THE ROLE OF *DROSOPHILA* NUCLEAR RECEPTOR *FTZ-F1* IN SOMATIC CELLS FOR GERM CELL DEVELOPMENT

By

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ABSTRACT

The production of gametes is a controlled synergistic relationship between germ cells and somatic cells. In *Drosophila* oogenesis, the NR5A subfamily nuclear receptor *ftz-f1* is expressed in the germline stem cells, germ cysts, somatic escort cells, and follicle cells. *Ftz-f1* has been identified for its roles in oocyte production to promote egg chamber survival, germline stem cell maintenance, and ecdysteroid signaling. This paper identifies *ftz-f1* in the escort cells as an important, but not a sole contributor, to germ cell encapsulation and oocyte specification. We also demonstrate that depletion of *ftz-f1* in the escort cells and neurons results in a loss of germline stem cells from the niche that can be rescued by external supplement of ecdysone. Interestingly, we find that *ftz-f1* is likely necessary in neural tissue only for germline stem cell maintenance. This study highlights the importance of somatic and germline cell communication and provides novel potential insight into long range signaling between the nervous system and reproductive systems.

THE ROLE OF *DROSOPHILA* NUCLEAR RECEPTOR *FTZ-F1* IN SOMATIC CELLS IS NECCESSARY FOR GERMLINE STEM CELL MAINTENANCE

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Sydney Sanders

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LIST OF SYMBOLS OR ABBREVIATIONS

GSC Germline Stem cell

CB Cystoblast

Ftz-f1 *Ftz-transcription factor-1*

BMP *Bone Morphogenetic Protein*

EGFR *Epidermal Growth Factor Receptor*

Dpp *Decapentaplegic*

Gbb *Glass bottom boat*

RNAi RNA interference

LRH-1 Liver Receptor homolog-1

SF-1 Steroidogenic Factor-1

Bam Bag of Marbles

Dcp-1 *Drosophila* cleaved caspase-1

Dad Daughters against Decapentaplegic

Tor Target of Rapamycin

Smurf Smad ubiquitination regulatory factors

EcI Ecdysone importer

EcR Ecdysone receptor

Phm Phantom

AstC-DN1p Allostatin C in the posterior dorsal neuron 1

AstC Allostatin C

JH Juvenile hormone

Dae Days after eclosion

CHAPTER 1: INTRODUCTION

Oogenesis is a combination of tightly regulated cell biological processes that synergize to generate a mature oocyte (Sánchez & Smitz, 2012). Vertebrate and invertebrate reproductive systems are composed of germline and somatic cells which are formed from unique cell lineages during development, but work in conjunction with one another to produce a viable egg. Haploid germ cells are integral to development and the only cell type that can generate an entirely new organism (Cinalli et al., 2008). Somatic cells in close proximity to the germ cells are essential for oogenesis. Cellular communication between companion somatic cells and primordial oocytes is necessary for providing the developing egg regulatory signals to promote developmental competence in mammals and insects (Sánchez & Smitz, 2012). Despite the extensive knowledge of the molecular control of both cell types separately, it is largely unclear how the somatic cells guide early germ cell division in the ovary (Antel & Inaba, 2020; Clarke, 2018; Elkouby & Mullins, 2017; Kirilly et al., 2011; Rodrigues et al., 2021). Moreover, somatic cells at great distances in the body can also influence germ cell proliferation and development. The most classic example is the mammalian hypothalamic-pituitary-ovary axis, in which the hypothalamus releases gonadotrophins to stimulate the pituitary gland to secrete other hormones including luteinizing hormone (LH) and follicle stimulating hormone (FSH) to stimulate the sexual maturation of the organism (Kaprara & Huhtaniemi, 2018). However, the respective contributions of local soma/germ cell signaling versus long-range signals on oocyte development are unknown.

Drosophila melanogaster is a valuable model organism for studying intercellular interactions due to the structure of the female ovary and the vast array of genetic tools available for tissue-specific depletion of gene products. Female *Drosophila* contain two ovaries, connected by an oviduct, each composed of 14-16 strands of ovarioles containing progressively

older egg chambers or follicles (Figure 1A) (Hinnant et al., 2020; McLaughlin & Bratu, 2015). At the anterior tip of each ovariole is a germarium that contains germline stem cells (GSCs) and developing germline cysts, surrounded by somatic support cells (cap cells, escort cells, and follicle cells) (Figure 1B). The GSCs are housed in the anterior region of the germarium where they are attached to cap cells via adherens junctions (Song et al., 2002). This area of the germarium is considered the self-renewal niche, in which GSC maintenance is regulated by the adjacent cap cells and escort cells (Hinnant et al., 2020). GSCs go through an asymmetric mitotic division to create a cystoblast committed to differentiation. Subsequently, the cystoblast goes through a series of four mitotic divisions with incomplete cytokinesis, forming 2-, 4-, 8-, and 16-cell cysts. Each of these cysts are ushered posteriorly through the germarium by escort cells until they become encapsulated by a monolayer of follicle cells. When a 16-cell cyst exits the germarium, it will become a single egg chamber that will develop further into a mature egg (Banisch et al., 2017).

Escort cells are a supportive somatic cell type known to promote germ cell proliferation and differentiation (Banisch et al., 2017). Escort cells possess unique actin-based cellular projections that physically contact the GSCs and developing cysts, aiding germ cell differentiation (Banisch et al., 2017; Kirilly et al., 2011). Previous studies have shown that escort cells have a variety of different and dynamic morphological features that are used to properly capture, release, and usher the germ cells through the germaria to be received by the follicle cells (Banisch et al., 2017). The positioning of escort cells within the germaria play a role in the specific processes necessary for the germ cells to mature into a functional oocyte (Banisch et al., 2017).

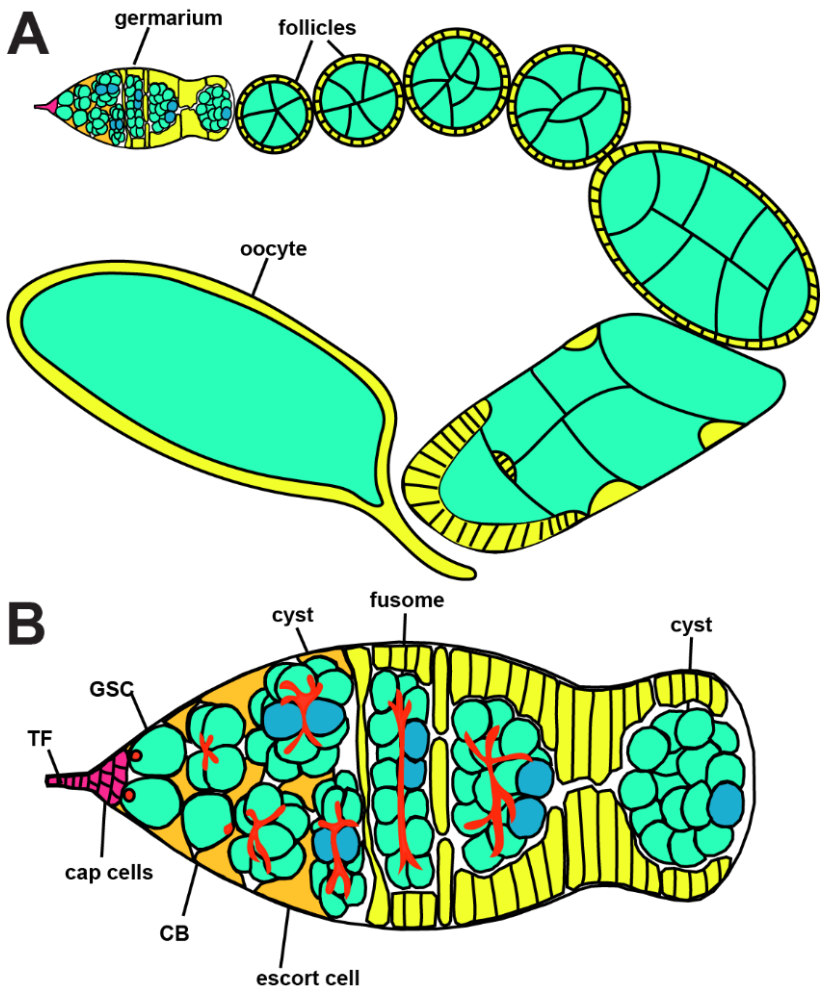

Figure 1. *Drosophila melanogaster* as a model for oogenesis.

The *Drosophila* ovary is composed of 14-16 ovarioles (A) consisting of progressively older egg chambers. Beginning at the transverse filament (TF) the cap cells anchor the germline stem cells (GSCs) housed at the anterior tip of the tip of germaria (B) which divide asymmetrically into the cystoblast (CB) with the assistance of the escort cells (orange). The CB then mitotically divides into a 2, 4, 8, and 16-cell cyst which is composed of 15 nurse cells and one oocyte (blue) connected by a branching fusome (red). The fusome degrades and the 16-cell cyst is encapsulated by a monolayer of follicle cells (yellow) forming the presumptive egg chambers to progress the developing oocyte into a mature egg ready for fertilization.

Live cell imaging studies have identified two characteristic types of escort cell extensions in the germarium: small highly dynamic projections and long persistent protrusions (Banisch et al., 2017). Escort cells in the self-renewal niche, anterior escort cells (aECs), have shorter projections and primarily function in the self-renewal and maintenance of the GSCs in an undifferentiated state (Tu et al., 2021; Xie, 2013). As cystoblasts/cysts move away from the self-renewal niche and begin the process of differentiation, the posterior escort cells lengthen their projections to traverse the germarium to adequately encapsulate the mitotically active germ cell cysts (Kirilly et al., 2011; Liu et al., 2015; Maimon et al., 2014). Posterior escort cells have protrusions which are characteristically longer and have been observed to not only contact the germ cells but each other. These structures are thought facilitate packaging and delivery of the cysts to neighboring follicle cells (Banisch et al., 2017). Other labs have attempted to further characterize various populations of escort cells based primarily on gene expression. For example, while one group identified two types of escort cells (anterior and posterior), which exhibit longer projections that orchestrate cyst division, differentiation, and 16-cell cyst-to-egg chamber formation (Shi et al., 2021), other studies using single cell transcriptomics and lineage tracing revealed three or five distinct escort cell populations (Rust et al., 2020; Tu et al., 2021). Although it remains unclear how different escort cells are from one another, their importance in terms of germ cell differentiation and physical movement through the germarium is undisputed.

