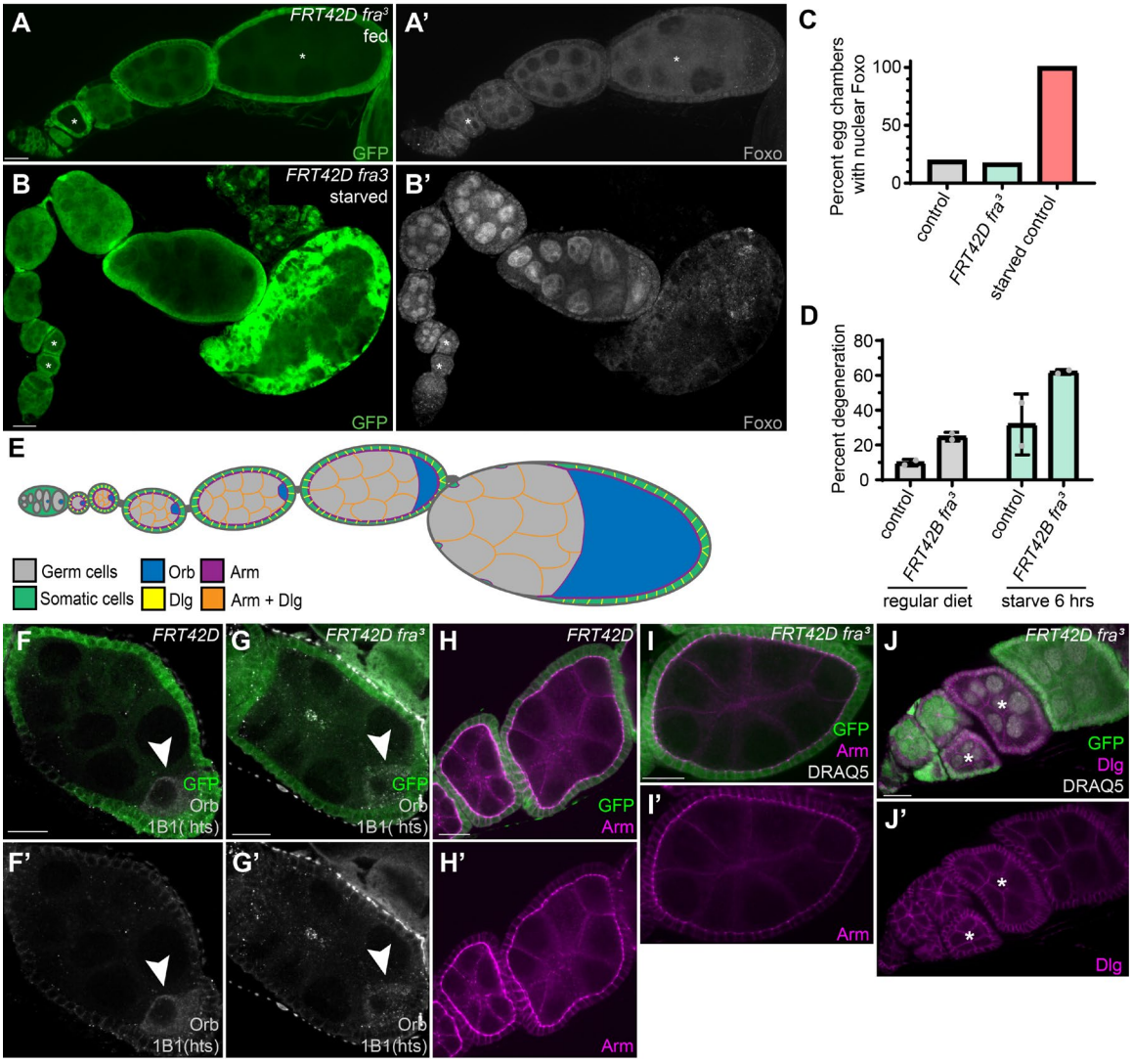

Figure 2. 4. **Loss of fra does not affect the ovarian response to diet or the polarity markers Orb, Armadillo, and Discs large**.

(A) Ovariole from a fed fly with negatively marked fra^3 germline clones (GFP-, white asterisks) stained for GFP (green) and (A') Foxo (grey) (B) Ovariole from a starved fly with negatively marked fra^3 germline clones (GFP-, white asterisks) stained for GFP (green) and (B') Foxo (grey). (C) Percentage of egg chambers with negatively-marked germline clones that had Foxo localized to the nurse cell nuclei. N= 47, 18, 19 egg chambers. (D) Percentage of control and fra^3 germline mutant ovarioles containing a

degenerating egg chamber. Flies were either fed a regular diet or starved for 6 hours preceding dissection. Ovarioles with fra^3 germlines still respond to diet. N=234, 137, 229, 159 ovarioles from two independent trials. (E) Schematic depicting normal localization of Orb, Arm, and Dlg in an ovariole. (F and G) Egg chamber with either a negatively-marked control germline clone (F, F') or a negatively marked fra^3 germline clone (G, G') stained for GFP (green), Orb and 1B1 (both grey). Orb is localized to the oocyte in both egg chambers (arrowhead). (H and I) Egg chambers with either negatively-marked control germline clones (H, H') or a negatively-marked fra^3 germline clone (I, I') stained for Armadillo (magenta) and GFP (green). (J,J') Ovariole containing negatively-marked fra^3 germline clones (white asterisks) stained for Discs large (magenta), GFP (green), and DRAQ5 (grey). Error bars represent s.d. Scale bars are 20 microns.

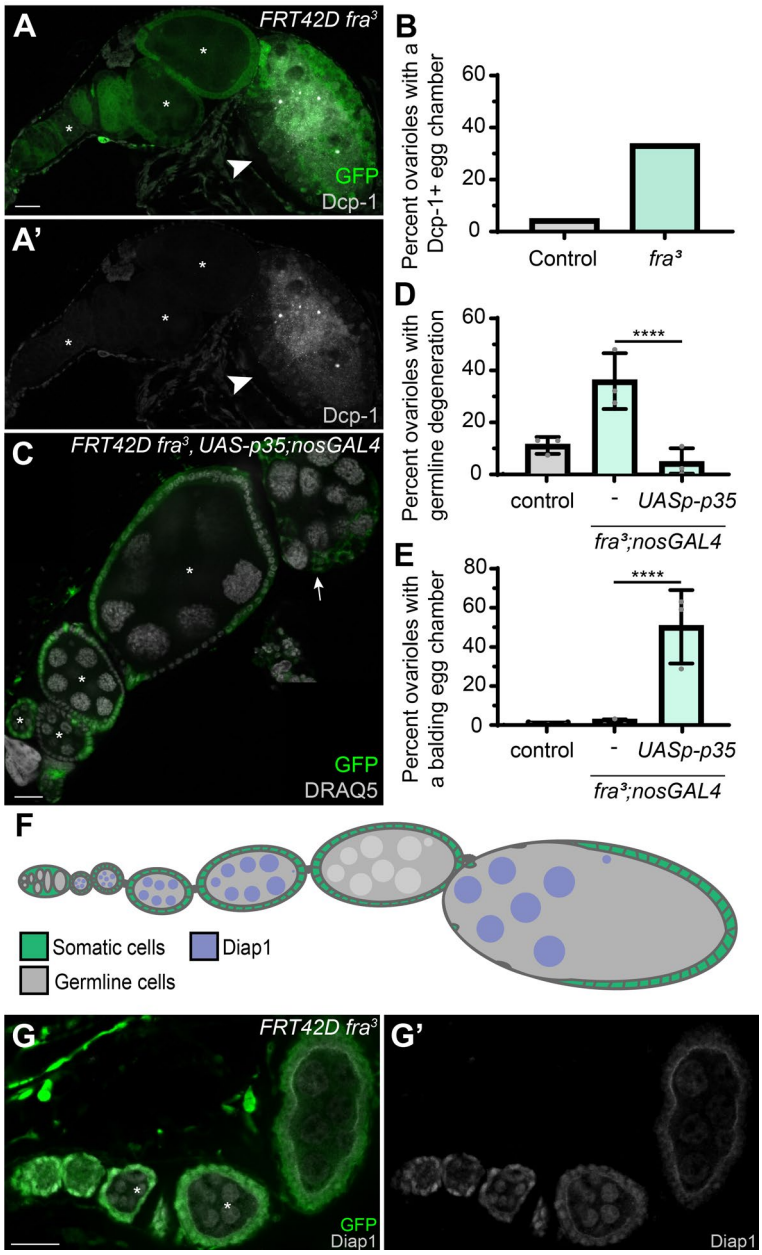

Figure 2. 5. **Fra is required in the germline to prevent apoptosis**.

(A) Ovariole with fra^3 germline clones (GFP-, white asterisks) stained for GFP (green) and activated Dcp-1 (grey). Ovariole contains a degenerating egg chamber with Dcp-1 expression (white arrowhead). (A') Dcp-1 channel from A. (B) Percentage of ovarioles with a Dcp-1 positive egg chamber. N=22, 33 ovarioles from a single experiment. (C)

Ovariole with fra^3 germline cysts (GFP-, white asterisks) and germline specific p35 to inhibit caspases stained for GFP (green) and DRAQ5 (grey). Arrow indicates follicle cell death. (D-E) Percentage of ovarioles containing germline clones with germline degeneration (D) and balding egg chambers (E) when caspases are inhibited in fra^3 germline cysts. For both graphs N=226, 164, 192 ovarioles scored across three independent trials. Error bars represent s.d. Statistical significance determined by Fisher's exact test, ****= p-value <0.0001. (F) Schematic depicting Diap1 expression in a wild type ovariole. (G, G') ovariole with fra^3 germline cysts (GFP-, white asterisks) wild-type germline cysts (GFP+) stained for GFP (G, green) and Diap1 (G and G', grey). Diap1 is not prematurely downregulated in fra^3 germline clones. Image is representative out of 44 ovarioles. Scale bars are 20 microns.

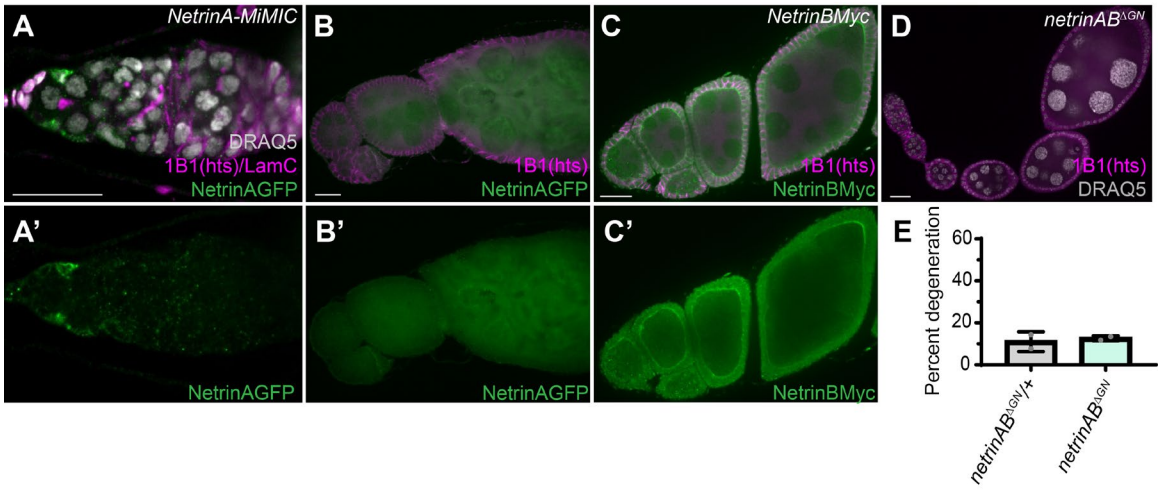

Figure 2. 6. **Netrin is expressed in the ovariole but not required for egg chambers to progress through mid-oogenesis**.

