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

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Summary statement: The transcriptional activation domain of Frazzled is required to promote germline survival independently of its canonical ligand Netrin.

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 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 lead to cleavage of the Dcc ICD by caspase 3, activating 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, *netrin* conditional knockout in rodent brains leads 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 tissues (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 *C. elegans* reproductive system innervation (Asakura et al., 2007), and Unc-6 secreted from neurons also shapes the developing reproductive system (Ziel et al., 2009). In other cases, Netrin and its receptors have clear tissue-intrinsic roles. For example, 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 germarium's anterior tip, 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) (Fig. 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 cell death effector caspase Dcp-1 cleavage and activation, then 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 (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 (Fig. 1B,B’). In the vitellarium, GFP-Fra is present on the membrane of somatic follicle cells, where it is enriched at the apical domain (Fig. 1B’, arrowhead). We also detect GFP-Fra on both nurse cell (arrow) and oocyte membranes in the germline. Additionally, 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 (Fig. 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 (Fig. 1E,F,G). As expected, GFP-negative cells do not contain Fra (Fig. 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: *fra*^{3} and *fra*^{4} (null alleles) and *fra*^{6} (a hypomorphic allele) (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) (Fig. 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 (Fig. 2B-D). This degeneration is easily recognized by the presence of pyknotic nurse cell nuclei (Fig. 2B’) and is accompanied by the apparent enlargement of some follicle cells (Fig. 2B’’), suggesting germline engulfment (Etchegaray et al., 2012). Consistent with differences in Fra 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 (Fig. 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 (Fig. 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 (Fig. 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 cytoplasmic GFP leakage, 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 (Fig. 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 (Fig. 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 (Fig. 3E). As expected, germline expression of the full-length Fra receptor rescues the *fra* mutant degeneration phenotype (Fig. 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 impinge 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, unphosphorylated FoxO is is transported into the nucleus to activate 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 and germline cells and is enriched at the follicle cell apical (Fig. 4E,H). Arm localization in follicle cells adjacent to *fra* germline cysts is indistinguishable from its localization in wild type ovarioles (Fig. 4I). Similarly, Discs large (Dlg), which localizes to lateral domains of follicle cells (Goode and Perrimon, 1997), is normally distributed in egg chambers with *fra* mutant germline cysts (4J). These results suggest that Fra does not regulate apicobasal or lateral follicle cell polarity non-autonomously. As expected, localization of both Arm and Dlg is unperturbed in *fra* mutant follicle cells (Fig. 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 (Fig. S2A,B), indicating that engulfment signaling in the follicle cells is active in degenerating egg chambers. Thus, while known triggers of the mid-oogenesis checkpoint appear unaffected in *fra* mutants, downstream degeneration appears similar.

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 (Fig. 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 (Fig. 5C,D), creating egg chambers with a persistent germline and missing follicle cells ("balding" egg chambers, Fig. 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; expression decreases at mid-oogenesis, then increases again after stage eight (Baum et al., 2007; Foley and Cooley, 1998) (Fig. 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 prevents degeneration caused by starvation (Baum et al., 2007; Mazzalupo and Cooley, 2006). We reasoned that if Fra were preventing Dcp-1 activation through its negative regulator Diap1, then *fra* germline cysts might have prematurely 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 (Fig. 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 has Netrin-independent and -dependent signaling 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 (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 in the germarium (Fig. 6A). We do not detect NetrinA outside of the germarium. In contrast, flies expressing NetrinB-Myc from its endogenous locus (Brankatschk and Dickson, 2006) have Myc signal throughout the ovariole (Fig. 6B,B'). Thus, while NetrinA is unlikely to signal through Fra in the vitellarium, the NetrinB expression pattern is consistent with such a role.

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 (Fig. 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 (Fig. 6D). Thus, Netrin is dispensable for progression of egg chambers through mid-oogenesis, and 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

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 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 none changed the rate of degeneration at mid-oogenesis when overexpressed in the germline of wild-type flies (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 (Fig. 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 (Fig. 7A,D). Since this E1354A point mutation disrupts the transcriptional activation domain in Fra without affecting 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 (Fig. 7B,E). Surprisingly, the Fra ICD alone fails to rescue degeneration in *fra* germline clones (Fig. 7C,D) despite rescuing 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 are necessary for Fra function in the germline but dispensable in the nervous system. 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 its only known target (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 sets of primers directed against *comm* cDNA on mRNA extracted from both *Drosophila* ovaries and embryos. While we detect *comm* mRNA in the ovary, this method cannot resolve its expression pattern (Fig. 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 (Fig. S3B). A positive control, where we induce transgenic Comm expression in follicle cells using *traffic jam-GAL4*, demonstrates that our probe can detect *comm* mRNA (Fig. S3C), indicating 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 low levels in certain follicle cells in the ovary but does not detect germline Comm (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 (Fig. S3D). Moreover, when we compared *fra*/+; *comm*/+ female flies to sibling controls, we observed no 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