One of the most important roles of somatic cells on germ cell function is to provide secreted ligands and signals necessary to maintain GSC self-renewal, cyst survival, and yolk deposition (Armstrong, 2019; Drummond-Barbosa, 2019). For example, intercellular signaling pathways allow escort cells to extrinsically control of GSC maintenance and progeny differentiation (Tu et al., 2021). In the self-renewal niche, the cap cells and anterior-most escort cells secrete the Bone Morphogenetic Protein (BMP) ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb) to

promote self-renewal of the GSC (Tseng et al., 2018). Reception of these signals in GSCs represses transcription of germ cell differentiation factors, such as Bag of marbles (Bam), in the GSCs, which is necessary for renewal of GSCs and suppression of differentiation (Xie, 2013). A recent genetic screen for escort cells for factors that modulate GSC self-renewal also suggest that preventing BMP signaling is a general function of the differentiation niche (Gao et al., 2019). As the daughter cell (cystoblast) moves away from the self-renewal niche, levels of TGF-β ligands are not sufficient to activate the BMP signaling pathway, permitting the transcription of Bam and subsequent differentiation. Bam is a ubiquitin-associated protein necessary for differentiation of the cystoblast and subsequent mitotic divisions (Ji et al., 2017; McKearin & Spradling, 1990; Ohlstein & McKearin, 1997). Escort cells also produce Dally, a protein that sequesters TGF-β ligands to keep signaling confined in the self-renewal niche. Regulation of BMP signaling is therefore pivotal to self-renewal of GSCs and differentiation of their daughter cells.

***The nuclear receptor ftz transcription factor-1 (ftz-f1*)**

Ftz transcription factor 1 (ftz- f1), a member of the NR5A superfamily, is required in *Drosophila* embryogenesis, anterior/posterior patterning, metamorphosis, axon pruning, and, in the ovary, egg chamber survival and ovulation (Beachum et al., 2021; Boulanger et al., 2011; Guichet et al., 1997; Knapp et al., 2020; Ueda et al., 1992). As a "orphan" nuclear receptor, *Ftz-f1* can bind phospholipid ligands or activate transcription without the presence of a ligand. *Ftz-f1* is conserved with mammalian orthologs implicated in reproductive functions, sex determination, gonadogenesis, and ovulation (Meinsohn et al., 2019; Yazawa et al., 2015). Mammalian orthologs *Liver Receptor Homolog-1* (*LRH-1*) and *Steroidogenic Factor-1* (*SF-1*) are required for the formation of corpus luteum as well as granulosa cell proliferation in mouse oogenesis. *Ftz-f1* is

structurally conserved and binds similar regulatory DNA sequences to LRH-1 and SF-1 (Bertolin et al., 2014; Duggavathi et al., 2008; Meinsohn et al., 2019). The *Ftz-f1* genomic locus encodes two distinct mRNA isoforms. α-*ftz-f1* is maternally loaded into the egg and plays a role in anterior/posterior patterning of the embryo. β-*ftz-f1* is required for metamorphosis, ecdysone induced gene expression, and cholesterol uptake with subsequent conversion to ecdysone, one of the major steroid hormones in *Drosophila* (Parvy et al., 2005; Talamillo et al., 2013). The structural and functional activities of *ftz-f1* and its mammalian homologs are indicative of a deeply rooted role in the complex processes of gonadal functions including gametogenesis.

Interestingly, *Ftz-f1* is expressed in a variety of ovarian cell types including the escort cells and may function there to guide cyst encapsulation downstream of ecdysone signaling activation (Beachum et al., 2021; Shi et al., 2021). Ecdysone is essential for a variety of important checkpoints in oocyte formation, including cyst formation and encapsulation, follicle cell growth and development, and migration of border cells and follicle cells (Bai et al., 2000; Buszczak et al., 1999; Carney & Bender, 2000; Jang et al., 2009; König et al., 2011; Morris & Spradling, 2012). Although ecdysone is synthesized by late stage follicle cells in more developed egg chambers, recent studies suggest that ecdysone may also be synthesized in escort cells (Gancz et al., 2011; Huang et al., 2008; Shi et al., 2021). Indeed, Shi and colleagues recently proposed the model that posterior escort cells receive ecdysone produced from the anterior escort cells, activating *ftz-f1* transcription to guide cyst encapsulation and transition to neighboring egg chambers (Shi et al., 2021).

Using the *Drosophila* ovary as a model system, this research addresses how *ftz-f1* in somatic cells interact with the germline to guide the self-renewal and maintenance of a resident stem cell population. Here we identify f*tz-f1* as an important non-autonomous regulator of germline

stem cell development in the ovary. We show that somatic *ftz-f1* is not a primary driver of germline cyst encapsulation, however, remains as an important regulator of the GSC niche. To determine if *ftz-f1* is required for ecdysone biosynthesis and GSC niche maintenance, we performed a rescue experiment and found that supplemental ecdysone partially rescues GSC number. We also deduce that the cell death is not due to ftz-f1 depletion, but instead is a function of experimental temperature shifting. Lastly, through the use of a newly available escort cell driver, we propose that *ftz-f1* is more likely needed in the neurons to maintain GSC populations, instead of the escort cells.

CHAPTER 2: Results

Somatic *ftz-f1* contributes to germline cyst encapsulation

During typical egg production, cystoblasts/cysts are surrounded by escort cell protrusions and moved posteriorly through the germarium, while synchronously dividing into 4, 8, and 16-cell cysts (Figure 1). They are then packaged by a monolayer of follicle cells before exiting the germaria into individualized egg chambers. Previous studies by our lab and others identified *ftz-f1* as a novel regulator of oogenesis (Beachum et al., 2021; Shi et al., 2021). In particular, Shi and colleagues proposed the model that *ftz-f1* is necessary in posterior escort cells to promote cyst differentiation (Shi et al., 2021). Preliminary studies using a pan-ovarian somatic cell driver supported this model, as egg chambers occasionally had more or less than 16 cells (an indicator of encapsulation defects) (Beachum et al., 2021). As a more definitive test of this model, I used the *UAS-Gal4/Gal80* system to specifically manipulate *ftz-f1* expression in somatic cells. The *UASGal4/Gal80* system is a valuable tool for controlling spatial and temporal expression of genes (Weaver et al., 2020). In this study, we used the *c587-Gal4;Gal80$^{t.s.}$* fly which expresses the yeast derived *Gal4* protein and temperature activated *Gal80* protein to drive expression of the *ftz-f1^{RNAi}* alleles in somatic tissue of the ovary. At 18°C, both the *Gal4* and the *Gal80* proteins are transcribed, subsequently bind together, and position themselves, binding to the *Upstream Activation Site* of the *ftz-f1^{RNAi}* locus, repressing the production of the short hairpin RNAs. Upon temperature shifting to 29°C, the *Gal80* is repressed, allowing the *Gal4* to bind to the *UAS-ftz-f1^{RNAi}* locus, permitting the production of the hairpins, which is particularly important to this study for restricting the RNAi until adulthood negating larval lethality (Figure 2). The *c587-Gal4;Gal80 $^{t.s.}$* has been used frequently in studies to manipulate gene expression specifically in the somatic cells within the *Drosophila* reproductive organs (Zhu & Xie, 2003). Many labs have used this driver to investigate

the implications of various knockdown alleles in the escort cells, including Shi and colleagues. However, recently it was discovered that the *c587-Gal4;Gal80$^{t.s.}$* driver not only expresses in the somatic escort cells, but also in a number of unreported cell types including the neurons, fat body, and later stage follicle cells (Weaver et al., 2020).

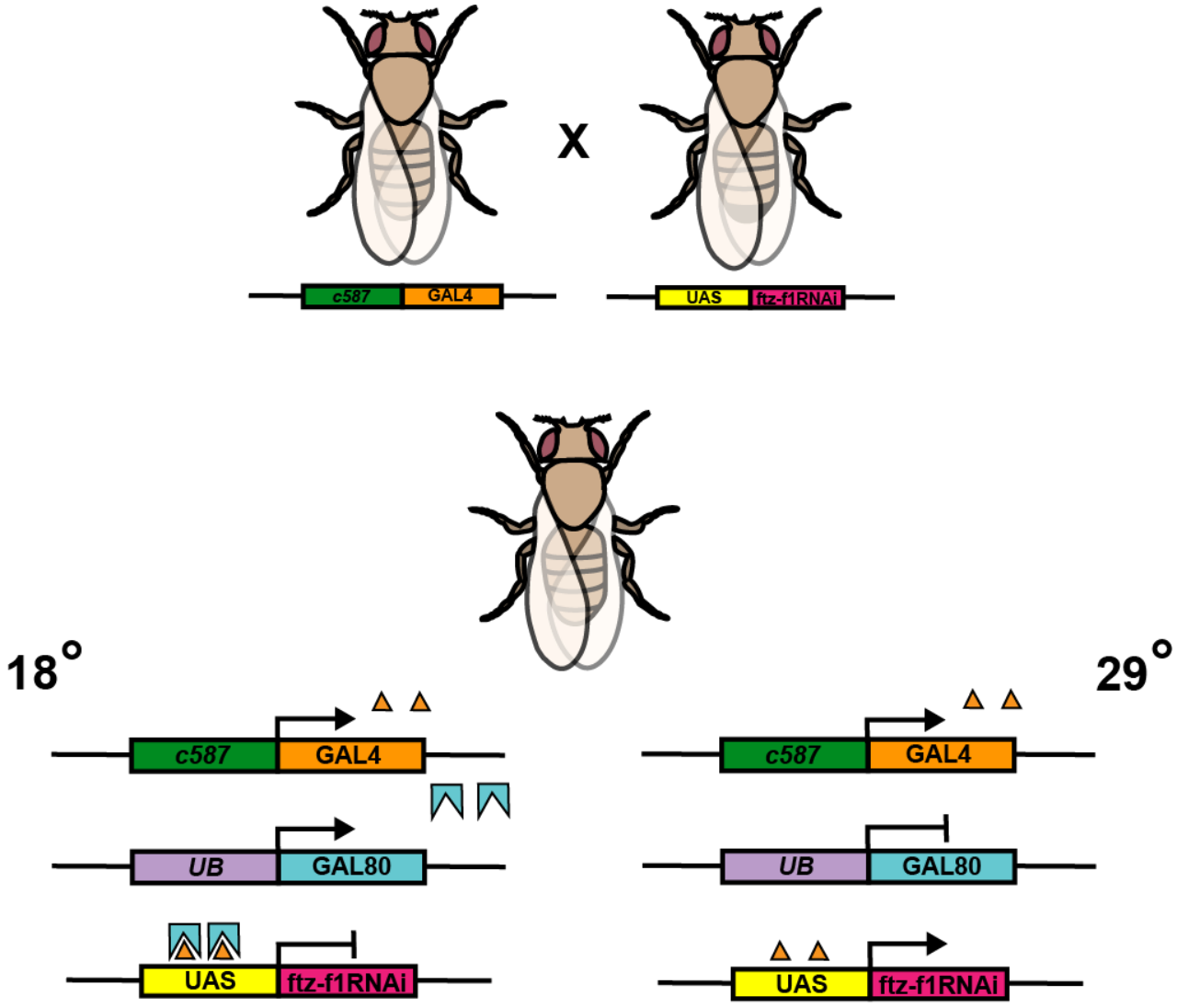

Figure 2. Breeding scheme for temporally and spatially controlled expression of RNAi transgenes.