(A) NetrinA-MiMIC germarium stained for GFP (NetrinA-GFP, green) and 1B1/LamC (cell membranes and cap cell nuclear envelopes, magenta). (A') GFP channel from A.

(B) NetrinBMyc ovariole stained for Myc (green) and 1B1 (magenta). (B') Myc channel from A. (C) Ovariole from a netrinAB$^{\Delta GN}$ escaper stained with 1B1 (cell membranes; magenta) and DRAQ5 (nuclei, green) (D) Percentage of netrinAB$^{\Delta GN}$ ovarioles containing a degenerating egg chamber compared to sibling heterozygotes. N= 280, 193 ovarioles from two independent trials. Error bars represent s.d. Scale bars are 20 microns.

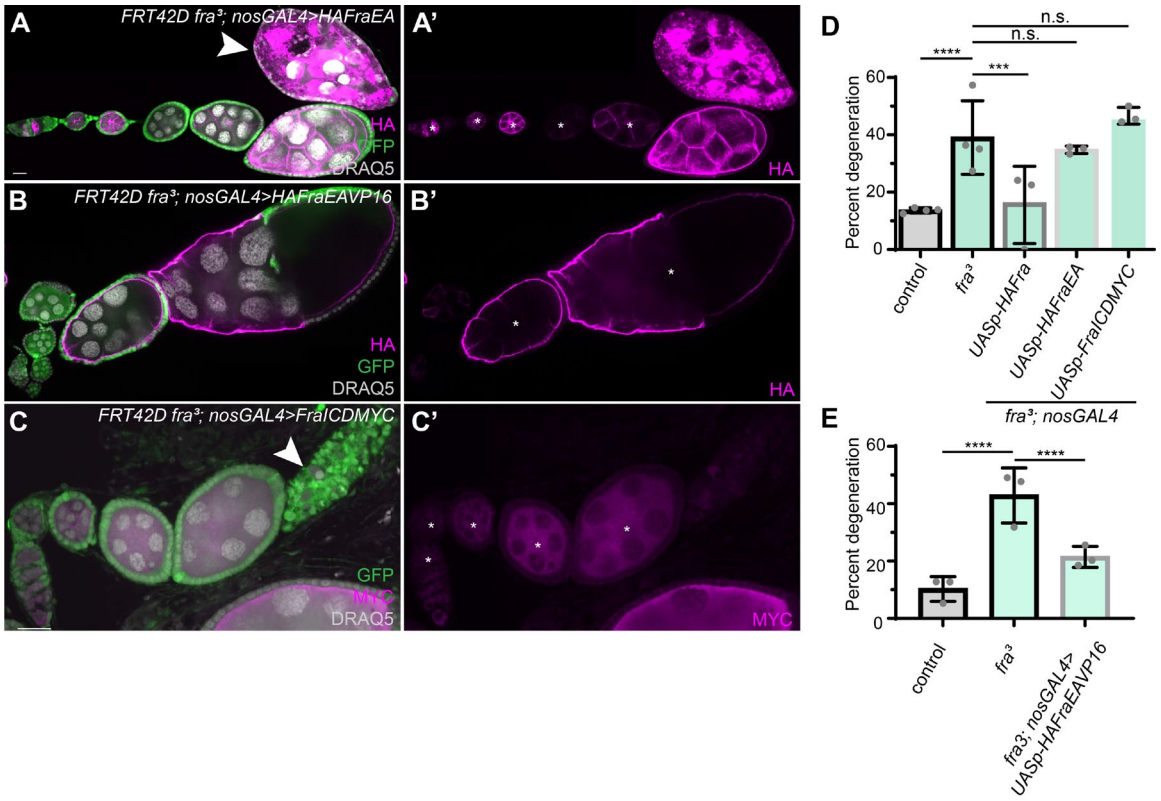

Figure 2. 7. **Fra's transcriptional activation domain is required for egg chamber progression through mid-oogenesis.**

(A-C) Ovarioles with fra3 germline clones (GFP-, white asterisks) and (A) HAFraE1354A, (B) HAFraE1354A-VP16, or (C) FraICDMYC driven by nanos-GAL4. Ovarioles stained for GFP (green), HA or MYC (tagged transgenes, magenta), and DRAQ5 (nuclei, white).

Arrowhead indicates degenerating egg chamber. (A'-C') HA (A',B') or MYC (C') from panels A-C. (D-E) Graphs showing the percentage of ovarioles containing a degenerating egg chamber in ovarioles containing fra3 mutant germline cysts. (D) HAFraE1354A and FraICDMYC are unable to rescue egg chamber degeneration. The first three genotypes of this graph also appear in Figure. 3F as these were tested simultaneously and use the same controls. N=208, 260, 116, 123, 80 ovarioles across at least three trials for each genotype. (E) Degeneration in ovarioles containing fra3 germline clones is rescued by HAFraE1354A-VP16. N=326, 184, 224 ovarioles across three independent trails. Statistical significance determined by Fisher's exact test and p-values were adjusted by Bonferroni-Dunn for multiple comparisons, ***= p-value= 0.0003, ****= p-value <0.0001. Error bars represent s.d. Scale bars are 20 microns.

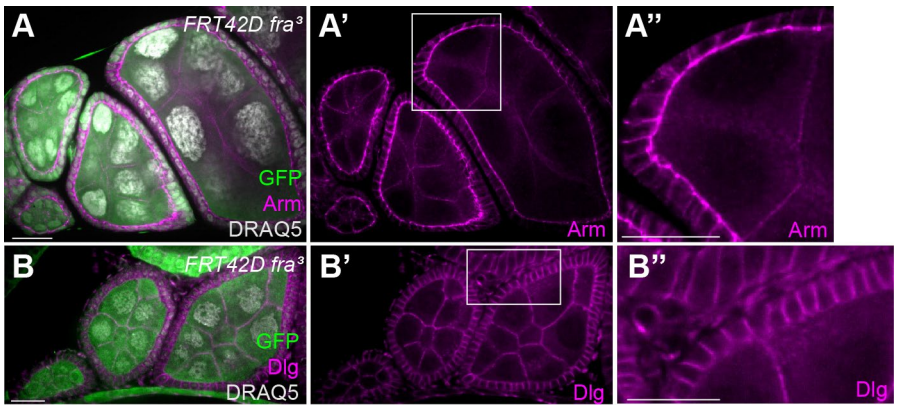

Figure 2. S 1. **Armadillo and Discs large do not appear to be affected by loss of fra from the germline or follicle cells.**

(A) Egg chambers that have wild-type germline cysts (GFP+) and some fra mutant follicle cells (GFP-) stained for GFP (green), Arm (magenta), and DRAQ5 (grey). (A') Arm channel from A. (A'') inset from A' with both GFP+ and GFP- follicle cells. (B) Egg

chambers with wild-type cysts (GFP+) and fra mutant follicle cells (GFP-) stained for GFP (green), Discs large (magenta), and DRAQ5 (grey). (C') Discs large channel from C. (C'') inset from C' with both GFP+ and GFP- follicle cells. Scale bars are 20 microns.

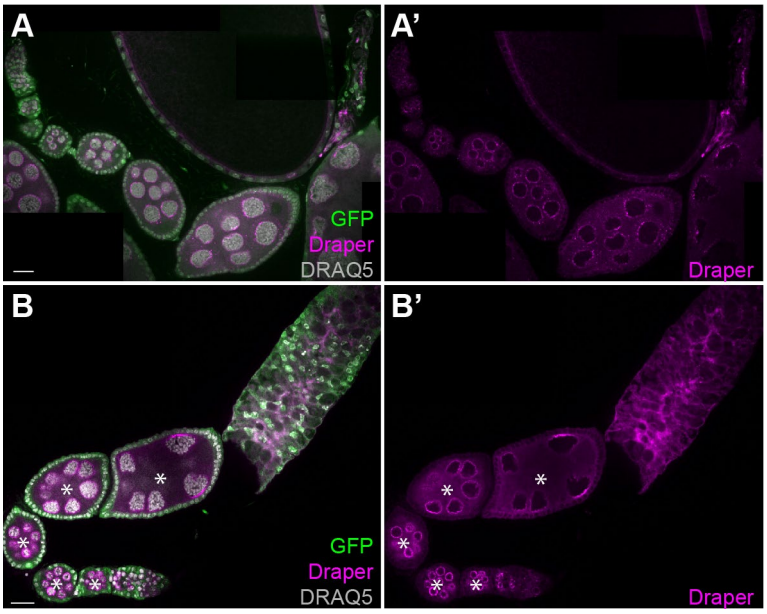

Figure 2. S 2. **Draper is expressed in dying egg chambers.**

(A) Wild type ovariole with a degenerating egg chamber stained for GFP (green), Draper (magenta), and DRAQ5 (grey). (B') Draper channel from A. (B) Ovariole with fra mutant germline cysts (GFP-, white asterisks) stained for GFP (green), Draper (magenta), and DRAQ5 (grey). (B') Draper channel from B. Scale bars are 20 microns.

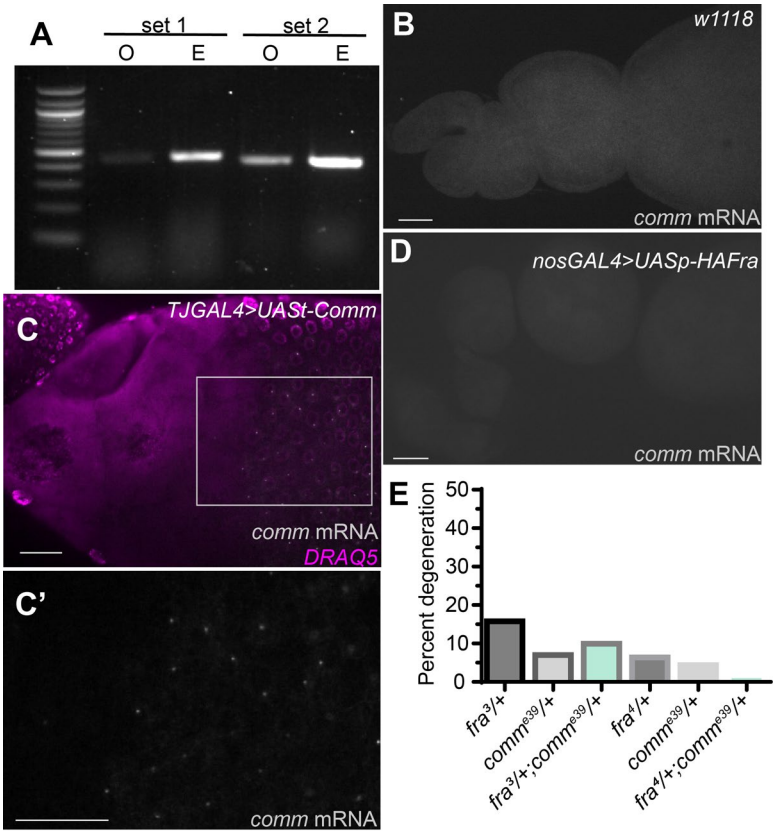

Figure 2. S 3. **Comm does not appear to be expressed in the germline.**

(A) RT-PCR for comm using two different primer sets on ovary (O) and embryonic (E) samples. (B) Optical section through the middle of a wild type ovariole. Small molecule fluorescent in situ hybridization (smFISH) for comm mRNA (grey), over-exposed. (C) Ovariole with Comm driven in follicle cells by traffic jam GAL4 (TJGAL4) stained with DRAQ5 (magenta) and comm probe (grey). (C') comm mRNA channel from boxed region in C. (D) Ovariole with HAFra driven in the germline with comm mRNA probe (grey). (E) Percentage of ovarioles containing a degenerating egg chamber. N=161, 190, 155, 104, 85, 93 ovarioles from one trial. Scale bars are 20 microns.

