Drosophila Ovarian Stem Cell Establishment is Regulated by

Nuclear Hormone Receptor *ftz-f1*

by

Hanna E. Berghout

June 2017

Director of Thesis: Elizabeth T. Ables, Ph.D.

Major Department: Biology

Stem cells are a fundamental underpinning of tissue biology. Loss of the self-renewing function of stem cells leads to conditions such as infertility and tissue wasting. Stem cells integrate a variety of signals to maintain their fate and proliferative capacity. Although intrinsic and local cues are well studied, less is known about how extrinsic signals, such as hormones, affect stem cell fate and function. The highly characterized *Drosophila melanogaster* steroid hormone ecdysone regulates germline stem cell (GSC) proliferation and self-renewal, as well as oogenesis and metamorphosis. Though many genes, including nuclear hormone receptor *ftz transcription factor 1 (ftz-f1)*, are thought to be targets of ecdysone signaling, it is unclear how these targets impact GSC fate and function. To explore the role of *ftz-f1* in ovarian stem cells, we used the *UAS-GAL4* system and RNA interference (RNAi) to reduce *ftz-f1* function specifically in germ cells or surrounding somatic cells. We demonstrate that *ftz-f1* is intrinsically required for the establishment of the proper number of GSCs during development. Reduced *ftz-f1* function in germ cells leads to a significant decrease in average number of GSCs. During larval stages, *ftz-f1*

depleted ovaries contain a number of PGCs located significantly further away from the terminal filament stacks. Our results also suggest that *ftz-f1* is required in ovarian somatic cells during development for proper movement of germ cells out of the germarium in adult stages. Taken together, we suggest *ftz-f1* function during juvenile stages is critical for the establishment of GSCs and development of their progeny.

Drosophila Ovarian Stem Cell Establishment is Regulated by

Nuclear Hormone Receptor *ftz-f1*

A Thesis

Presented to the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Molecular Biology and Biotechnology

by

Hanna E. Berghout

June 2017

Drosophila Ovarian Stem Cell Establishment is Regulated by Nuclear Hormone Receptor

ftz-f1

by

Hanna E. Berghout

APPROVED BY:

DIRECTOR OF THESIS: _____
Elizabeth Ables, Ph. D.

COMMITTEE MEMBER: _____
Eric Anderson, Ph. D.

COMMITTEE MEMBER: _____
Anthony Capehart, Ph. D.

COMMITTEE MEMBER: _____
Randall Renegar, Ph. D.

CHAIR OF THE DEPARTMENT
OF BIOLOGY: _____
Jeffrey S. McKinnon, Ph. D.

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, Ph. D.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Thesis Proposal: The Role of *ftz-f1* in *Drosophila* Ovarian Stem Cell Fate and Function.

Drosophila Ovarian Stem Cell Establishment is Regulated by Nuclear Hormone Receptor

ftz-f1

LIST OF FIGURES

LIST OF SYMBOLS OR ABBREVIATIONS

AB	Antibody
AEL	After egg laying
BAB1	Bric-a-brac 1
Bam	Bag of marbles
BMP	Bone morphogenetic protein
CB	Cystoblast
CKN	Chicken
DAE	Days after eclosion
Dpp	Decapentaplegic
DSHB	Developmental Studies Hybridoma Bank
EBR	Ephrussi-Beadle Ringers
EcR	Ecdysone Receptor
FA	Formaldehyde
FSC	Follicle stem cell
GFP	Green fluorescent protein
GSC	Germline stem cell
HSC	Hematopoietic stem cells
LL3	Late third instar
Mad	Mothers against Dpp

ML3	Mid third instar
MSE	Mouse
NC	Nurse cell
OO	Oocyte
PBS	Phosphate-buffered saline
PGC	Primordial germ cell
RBT	Rabbit
RC	Ring Canal
TF	Terminal filament
TJ	Traffic Jam

CHAPTER 1

Thesis Proposal: The Role of *ftz-f1* in *Drosophila* Ovarian Stem Cell Fate and Function.