The *UAS-Gal4* system allows for tissue specific knockdown of *ftz-f1*^{RNAi} transgenes. Using the additional *Gal80* temperature sensitive (t.s.) element, the *ftz-f1*^{RNAi} transgene can be repressed in larval *Drosophila*, then later activated in the adult fly at 29°C.

We specifically depleted *ftz-f1* mRNA from escort cells, using three well-described RNAi transgenes (Beachum et al., 2021; Knapp et al., 2020). Each RNAi allele targets different regions of the *ftz-f1* locus, but are expected to affect expression of both the α and β isoforms (Boulanger et

al., 2011; Broadus et al., 1999; Yamada et al., 2000). The three different alleles used in this study produce different length short hairpin RNA interference sequences with intentions to disrupt endogenous production of *ftz-f1*. Knockdown of *ftz-f1* in escort cells, using the *c587-Gal4;Gal80$^{t.s.}$* driver, resulted in an increase of encapsulation defects within the germaria, where the germ cyst is not properly ushered to the follicle cells for packaging into the egg chamber (Figure 3). Encapsulation defects were characterized as a variety of phenotypes where the germ cysts are not properly enveloped by the follicle cells, coined as follicle cell disorganization, and "slowed pinching," where the cyst is delayed and improperly encapsulated as it buds off into subsequent egg chambers. These phenotypes can result in an improper number of cells within the egg chambers and consequently fewer eggs laid and hatched. Although all RNAi transgenes exhibited encapsulation defects, the *ftz-f1^{KK}* allele had the most notable and frequent phenotypes (Figure 3C). However, encapsulation defects were modest and incompletely penetrant, occurring in less than 20% of all germaria examined (Figure 3E). This suggests that while the depletion of *ftz-f1* may be contributor of encapsulation defects, we cannot rule out the possibility that there are other mechanisms mediating germ cell encapsulation.

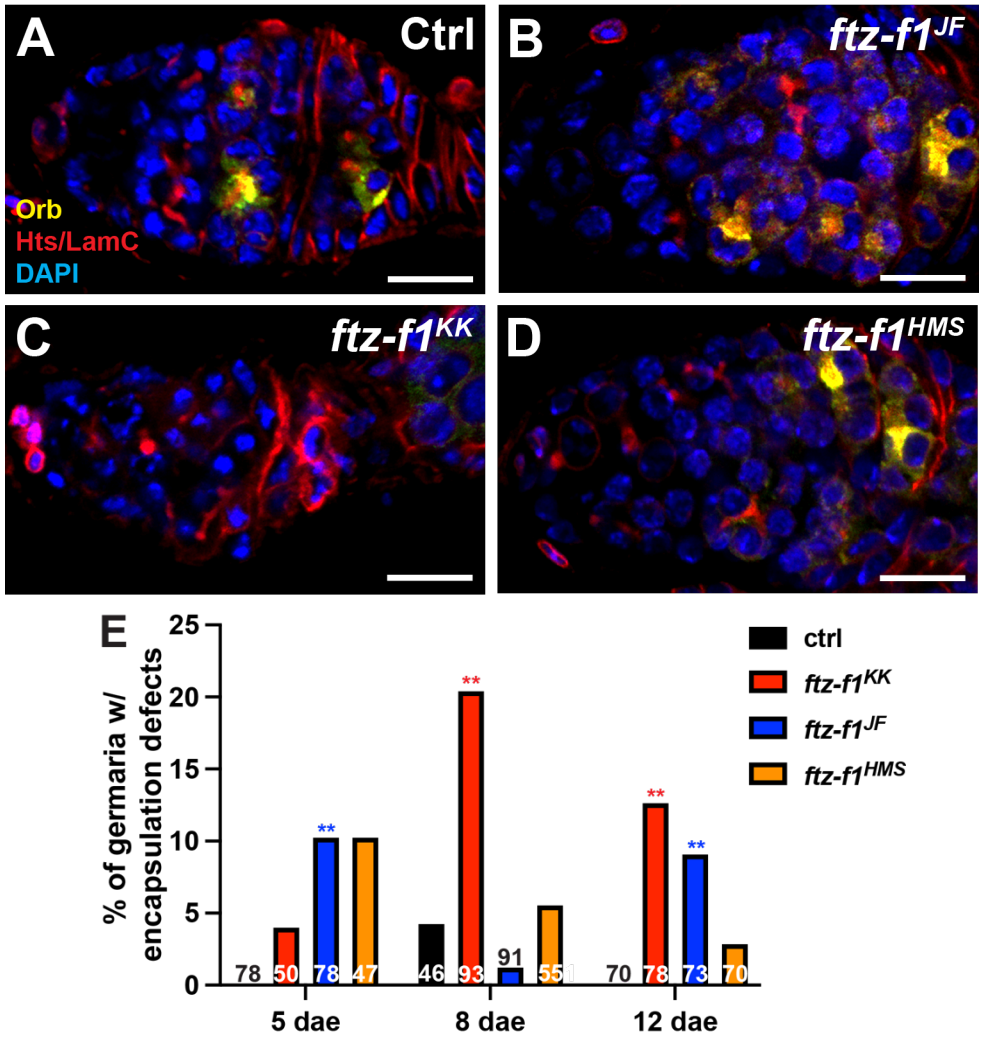

Figure 3. *Ftz-f1* in somatic cells partially contributes to proper cyst encapsulation.

(A) Representative control germaria, (B) *ftz-f1*^{JF}, (C) *ftz-f1*^{KK}, and (D) *ftz-f1*^{HMS} at 8 days after eclosion immunostained with anti-Orb (yellow; oocyte), anti-Hts (red; fusome), anti-LamC (red; nuclear lamina), and DAPI (blue; nuclei). Scale Bars, 10 μm. (E) Percent of ovarioles containing encapsulation defects described as slowed pinching and follicle cell disorganization. Numbers in bars represent numbers of germaria quantified for encapsulation defects. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Chi-squared test.

Somatic Ftz-f1 expression is not necessary for oocyte differentiation

Oocyte development requires a variety of intricate events to generate a fully functional egg ready for fertilization. Determination of the oocyte occurs as the cyst transverse the germarium being one of such events (González-Reyes et al., 1997). Cell fate determination occurs during early mitotic divisions and two pro-oocytes are established in a single cyst. The daughter cells of the GSCs go through four rounds of incomplete cell divisions resulting in the 16-cell cyst containing a single cell that becomes the oocyte and the remaining 15 cells that have adopted nurse cell fate (Huynh & St Johnston, 2004). As the cyst migrates posteriorly, the oocyte polarizes (Bastock & Johnston, 2008). The nucleus condenses to become the karyosome, the centrosome proteins and RNAs become posteriorly localized. In *Drosophila,* oocyte specification is indicated by the accumulation of Orb, which is present in the cytoplasm of the developing oocyte and is first evident in the 8- and 16-cell cyst as it becomes restricted to the presumptive oocyte fate (Lantz et al., 1994).

Upon determining that *ftz-f1* may play a role in proper cyst encapsulation, we then shifted our focus to the production of 16-cell cysts in *ftz-f1* depleted germaria. Assuming there are additional modulators of proper encapsulation other than somatic *ftz-f1*, the next target of investigation was determining if there is an increase or decrease in 16-cell cysts, resulting in cyst stagnation and improper ushering of germ cells into respective egg chambers, which has been previously documented (Shi et al., 2021). To count 16-cell cysts, the Hts-RC antibody was utilized, as it is a marker for ring canals and is only active in the 16-cell cysts. Upon image analysis we noted there is a significant loss of 16-cell cysts in the $ftz\text{-}f1^{KK}$ mutant (Figure 4D). Interestingly, the $ftz\text{-}f1^{JF}$ mutant germaria presented an increase in 16-cell cysts. Taken together, these results suggest that *ftz-f1* is dispensable for proper encapsulation and oocyte specification of germ cells by follicle cells.

In addition to the encapsulation defects and varying numbers of 16-cell cysts, there is an increase in the percent of germaria lacking an Orb-positive oocyte in the *ftz-f1*^{KK} allele, when compared to the control germaria that normally exhibits 1-2 Orb positive cysts, respectively (Figure 5A). Alternatively, there is no significant amount of oocyte defects in the *ftz-f1*^{JF}or *ftz-f1*^{HMS} alleles (Figure 5E). The lack of oocytes present in the germaria may be the result of reduced number of 16-cell cysts in the *ftz-f1*^{KK} allele, leading to encapsulation errors. However, we cannot definitively conclude that *ftz-f1* is necessary for oocyte specification in the germarium.

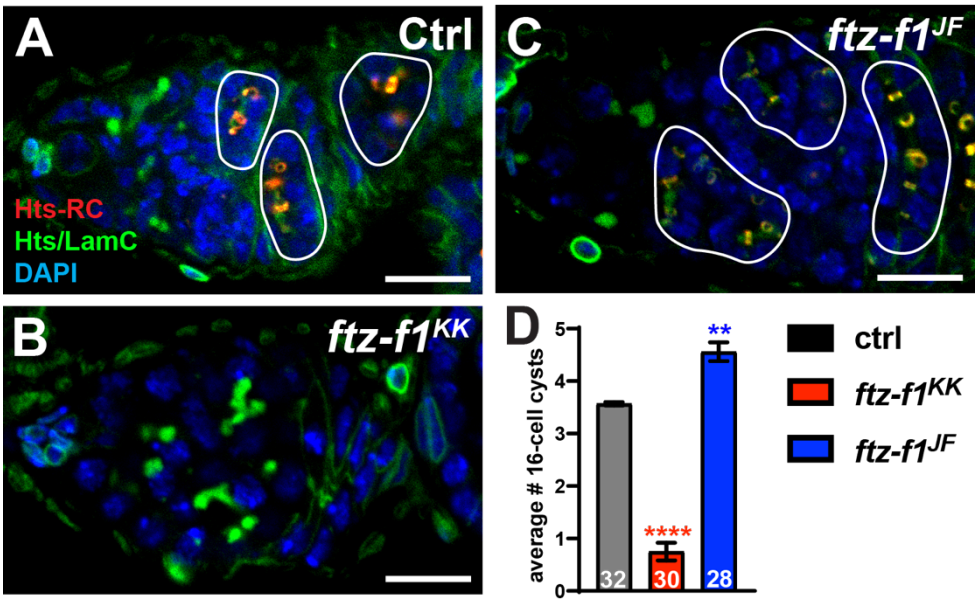

Figure 4. Loss of ftz-f1 in somatic cells alters the number of cysts produced.

(A) Control germaria, (B) *ftz-f1*^{KK}, and (C) *ftz-f1*^{JF} immunostained with anti-Hts-RC (red; ring canals), anti-Hts (green; fusome), anti-LamC (green; nuclear lamina), and DAPI (blue; nuclei). White outline represents Hts-RC positive 16-cell cysts (cc). Scale Bars, 10 μm. (D) Average number of 16-cell cysts per germaria. Number in bars represent number of germaria quantified. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Student's ANOVA test; error bars are SEM.