Here, we explore the role of Fra in the ovary and demonstrate that Fra intrinsically promotes germline survival independently of Netrin. Fra localized to the cell membrane of nurse cells, oocytes, and follicle cells. Loss of germline, but not follicle cell, *fra* leads to 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 have wild-type starvation-induced degeneration response and FoxO localization, suggesting that Fra is not involved in nutrient sensing. Furthermore, the normal localization of Orb, Armadillo, and Discs large in egg chambers with *fra* mutant germline cysts indicates that loss of *fra* is unlikely to affect germline or apicobasal/lateral follicle cell polarity. In *fra* mutant germlines, Dcp-1 expression is elevated, suggesting that the degeneration observed at mid-oogenesis is triggered by the activation of apoptosis. Accordingly, specifically inhibiting caspases with the *UASp-p35* transgene results in a robust rescue of the *fra* mutant degeneration phenotype. 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 at the mid-oogenesis checkpoint. Consistent with a Netrin-independent role for Fra here, 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 vitellogenesis requires significant energy input, this checkpoint may prevent a costly investment in a low-quality oocyte (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 localization indicates 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 regulate germline stem cell maintenance (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 several contexts, Dcc has been shown to 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 limited to 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. Removing Netrin has no effect on egg chamber degeneration. However, Fra loss 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, Fra ICD cleavage generates multiple 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, Fra's transcriptional activation domain is required for egg chamber progression 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 eliminate the possibility that it enters 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 we do not detect Comm 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). Our work in the ovary 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.

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 teased apart, then 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 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 overnight at 4°C with rocking. Antibodies were washed off with PBT, then samples were stained with secondary antibodies and/or stains diluted in block for at least one hour at room temperature with rocking. After washing, samples were cleared in 90% glycerol with antifade (20 µg/mL propyl gallate) overnight, then mounted onto slides. Ovaries were analyzed 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: chick anti-GFP (1:1000, abcam #13970), mouse anti-1B1 (1:100, DSHB concentrate), mouse anti-LamC (1:20, DSHB supernatant), 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 (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 prior to dissection.

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.

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. RNA was extracted from both ovaries and embryos using the Qiagen RNeasy mini kit protocol. 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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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

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

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

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

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

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 (Fig. 7).

Fig. 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 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, 20 microns.

Fig. 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 illustrates 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 wild-type and 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, 20 microns.

Fig. 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, 20 microns.

Fig. 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, 20 microns.

Fig. 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 of 44 ovarioles. Scale bars, 20 microns.

Fig. 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, 20 microns.

Fig. 7.

Fra's transcriptional activation domain is required for egg chamber progression through mid-oogenesis. (A-C) Ovarioles with *fra*^{3} 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 Fig. 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 *fra*^{3} 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, 20 microns.

Supplementary Fig. 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, 20 microns.

Supplementary Fig. 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, 20 microns.

Supplementary Fig. 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. 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, 20 microns.