Introduction

Stem cells have been of heightened interest and are emerging as one of the fundamental underpinnings of tissue biology because of their potential to replenish, regenerate, and repair damaged tissues, including adult tissues (Lawson et. al., 2009). Stem cells have become an integral part in current therapies for human diseases, including neurological disorders, congenital heart defects and chronic lung disease (Ables et. al, 2012). Stem cells are undifferentiated cells, defined by their ability at the single cell level to both self-renew and proliferate to produce mature daughter cells, both nonrenewing and terminally differentiated (Wagers & Weissman, 2004).

There are two major types of stem cells: embryonic stem cells, which are able to give rise to all embryonic and extra embryonic cell types and adult tissue resident stem cells, which have a predetermined fate and will only form mature cells of the tissue from which they originate (Rippon and Bishop, 2004). Stem cells allow blood, bone, gametes, epithelia, nervous system, muscles, and other tissues to be replenished by fresh cells throughout life (Morrison and Spradling, 2008). Loss of the self-renewing function can lead to various conditions such as infertility, anemia, and immunodeficiency, whereas over proliferation can lead to disruption of normal tissue homeostasis, possibly contributing to tumor formation and growth (Morrison and Spradling, 2008).

Stem cell proliferation and self-renewal are tightly regulated by local signals from their niche, intrinsic cues, and long-range signals, such as nutrition and hormones that maintain stem cell fate and proliferative capacity (Drummond-Barbosa, 2008). Figure 1 shows a stem cell

integrating a variety of intrinsic and extrinsic cues. This diagram highlights potentially beneficial

outcomes of stem cell research, like disease modeling or prevention and treatment of birth
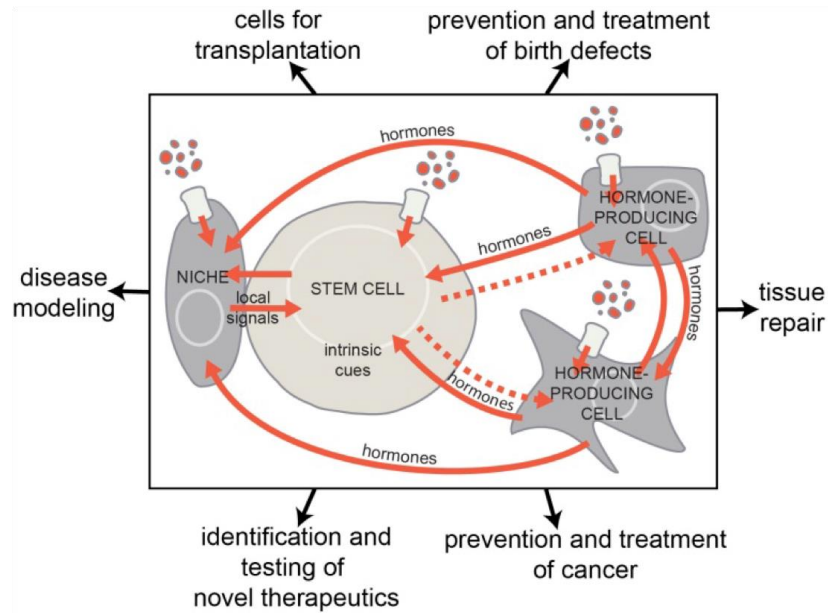
Figure 1: Stem Cell and its Corresponding Signals (Ables et al, 2012)

defects. Many studies have addressed how intrinsic and local regulators maintain stem cell

identity and proliferative potential, but much less is known about the direct actions of systemic

hormones on stem cells and the niche cells that support them, despite the therapeutic relevance of

exploring this level of regulation (Ables and Drummond-Barbosa, 2010). Moreover, we know

remarkably little about how systemic hormones influence the development of tissue-resident

stem cell populations (Gilboa, 2015).