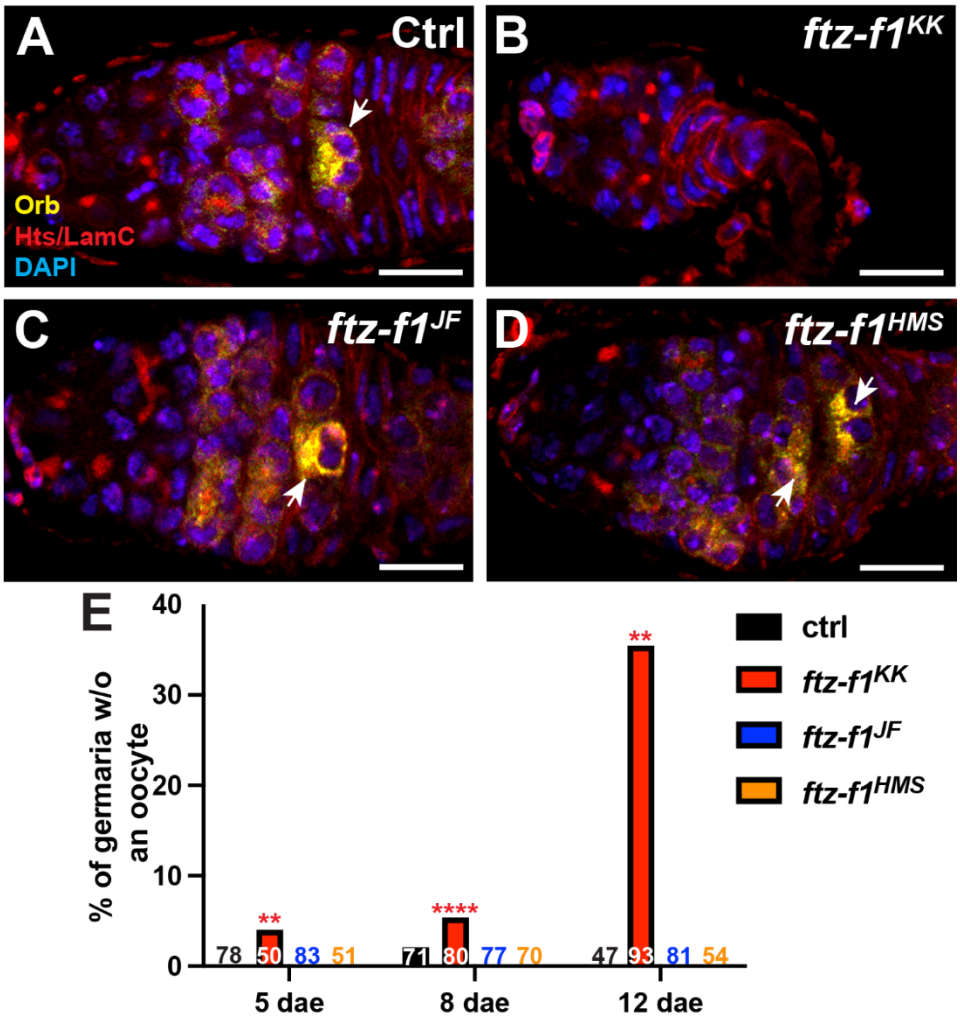

Figure 5. Somatic *ftz-f1* is not required for oocyte specification.

(A) Control germaria, (B) *ftz-f1*^{KK}, and (C) *ftz-f1*^{JF}, and (D) *ftz-f1*^{HMS} immunostained with anti-orb (yellow; oocyte), anti-Hts (red; fusome), anti-LamC (red; nuclear lamina), and DAPI (blue; nuclei). Scale Bars, 10 μm. (E)Percent of ovarioles lacking an Orb positive oocyte. Numbers in bars represent number of germaria quantified for oocyte defects. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Chi-squared test.

Ftz-f1 depletion causes reduced EGFR signaling activity

Epidermal Growth Factor Receptor (EGFR) signaling in the escort cells have been documented as a critical regulator of germ cell cyst differentiation (Mao et al., 2019). EGFR signaling works in opposition to BMP signaling by repressing *dally,* a proteoglycan facilitating Dpp diffusion (Liu et al., 2015). The EGFR signaling pathway begins when growth factors secreted from germ cells which are received by surface receptors on escort cells. The surface receptors then activate the MAP kinase signal cascade including ERK, p38, and JNK (Banisch et al., 2017; Lusk et al., 2017). This signaling pathway activates gene transcription and subsequent actin-cytoskeletal remodeling, crucial for escort cell projections and enveloping and differentiation of the cysts.

Upon depletion of *ftz-f1* in the escort cells, there appears to be a decrease in the percentage of germaria that contain MAPK positive escort cells projections throughout the middle of the germarium at 8 days after eclosion (Figure 6) (Table 2). However, this was quantified by the identification of MAPK positive escort cells, without the use of a secondary escort cell marker. Therefore, the depletion in escort cell protrusions could be due to decrease in the MAPK signaling. Without a secondary escort cell marker, we cannot definitively confirm an absence in projections (Figure 6). Further testing would need to be conducted, specifically utilizing secondary escort cell markers, to determine how the structure and functionality of the escort cell protrusions are altered when *ftz-f1* is abrogated. While we did not see consistent encapsulation or oocyte defects across each *ftz-f1* mutant allele, this data does however suggest that *ftz-f1* is necessary for proper EGFR signaling and will requiring future experimentation.

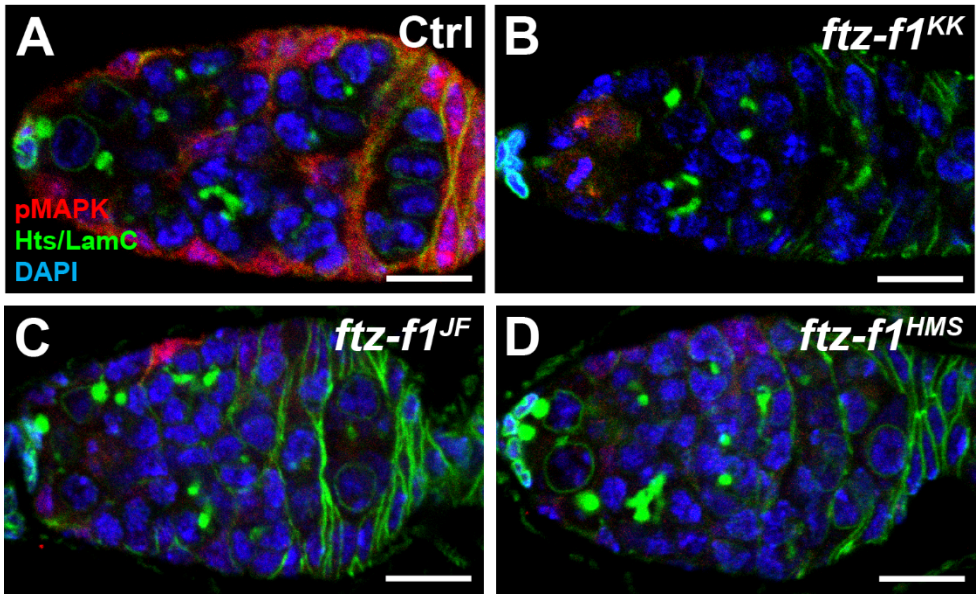

Figure 6. Ftz-f1 depletion in escort cells reduces EGFR activity.

(A) Representative images of control germaria, (B) *ftz-f1*^{KK}, and (C) *ftz-f1*^{JF}, and (D) *ftz-f1*^{HMS} immunostained with antipMAPK (red), anti-Hts (red; fusome), anti-LamC (red; nuclear lamina), and DAPI (blue; nuclei). Scale Bars, 10 μm.

Table 1. Percent germaria displaying pMAPK positive escort cells.

	Control		*Ftz-f1*^{KK}		*Ftz-f1*^{JF}		*Ftz-f1*^{HMS}	
dae	% of germaria with pMAPK+ EC	N	% of germaria with pMAPK+ EC	N	% of germaria with pMAPK+ EC	N	% of germaria with pMAPK+ EC	N
8	94.4%	36	14.3% ****	35	20% ****	30	6.7% ****	30

*p < 0.05, **p < 0.01, ****p* < 0.001, *****p* < 0.0001. Chi-squared test.

Ftz-f1 is necessary in somatic cells to maintain the size of the GSC population

A recent study suggests that *ftz-f1* functions in the escort cells to promote germ cyst differentiation yet lacks a role in self renewal of the GSC (Shi et al., 2021). However, based on preliminary data from our lab, I hypothesized that *ftzf1* functions in escort cells to promote GSC self-renewal. To reconcile my results with the published study, I depleted *ftz-f1* from the escort cells, and looked for the presence of germline stem cells. We used the *c587-Gal4;Gal80$^{t.s.}$* driver to perform tissue specific knockdown of *ftz-f1* in the escort cells in conjunction with the each of the three *ftz-f1* mutant alleles as previously described (Table 2). The number of germline stem cells within the self-renewal niche is one of the methods of determining oogenesis efficiency and productivity, with control germaria generally exhibiting 2-3 GSCs. Loss of GSCs is indicative of fewer oocytes being produced, and a subsequent decrease in reproductive fitness. Upon depletion of *ftz-f1* from the escort cells using the *c587-Gal4;Gal80$^{t.s.}$* driver, there was a significant loss of GSCs from the niche at 5, 8, and 12 days after eclosion (Figure 7). It is important to note that there is a natural decrease in GSCs as the flies age. These findings suggested there is a significant difference in the average number of GSCs present in the germarium when *ftz-f1* is depleted that cannot be explained by the aging process alone. Therefore, these findings suggest that somatic *ftz-f1* is required for proper GSC maintenance (Figure 7E).