Table 2. 1. Loss of Fra causes egg chamber degeneration around mid-oogenesis (stage eight).

Stage preceding degeneration	Percent of ovarioles [a]
Four	1.28
Five	6.41
Six	12.82
Seven	16.67
Eight	52.56
Nine	7.69
Ten+	2.56

a: Ovarioles with mosaic *fra* germline clones with a degenerating egg chamber. Data is from four independent trials (N=78).

Table 2. 2. Expressing Fra transgenes in a wild-type germline does not affect egg chamber degeneration.

Genotype	Degeneration [a] (%)	Ovarioles
nos-GAL4	5.19	154
nos-GAL4>UASp-HAFra	12.12	33
nos-GAL4>UASp-FraICDMyc	4.62	65
nos-GAL4>UASp-HAFraE1354A	4.26	141

a: Percentage of ovarioles containing a degenerating egg chamber. Data is from one trial and flies were 5-8 days old and were a similar age to those used in the rescue experiments (Figure. 7).

Materials and Methods

Fly stocks

Fly lines used in this study: *w***; P{FRT(w*^{hs}*)}G13 fra*^{4}*/CyO, P{lacZ.w*$^{+}$*}276* [Bloomington Drosophila Stock Center (BDSC) #8743], *w***; P{FRT(w*^{hs}*)}G13 fra*^{3}*/CyO, P{lacZ.w*$^{+}$*}276* (BDSC #8813), *w[*]; P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=ovoD1-18}2R/T(1;2)OR64/CyO* (BDSC #4434), *y*^{1} *w*^{67c23}*; Mi{PT-GFSTF.1}fra*$^{MI06684\text{-}GFSTF.1}$ (BDSC #59835), *NetAB*$^{\Delta GN}$*/FM7* (provided by Thomas Kidd), *w[*]; Bac{w[+mW.hs]= GreenEye.nosGAL4}Dmel6* (BDSC #32180), *hsFLP1; FRT42D Ubi-GFP/CyO* (provided by Elizabeth Ables), *y*^{1} *w** *Mi{PT-GFSTF.1}NetA*$^{MI04563\text{-}GFSTF.1}$*/FM7j* (BDSC #59409), *NetB-MYC* (Brankatschk and Dickson, 2006), *FRT42B (FRT G13) fra*^{6}, *FRT42D fra*^{3}, *hsFlp1; FRT42D UbiGFP; nos-GAL4*, *UASp-p35* (provided by Andreas Bergmann). Transgenic fly lines generated and used in this study: *UASp-HA-Fra*, *UASp-HA-FraE1354A*, *UASp-FraICD-MYC*, *UASp-HA-FraE1354A-VP16.*

Generation of UASp Fra transgenes for germline expression

To generate *UASp-FraICD-MYC*, *FraICD-MYC* was amplified from *UASt-FraICD-MYC* by PCR and subcloned into *pUASp-attB* (DGRC #1358). To generate *UASp-HA-Fra*, *HA-Fra* was amplified from *UASt-HA-Fra* by PCR and subcloned into *UASp-attB*. To generate *UASp-HA-FraE1354A*, the c-terminal end of Fra was cut from the *UASp-HA-Fra* plasmid using XbaI, and the c-terminal end of *HA-FraE1354A* (containing the E1354A mutation) was cut from the *UASt-HA-FraE1354A* plasmid by XbaI and inserted into the cut *UASp-HA-Fra* plasmid. To generate UASp-HA-FraE1354A-VP16, the construct was cut from *UASt-HA-FraE1354A-VP16* using NotI and inserted into *UASp-*

attB. Constructs were verified by sequencing at the Penn Genomics Core. Transgenic flies were generated by phiC31 targeted insertion into the 86F8 site by BestGene Inc. (Chino, CA).

Immunostaining and imaging

Ovaries were processed as described (Laws and Drummond-Barbosa, 2015) with minor modifications. Briefly, ovaries were dissected in cold phosphate buffer saline (PBS, Roche), and carefully teased apart. They were fixed for 13-15 min in 5.3% PFA in PBS (Electron Microscopy Services). The fix was washed off with 0.01% Triton-X100 in PBS (PBT), and the ovaries were blocked overnight in PBT with 5% bovine serum albumin (w/v) and 5% normal goat serum. Primary antibodies were diluted in blocking solution and incubated with samples on a nutator overnight at 4°C. Antibodies were washed off with PBT at room temperature. Secondary antibodies and/or stains were diluted in block and placed on a nutator at room temperature for at least one hour. After washing off secondary antibodies, samples were cleared in 90% glycerol with antifade (20 µg/mL propyl gallate) overnight, then mounted onto slides. Ovaries were analyzed and scored for degeneration on a Nikon Ti-U inverted microscope with a Nikon OFN25 40X objective and imaged on a PerkinElmer spinning disk confocal system with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head with Volocity imaging software. Images were tiled using a FIJI pairwise stitching macro (Preibisch et al., 2009) and equally and minimally adjusted using FIJI and Adobe Illustrator.

Antibodies and stains

Primary antibodies used in this study: chick anti-GFP (1:1000, abcam #13970), mouse anti-1B1 (1:100, DSHB concentrate), rabbit anti-Fra (1:100, provided by Michael

Murray), mouse anti-MYC (1:250, DSHB #9E10 concentrate), mouse anti-HA (1:250, Biolegend #901502), rabbit anti-dFoxO (1:500, provided by Pierre Leopold), rabbit anti-cleaved Dcp-1 (1:50, Cell Signaling #9578S), mouse anti-Diap1 (1:100, provided by Bruce Hay), and mouse anti-Draper (1:20, DHSB 5D14 supernatant).
Secondary antibodies used in this study (all at 1:200): goat anti-chick 488 (Invitrogen #A11039), goat anti-mouse 488 (Invitrogen #A11209), goat anti-mouse CY3 (Jackson Immuno #115-165-003), and Goat anti-rabbit CY3 (Jackson Immuno #111-165-144). DRAQ5 (1:1000 Cell Signaling #40845) was included with secondary antibodies.

Genetic mosaic analysis

ovo^{D} clones were generated by heat shocking late second/early third instar larvae (*hsFlp1; FRT42B/ovo^{D}, FRT42B, hsFlp1; fra^{3}, FRT42B/ovo^{D}, FRT42B, hsFlp1; fra^{4}, FRT42B/ovo^{D}, FRT42B, hsFlp1; fra^{6}, FRT42B/ovo^{D}, FRT42B*) in vials for 1 hour in a 37°C water bath. Female flies 0-3 days old were collected and cultured with healthy males in vials with yeast paste for two days prior to dissection.

Negatively-marked clones were generated by heat shocking progeny ([*hsFlp1; FRT42D UbiGFP /FRT42D *], [hsFlp1; FRT42D UbiGFP /FRT42D*; UASp-HA-Fra/nos-GAL4], [hsFlp1; FRT42D UbiGFP /FRT42D *; UASp-HA-FraE1354A/nos-GAL4], [hsFlp1; FRT42D UbiGFP /FRT42D *; UASp-FraICD-Myc/nosGAL4], [hsFlp1; FRT42D UbiGFP /FRT42D *; UASp-HA-FraE1354A-VP16/nosGAL4], [hsFlp1; FRT42D UbiGFP /FRT42D fra^{3}, UAS-p35; nosGAL4/+]* where * is a wild-type or mutant allele of *fra*) in vials for 1 hour in a 37°C water bath once each day for three consecutive days (approximately days 5, 6, and 7 after egg-laying). Female flies 0-3 days old were cultured with healthy males

in vials with yeast paste. Flies were fed with fresh yeast paste every 1-2 days for four days and dissected on the fifth day.

Scoring degeneration or follicle cell death

Ovarioles were scored blind to genotype. Degeneration was scored by the presence or absence of pyknotic nuclei visualized by the nuclear stain DRAQ5. Follicle cell death was scored by the absence of follicle cells surrounding nurse cell nuclei that are not condensed.

Diet experiment

Female flies with *fra*^{3} germline clones (See *ovo*^{D} clone generation in "Genetic mosaic analysis" above) were collected at 0-3 days old and placed on wet yeast paste with healthy males. Half of the flies were placed in a vial with a wet Kimwipe and no food for 6 hours prior to dissection. Flies were dissected and processed as described above.

RT-PCR

Approximately 25 female flies were fed yeast paste for three days prior to dissection in RNase-free PBS and put on ice. *w*^{1118} fly embryos were collected from apple juice plates after adding 50% bleach for three minutes and washing with distilled water. The Qiagen RNeasy mini kit protocol was followed for RNA extraction from both ovaries and embryos. Qiagen One-step RT-PCR kit protocol was used for RT-PCR. Two sets of primers were used to detect *comm* mRNA: Set 1 FWD CTCTCCAAGTCGGTGGTTCT, REV TTCATGCCGTAGGCAAAGTG, Set 2 FWD ATCTGTGGATCGGAGTGGTC, REV TTATTCAGCGGCTCCTGCTT.

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Competing Interests

The authors declare no competing interests.

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Data Availability

All data generated in this study are included in the manuscript and supporting files.

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CHAPTER 3: A yeast two-hybrid screen to identify Fra-interacting proteins

During early embryonic development, axons are guided to their synaptic targets to form functional neural circuits. Axon guidance is tightly regulated by a set of conserved axon guidance signaling pathways. Typically, axon guidance is mediated by a ligand-receptor interaction, which recruits cytoplasmic proteins to the intracellular domain (ICD) of the receptor and affects the cytoskeleton at the growth cone of the axon. The effect of this is that an axon is attracted to or repelled from the ligand expressed. Often both attractive and repulsive signaling cues are expressed in the same tissue, and axons must regulate their response to these cues to be guided accurately (Zang et al., 2021).