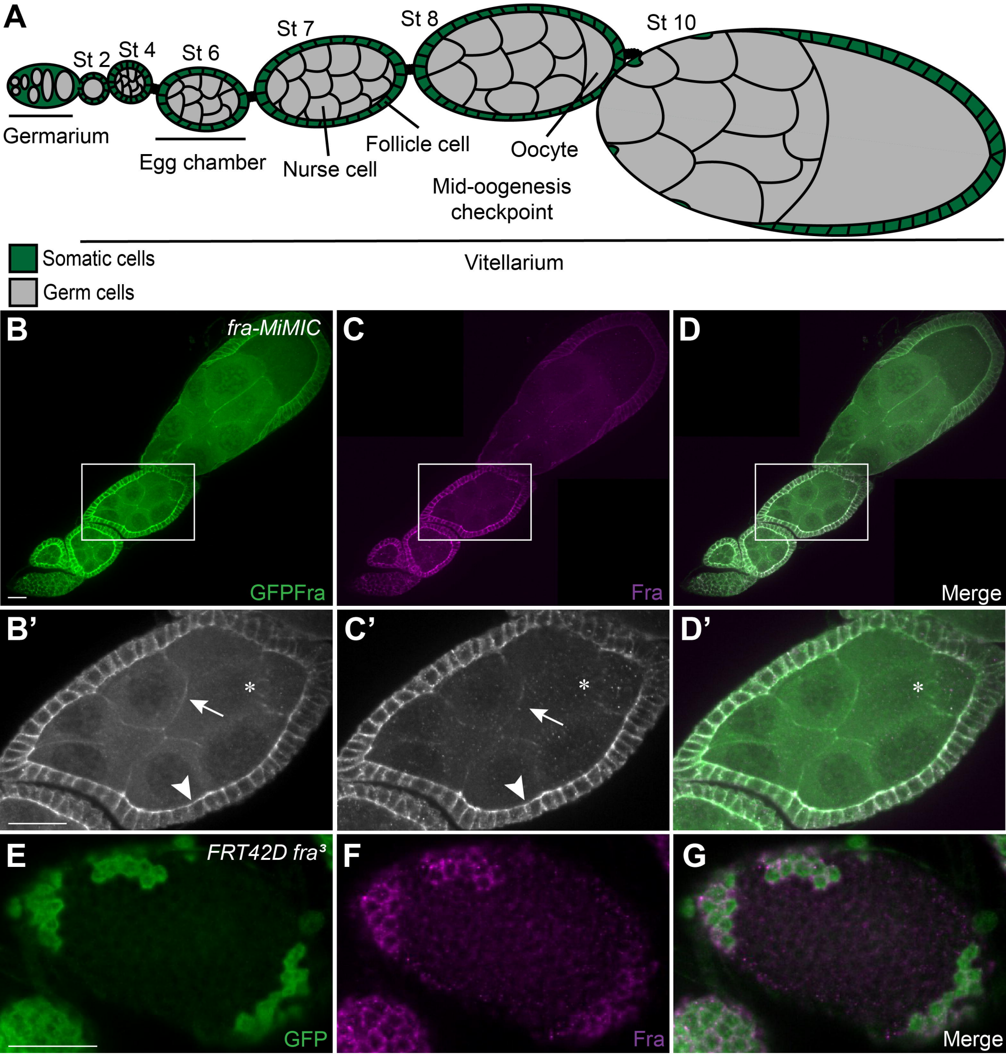
A
St 2
St 4
St 6
St 7
St 8
St 10
Germarium
Egg chamber
Nurse cell
Follicle cell
Oocyte
Mid-oogenesis checkpoint
Vitellarium
Somatic cells
Germ cells
B
fra-MiMIC
GFPFra
C
Fra
D
Merge
B'
C'
D'
*
E
FRT42D fra³
GFP
F
Fra
G
Merge

Fig. 1.