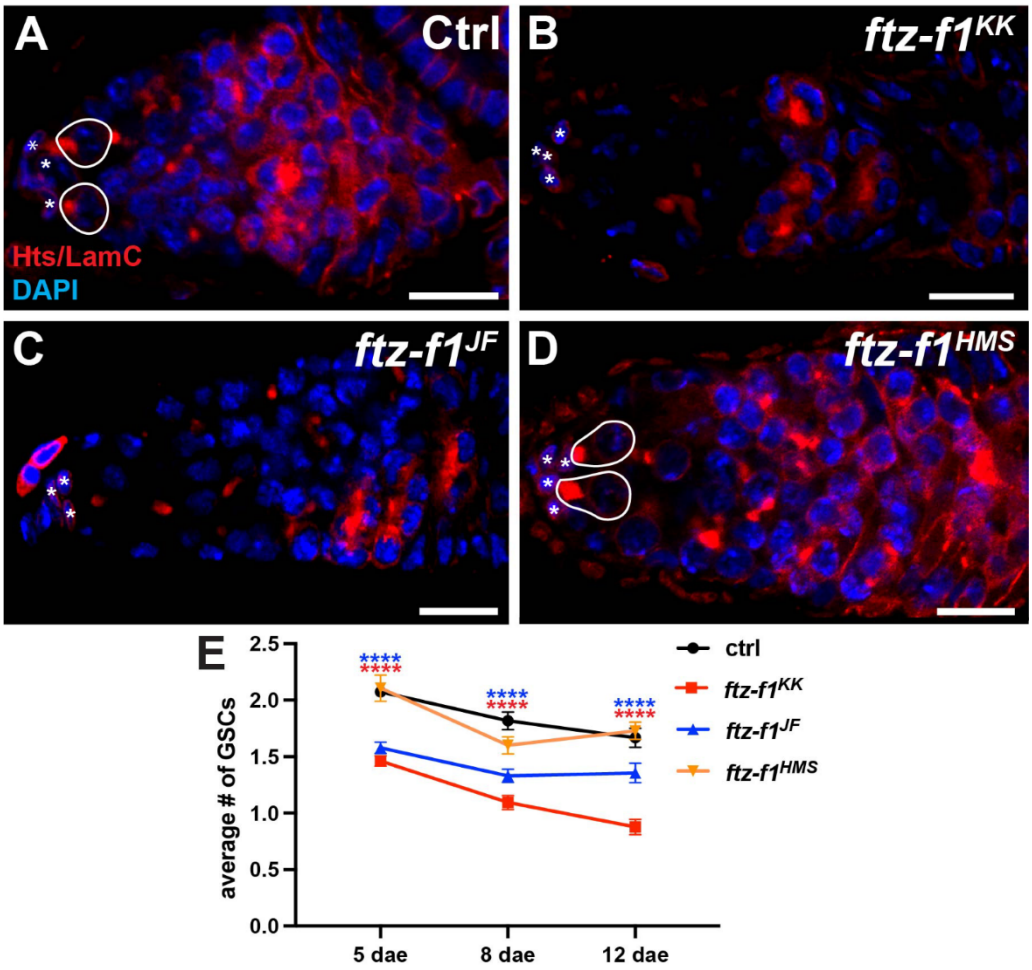

Figure 7. Knockdown of *ftz-f1* in escort cells results in fewer germline stem cells.

(A) Control germaria, (B) *ftz-f1*^{KK}, and (C) *ftz-f1*^{JF}, and (D) *ftz-f1*^{HMS} immunostained with anti-Hts (red; fusome), anti-LamC (red; nuclear lamina), and DAPI (blue; nuclei). Solid white lines outline GSCs present in the germarium. Asterisks denote cap cells. Scale Bars, 10 μm. (E) Average number of GSCs per germarium at 5, 8, and 12 days after eclosion (dae). For sample sizes, see Table 2. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Student's t-test; error bars are SEM.

Table 2. Summative GSC loss in *ftz-f1RNAi alleles across 3 time points.*

	Control		*Ftz-f1*^{KK}		*Ftz-f1*^{JF}		*Ftz-f1*^{HMS}	
dae	Avg # of GSCs	N	Avg # of GSCs	N	Avg # of GSCs	N	Avg # of GSCs	N
5	2.07	321	1.46 ****	247	1.58 ****	249	2.11	47
8	1.82	82	1.09 ****	127	1.33 ****	121	1.6	85
12	1.67	99	0.88 ****	106	1.41 *	98	1.73	70

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Student's t-test.

***Ftz-f1* is predominantly required in neurons to maintain the GSC population**

The *UAS/Gal4/Gal80* system is widely used for tissue-mutations within *Drosophila* research. Recently many published *Gal4* drivers have reported secondary sites of expression beyond their published cell type and tissue expression pattern (Weaver et al., 2020). The *c587-Gal4;Gal80$^{t.s.}$* driver is commonly used withing the *Drosophila* community to drive expression of various knockdown alleles in the escort cells. However, recent studies have identified *c587-Gal4* expression present in not only the escort cells, but also in the brain, fat body, and occasionally in later follicle cells (Weaver et al., 2020). Acknowledging this, we then sought to use a *Gal4* driver that drives only in the escort cells to determine if the reduction of GSCs is due to *ftz-f1* depletion in the escort cells and not anywhere else within the fly. Recently, a new driver, *c587-Gal4;n-syb-Gal80;Gal80$^{t.s.}$-* (*c587-Gal4;n-syb-Gal80*) has been made available that exclusively drives expression solely in the escort cells.

To determine how the differences in expression alter the results demonstrated, *c587-Gal4;n-syb-Gal80* was crossed with the each *ftz-f1* mutant alleles and GSCs were quantified 5 days after eclosion. Interestingly, there were no statistical differences between the *c587-Gal4;n-syb-Gal80* control and the RNAi transgenes at five days after eclosion (Figure 8). This data suggests that *ftz-f1* is not needed in the escort cells but instead it is likely required in the neurons to promote GSC maintenance in the germarium.

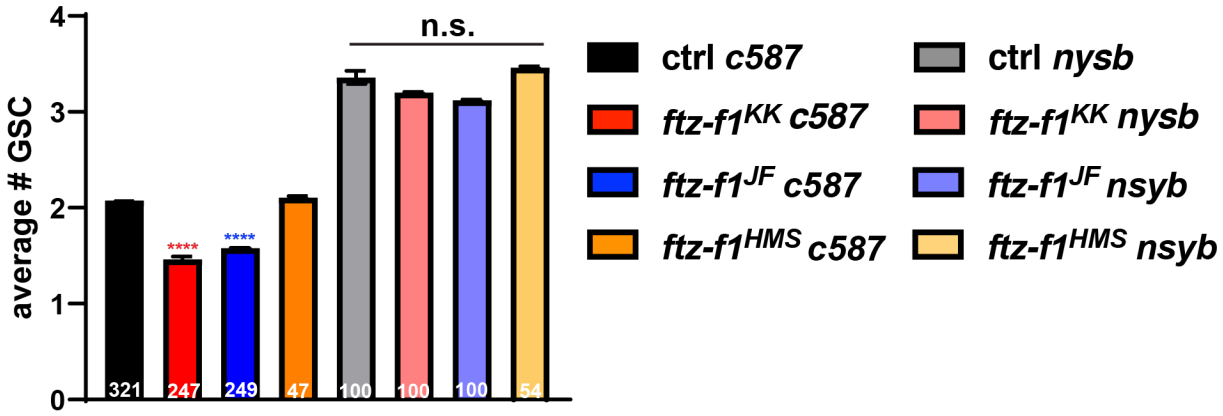

Figure 8. *Ftz-f1* is likely necessary in the neurons for germline stem cell maintenance. Average number of GSCs per germarium in the traditional *c587-Gal4;Gal80$^{t.s.}$* control and the *c587-Gal4;n-syb-Gal80* control, both crossed to *ftz-f1^{KK}*, *ftz-f1^{JF}*, and *ftz-f1^{HMS}*. Numbers in bars represent number of germaria quantified for oocyte defects. Not Significant (n.s.), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Student's ANOVA test; error bars are SEM.

Loss of GSCs in the absence of somatic ftz-f1 is not due to increased cell death

Now cognizant that the original *c587-Gal4;Gal80$^{t.s.}$* driver displays secondary expression sites, we then sought to understand why there were a loss of GSCs when we depleted *ftz-f1* using this driver. GSCs are frequently lost from the niche for a few reported reasons such as a poor diet, loss of E-cadherin, experimental ablation, and aging (Hsu et al., 2019). Another possibility for GSC loss from the niche is that in the absence of *ftz-f1*, GSCs die prematurely. To test this hypothesis, I stained ovaries with α-*Drosophila* cleaved caspase-1 (α-Dcp-1), a marker of apoptotic cell death. To properly consider the effect temperature shifting the flies would have on my phenotype, I set a temperature control cross for the driver and RNAi lines where the files were not temperature shifted from 18 to 29°C. We found increased Dcp-1-positive germaria in every temperature-shifted group including driver controls (Figure 9). Cell death occurred in both somatic and germ cell types including germ cell cysts, follicle cells, and suspected escort cells; though, I cannot definitively conclude death occurred in the escort cells without the presence of a secondary specific escort cell marker. This data suggests that cell death is not occurring from the knockdown of *ftz-f1*. Instead, the cell death is a result of the temperature shift, as the experimental flies are not statistically different from the temperature shifted controls (Figure 9).

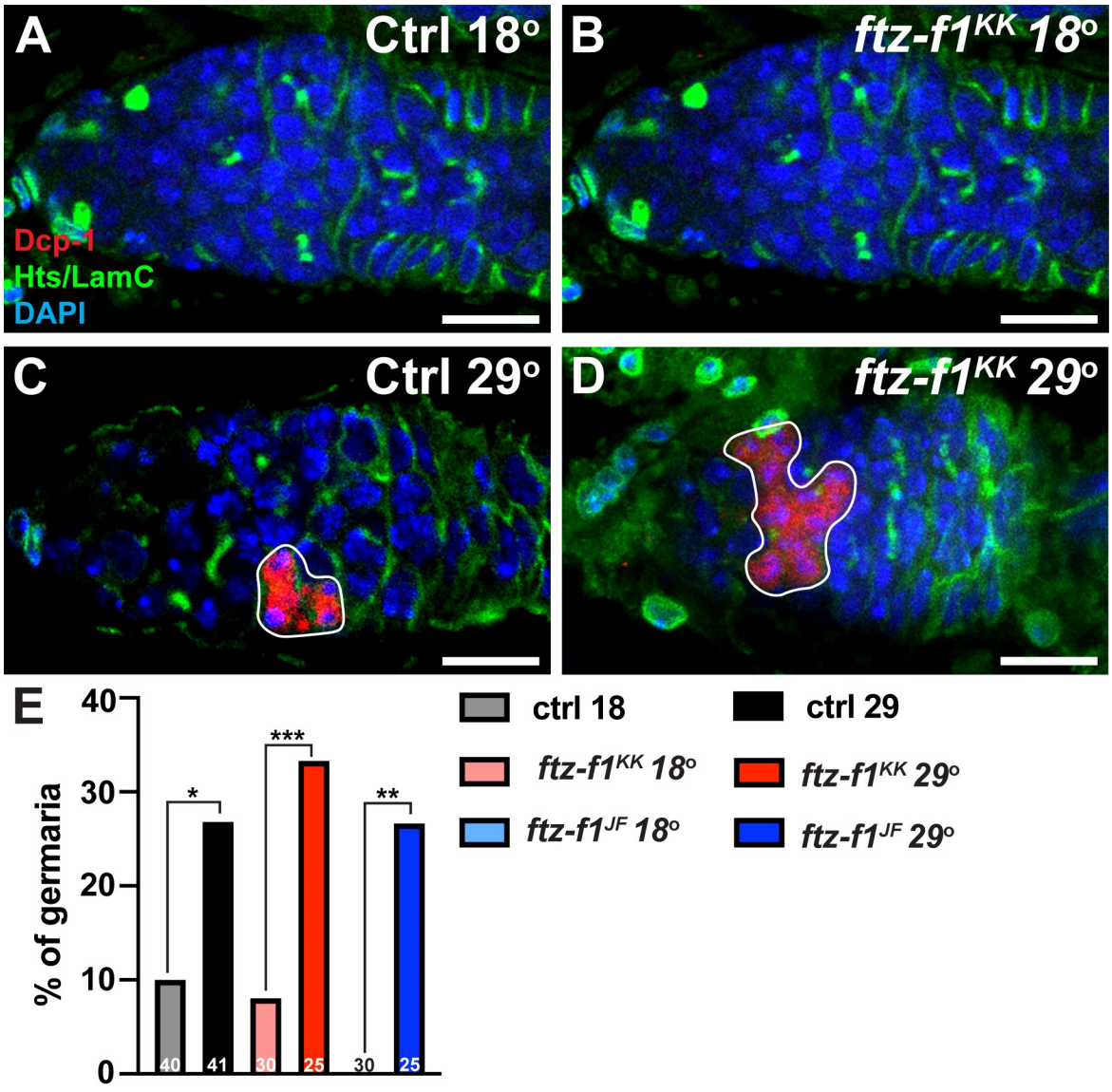

Figure 9. Cell death in *ftz-f1* depleted germaria is due to temperature shifting