At the *Drosophila* ventral nerve cord, a structure which is analogous to the vertebrate spinal cord, midline glial cells secrete both attractive and repulsive axon guidance cues (Harris et al., 1996; Mitchell et al., 1996; Kidd et al., 1999). Axons that cross the midline, also known as commissural axons, must first be attracted towards the midline, and then repelled to exit the midline and be prevented from re-crossing. Midline glial cells secrete both Slit ligands, which signal repulsion through Roundabout (Robo) receptors (Kidd et al., 1999; Kidd et al., 1998), and Netrin ligands, which signal attraction through the Frazzled (Fra) receptor (Harris et al., 1996; Mitchell et al., 1996; Kolodziej et al., 1996). In vertebrates, Netrin signals attraction through homologues of Fra, the Dcc receptor and Neogenin (Neo) receptor (Keino-Masu et al., 1996). One way for axons to mediate their response to guidance cues they encounter is by regulating the expression of axon guidance receptors on their growth cone. In both vertebrates and invertebrates, down-regulation of Robo signaling is required for commissural axons to cross the midline

(Sabatier et al., 2004; Kidd et al., 1998). In *Drosophila*, commissural axons have reduced expression of Robo on their growth cone as they are crossing the midline (Kidd et al., 1998). Robo is reduced because of the protein Commissureless (Comm), which is expressed specifically and transiently in commissural neurons as their axons are crossing the midline (Keleman et al., 2005; Keleman et al., 2002). Comm binds to newly made Robo and promotes the degradation of Robo by sending it to the late endocytic pathway, preventing the newly translated Robo from reaching the cell membrane of the growth cone (Kidd et al., 1998; Keleman et al., 2005; Keleman et al., 2002; Tear et al., 1996). Thus, commissural neurons are not repelled by Slit, while Netrin still signals attraction through Fra expressed on the growth cone. In this way, commissural axons respond to attractants, but not to repellants, to enter the midline. Post-crossing, Comm expression is reduced, Robo is localized to the growth cone, and the axon is repelled from the midline and prevented from re-crossing by Slit.

Interestingly, Fra also has a Netrin-independent function as a transcription factor that activates expression of *comm* (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009). Thus, Fra is able to promote midline crossing by both affecting local cytoskeletal rearrangements in response to Netrin to promote attractive signaling (Zang et al., 2021), and antagonizing repulsive signaling by activating transcription of *comm* independent of Netrin (Neuhaus-Follini and Bashaw, 2015; Yang et al., 2009). Both Dcc and Neo ICDs have been found to activate transcription of a luciferase reporter (Taniguchi et al., 2003; Goldschneider et al., 2008).

Fra is a type 1 transmembrane protein that is part of the Immunoglobulin (Ig) superfamily. The extracellular domain (ECD) of Fra consists of four Ig domains followed by six fibronectin (FN) type III repeats (Kolodziej et al., 1996). The intracellular domain

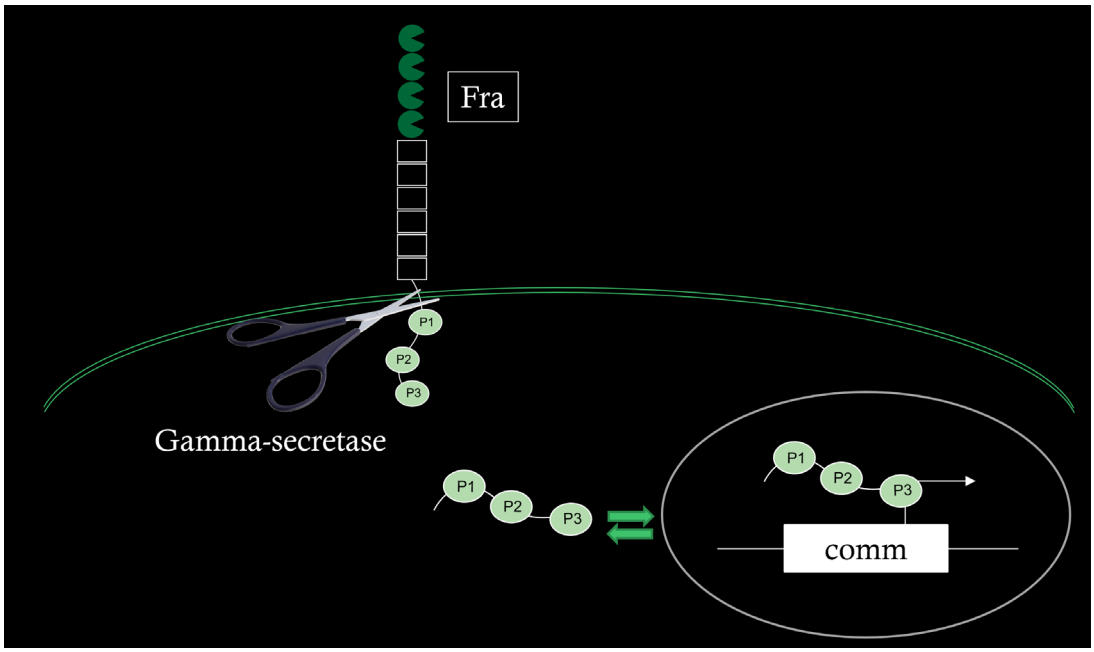

Figure 3. 1. **Current model of Fra as a transcription factor.**

Gamma-secretase cleaves the intramembrane region of Fra, releasing the intracellular domain. This allows the ICD to enter the nucleus and activate transcription. In the nerve cord, Fra activates transcription of comm to promote midline crossing.

(ICD) of Fra is made up of three P motifs (P1, P2, and P3) that are conserved between Fra and its homologs (Dcc, Neo, UNC-40), and less conserved regions between the P motifs (Fra structure depicted in Figure 3.1). In our model of how Fra activates transcription, Fra interacts with a putative ligand that induces ecto-domain shedding of Fra by an unknown metalloprotease. It was previously shown that Fra is cleaved by gamma-secretase, a multi-protein protease complex that cleaves type 1 transmembrane receptors within their transmembrane region (Struhl and Adachi, 2000), releasing the intracellular domain (ICD)(Neuhaus-Follini and Bashaw, 2015). The Fra ICD can translocate to the nucleus. and activate transcription of *comm*, and potentially other unknown targets. The P3 motif within the Fra ICD contains a transcriptional activation domain that is necessary to activate transcription of *comm* mRNA (Neuhaus-Follini and Bashaw, 2015). However, how the Fra ICD regulates transcription is still not completely understood. Interestingly, the Fra ICD does not contain a canonical DNA-binding motif, indicating that Fra may directly interact with a DNA-binding protein to affect transcription of target genes.

I conducted a yeast two-hybrid screen to identify DNA-binding proteins that interact with the Fra ICD (Golemis et al., 2008). Since transcriptional activation is the output of a yeast two hybrid interaction, I used a Fra ICD with a point mutation in the P3 region that prevents the activation of transcription of reporter genes in yeast, and prevents the activation of *comm* mRNA expression in the developing *Drosophila* nerve cord (Neuhaus-Follini and Bashaw, 2015). This FraICDE1354A protein was fused to a LexA binding domain. For potential interactors, I used a library of cDNA plasmids from 0-24hr embryonic lysates. These cDNAs generate proteins fused to a B42 activation domain. The plasmids were transformed into yeast cells with the LEU2 gene under a LexA operator. These yeast cells are unable to express LEU2, and die when plated on

media lacking leucine. In cells where the cDNA plasmid encodes a protein that interacts with the Fra ICD, both proteins will be recruited to LEU2 via the LexA binding domain, activate transcription of LEU2 and allow growth on media lacking leucine (Figure 3.2). To identify proteins that interact with the Fra ICD, I isolated the cDNA from colonies that grew on media lacking leucine. Then, I sequenced the cDNA, and identified the gene using the basic local alignment search tool (BLAST) against the *Drosophila* genome. This yeast two-hybrid screen identified 68 proteins that potentially interact with Fra (Table 3.1). The proteins identified include DNA-binding/transcriptional proteins, cytoskeletal interactors, kinases, proteins involved in translation, RNA-binding proteins, proteins involved in transport, and metabolic proteins. The ribosomal proteins and Focal adhesion kinase (Fak) help to validate the screen since their vertebrate homologs are known to interact with Dcc (Tcherkezian et al., 2010; Rajasekharan et al., 2009).

Pleiohomeotic-like and Clawless

I selected Clawless (C15/Cll) as a DNA-binding protein to follow-up on because it has such a restricted expression pattern in the embryonic nerve cord (Berkley *Drosophila* Genome Project- BDGP). Cll/C15 is a homeobox DNA-binding domain containing protein that is homologous to Hox11 genes in vertebrates. Cll/C15 has been implicated in both the activation and repression of genes, which indicates that it could be involved in the activation of *comm* expression. Using a Cll antibody as well as *in situ* probe, I found that Cll/C15 is expressed in two populations of neurons, (1) neurons within the EG subtype of eagle neurons, and (2) a pair of neurons that are labelled with the Cll-gal4 (E04) line (Figure 3.6). Both the antibody and the probe share similar expression patterns (Figure 3.6), suggesting that the antibody and mRNA probe are labelling Clawless gene products.

DNA-binding/ transcription	Pleiohomeotic-like (Phol), Rough (Ro), Hat-trick (Htk), Clawless (Cll/C15), Chromator (Chro/Chriz), Mediator subunit 30 (Med30), Mnt, SET domain containing 2 (Set2), CG9175, Asense, B52, CG12391
Cytoskeletal/ adhesion	WASp, CG1890, Cindr, Mars/Hurp, Unc-45, Drop out (dop), Myosin81f, dim gamma-tubulin2 (Dgt2) Multiplexin (Mp), Contactin (Cont)
Kinase	Focal adhesion kinase (Fak), Calcium/Calmodulin dependent protein kinase I (CaMKI), CG33671/CG33672, cyclinG (CucG-regulates kinase activity)
Translation	Eukaryotic initiation factor 1A (eIF-1A), Ribosomal proteins RpL10, RpL7A, RpS15, Receptor of activated protein kinase C1 (Rack1)
RNA-binding	CG11414, kep1/qKr58E-3, CG15432, Syncrip
Metabolic	Cytochrome c heme lyase (Cchl), Plasma membrane calcium ATPase (PMCA), Ecdysone-inducible gene L3 (ImpL3), superoxide dismutase (sod), Glutathione synthetase (Gss), Glutathione S transferase D1 (GstD1), Na pump alpha subunit (ATP alpha), Phospohoglucose mutase (Pgm), Spermidine synthase (SpdS), Malate dehydrogenase 2 (Mdh2), Ugt36Bc, Cytochrome C oxidase subunit 6B (Cox6B), Ornithine decarboxylase antizyme (Oda), Succinate dehydrogenase, subunit D (SdhD), CG6734, CG5326, CG8207, CG3887, CG8630
Transport	Alpha soluble NSF attachment protein (AlphaSNAP), Sec24AB, Translocase of outer membrane (Tom40),
Other/Unknown	Osiris6 (Osi6), Gasp, TweedleL, TweedleD, CG11700, CG10338, CG2915, CG31198, Globin1 (Glob1), CG8547, CG13047, CG31813, CG8818, CG11122

Table 3. 1**.** Proteins identified to interact with the Fra ICD in a yeast-two hybrid screen. The Fra ICD was fused to a LexA DNA-binding domain and was transformed into yeast cells along with cDNA from 0-24hr embryonic lysates that were fused to a B42 activation

domain. An interaction between the Fra ICD and another protein activates transcription of Leu, allowing the cells to grow on plates without Leu. DNA was isolated from the colonies that grew and DNA was sequenced to identify which proteins interact with Fra.

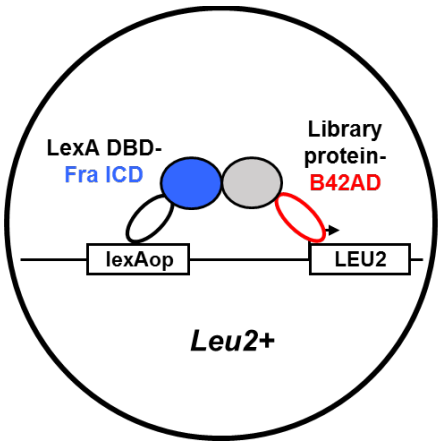

Figure 3. 2**. How the yeast two-hybrid screen works.**

When the Fra ICD and a protein encoded by the library cDNA interact, LEU2 is transcriptionally activated, and yeast can grow on media without leucine.