Drosophila melanogaster Germline Stem Cells as a Model System

The *Drosophila melanogaster* ovary provides an excellent model for the study of tissue-resident

stem cell development and function. This system provides a way to easily visualize stem cells in

A. Larval Ovary

Cap Cell Precursor

PGC

Terminal Filament

Escort Cell Precursor

B. Adult Ovary

Follicle Cells

Terminal Filament

Cap Cells

GSC

Escort Cells
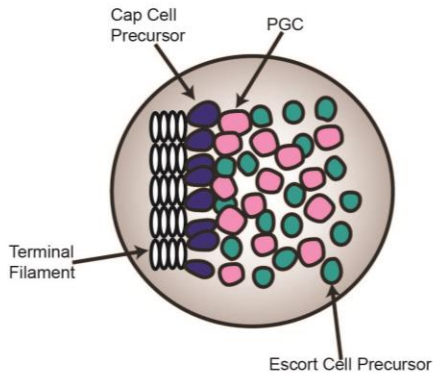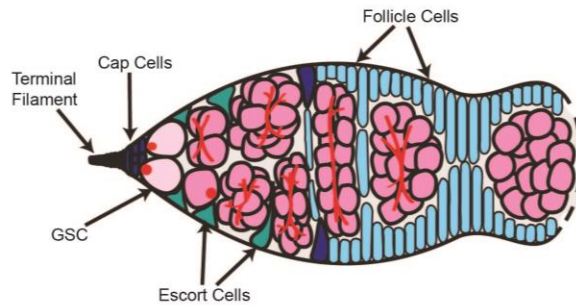
Figure 2: The Establishment of GSCs in Their Niche

their microenvironment while also utilizing the elegant genetic and experimental techniques available in the *Drosophila* system. The *Drosophila* ovary forms during embryogenesis by association of somatic gonadal precursors and primordial germ cells (PGCs) (Gilboa, 2015). During early stages of ovary development, PGCs, which are the precursors of the germline stem cells (GSCs), along with other somatic cell precursors proliferate and begin the establishment of the GSCs in their niche (Figure 2) (Gancz and Gilboa, 2013). PGCs are the first germ cell population established during development and are the immediate precursor for oocytes (Alberts et. al, 2002).

The adult female *Drosophila* has two ovaries (Figure 3A-B) that are composed of 14-16 ovarioles (Figure 3C) (Spradling, 1993). Each ovariole functions as an independent production line for eggs (Spradling, 1993). At the anterior tip of each ovariole (boxed) is the germarium, which contains GSCs (Figure 3D). These GSCs reside in a specialized somatic cell niche, which modulates their behavior. The GSCs are anchored to the anterior end of the germarium and are recognized by their anteriorly localized fusomes, which easily distinguish the GSCs from their

differentiated daughter cells, or cystoblasts. Stem cell function can be genetically manipulated and visualized *in vivo*, as all of the GSC progeny are arranged in a linear fashion.
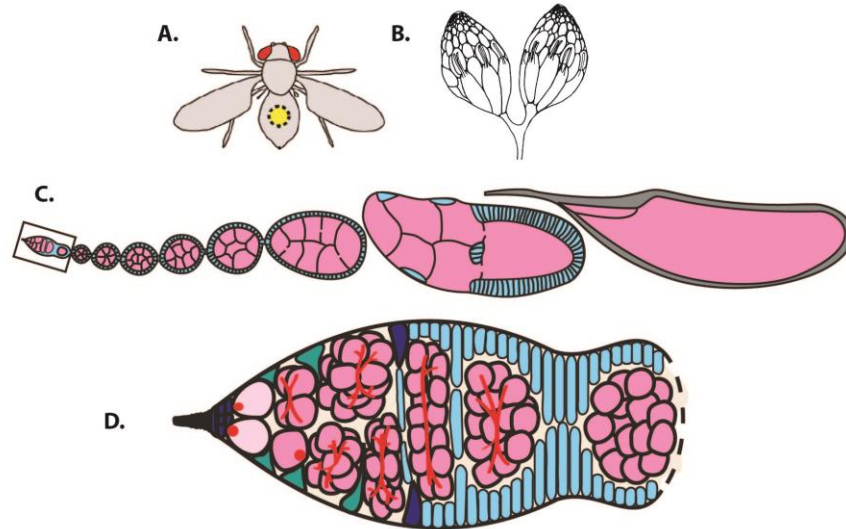
Figure 3: *Drosophila melanogaster* **Oogenesis Anatomy**