Maximum intensity projection (5 µm subset) images of (A) Control germaria at 18°, (B) *ftz-f1*^{KK} at 18°, (C) control germaria at 29°C, and (D) ftz-*f1*^{KK} at 29°C immunostained with anti-Dcp-1 (red; activated caspase), anti-Hts (green; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelopes), and DAPI (blue, nuclei). Scale Bars, 10 µm. (E) Percent of ovarioles containing Dcp-1 positive germ cell cysts or somatic cells. Numbers in bars represent the total number of germaria analyzed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Chi-squared test.

Knockdown of ftz-f1 causes differential expression of Dad-LacZ

Another avenue we used to investigate GSC loss in the original experiments using the *c587-gal4;Gal80$^{t.s.}$*, was the BMP signaling pathway. BMP signaling ligands *Decapentaplegic* (*Dpp)* and *Glass bottom boat (Gbb)* originate from the cap cells received by receptors on the GSCs activating the BMP signaling cascade. This signaling pathway is essential for stem cell self-renewal via the repression of Bam (Figure 10A). Daughters against Decapentaplegic *(Dad)* is a key developmental morphogen and is expressed in the GSCs and CBs. To investigate whether BMP signaling is abrogated when somatic *ftz-f1* is depleted, I used the *c587-Gal7;Gal80$^{t.s.}$;dad-lacZ* reporter line in conjunction with the *ftz-f1^{RNAi}* alleles and quantified the intensity levels of BMP signaling occurring in the GSCs. When depleted from the escort cells, *ftz-f1^{KK}* allele had significantly less intense *dad-lacZ* expression in comparison to controls (Figure 10D). While the *ftz-f1^{JF}* mutant had significantly higher levels of *dad-lacZ* expression compared to the controls (Figure 10C). There may be a variety of reasons to explain the differential expression of LacZ in in RNAi-expressing germaria. This suggests there may be other mechanisms disrupting BMP signaling within the germ cells such as the known off target of the *ftz-f1^{KK}* allele, Target of Rapamycin (*Tor*) signaling.

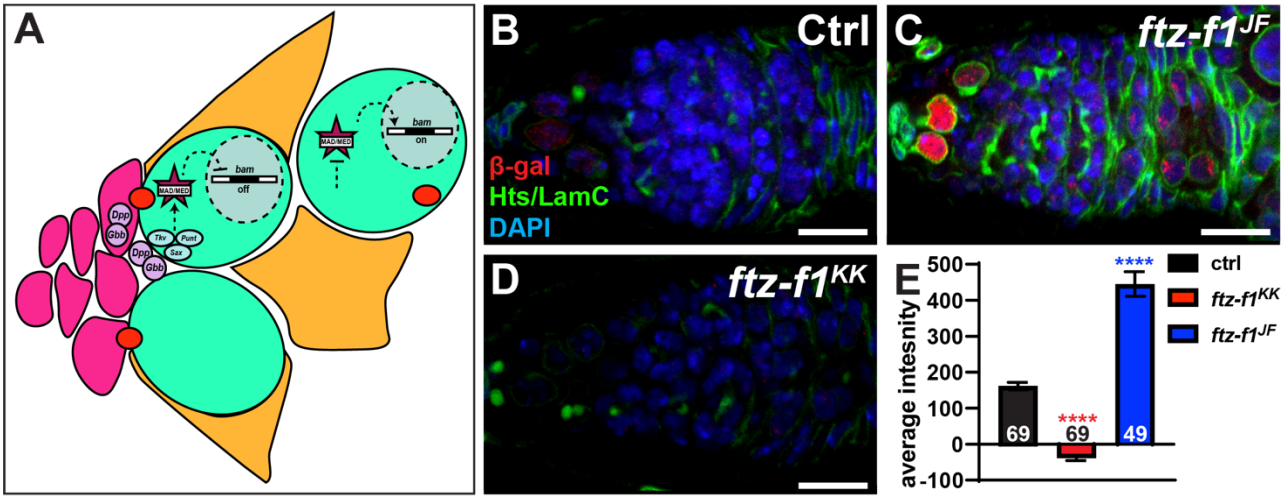

Figure 10. Knockdown of *ftz-f1* causes differential expression of Dad-LacZ.

Schematic of BMP signaling cascade in the germarium. BMP signal ligands: Decapentaplegic (Dpp) and glass bottom boat (Gbb) originate from the cap cells and bind to BMP receptors thick veins (tkv), sax, and Punt, activating the BMP signal cascade. Signaling intermediates Mad/Medea is phosphorylated repressing bam, preventing premature GSC differentiation. As the asymmetrically divides to a cystoblast, BMP ligands are not present in a high enough titer to repress bam expression, activating differentiation of the CB. (B) Control germaria, (C) *ftz-f1*^{JF}, and (D) *ftz-f1*^{KK} immunostained with anti-β-galactosidase (red; Dad expression), anti-Hts (green; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelopes), and DAPI (blue, nuclei). Scale Bars, 10 μm. (E) Average intensity of GSCs expressing b-galactosidase. Numbers in bars represent the total number of GSCs analyzed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Student's t-test; error bars are SEM.

GSCs can be partially rescued in *ftz-f1* depleted germaria with supplement of 20E

Drosophila steroid hormone ecdysone is widely known for its implications during development, growth, nutritional and circadian cycles through insulin, nitric oxide, and TGF-B activation of ecdysone receptor heterodimer complex with Ultraspherical (Cáceres et al., 2011; McBrayer et al., 2007; Rulifson et al., 2002). Recent developments within the *Drosophila* community have identified local production of ecdysone to occur within the escort cells, assisting the cystoblast through a series of synchronous cell divisions (Shi et al., 2021). It has been suggested that ecdysone biosynthesis occurs in the anterior escort cells and is received by ecdysone receptor (EcR) in posterior escort cells important for 16-cell cysts transition to egg chambers. They found that depletion of EcR and ftz-f1 in the pECs results in similar phenotypes consisting of swollen germaria, 16-cell cyst stagnation, and improper encapsulation by follicle cells.

Due to the phenotypic similarities between *EcR* and *ftz-f1* knock-down, and the hypothesis that *ftz-f1* is a downstream target of EcR, I hypothesized that the addition of ecdysone back into the system would rescue the phenotypes observed. Focusing on GSC loss phenotype, I found that supplemental 20E in mutant flies partially abrogates the effects of knocking down *ftz-f1* in the escort cells

(Figure 11). This suggests that *ftz-f1* may be necessary for ecdysone biosynthesis.

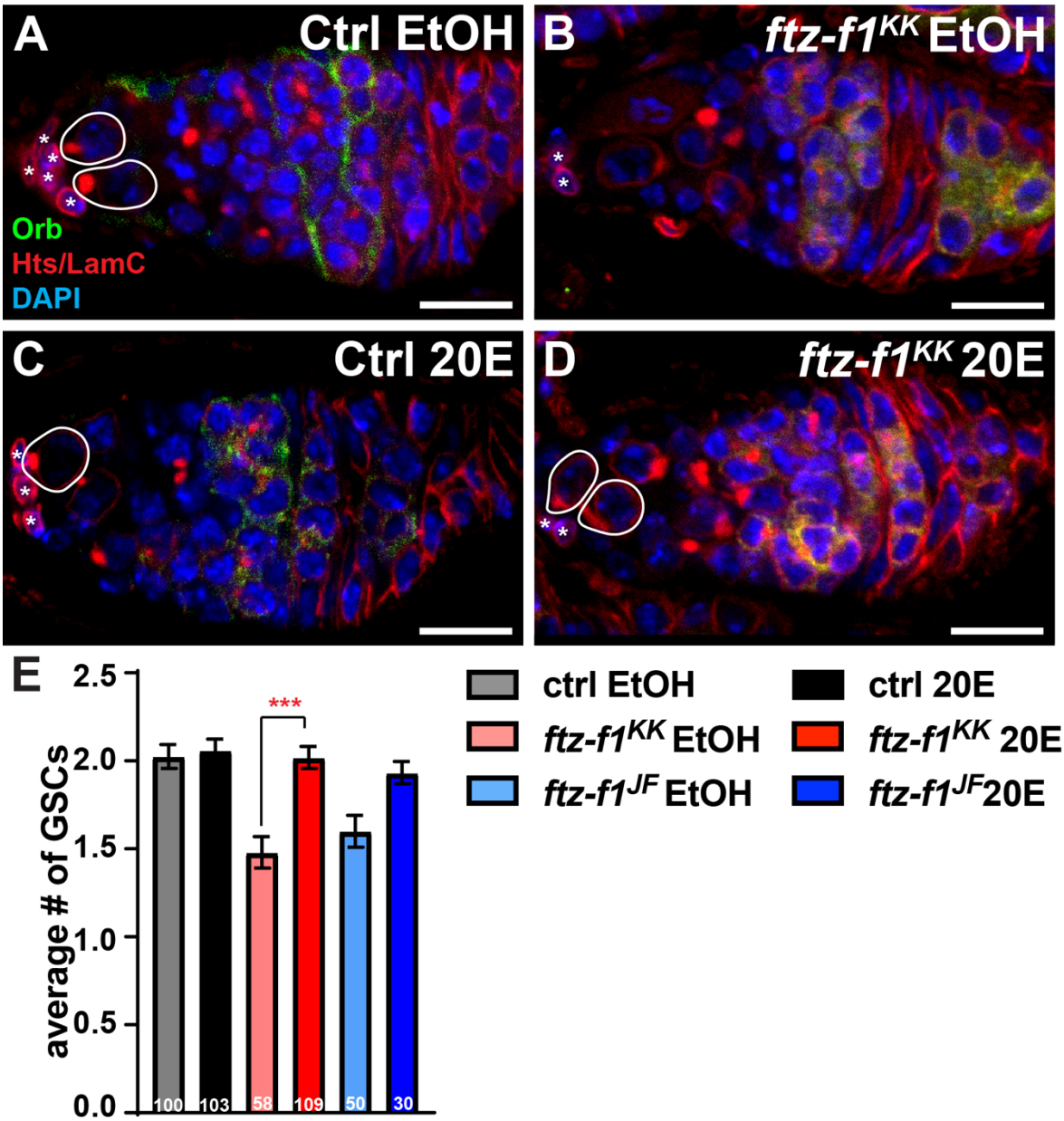

Figure 11. GSC maintenance can be partially rescued in *ftz-f1* depleted germaria with supplement of 20E.