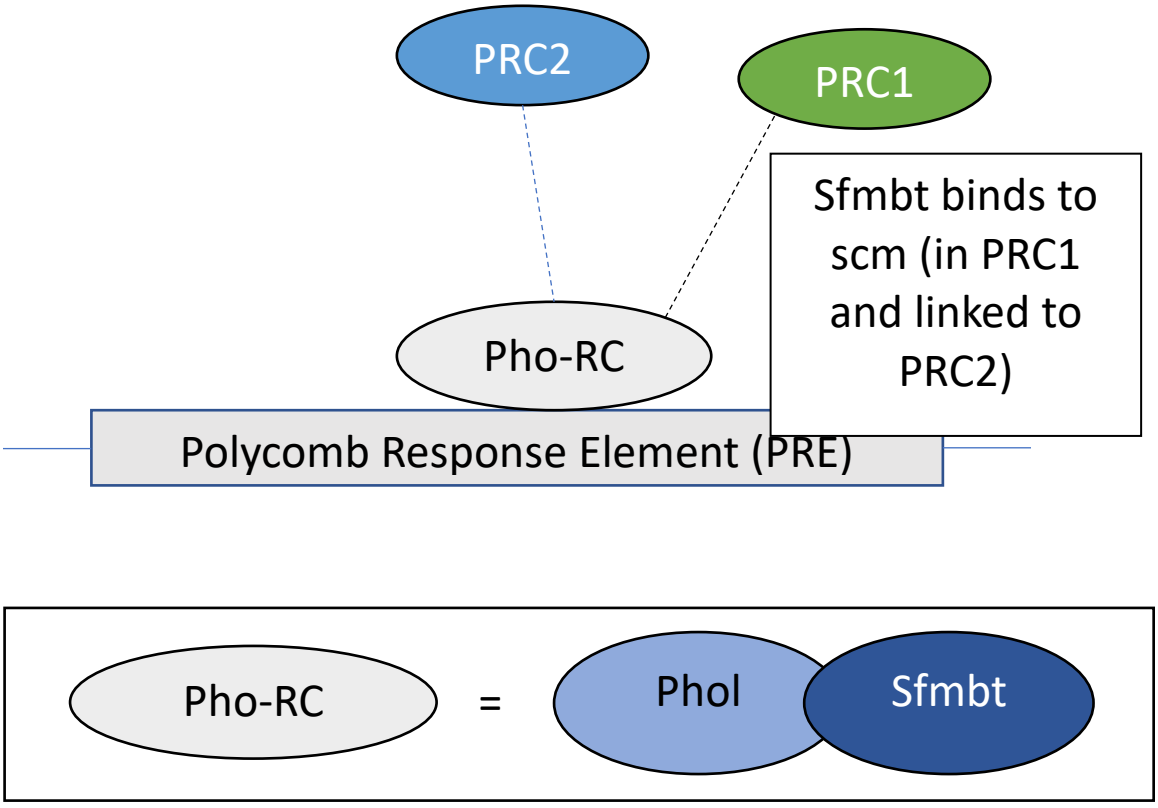

Figure 3. 3. **Depiction of how Pleiohomeotic-like (Phol) binds to DNA in a repressive complex and interacts with polycomb response complexes.**

Phol binds to Sfmbt to form a repressive complex. This repressive complex is linked to the polycomb response complexes through Sfmbt.

The FraΔC sensitized background uses a dominant negative Fra receptor that lacks its C-terminal domain. This sensitized background has been used previously in the lab to identify proteins that are involved in axon guidance (Garbe et al., 2007; O'Donnell and Bashaw, 2013; Hernandez-Fleming et al., 2017). While I tested some of the DNA-binding proteins from this screen, including Ro and Htk, Pleiohomeotic-like (Phol) is the only one that gave a positive result (Figure 3.4). Phol is a polycomb group (PcG) protein that often acts redundantly with its homolog Pleiohomeotic (Pho). Pho and Phol form a repressive complex when either are bound to the protein Sfmbt, and interact with the polycomb response complexes (Figure 3.3). These proteins are negative regulators that commonly maintain repression of homeotic genes. Still there is some evidence that Phol and Pho maintain expression of the gene even-skipped, indicating that Phol could activate/maintain *comm* expression. The sensitized FraΔC background uses one copy of a dominant negative Fra construct, which results in a low level of axons failing to cross the midline of the embryo when all of these axons would normally cross (Garbe et al., 2007). In flies with only the dominant negative construct being expressed, I see 31% of axons fail to cross the midline. When one copy of *phol* is removed in this sensitized background, the percentage of axons that fail to cross the midline significantly increases to 44% (Figure 3.4), suggesting that Phol has a role in axon guidance. However, *phol* zygotic mutants in a wild-type background have neither detectable crossing defects in a subset of commissural neurons, eagle neurons, nor in all commissural neurons (Figure 3.4). In addition, these mutants have no effect on *comm* expression in eagle neurons

(data not shown), indicating that while Phol appears to have a role in axon guidance, it is not involved in the transcriptional activation of *comm.* Alternatively, it is possible that

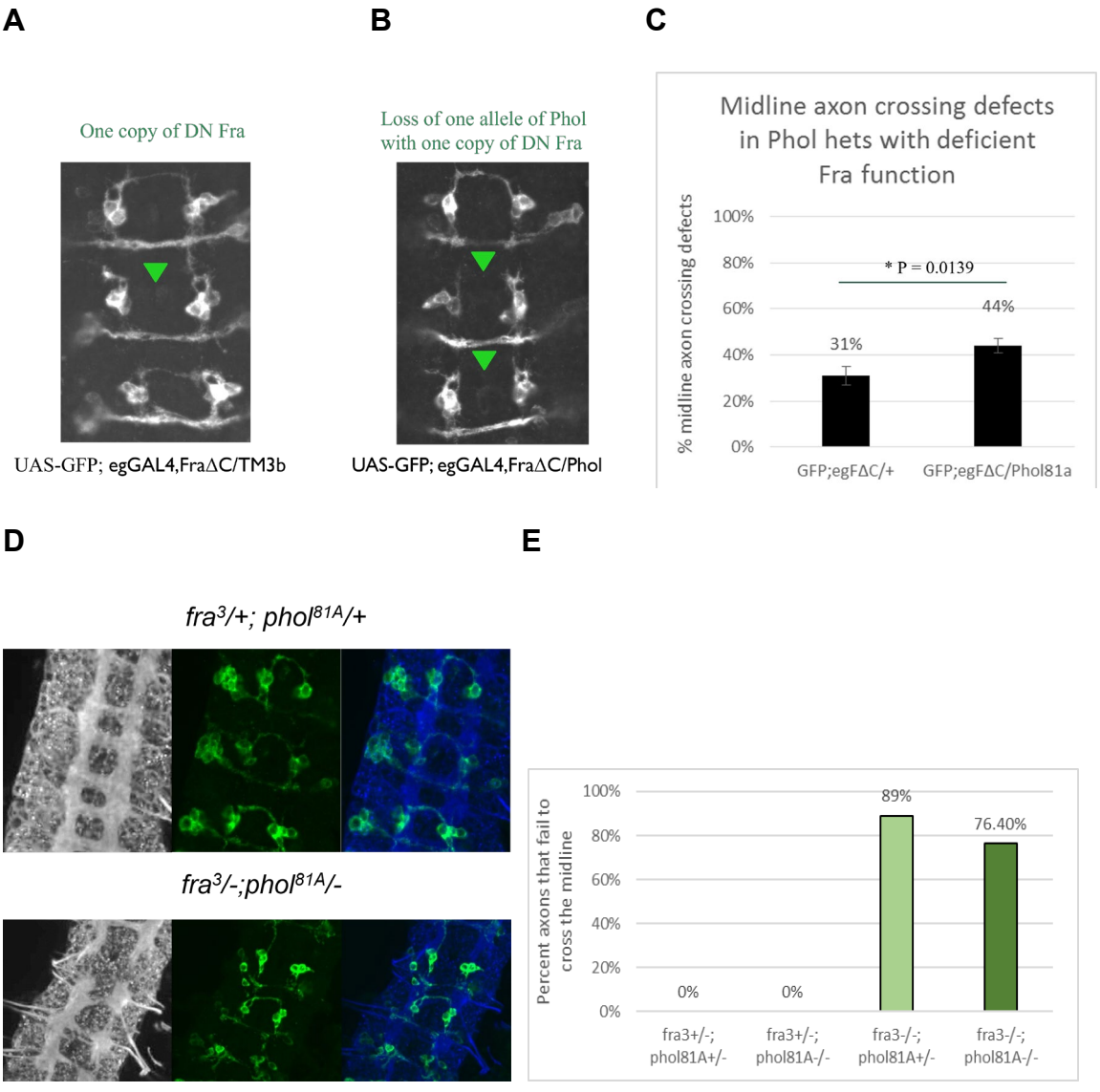

Figure 3. 4**. Loss of phol causes defects in midline-crossing in sensitized backgrounds.**

(A, B) Representative images of the genotype with three hemi-segments of the nerve cord labelling the eagle neurons and their axons with GFP. (A) One copy of FraΔC is expressed in eagle neurons, and in these three hemi-segments, one out of the three sets of EW eagle neurons fails to cross the midline (marked by the green arrowhead). (B) When one copy of Phol is removed ($phol^{81A}$/+) in the FraΔC background, there is an increase in EW axons that fail to cross the midline. (C) Quantification of EW axons that fail to cross the midline in stage 15-16 embryos (n=22, 23). Statistics done with student's t-test. (D) Representative images of the genotype indicated. Three hemi-segments of the embryonic nerve cord at stages 15-16. From left to right, HRP stain, GFP labelling eagle neurons and their axons, and merged channels (HRP in blue and GFP in green). (E) Quantification of EW axons that fail to cross the midline in genotypes specified (n=14, 6, 6, 11).