Ecdysone Signaling is required in *Drosophila* Oogenesis and Metamorphosis

Steroid hormones are critical regulators of tissue-resident stem cell development and function. The predominant steroid hormone in *Drosophila* is ecdysone, which is structurally similar to the human sex steroid estrogen (Mangelsdorf et. al., 1995). Ecdysone has been widely characterized in *Drosophila* oogenesis and has been implicated in various processes (Belles and Piulachs, 2015). Ecdysone is required for follicle development (Carney and Bender, 2000), regulates border cell migration (Bai et al., 2000), and is also involved in other critical steps of oogenesis, such as vitellogenesis or yolk deposition, shown (Figure 4) (Jang et al., 2009). Most importantly, ecdysone signaling is critical for the development and maintenance of GSCs and their surrounding niche cells. Ecdysone signaling maintains the structure of the GSC niche and allows somatic niche cells to support a normal rather than a reduced number of GSCs (Morris
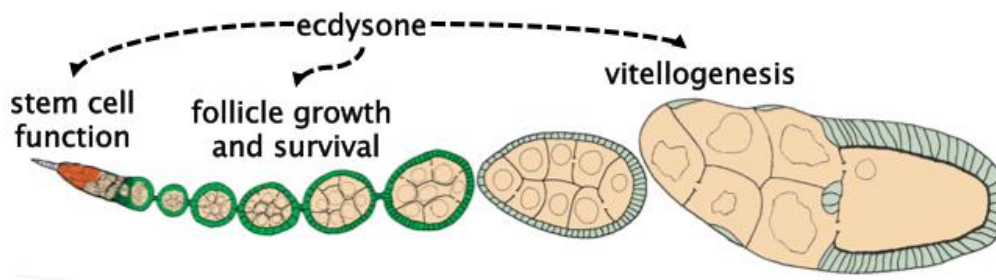
Figure 4: Ecdysone and its effects in *Drosophila* Oogenesis

and Spradling, 2012). Ecdysone signaling also directly stimulates GSC proliferation and directly promotes maintenance, or the ability to self-renew, of GSCs (Ables and Drummond-Barbosa, 2010).

Ecdysone is also required for *Drosophila* growth and metamorphosis at all stages of development. Ecdysone induces the histolysis of nearly all of the larval tissues, along with promoting differentiation and morphogenesis of the structures composing the adult fly, including the formation of the adult ovary (Baehrecke, 1996; Gancz et. al, 2011). Previous literature has shown ecdysone and *ftz-f1* work in cohort to control growth and metamorphosis (Broadus et. al, 1999; Sultan et. al, 2014). Ecdysone signaling induces the onset of several early genes, one of them being *ftz-f1*.

Ftz-f1 is required for multiple developmental processes

Nuclear hormone receptor *ftz transcription factor 1 (ftz-f1)* is also required for multiple developmental processes. *Ftz-f1* has been shown to be a competence factor for stage-specific responses to steroid hormone ecdysone during *Drosophila* metamorphosis (Broadus et al, 1999). Also, when *ftz-f1* is knocked down in the *Drosophila* testis, it induces GSC loss, similar to the phenotype resulting from the knockdown of ecdysone receptor (EcR) (Li et al, 2014).