(A) Control germaria fed ethanol (EtOH) yeast paste, (B) *ftz-f1*^{KK} fed EtOH yeast paste and (C) Control germaria fed 20E yeast paste, and (D) *ftz-f1*^{KK} fed 20E yeast paste at 5dae immunostained with anti-Orb (yellow; oocyte), anti-Hts (red; fusomes and follicle cell membranes) and anti-LamC

(red; nuclear envelopes), and DAPI (blue, nuclei). (E) Average number of GSCs in flies fed with EtOH and 20E. Numbers in bars represent the total number of germaria analyzed. Scale bar, 10 μm. *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$; Student's t-test; error bars are SEM.

CHAPTER 3: DISCUSSION

While *ftz-f1* in somatic cells has been characterized for its involvement in intrinsic and extrinsic influence of germ cell development and oogenesis, the mechanisms by which this is accomplished remains unclear. In this study, I have demonstrated that somatic *ftz-f1* is necessary for adequate GSC production and maintenance. I also show that depletion of ftz-f1 in somatic cells does not result in increased cell death but is attributed to temperature shifting. I have demonstrated that the $ftz\text{-}f1^{KK}$ allele produces off-target effects when depleted from the somatic tissues including encapsulation defects, loss of oocyte within germaria attributed to fewer 16-cell cysts produced. Through a rescue experiment, I demonstrated that supplemental ecdysone abrogates the effects of ftz-f1 depletion in the $ftz\text{-}f1^{KK}$ allele and is trending toward the same phenotype in the $ftz\text{-}f1^{JF}$ allele. Lastly and intriguingly, I found the *c587-Gal4;n-syb-Gal80* driver shows no significant decreases in GSC quantity in comparison to the $c587\text{-}Gal4;Gal80^{t.s.}$ driver suggesting *ftz-f1* is actually necessary in neurons for GSC maintenance. Taken together with recent studies about *ftz-f1* and escort cell influence on germ cell tissues, this research poses interesting follow up experimentation to tease apart the role *ftz-f1* plays in somatic tissues to promote germ cell self-renewal and differentiation.

My studies investigating *ftz-f1* depletion using the $c587\text{-}Gal4;Gal80^{t.s.}$ driver resulted in the loss of GSCs from the niche in the $ftz\text{-}f1^{KK}$ and $ftz\text{-}f1^{JF}$ mutant alleles. This phenotype however was not exhibited in the $ftz\text{-}f1^{HMS}$ allele suggesting there are various efficiencies of knockdowns across the mutants. This variation may be due to the different length hairpins produced by each $ftz\text{-}f1^{RNAi}$ mutant allele. However, without testing to confirm the efficacy of each of the RNAi alleles, we are unable to determine how potent of a knockdown each allele produces within the escort cells.

Further experimentation is necessary to determine the effectivity of the RNAi lines throughout this study.

We also had suspicions that somatic *ftz-f1* depletion would result in increased instances in of notable encapsulation defects across each of the mutant alleles based off of previous data collected in the lab. However, the modest phenotypic displays of encapsulation defects in each of the mutants and control tissues suggested that *ftz-f1* depletion is not a primary driver of proper encapsulation. The *ftz-f1*^{KK} mutant was the only mutant that had a decrease in Orb accumulation and a decrease in 16-cell cyst quantity. Interestingly, the *ftz-f1*^{JF} mutant displayed some encapsulation defects and significantly more 16-cell cysts. These findings coincide with the results published by the Shi lab, where *ftz-f1*-depleted germaria display cyst stagnation and an overabundance of 8- and 16-cell cysts.

Finding that only *ftz-f1*^{KK} RNAi transgene displayed encapsulation defects, loss of Orb accumulation, fewer 16-cell cysts, and decreased *Dad* signaling led us to believe there may be other pathways modulated specifically when using the *ftz-f1*^{KK} mutant. After further research we found that the *ftz-f1*^{KK} allele exhibits one off target, *Target of Rapamycin* (*Tor*). . Tor is an evolutionarily conserved nutrient sensing protein kinase that regulates growth and metabolism in response to environmental stress and aging and is implicated in larval development. (Chang & Neufeld, 2009; Demontis & Perrimon, 2009; Oldham et al., 2000; Tiebe et al., 2015; Y. Zhang et al., 2001). Tor mutations have shown significant decreases in the number of 8- and 16-cell cysts and decreased cyst growth rates (LaFever et al 2010). Now aware of the off-target effects of *ftz-f1*^{KK} mutant can cause a variety of growth and developmental defects, we attributed the encapsulation defects, lack of 16-cell cysts and Orb accumulation to Tor signaling interference and

not solely the result of ftz-f1 knockdown in the KK allele. However, this could be a potentially interesting result, suggesting that *ftz-f1* and *Tor* work synergistically in somatic cells to promote cyst cell proliferation and/or differentiation. Future studies should further investigate this potentially novel signaling cross-talk.

My data suggests that knockdown of *ftz-f1* in the somatic cells results in a decrease in the number of GSCs. I expected to continue to see a loss of GSCs from the self-renewal niche as well as a decrease in BMP signaling indicated by a decrease in *dad-lacZ* expression. I hypothesized seeing less intense dad expression and fewer GSCs in *ftz-f1* mutants, indicating a decrease in BMP signaling and therefore premature differentiation in the $ftz\text{-}f1^{KK}$ and $ftz\text{-}f1^{JF}$ mutants. Again, I observed a decrease in *dad* expression in only the $ftz\text{-}f1^{KK}$. Further experimentation will need to be conducted in order to determine if *ftz-f1* depletion is the sole contributor of BMP signaling reduction, or if there are other mechanisms interfering with this signaling pathway. Alternatively, the BMP signaling pathway is also negatively regulated in the self-renewal niche by HECT domain ubiquitin E3 ligase Smad ubiquitination regulatory factors, or Smurf, regulating the output of BMP signaling (Kavsak et al., 2000; Lim et al., 2000; Y. Zhang et al., 2001; H. Zhu et al., 1999). Smurf in female *Drosophila* plays an important role in Dpp responsive germ cells, promoting differentiation of the cystoblast (Casanueva & Ferguson, 2004; Xia et al., 2010). I hypothesized that *ftz-f1* depletion in the escort cells has an indirect effect on BMP signaling from the cap cells via modification of BMP signal transduction regulators, such as Smurf, in the self-renewal niche, and still believe this is likely the case. Further experimentation is necessary to determine if *ftz-f1* is a regulator of Smurf activity.

Previously, knockdown of *ftz-f1* in the somatic cells has been observed to cause programmed cell death in egg chambers (Beachum et al., 2021). I replicated this experiment to determine if *ftz-*

fl depletion in the escort cells results in similar cell death phenotypes displayed in this study. Due to growing concern regarding the 29°C incubator not correctly maintaining its temperature during experimentation or overcompensating temperature deficits from opening and closing the incubator, a temperature control was set in order to determine the differences in GSC numbers and Dcp-1 positive cells in ovarian tissue. A recent publication in the field has also confirmed that temperature shifting flies is problematic, resulting in an increase of cell death in control tissues and decreased numbers of germ cells, consistent with our findings in this study (Gandara and Drummond-Barbosa 2022). While there are currently no alternatives to the well described and widely used *Gal4;Gal80* temperature shifting system in *Drosophila*, I think future experiments using this system should be tightly regulated and additional controls should be set in order to account for the increased cell death and loss of germ cell tissue. This would require the incubators used for temperature shifting to remain at stable temperatures with minimal variation optimal for inducing mutation, decreased times in which the flies are removed from the incubators for feeding, and temperature controls to be set with each experiment to observe the phenotypic difference between temperature shifted flies and flies that are not temperature shifted flies.

Future ftz-f1 directions: neuronal expression and experiments

One of the most interesting findings from this study is that *ftz-f1* may not be necessary in the escort cells but instead necessary in the neurons for GSC maintenance (Figure 8). The original *c587-Gal4;Gal80$^{t.s.}$* driver used throughout these experiments is well-known for its expression in the escort cells, but more recently recognized to drive expression in the neurons, fat body, and late-stage follicle cells (Weaver et al., 2020). Upon receiving the *c587-Gal4;n-syb-Gal80* driver, which expresses only in escort cells (Weaver et al., 2020), I hypothesized that observable effects of the knockdown would coincide with previous GSC loss data. However, the *c587-Gal4;n-syb-Gal80*

knockdown experiments displayed no loss in GSCs and notably higher GSCs present in control and mutant germaria. This data suggests that neuronal *ftz-f1* may contribute to regulation of GSC self-renewal. Further experimentation will need to be conducted to support this potentially novel result, as there may be a connection between *ftz-f1*, ecdysone biosynthesis, and a neuronal component influencing oogenesis that has not yet been fully investigated.

A genetic screen in 2018 identified Ecdysone importer (EcI) as a necessary component for cellular uptake of ecdysone, challenging the widely accepted notion that steroid hormones passively diffuse across cell membranes to cause transcriptional change (Okamoto et al., 2018). This lab also identified ecdysone is not released via simple diffusion, however, it requires high concentrations of vesicles to release ecdysone with and against concentration gradients (Yamanaka et al., 2015). Here, they identified Oatp74D, a member of the solute carrier organic ion superfamily (SLCO), which localizes at the plasma membrane of tissues receiving ecdysone. A series of experiments was conducted to determine the effects of knocking down the EcI and ecdysone receptor, which resulted in larval lethality. Following up, a rescue experiment was conducted to negate larval arrest via supplement of 20E, which I adapted my rescue experiment from in addition to a protocol received from friends at Indiana University. While ecdysone supplement did not rescue larval arrest in EcI and EcR mutants, it did, however, rescue developmental arrest of phantom (phm, a gene encoding P450 enzyme necessary for ecdysone biosynthesis) RNAi larvae, suggesting EcI functions downstream of ecdysone production and upstream of EcR action (Okamoto et al., 2018).