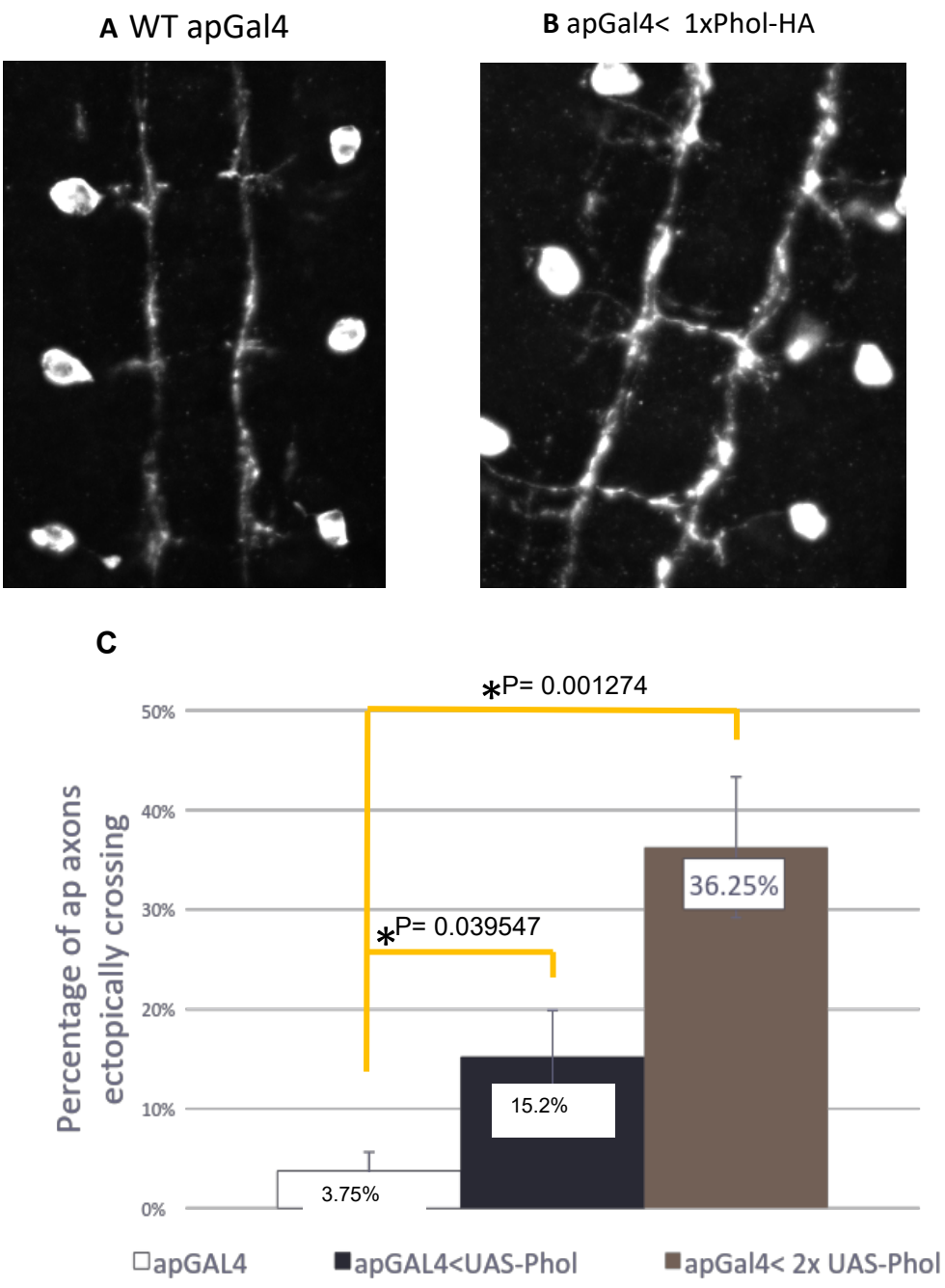

Figure 3. 5**. Phol induces ectopic crossing in apterous neurons in a dose-dependent manner.**

A UAS-Phol-HA transgene is expressed in apterous neurons using Apterous-Gal4 and UAS-TauMycGFP to label axons. (A) A section of a wild-type nerve cord with three pairs of apterous neurons labelled with GFP, where apterous neurons do not cross the midline. (B) A section of a nerve cord with one copy of Phol-HA expressed in apterous neurons where two of the three pairs of neurons have ectopic crossing events. (C) The percentage of apterous neurons that have a crossing event. Wild-type flies have very minimal ectopic crossing events, while expressing either one or two copies of Phol-HA significantly increases the percentage of apterous neurons with ectopic crossing events (n= 10, 14, 10). Statistics were done with a student's t-test.

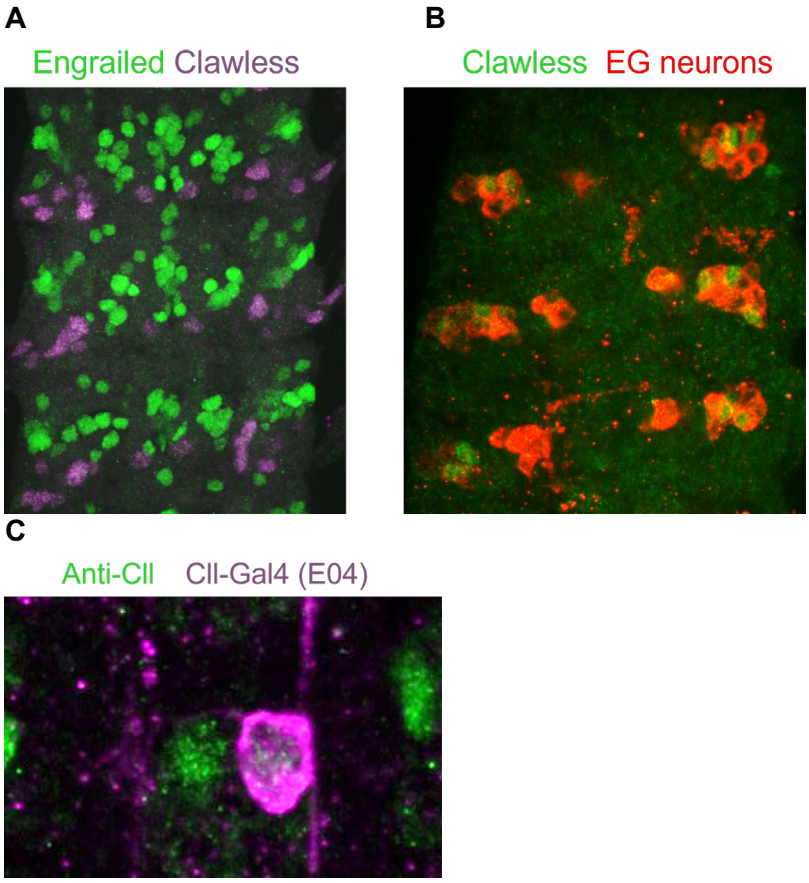

Figure 3. 6. **Clawless expression in two commissural neuron populations.**

(A) Embryonic nerve cord stained for Engrailed (green) and Clawless protein (magenta). (B) Embryonic nerve cord with eagle neurons labelled with eagle-Gal4> UAS-Tau-Myc-GFP and stained for GFP (red) and clawless mRNA (green). (C) Image of a single hemi-segment in the nerve cord at the midline of a neuron with an axon extending across the midline labelled with Cll-Gal4 (E04-Gal4)> UAS-Tau-Myc-GFP stained for GFP (magenta) and Clawless (green).

redundancy with Pho, or maternal Phol contribution may be masking axon guidance defects and *comm* expression defects in *phol* zygotic mutants.

To further examine the role of Phol in the nerve cord, I examined embryos that were heterozygous or mutant for *fra,* and heterozygous or mutant for *phol.* In the double heterozygous embryos, or embryos that were heterozygous for *fra* and mutant for *phol,* all of the eagle neuron axons cross the midline (Figure 3.4). On the other hand, in embryos that are mutant for *fra* and are either heterozygous or mutant for *phol,* strikingly, 80-90% of axons fail to cross the midline (Figure 3.4). This is much greater than the previously scored 30-50% of axons that fail to cross the midline in *fra* mutants (Hernandez-Flemming et al., 2017). These preliminary results need to be repeated to be confirmed, but these results indicate that pleiohomeotic-like is acting via multiple pathways to affect midline crossing.

It is possible that Phol could be affecting axon crossing non-cell autonomously in these sensitive backgrounds. To determine whether Phol has a cell-autonomous role, we expressed an HA-tagged Phol in a subset of neurons whose axons never cross the midline, the apterous (ap) neurons. Expressing one copy of Phol-HA in the apterous neurons induced axon crossing from 3.75% to 15.2%, and two copies of Phol-HA further increased axon crossing to 36.25% (Figure 3.5). This suggests that Phol can act cell autonomously, and induces crossing in a dose-dependent manner.

RNAi screen in the ovary

Since the cDNA library used in the yeast-two hybrid screen was created from whole embryonic lysates, it is likely that some of the proteins identified interact with Fra in tissues outside of the nervous system. We now know that Fra is required in the ovary for egg chambers to progress through mid-oogenesis, and this most likely leads to a

decrease in egg-laying, although we are currently testing whether knockdown of *fra* in the germline with RNAi impacts egg-laying. The ovary represents a useful system to test the requirement of interactors from the yeast-two hybrid screen for egg-laying, since RNAi works effectively in the ovary and provides a quick screening method. Together with a postdoc in the lab, Dr. Kate Laws, we knocked down several genes in the germline, using multiple RNAi lines when possible, and calculated the number of eggs laid per female per day (Table 3.2). Some of the genes that appear to have an effect on egg-laying include Cll, CG12391, and Rack1. Follow-up using RNAi or clonal analysis in the ovarian germline is necessary to determine why the loss of these proteins decrease the number of eggs laid. If these proteins are required for Fra function at mid-oogenesis, we would expect to see degeneration at mid-oogenesis.

Overall, many classes of proteins were identified that are of relevance to Fra signaling, including DNA-binding/transcriptional proteins, cytoskeletal binding proteins, and translational/ribosomal proteins. Following up on these proteins by confirming their interaction with Fra, knocking them down in the ovarian germline, and testing for their requirement using the FraΔC sensitized screen will be the first steps in determining if they are important for Fra signaling. Clawless is expressed in two neuronal populations, and may be important in the ovarian germline for egg-laying. Preliminary results of Phol indicate that it is important for axon guidance and has a role in promoting midline crossing, although it also appears to have pleiotropic effects in the nerve cord.

	Gene name	RNAi line	Day 5 eggs/female/day	Day 10 eggs/female/day
Group 1	GFP		119.59	56.44
	Cll	pVal10	82.82 (2)	4.89 (2)
		pVal20	60.61	24.74
	Htk		125.8	93.26
Group 2	GFP		119.49	48 (2)
	CG12391	pVal10	69.54	39.06
		pVal20	58.43	11.30
	WASp	pVal22	105.50	40.84
	Chro	pVal22	115.67	55.14
		pVal20	37.14	21.71
Group 3	GFP		111.10	43.01
	CG1890	JF01146	93.37	33.31
		HMJ21731	88.05	42.60
	Cindr	HMJ01892	101.33	33.64
		HMS01795	100.52	9.92
		GLV21035	102.05	26.95
	Mp	HMJ21668	104.31	37.29 (2)
Group 4	GFP		80.86	23.93
	Rack1	GL00630	67.43	18.39
		HMS01123	16.89	6.12
	CaMKI	GL01332	66.68	34.74
		GL00274	108.83	32.64
	CG10338	HMJ21246	51.28	36.22
	CG2915	HMJ30194	68.44	29.41

Table 3. 2**.** Germline RNAi knockdown of potential Fra interactors effect on egg-laying.

Each genotype was set up in triplicate. Day 5 and Day 10 values were averaged across three cages unless otherwise noted.

Materials and Methods

Yeast two-hybrid screen

The cDNA library used was generated by H. Araj (see Terman et al., 2002). Yeast two-hybrid screen followed protocol outlined in Golemis et al., 2008.

Fluorescent *in situ* hybridization (FISH)

FISH was done on *Drosophila* embryos to label *clawless* mRNA as described in Labrador et al., 2005.