Furthermore, mosaic analysis confirmed that these two factors are cell autonomously required for cyst stem cell maintenance (Li et al, 2014). Over-expression of *ftz-f1* in the somatic cells of the ovary results in precocious PGC differentiation (Gancz and Gilboa, 2011). From prior literature, there is a connection between *ftz-f1* and ecdysone but it is unclear how *ftz-f1* specifically impacts female GSC fate and function. Given the connection between *ftz-f1* and ecdysone in the previous literature and my preliminary experiments, **I hypothesize that *ftz-f1*, a downstream target of ecdysone, is necessary for proper GSC function and for the establishment of tissue resident stem cells in their niche in *Drosphila* oogenesis.**

Objectives

My hypothesis will be tested in two separate specific aims. For the first aim and with the knowledge that ecdysone signaling is required in GSCs (Ables & Drummond-Barbosa, 2010) and in escort cells (Morris & Spradling, 2012) for proliferation of both GSCs and their daughter cells, I will test if *ftz-f1* is required in escort cells for germ cell differentiation and in GSC for maintenance and proliferation. The second aim will use *Drosophila* primordial germ cells (PGCs) to see how reduced *ftz-f1* function will affect the establishment of the GSCs in their niche.

Specific Aims

1. Ecdysone signaling is required in Germline Stem Cells (GSCs) (Ables & Drummond-Barbosa, 2010) and in escort cells (Morris & Spradling, 2012) for germ cell differentiation.

    a. Is *ftz-f1* required in the somatic cell niche for germ cell differentiation?

    b. Is *ftz-f1* required for stem cell maintenance and proliferation?

2. Is *ftz-f1* is necessary for the establishment of tissue resident stem cells in their niche.
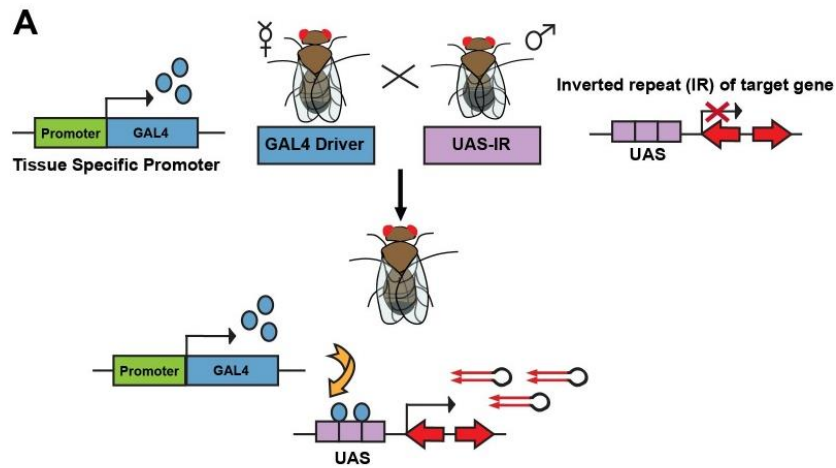
Figure 6: UAS-GAL4 System (Helms et. al, 2017)

Specific Aim 1: Experimental Approach

To test Specific Aim 1A, I will use the UAS-GAL4 system (Figure 6) in combination with short hairpin RNA interference (RNAi) to reduce *ftz-f1* function in the ovarian somatic cells. By crossing *ftz-f1^RNAi* mutants with various ovarian somatic cell drivers, I will examine the effect of reduced somatic cell niche *ftz-f1* on the average number of GSCs.

To test Specific Aim 1B, I will use the UAS-GAL4 system to cross *ftz-f1^RNAi* mutants with well characterized germ cell-specific driver, *nosGal4::VP16* (Rørth, 1998) and examine the resulting phenotype following reduced *ftz-f1* function in GSCs and their progeny.