Other literature suggests that ecdysone is produced in the anterior escort cells and is received by EcR in posterior escort cells for *ftz-f1* mediated signaling promoting cyst differentiation (Shi et al., 2021). Considering new developments surrounding potential neuronal input from EcI, local ecdysone production, and new insight provided by an escort cell specific driver, and off-target

effects produced it is difficult to conclude what exactly is occurring in mutant tissues resulting in GSC loss without further experimentation.

While there is much still unknown about the role of *ftz-f1* in the escort cells, and now potentially the neurons, there is room for further investigation. Moving forward, I would investigate death via Dcp-1 and EGFR signaling in the *n-syb-gal4* mutants. I hypothesize that the germaria would not exhibit phenotypes originally documented in the *ftz-f1* mutants due to the lack of neuronal expression. However, another avenue beyond the scope of this thesis project would be exploring the role of *ftz-f1* in neural tissue and the subsequent effect on oogenesis. Future investigators could use a variety of neuronal cell drivers to investigate the effects of *ftz-f1* depletion in the brain and the relationship to the egg production and quality.

Recent studies have identified pacemaker neurons in the fly brain produce neuropeptide allostatin C in the posterior dorsal neuron 1 (AstC-DN1p) (Zhang et al., 2021). In *Drosophila*, insulin signaling originates from the pars intercerebralis region of the brain, which is critical for oogenesis, directly stimulating GSC division and oocyte development. Not only is it critical for its role in oogenesis, but it is also integral for the synthesis of ecdysteroid 20E and juvenile hormone (JH) (Tatar et al., 2001; Tu et al., 2002, 2005). Allostatin C was identified as a regulator of JH and its function is conserved across many species. In this study, they used an *AstC-gal4* driver to evaluate female fecundity by an egg laying assay to assess reproductive health of the flies. They found a decrease in eggs laid in the flies with *AstC* suppressed neurons. I would like to propose a similar set of experiments using the AstC-Gal4, and other neuronal *gal4* drivers, with the *ftz-f1* mutants to determine if ftz-f1 depletion in the brain results in similar loss of function phenotypes, to parse out where *ftz-f1* is necessary in various regions of the brain. Another potential avenue the *ftz-f1* project could go down is a genetic screen to determine if ftz-f1 loss of function phenotypes

reflect a general role in neurons for oogenesis. Here we could attempt to identify which neuronal cell types are integral for egg production and quality.

CHAPTER 4: MATERIALS AND METHODS

Drosophila husbandry and culture

All *Drosophila* stocks were maintained on standard cornmeal/molasses/yeast medium (Genesee Scientific, Nutri-Fly-MF) at 22°C–25°C. Genes/alleles with multiple names are referenced using FlyBase nomenclature for simplicity. Except where noted, female flies were collected one to two days after eclosion and maintained on standard medium supplemented with wet yeast paste for 5-12 days (changed daily) at 29°C prior to ovary dissection.

Tissue-specific RNA interference

I depleted *ftz-f1* mRNA from somatic cells using three well-described RNAi transgenes (Beachum et al., 2021; Knapp et al., 2020): y^{1} v^{1}*; P{TRiP.JF02738}attP2* (*ftz-f1*^{JF}; Bloomington stock #27659), *P{KK108995}VIE-260B* (*ftz-f1*^{KK}; Vienna stock #104463), and *P{TRiP.HMS00019}attP2* (*ftz-f1*^{HMS}; Bloomington stock #33625*)*. To limit *Gal4* expression specifically to adult follicle cells (thus circumventing developmental lethality), we used the *Gal4/Gal80*^{ts} system (McGuire et al., 2004).The *c587-Gal4* driver allowed me to drive RNAi expression specifically in escort cells. However, knockdown of *ftz-f1* in larval *Drosophila* is lethal. To negate this, we combined the *c587-Gal4* driver with the temperature sensitive *Gal80*$^{t.s.}$, repressing the RNAi expression during larval stages and only activating RNAi knockdown in the adults by switching the progeny to a permissive 29°C at eclosion. The UAS/Gal4:Gal80 system is an efficient tool for tissue specific knockdown, with knockdown occurring as soon as three hours after temperature shift and is used throughout the duration of this study (McGuire et al., 2004). Flies bearing *yw;nysyb-gal80-c587gal4/fn7a;tub-gal80ts/tm6* were raised at 18°C and then shifted to 29°C at eclosion to induce expression of the *UAS-RNAi* constructs (kindly

provided by Dr. Leslie Weaver at Indiana University). Driver expression was confirmed using *y[1] w*; P{w[+mC]=UAS-mCD8::GFP.L}LL5* (*UAS-mCD8::GFP*; Bloomington stock #5137).

I performed a driver control experiment to confirm *c587-Gal4;Gal80* flies drive expression in the escort cells, and the RNAi control. All crosses were set at room temperature on the same day and will be allowed to mate for 2 days allowing the females to lay approximately 200-300 eggs per vial before being tossed into new vials for temperature shifting. The progeny were shifted to the 18° C incubator until eclosion, where the flies were then shifted to the 29° Celsius incubator. The flies were fed daily with wet yeast paste until dissection. Ideally each vial will contain about 10-15 females to dissect. Dissecting the samples at three time points will allow for a comprehensive view of development as the flies age.

20E Rescue experiment

To test whether excess ecdysone could rescue the phenotypes resulting from ftz-f1 knockdown, females were fed wet yeast paste containing a final concentration of 100 μM 20Hydroxyecdysone (Sigma #H5142) in 5% ethanol for 5 days before dissections, as described {Ono, 2006 #141}. Samples were dissected at 5 days after eclosion (dae).

Immunostaining and microscopy

Each sample set was dissected and stained with α-Orb (oocyte marker), α-Hts (fusome/cell boundary marker), α-LamC (nuclear lamina marker), and DAPI (nuclei marker). Each of these antibodies allow for the overall visualization of the GSCs, developing cysts, and specified oocyte. I was able to identify GSC quantity across various time points to determine if there is a significant loss of GSCs due to the mutation and age. I was able to identify the number, quantity, and structure of germ cell cysts, as well as if the oocyte is being formed in control and mutant germaria. Data

collection was performed on the LSM700 confocal microscope where I quantified the number of GSCs. Ovaries were prepared for immunofluorescence microscopy as described (Ables & Drummond-Barbosa, 2010). Ovaries were dissected and ovarioles teased apart in Grace's medium without additives (Caisson Labs) and fixed in 5.3% formaldehyde (Ted Pella Inc, 18505) in Grace's medium for 13 min at room temperature. They were then washed extensively in phosphate buffered saline (PBS, pH 7.4; Fisher) with 0.1% Triton X-100 and blocked for 3 hours in blocking solution [5% bovine serum albumin (Sigma), 5% normal goat serum (MP Biomedicals), and 0.1% Triton X-100 in PBS] at room temperature. The following primary antibodies were used overnight at 4°C: mouse anti-Hts [1B1, Developmental Studies Hybridoma Bank (DSHB); 1:10], mouse anti-Lamin C (LamC) (LC28.26, DSHB; 1:100), chicken anti-GFP (ab13970, Abcam; 1:2000), mouse anti-Orb (4H8/6H4, DSHB; 1:100), rabbit anti-Dcp1 (37729, Cell Signaling; 1:100), and chicken anti-β-Galactosidase (ab9361, Abcam; 1:2000), mouse anti-Hts-RC (DSHB; 1:10). Following an overnight incubation at 4°C with Alexa Fluor 488-, 568-, or 633- conjugated goat species-specific secondary antibodies (Life Technologies; 1:200). Ovaries were counter-stained with 0.5 mg/ml 49-6-diamidino-2-phenylindole (DAPI) (Sigma) to identify nuclei or phalloidinAlexaFluor-647 (Life Technologies; 1:400) to visualize F-actin. Ovaries were mounted in 90% glycerol containing 20 mg/ml n-propyl gallate (Sigma). Confocal Z-stacks (1 μm optical sections) were collected with a Zeiss LSM700 microscope using Zeiss ZEN software. Images were analyzed, and minimally and equally enhanced via histogram using Zeiss ZEN software.

Ovaries were stained with 0.5 μg/ml 4'-6 diamidino-2-phenylindole (DAPI; Sigma) in 0.1% Triton X-100 in PBS. Ovaries were mounted in 90% glycerol in conjunction with 20% npropyl-gallate (Sigma). Confocal z-stacks (1 μm optical sections) were collected with either the Zeiss LSM700 laser scanning microscope using ZEN Black 2012 software or ZEN Blue 2012 software.

Images were analyzed using Zeiss ZEN software, and minimally and equally enhanced via histogram using ZEN and Adobe Photoshop Creative Suite.

Statistical Analysis

All experiments were performed in triplicate from independent genetic crosses, using at least 10 ovaries per replicate. Statistical analysis was performed in Prism (GraphPad, Inc.) and Excel (Microsoft) software. Statistical differences between one control group and one experimental group were analyzed by Student's two-tailed t-test or Chi-square analyses (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Bar graphs show averages plus/minus the standard error of the mean (SEM). Sample values (n) are presented on graphs in or above bars and represent the number of cells, ovarioles, or germaria examined as indicated.

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APPENDIX A

Table of Fly Stocks Used

Genotype	**Received From**	**Stock #**	**Reference**
y[1] w[*]; P{w[+mc]=UAS-mCD8::GFP.L}LL5	Kyoto	108058	
ftz-f1^{KK}	VDRC	104463	
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02738}attP2	Bloomington	27659	
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00019}attP2/TM3, Sb[1]	Bloomington	33625	
C587-Gal4 (x);tubPGal80[ts] (II)	D. Drummond-Barbosa		
C587; Dad-LacZ/TM3,sb	T. Xie		
yw;nysyb-gal80-c587gal4/fn7a;tubgal80ts/tm6	L. Weaver		
Yellow white	D. Drummond-Barbosa		
y[1]v[1];ftz-f1^{KK};gal80ts	SOS		This study
y[1]v[1];gal80ts;ftz-f1^{JF}	SOS		This study

APPENDIX B

Table of Antibodies Used

Antibodies Used	Dilution	Species	Stains	Protein	Received from
Orb (4H8)	1:500	Mouse	oocytes	oo18 RNA-binding protein	DSHB
Orb (6H4)	1:500	Mouse	oocytes	oo18 RNA-binding protein	DSHB
Hts-RC	1:10	Mouse	Ring canals	Hu-Lo Tai Shou	DSHB
1B1 or Hts	1:10	Mouse	Fusomes, follicle cells	Hu-Lo Tai Shou	DSHB
Lamin C	1:100	Mouse	Nuclear envelope	Laminin C	DSHB
DAPI	0.5 µg/ml	n/a	DNA (nuclei)	4'-6-diamidino-2-phenylindole	Sigma
B-galactosidase	1:2000	Chicken	LacZ	LacZ	Abcam
pMAPK	1:200	Rat	EGFR signaling molecules	Erk	Abcam
Dcp-1	1:200	Rabbit	Pre-apoptotic cells	Cleaved caspase-3	Abcom