Immunostaining and imaging

Embryo fixation and staining was done as described in Kidd et al., 1998. The following antibodies were used: rat anti-C15 (From Gerard Campbell, 1:1000 but re-used 3-7 times), mouse anti-βgal (DSHB, 1:150), mouse anti-HA (Covance#MMS-101P, 1:250), rabbit anti-GFP (Invitrogen #A11122, 1:500), rabbit anti-c-Myc (Sigma #C3956, 1:500), chick anti- βgal (Abcam #9361, 1:500), Cy3 goat anti-mouse (Jackson #115-165-003, 1:500), Alexa-488 goat anti-rabbit (Molecular Probes #A11008, 1:500). Imaging was done on a Nikon Ti-U inverted microscope with a Nikon OFN25 40X objective and imaged on a PerkinElmer spinning disk confocal system with a Hamamatsu C10600-10B CCD camera and Yokogawa CSU-10 scanner head with Volocity imaging software. Images were equally and minimally adjusted using FIJI.

Genetics

The following alleles were used: *fra*^{3}, *phol*^{81A}(Bloomington stock #24164), *UAS-Phol-HA* (FlyORF F000268)*, apGal4*, *egGal4*, *CllGal4* (E04), *UAS-Tau-Myc-GFP*, RNAi lines listed in Table 3.2.

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CHAPTER 4: Discussion and future directions

In the *Drosophila* embryonic nervous system, Frazzled (Fra) functions via two signaling modes to promote axon growth across the midline: Netrin-dependent local cytoskeletal changes (Zang et al., 2021), and Netrin-independent transcriptional activation (Neuhaus-Follini and Bashaw, 2015). Indeed, these two types of signaling mechanisms even occur in the same subsets of commissural neurons (Garbe et al., 2007; Neuhaus-Follini and Bashaw, 2015). The Netrin-dependent signaling mode of Fra has been well studied, especially in the nervous system. Briefly, Netrin interacts with the extracellular domain of Fra, which recruits cytoplasmic proteins to the Fra intracellular domain. This leads to cytoskeletal changes within the growth cone that affect growth cone turning and outgrowth (Zang et al., 2021). However, Fra also acts independently of Netrin to promote axon growth across the midline (Neuhaus-Follini and Bashaw, 2015; Long et al., 2009). For this mechanism, a putative ligand interacts with Fra, which most likely recruits a metalloprotease that cleaves Fra, causing ectodomain shedding. We know that gamma-secretase cleaves the intramembrane region of Fra, and that this is required for the Fra intracellular domain to enter the nucleus and activate transcription (Neuhaus-Follini and Bashaw, 2015). Fra contains an activation domain within the conserved P3 region that is necessary to activate transcription of *commissureless (comm)*(Neuhaus-Follini and Bashaw, 2015). Comm functions cell-autonomously to promote axon growth across the midline by downregulating the axons response to the repulsive cue Slit (Kidd et al., 1998; Keleman et al., 2005; Keleman et al., 2002; Tear et al., 1996). Interestingly, Fra lacks a DNA-binding domain, indicating that Fra might interact with other proteins to associate with its target genes.

Each female *Drosophila* contains a pair of ovaries that consist of 15-20 strings of developing eggs, called ovarioles. The germarium, which houses the stem cell populations for the germline cells and the somatic cells, resides at the anterior end of the ovariole (Kirilly and Xie, 2007). The germline stem cells will give rise to germline cysts that are encapsulated by somatic follicle cells, and this unit is called an egg chamber. This egg chamber buds from the germarium and grows in size. Stage eight marks mid-oogenesis, or vitellogenesis, and the oocyte within the egg chamber begins to take up yolk proteins and lipids (McLaughlin and Bratu, 2015). *Drosophila* egg production is a highly energy-dependent process, and there are checkpoints in place during oogenesis to ensure production of viable eggs. One of these checkpoints occurs at mid-oogenesis, where cell death occurs in response to poor nutrient conditions or abnormal egg chambers (Drummond-Barbosa and Spradling, 2001; Beachum et al., 2021; Tanentzapf et al., 2000; Chao and Nagoshi, 1999). Poor nutrient conditions cause a partial block in ovulation (Drummond-Barbosa and Spradling 2001), in part due to germline death at mid-oogenesis (Drummond-Barbosa and Spradling 2001; Mazzalupo and Cooley, 2006; Pritchett et al., 2009; Buszczak et al., 2002; Terashima and Bownes, 2006).

Here, I found that Fra most likely regulates transcription in the ovary, and identified DNA-binding proteins that might help Fra associate with target genes. In the ovary, Fra is expressed in both the somatic follicle cells and the germline cells, although Fra is specifically required in the germline for egg chambers to progress through mid-oogenesis. Fra does not affect diet-dependent signaling, apical polarity in follicle cells, or germline polarity, suggesting that Fra impinges on some unknown factor to trigger the checkpoint at mid-oogenesis. Loss of *fra* causes Dcp-1 activation, indicating that Fra has an anti-apoptotic role in the ovary. Netrin is not required at mid-oogenesis, and the

transcriptional activation domain within Fra is required, suggesting that Fra is regulating transcription in the ovary.

Fra activates transcription in the embryonic nerve cord, but its lack of a DNA binding domain suggests that it might be recruited to gene targets via a DNA-binding protein. I conducted a yeast-two hybrid screen to identify proteins that interact with the Fra intracellular domain. Of 68 interactors, eight were DNA-binding proteins. One of these proteins, Clawless/C15 (Cll), has a very restricted expression pattern within two neuronal populations in the nerve cord. Another DNA-binding protein identified, Pleiohomeotic-like (Phol), increases axon guidance defects when one allele is removed in a FraΔC sensitized background. Over-expression of Phol in apterous neurons induces ectopic crossing in a dose-dependent manner, indicating that Phol can function cell-autonomously. Embryos mutant for both *phol* and *fra* have severe axon guidance defects, suggesting that Phol may act in a pleiotropic manner in the nerve cord. Overall, this work has identified proteins that may interact with Fra to regulate transcription, and determined that Fra functions independently of Netrin to promote germline survival during oogenesis.

All pathways lead to death

To ensure the production of high-quality eggs, there is a checkpoint at mid-oogenesis that when activated results in egg chamber degeneration. The checkpoint at mid-oogenesis is triggered either because the egg chamber is of low quality, or due to low energy resources, such as poor nutrient conditions. Many diet-dependent pathways function at this checkpoint, both ovary/germline-intrinsic and tissue extrinsic (Laws and Drummond-Barbosa, 2017). Abnormal egg chambers, such as disruptions to egg chamber polarity, disrupted chromatin structure, and follicle cell death also trigger the

mid-oogenesis checkpoint (Bass et al., 2007; Beachum et al., 2021; Tanentzapf et al., 2000; Chao and Nagoshi, 1999). I have shown that the loss of *fra* in the germline causes egg chamber degeneration at mid-oogenesis (Figure 2.2). The adult ovary has a robust and well-characterized response to nutrient-poor conditions, and flies fed a diet lacking yeast causes degeneration at mid-oogenesis (Drummond-Barbosa and Spradling, 2001). During mid-oogenesis, oocytes take up yolk proteins and lipids, which is an energy-intensive process. Several nutrient-sensing pathways, including insulin signaling are required at mid-oogenesis (LaFever and Drummond-Barbosa, 2005). To determine whether the loss of *fra* in the germline may affect diet-dependent pathways, I tested whether *fra* mutant germlines were responding to diet normally. While the transcription factor FoxO is not necessary for insulin signaling at mid-oogenesis, it can still be used as a readout for insulin signaling (LaFever et al., 2010). Under well fed conditions, Insulin signaling causes the phosphorylation and activation of the serine/threonine kinase Akt1. Akt1 is then able to negatively regulate FoxO, causing it to remain in the cytoplasm (Manning and Toker, 2017; Nassel et al., 2015). However, under poor diet or starvation conditions, Akt1 is not activated, and FoxO localizes to the nucleus and affects transcription. If the loss of *fra* affected insulin signaling, we would expect to see FoxO in the nucleus of the nurse cells. However, in both wild-type and *fra* mutant germlines, FoxO remains in the cytoplasm (Figure 2.4), indicating that Fra does not affect insulin signaling. I explored the possibility that Fra acts independently of Insulin signaling to regulate the response to diet, and tested the ovarian diet response in *fra* germline mutants. When we starve flies for six hours, we see an increase in degeneration at mid-oogenesis between 20-30% when compared to well-fed wild-type flies (Figure 2.4). If Fra were affecting the ovarian response to diet, degeneration in *fra* mutants could be due to the failure of the germline to sense nutrient levels properly. If this were the case, when

flies with *fra* germline mutants are starved, we would expect to see a small or limited increase in degeneration as compared to well-fed flies with *fra* mutant germlines. However, when we starve flies with *fra* mutant germlines, we see that degeneration increases by more than 30% (Figure 2.4), suggesting that the *fra* mutant germline is still competent to respond to starvation/dietary manipulation. Taken together with our FoxO observations, this suggests that Fra is not a mediator of the ovarian response to diet/nutrient-dependent gating of the mid-oogenesis checkpoint.

In addition to the tight coupling of oocyte development to the nutrient environment, there is a level of stochastic degeneration at mid-oogenesis that occurs in well-fed, healthy, wild-type flies. While the precise mechanisms of stochastic degeneration remain unclear, several studies indicate that low-quality oocytes may be targeted for degeneration. For example, inducing follicle cell death using chemicals was found to cause egg chamber death before the follicle cells died (Chao and Nagoshi, 1999). Furthermore, disrupting egg chamber polarity or chromatin structure also leads to degeneration at mid-oogenesis (Beachum et al., 2021; Tanentzapf., 2000; Bass et al., 2003). We found that *fra* germline mutants had no effect on apical polarity and germline polarity (Figure 2.4), indicating that Fra must impinge on something other than diet-dependent pathways and these axes of polarity. We cannot exclude other axes of polarity, although testing whether loss of *fra* from the germline has an effect on hatch-rate could allow us to gauge whether Fra might be impinging on polarity. Disruptions to egg chamber polarity can result in a mature oocyte that fails to hatch properly (Cha et al., 2017). We are currently determining whether the loss of *fra* from the germline via RNAi knockdown has any effect on hatch rate. If the loss of *fra* from the germline causes a reduction in hatch-rate, this could indicate some other axis of polarity is disrupted. Why does the loss of *fra* result in degeneration at mid-oogenesis? Dcc can interact directly

with caspase proteins in vertebrate tissues (Mehlen and Mazelin 2003, Goldschneider et al 2010; Mehlen et al 1998, Forcet et al 2001), and when *fra* is lost in the germline, the death effector caspase Dcp-1 is activated. Perhaps Fra directly interacts with Dcp-1 to induce apoptosis. Alternatively, given the requirement for its transcriptional activation domain, the answer could lie in the genes that Fra may be transcriptionally activating in the germline.