To assess the involvement of *ftz-f1* on GSC differentiation, I will examine the phenotype of *nos-Gal4 > ftz-f1^RNAi* and compare to *nos-Gal4 >yw*. Crosses will be set at the same time. Once the progeny have eclosed, they will be fed on yeast paste for 5 days, 8 days, and 12 days. Ovaries will then be dissected, fixed, and immunostained to easily identify GSCs using (as

described in Ables and Drummond-Barbosa, 2010) a nuclear marker, 4',6-diamidino-2-phenylindole (DAPI), a fusome marker, mouse anti-Hts (1B1), and a cap cell nuclear envelope marker, mouse anti-LaminC (LamC). All images will be obtained by mounting the samples on slides and imaging with a Zeiss confocal microscope. Quantification will be done by counting all GSC in control and mutant germaria and calculating the average number per germaria for use in statistical analysis. If *ftz-f1* is required for GSC differentiation, a decrease in the total number of GSC per germaria will be observed in the mutant germaria.

Specific Aim 1: Preliminary Data

My preliminary results suggest there are cell type-specific functions of *ftz-f1* in the *Drosophila* ovary. Knockdown of *ftz-f1* in the germline stem cells caused a significant decrease in the average number of GSCs, (Figure 7), while knockdown of *ftz-f1* in somatic cells slightly increased the average number of germline stem cells (Figure 8). There was a trend but means were not significantly increased with this small sample size.

Even more interestingly, knockdown of *ftz-f1* in both germ cell and somatic cell populations caused a phenotype that maintain an average number of germline stem cells consistent over my three time points, 5, 8, and 12 Days. These results suggest that a knockdown of *ftz-f1* causes a problem in development/establishment of the GSCs in their niche, and rather than maintenance defects, supporting my original hypothesis.
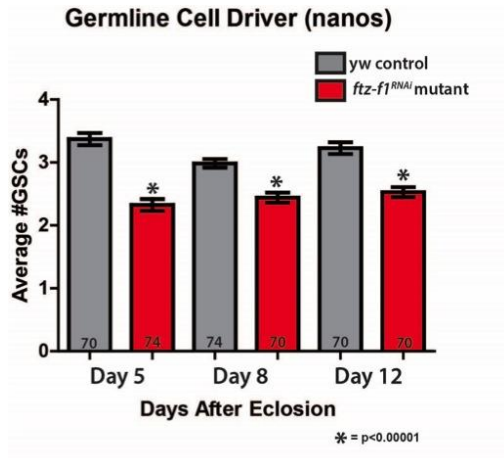
Figure 7: Ftz-f1^{RNAi} x Germline Cell Driver (nanos)
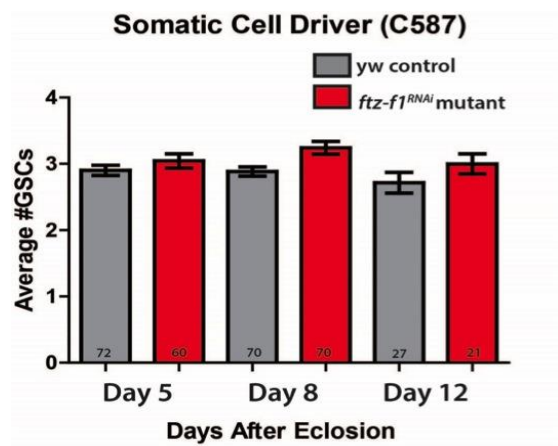
Figure 8: Ftz-f1^{RNAi} x Somatic Cell Driver (C587)

Specific Aim 2: Experimental Approach

To test Specific Aim 2, I will use the UAS-GAL4 system to cross *ftz-f1^{RNAi}* mutants with both germline and somatic drivers and this will allow me to clearly examine the effect of reduced *ftz-f1* function in PGCs (Figure 2A). I will mount and stain the mutant larval ovaries and compare them to controls of the same age and determine, based on the average number of PGCs, if *ftz-f1* is required (as described in Maimon and Gilboa, 2011). All images will be obtained by mounting the samples on slides and imaging with a Zeiss confocal microscope. Quantification will be done by calculating the average number of PGCs in control, germ cell knockdown and somatic cell knockdown mutant ovaries. If *ftz-f1* expression is necessary for establishment of PGCs in either the PGCs or the supporting somatic cells, there will be a reduction in the average number of PGC correctly situated in their somatic cell niche (adjacent to the cap cell precursors, shown in Figure 2A).