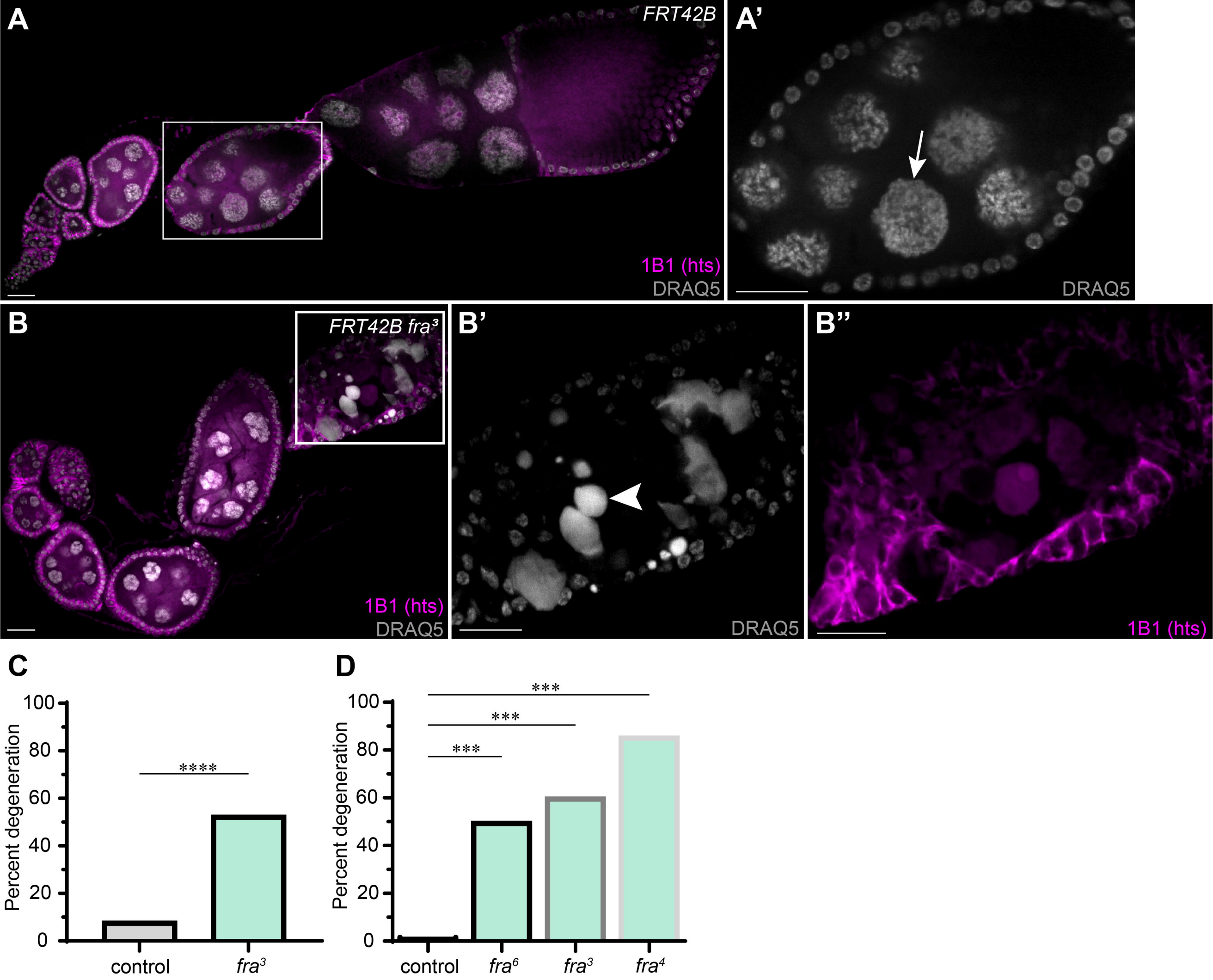
A
FRT42B
1B1 (hts)
DRAQ5
A’
DRAQ5
B
FRT42B fra³
1B1 (hts)
DRAQ5
B’
DRAQ5
B’’
1B1 (hts)
C
Percent degeneration
0
20
40
60
80
100
****
control
fra³
D
Percent degeneration
0
20
40
60
80
100
***
***
***
control
fra⁶
fra³
fra⁴

Fig. 2.