Fra/Dcc and apoptosis

Dcc has long been known as a tumor suppressor, and is often lost in colorectal carcinomas and some neuroblastomas (Chen et al., 1999; Forcet et al., 2001; Mehlen et al., 1998). In the absence of Netrin, Dcc is cleaved by caspase 3 and this leads to caspase activation and apoptosis (Mehlen and Mazelin 2003, Goldschneider et al 2010; Mehlen et al 1998, Forcet et al 2001). This signaling mode of Dcc has been found in neuronal and cancer cell lines, as well as some cells in the vertebrate nervous system (Castets et al., 2012; Furne et al., 2008; Jasmin et al., 2021; Chen et al., 1999; Forcet et al., 2001; Mehlen et al., 1998). This function is known as the “dependence receptor” model, where Dcc depends on its ligand Netrin to prevent apoptosis. However, whether Fra might interact with caspase machinery or have a pro-apoptotic role is unknown. In the absence of germline *fra,* egg chambers degenerate (Figure 2.3), indicating that Fra promotes egg chamber survival in the ovary. The death effector Dcp-1 is activated in *fra* germline mutants (Figure 2.5), suggesting that the loss of *fra* results in apoptosis. Blocking apoptosis in the germline has been shown to prevent the degeneration seen at the mid-oogenesis checkpoint (Peterson et al., 2003; Mazzalupo and Cooley, 2006). Preliminary analysis of *fra* germline mutants with germline expression of the anti-apoptotic baculovirus p35 protein blocks germline death, indicating that *fra* mutant

germlines undergo apoptosis. Thus, in contrast to Dcc's well-known pro-apoptotic role, Fra has an anti-apoptotic role in the *Drosophila* ovary. In vertebrates, Dcc interacts directly with caspase proteins and its intracellular domain is cleaved by caspase 3 (Mehlen and Mazelin 2003, Goldschneider et al 2010). The caspase cleavage site in Dcc is not conserved in Fra, but the Fra intracellular domain is cleaved multiple time *in vivo* (Neuhaus-Follini and Bashaw, 2015). Thus, it is possible that Fra could be cleaved by a caspase, such as Dcp-1. It will be interesting to determine if Fra interacts directly with Dcp-1 by conducting co-immunoprecipitations in S2R+ cells with both the inactive and active forms of Dcp-1. Furthermore, one could test whether Dcp-1 might cleave Fra in the ovary.

Netrin-Independent Fra signaling

Typically, Netrin interacts with the extracellular region of Fra, and this interaction recruits cytoskeletal effectors to its intracellular domain, leading to local cytoskeletal changes (Zang et al., 2021). However, Fra also acts independently of Netrin to affect cellular processes by regulating transcription (Yang et al., 2009; Neuhaus-Follini and Bashaw 2015; Goldschneider et al., 2008; Taniguchi et al., 2003). Fra transcriptionally activates the axon guidance gene *commissureless* independently of Netrin in the *Drosophila* embryonic nervous system (Yang et al., 2009; Neuhaus-Follini and Bashaw 2015). Whether Fra transcriptionally regulates genes independently of Netrin in other contexts is unknown. In the ovary, we see that Fra is required independently of Netrin for egg chambers to progress through mid-oogenesis (Figure 2.2, 2.3, 2.6), indicating that Netrin-independent Fra signaling is required in multiple tissues. In this context, the transcriptional activation domain within Fra is required for egg chamber survival (Figure 2.7), suggesting that Fra regulates transcription in the ovarian germline. It is possible

that the transgene expressing the Fra construct with an inactive transcriptional activation domain (FraE1354A) fails to rescue *fra* germline mutants because the point mutation interrupts the interaction between Fra and another unknown protein. Thus, I am currently determining whether FraE1354A fused to a VP16 activation domain (FraE1354A-VP16) is able to rescue *fra* germline mutants. If this transgene fails to rescue the degeneration in *fra* germline mutants, this will present a novel Netrin-independent signaling activity of Fra, and future work would be necessary to determine the upstream and downstream components involved.

If the FraE1354A-VP16 transgene rescues, this would mean that the transcriptional activation domain within Fra is required for egg chambers to progress through mid-oogenesis. If this is the case, it will be interesting to identify Fra transcriptional targets in the ovary and compare how similar they are to the genes that Fra regulates in the nerve cord. Since *comm* does not appear to be expressed in the germline, and *fra/+;comm/+* do not have an increase in degeneration at mid-oogenesis, Fra most likely does not regulate *comm* expression in the ovary (Figure 2.S1). This indicates that fra is likely to activate different genes in the ovary as compared to the nervous system. Using parallel approaches in both the ovary and the nervous system to explore Fra signaling could lead to significant mechanistic insights into Fra's transcriptional activity. For example, since RNAi works effectively in the ovary, but not in the embryonic nerve cord, this will allow us to use RNAi to screen through genes of interest in the ovary, and follow-up on genes that have an effect on egg-laying in both the ovary and the nervous system. Finally, determining whether the mechanism of Fra as a transcription factor is similar or different between the ovary and the nervous system can give us insight into how Fra might regulate transcription in other tissues.

Upstream regulation of Netrin-independent Fra signaling is still unclear. Since gamma-secretase function is typically preceded by metalloprotease cleavage (Sardi et al., 2006; Bai and Pfaff, 2012), and both vertebrate homologs of Fra are cleaved by metalloproteases (Bai et al., 2011; Okamura et al., 2011), metalloprotease cleavage of Fra is most likely required for this function. Presumably, another ligand interacts with Fra, which recruits the metalloprotease that cleaves the extracellular domain of Fra prior to gamma-secretase. In HEK293T cells, Neo is cleaved by the metalloprotease Tace/Adam17 (Okamura et al., 2011). Since Tace is conserved in Drosophila, it will be interesting to determine if Tace cleaves the extracellular domain of Fra in the ovary, and if this required for Fra function at mid-oogenesis. The ovary is a useful system to determine upstream regulators, and downstream interactors and transcriptional targets, especially since Fra functions only independently of Netrin to promote germline survival at mid-oogenesis. This allows us to better dissect Netrin-independent Fra transcriptional regulation without having Netrin-dependent signaling complicating the results. In addition, it will be interesting to determine if upstream regulation of Fra is the same between the nervous system and the ovary.

Potential Fra interactors in the nerve cord

During nervous system development, Fra functions as a transcriptional activator. Interestingly, Fra does not contain any known DNA-binding motifs, suggesting that DNA-binding proteins interact with and recruit Fra to gene targets. To identify DNA-binding proteins that interact with Fra, as well as other proteins that interact with the Fra intracellular domain (ICD), I conducted a yeast-two hybrid screen. I used a Fra ICD with an inactive transcriptional activation domain fused to a LexA binding domain. For potential interactors, I used a library of cDNA plasmids from 0-24hr embryonic lysate that

generate proteins fused to a B42 activation domain. Testing the interaction between Fra and proteins identified in the yeast-two hybrid screen by doing co-immunoprecipitations in S2R+ cells will be necessary to confirm that these proteins interact with Fra.

The screen identified several categories of proteins that are interesting in regard to understanding Fra signaling, including ribosomal proteins, cytoskeletal-binding proteins, DNA-binding proteins and nuclear proteins, RNA-binding proteins, and metabolic proteins (Table 3.1). While ribosomal proteins are often false-positive interactors in a yeast-two hybrid screen, there is evidence that Dcc directly interacts with ribosomal proteins to regulate local translation in axons (Tcherkezian et al., 2010), and thus these candidates might be interesting to explore in future studies. The cytoskeletal proteins identified, including Mars, Cindr, WASp, Dop, and Dgt2, may be novel proteins that interact with Fra following Netrin binding. The DNA-binding and nuclear proteins, including Phol, Cll, Ro, Htk, Asense, Med30, Mnt, and Set2 may be required for Fra to regulate transcription. Since the cDNA library was collected from whole embryos, it is likely that some of the proteins identified may interact with Fra in tissues outside of the nervous system. Indeed, Ro is not expressed in the nerve cord, and Phol and Set2 are expressed ubiquitously in the embryo based on expression data from the Berkeley *Drosophila* Genome Project (BDGP).

Cll has a highly restricted expression pattern in two subsets of commissural neurons (Figure 3.6). The first population is restricted to cells within the EG eagle subpopulation, and the second is a pair of commissural neurons labelled by a Cll-Gal4 line (Figure 3.6). Unfortunately, this Gal4 line turns on shortly after stage 14, and *comm* mRNA expression is faint and inconsistent in these neurons at this time (unpublished), making it difficult to determine if *cll* or *fra* mutants might affect *comm* mRNA expression in these neurons. This is consistent with what we know about *comm* mRNA, which is

spatially and temporally restricted to neurons as their axons are crossing the midline (Keleman et al., 2005; Keleman et al., 2002). In addition, these two populations are not affected by the sensitized background (FraΔC) that the lab has used in the past to identify proteins that are important for midline axon guidance (Garbe et al., 2007; O'Donnell and Bashaw, 2013; Hernandez-Fleming et al., 2017). This makes it challenging to easily determine if Cll has a role in axon guidance. Still, Fra is important for axon growth across the midline, and *fra* mutants affect many populations of commissural neurons (Kolodziej et al., 1996; Hernandez-Fleming et al., 2017). Thus, it is likely that Cll-Gal4 neurons have Fra expressed on their growth cones and require Fra for axon growth across the midline. This can be determined by counting crossing defects in *fra* mutant embryos using the Cll-Gal4 and a reporter that labels axons. In addition, testing whether *cll* mutants affect axon guidance in either of these neuronal populations, Cll-Gal4 or EG neurons, will resolve whether Cll has a role in axon guidance. As for using *comm* mRNA expression as a readout for Fra transcriptional function in EG neurons, *comm* mRNA levels are decreased in these neurons in *fra* mutants (Yang et al., 2009). However, Cll is only expressed in some of the EG neurons, which would make scoring for *comm* expression difficult if there is not a striking difference between sibling controls and *cll* mutants. Thus, it would be interesting, although challenging, to determine if *comm* levels in the EG neurons are affected in *cll* mutants. Because Cll is restricted to two small neuronal subpopulations, if it is important for *comm* expression in EG neurons, this would support the idea that Fra interacts with different DNA-binding proteins in different cells.

Unlike Cll, Phol has a broad expression pattern in the embryonic nerve cord. I used the sensitized FraΔC screening background to determine if Phol has a role in axon guidance. I saw that removing one copy of *phol* does increase axon guidance defects

(Figure 3.4), indicating that Phol is involved in axon guidance. Still, it is very likely that Phol may be acting non-cell-autonomously. Thus, I over-expressed Phol-HA in a subset of neurons that never cross the midline, the apterous neurons, to determine if Phol-HA has a pro-crossing role in axon guidance. I saw that Phol-HA induces ectopic crossing in a dose-dependent manner when expressed in apterous neurons (Figure 3.5), suggesting that Phol can function cell-autonomously to promote axon growth across the midline. Interestingly, *fra-/-;phol-/-* and *fra-/-;phol+/-* embryos have very severe axon guidance defects as compared to the *fra+/-;phol+/-* and *fra+/-;phol-/-* embryos, indicating that Phol has a Fra-independent function (Figure 3.4). These experiments need to be repeated to increase the sample size to be more confident in the conclusions indicated by these preliminary results. Nevertheless, the severity of axon guidance defects in the *fra-/-;phol-/-* mutants (Figure 3.4) suggests that if Phol does function with Fra, then it also has other functions in the nerve cord, and most likely has pleiotropic effects. Because Phol is a DNA-binding protein that interacts with PRC1 and PRC2 complexes to maintain gene expression of polycomb genes, this is a real possibility (Chen et al., 2010; Schuettengruber et al., 2009; Kim et al., 2008).

These experiments have contributed to our understanding of how Fra acts as a transcription factor. Furthering our knowledge of Fra signaling in oogenesis can help us to better understand how Fra functions in axon guidance. In addition, future work could further our understanding about how axon guidance receptors function as transcription factors during axon guidance, and may provide insight into the mechanisms that drive axon regeneration after injury. More broadly, insights into this pathway in both oogenesis and axon guidance will give us a better understanding of how cells communicate to regulate tissue morphology in the different tissues where Fra/Dcc, and other guidance receptors, are expressed.

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