To further test Specific Aim 2, I will use *ftz-f1¹⁷* null mutants, which have complete loss of *ftz-f1* in both germ and somatic cell populations (Broadus et. al, 1999). This mutant has a P-

10

element insertion, resulting in interruption of the gene expression (Levis et. al, 1993). These mutants only survive until larval stage. I will be able to compare these mutants to the flies with *ftz-f1* knockdown in germ or somatic cells, using the same feeding, dissecting, staining, mounting, viewing, and quantification procedures as the *ftz-f1*^{RNAi} PGCs. If *ftz-f1* is necessary for the establishment of the PGCs, then the developing mutant ovary will have a decrease in the average number of PGCs correctly situated in their somatic cell niche.

Summary

The signals that control stem cell maintenance and development, though addressed, are not fully understood. By understanding the signaling pathways involved in stem cell fate, we could potentially create therapies to regenerate and repair damaged tissues. There are many recognized regulators of stem cell function, however, *ftz transcription factor 1 (ftz-f1)*, although previously implicated as a potential regulator of stem cell function, has yet to be fully characterized. Through the study the function of my *ftz-f1*, I will provide fundamental insights into the molecular mechanisms that ultimately regulate stem cell function and fate.

With these proposed experiments, I will be able to elucidate the function of *ftz-f1* in *Drosophila* oogenesis, and determine its role in the establishment of GSCs in their niche. Given the importance of stem cells, this research will be beneficial in understanding all aspects of stem cell function regulation through long range signals, particularly steroid hormones and their nuclear receptors. This research will aid in the goal of being able to have a complete grasp on the molecular mechanisms underlying stem cell functions, which ultimately can allow us to create regenerative and repairing therapies.

CHAPTER 2

Drosophila Ovarian Stem Cell Establishment is Regulated by Nuclear Hormone Receptor *ftz-f1*

Hanna E. Berghout and Elizabeth T. Ables

This Chapter has been formatted according to the requirements necessary for submission

to the scientific journal *Development.*

KEYWORDS

SUMMARY STATEMENT

ftz-f1 is required for establishment of germline stem cells in their somatic cell niche.

ABSTRACT

Stem cells are a fundamental underpinning of tissue biology. Loss of the self-renewing function of stem cells leads to conditions such as infertility and tissue wasting. Stem cells integrate a variety of signals to maintain their fate and proliferative capacity. Although intrinsic and local cues are well studied, less is known about how extrinsic signals, such as hormones, affect stem cell fate and function. The highly characterized *Drosophila melanogaster* steroid hormone ecdysone regulates germline stem cell (GSC) proliferation and self-renewal, as well as oogenesis and metamorphosis. Though many genes, including nuclear hormone receptor *ftz transcription factor 1(ftz-f1),* are thought to be targets of ecdysone signaling, it is unclear how these targets impact GSC fate and function. To explore the role of *ftz-f1* in ovarian stem cells, we used the *UAS-GAL4* system and RNA interference (RNAi) to reduce *ftz-f1* function specifically in germ cells or surrounding somatic cells. We demonstrate that *ftz-f1* is intrinsically required for the establishment of the proper number of GSCs during development. Reduced *ftz-f1* function in germ cells leads to a significant decrease in the average number of GSCs. At larval stages, *ftz-f1* depleted ovaries contain a number of undifferentiated PGCs located significantly further away from the terminal filament stacks. Our results also suggest that *ftz-f1* is required in ovarian somatic cells during development for proper movement of germ cells out of the germarium in

adult stages. Taken together, these data indicate that *ftz-f1* function during juvenile stages is

critical for the establishment of GSCs and development of their progeny.