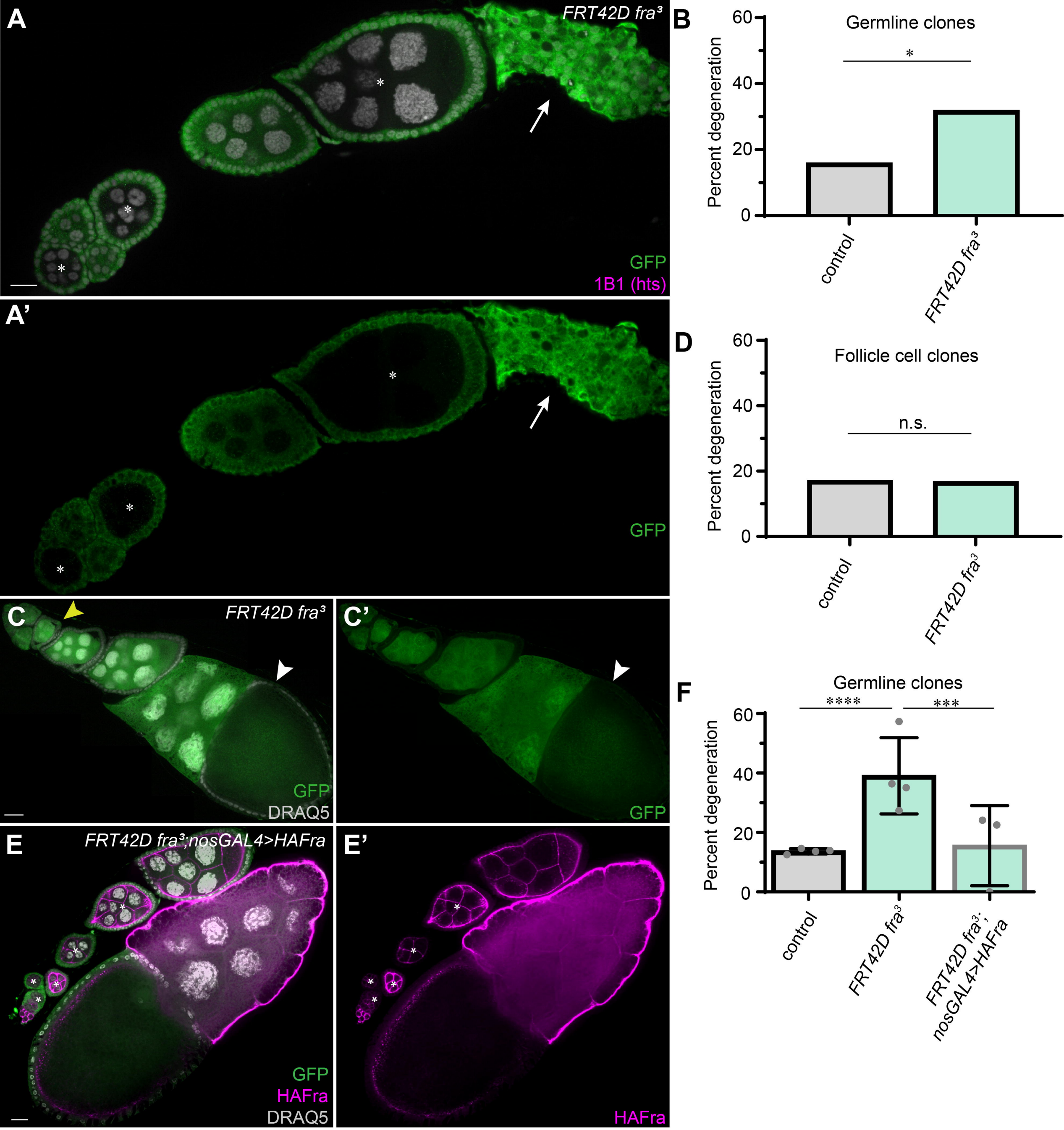
A
FRT42D fra³
GFP
1B1 (hts)
A'
GFP
B
Germline clones
Percent degeneration
60
40
20
0
*
control
FRT42D fra³
D
Follicle cell clones
Percent degeneration
60
40
20
0
n.s.
control
FRT42D fra³
C
FRT42D fra³
GFP
DRAQ5
C'
GFP
E
FRT42D fra³;nosGAL4>HAFra
GFP
HAFra
DRAQ5
E'
HAFra
F
Germline clones
Percent degeneration
60
40
20
0
****
***
control
FRT42D fra³
FRT42D fra³; nosGAL4>HAFra

Fig. 3.

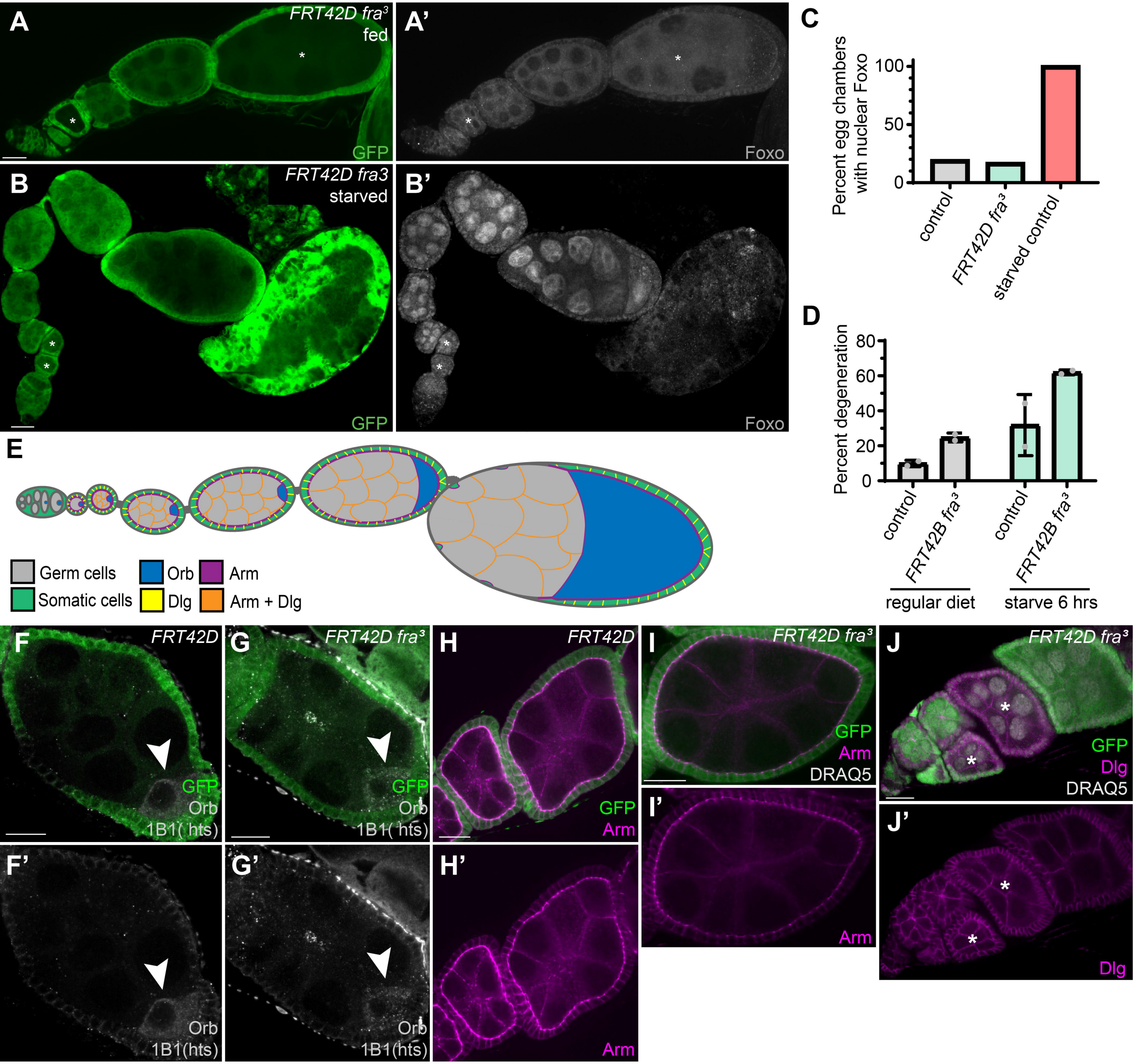
A
FRT42D fra^3
fed
*
*
GFP
A’
*
*
Foxo
B
FRT42D fra3
starved
*
*
GFP
B’
*
*
Foxo
C
Percent egg chambers with nuclear Foxo
100
50
0
control
FRT42D fra^3
starved control
D
Percent degeneration
80
60
40
20
0
control
FRT42B fra^3
control
FRT42B fra^3
regular diet
starve 6 hrs
E
Germ cells
Orb
Arm
Somatic cells
Dlg
Arm + Dlg
F
FRT42D
GFP
Orb
1B1(hts)
F’
Orb
1B1(hts)
G
FRT42D fra^3
GFP
Orb
1B1(hts)
G’
Orb
1B1(hts)
H
FRT42D
GFP
Arm
H’
Arm
I
FRT42D fra^3
GFP
Arm
DRAQ5
I’
Arm
J
FRT42D fra^3
*
*
GFP
Dlg
DRAQ5
J’
*
*
Dlg

Fig. 4.

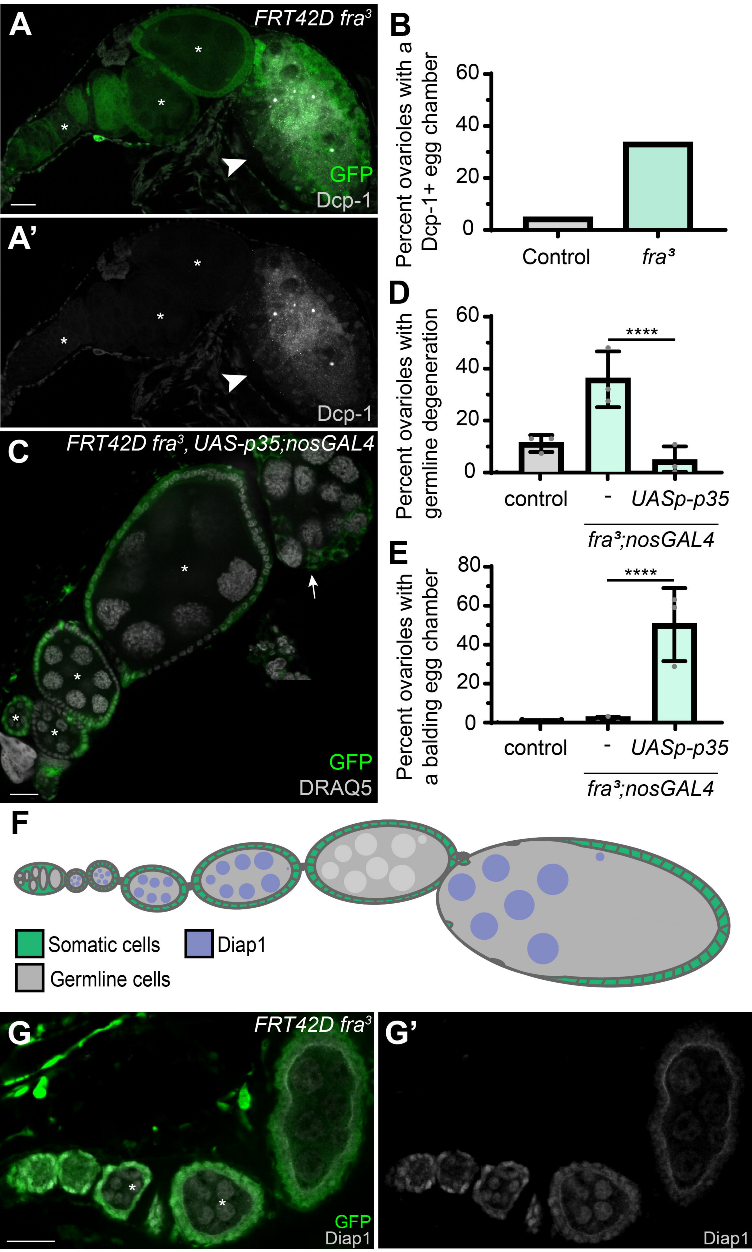
A
FRT42D fra³
GFP
Dcp-1
A'
Dcp-1
B
Percent ovarioles with a Dcp-1+ egg chamber
60
40
20
0
Control
fra³
C
FRT42D fra³, UAS-p35;nosGAL4
GFP
DRAQ5
D
Percent ovarioles with germline degeneration
****
control
-
UASp-p35
fra³;nosGAL4
E
Percent ovarioles with a balding egg chamber
80
****
F
Somatic cells
Diap1
Germline cells
G
FRT42D fra³
GFP
Diap1
G'
Diap1

Fig. 5.

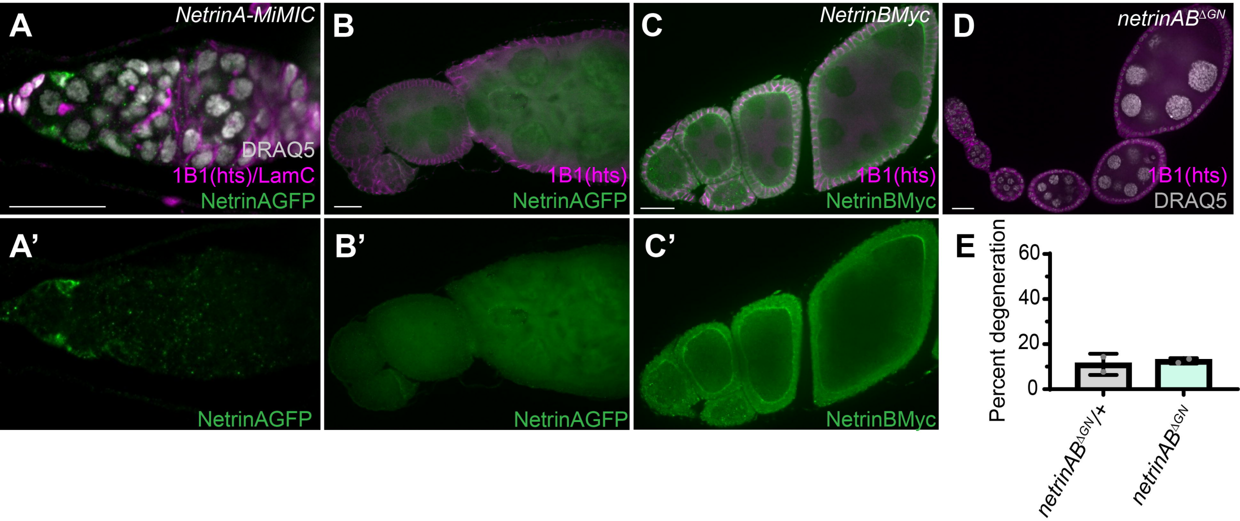
A
NetrinA-MiMIC
DRAQ5
1B1(hts)/LamC
NetrinAGFP
B
1B1(hts)
NetrinAGFP
C
NetrinBMyc
1B1(hts)
NetrinBMyc
D
netrinAB$^{\Delta GN}$
1B1(hts)
DRAQ5
A'
NetrinAGFP
B'
NetrinAGFP
C'
NetrinBMyc
E
Percent degeneration
60
40
20
0
netrinAB$^{\Delta GN}$/+
netrinAB$^{\Delta GN}$

Fig. 6.

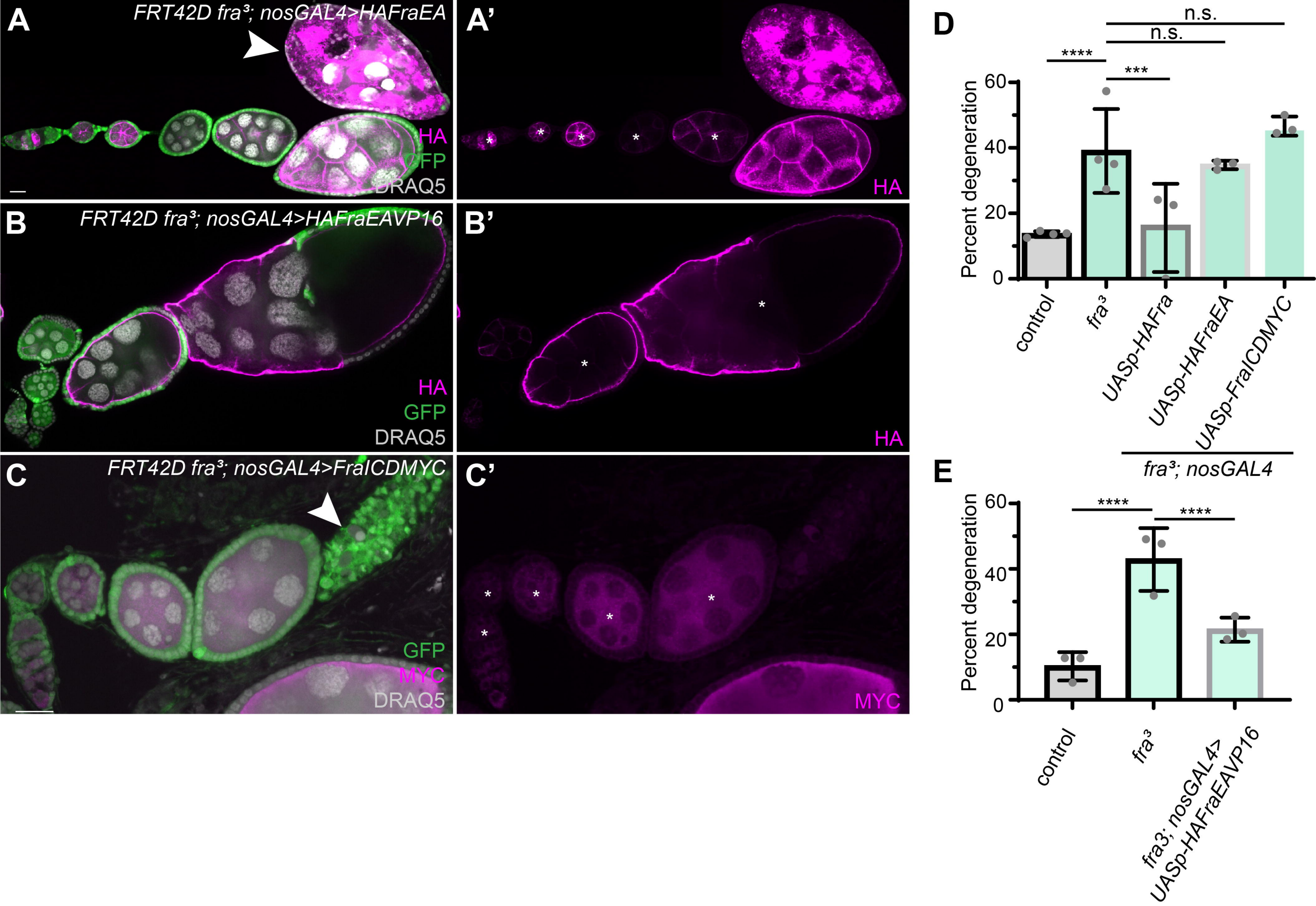
A
FRT42D fra³; nosGAL4>HAFraEA
HA
GFP
DRAQ5
A'
HA
B
FRT42D fra³; nosGAL4>HAFraEAVP16
HA
GFP
DRAQ5
B'
HA
C
FRT42D fra³; nosGAL4>FraICDMYC
GFP
MYC
DRAQ5
C'
MYC
D
Percent degeneration
0
20
40
60
****
***
n.s.
n.s.
control
fra³
UASp-HAFra
UASp-HAFraEA
UASp-FraICDMYC
fra³; nosGAL4
E
Percent degeneration
0
20
40
60
****
****
control
fra³
fra3; nosGAL4>
UASp-HAFraEAVP16

Fig. 7.