INTRODUCTION

Stem cells have emerged as one of the fundamental underpinnings of tissue biology because of their potential to replenish, regenerate, and repair damaged tissues, including adult tissues (Lawson et. al., 2009). Stem cells are undifferentiated cells, defined by their ability at the single cell level to self-renew and proliferate to produce mature daughter cells, both nonrenewing and terminally differentiated (Wagers & Weissman, 2004). Stem cells have become an integral part in current therapies for human diseases, including neurological disorders, congenital heart defects, and chronic lung disease (Hassan et. al., 2009; Bernstein and Srivastava, 2011; Gomperts and Strieter, 2006). To effectively wield stem cells for therapeutic interventions, such as disease modeling, tissue repair, and the prevention and treatment of birth defects, it is essential to further explore their regulation and function.

Stem cell proliferation and self-renewal are tightly controlled by many layers of regulation, including local signals from their niche, intrinsic cues, and long-range signals, such as nutrition and hormones, that maintain stem cell fate and proliferative capacity (Drummond-Barbosa, 2008). Many studies have addressed how intrinsic and local regulators maintain stem cell identity and proliferative potential, but much less is known about the direct actions of hormones, particularly steroid hormones, on stem cells and the niche cells that support them, despite the therapeutic relevance of exploring this level of regulation (Ables and Drummond-Barbosa, 2017 review). Steroid hormone signaling is critical for a wide variety of biological processes. Among other actions, steroid hormone signaling modulates gene transcription via interaction with intracellular nuclear receptors, and regulates expression of various genes in a network-like manner to initiate complex events involved in nearly every aspect of development and physiological responses (Evans, 1988; Beat et al., 1996; Beat and Klug, 2000).

16

Steroid hormone signaling has a critical role in various mammalian stem cells. The mammary stem cell niche responds readily to hormonal stimuli, including stem cell expansion and tissue growth (Joshi et al., 2012). The steroid hormone estrogen regulates the cell cycle activity of hematopoietic stem cells (HSCs) in the bone marrow (Nakada et al., 2014). Estrogen is also involved in establishment of adult quiescent satellite cell populations at puberty and their re-establishment during regeneration (Kim et. al., 2016). Although critical roles for stem cells have been identified in a variety of tissues, we know remarkably little about how steroid hormones influence the development and establishment of tissue-resident stem cell populations (Gilboa, 2015).

The *Drosophila melanogaster* ovary provides an excellent model for the study of tissue-resident stem cell development and function. Stem cells can be easily visualized in their microenvironment while also utilizing the elegant genetic and experimental techniques available in the *Drosophila* system. The adult female *Drosophila* has two ovaries that are composed of 16-20 ovarioles (Spradling, 1993). Each ovariole functions as a string of progressively more mature follicles, with every follicle containing a developing oocyte (Spradling, 1993) (Figure 1 A). At the anterior tip of each ovariole is the germarium, which contains GSCs (Figure 1 A boxed and D). These GSCs reside in a specialized somatic cell niche, composed of cap cells and terminal filament cells, which modulates GSC behavior (Xie, 2013). The GSCs are anchored to the anterior end of the germarium and are recognized by their anteriorly localized fusomes, which easily distinguish the GSCs from their differentiated daughter cells, or cystoblasts (Song and Xie, 2002). A GSC divides asymmetrically to generate one stem cell and one cystoblast. The cystoblast divides precisely four times to produce 16 interconnected cystocytes that are then encapsulated by a layer of follicle cells to form an egg chamber (King, 1970; Mahowald and

Kambyselllis, 1980; Spradling, 1993). Stem cell function can be genetically manipulated and visualized *in vivo*, as all of the GSC progeny are anatomically arranged in a linear fashion

The *Drosophila* ovary forms during embryogenesis by association of somatic gonadal precursors and primordial germ cells (PGCs) (Figure 1 B) (Bhat and Schedl, 1997). During early stages of ovary development, PGCs, including potential germline stem cell (GSC) precursors, along with other somatic cell precursors proliferate and differentiate, beginning the establishment of the GSC niche (King, 1970). Somatic proliferation is needed during these stages to allow correct formation of 16-20 ovarioles (Gilboa and Lehmann, 2006). The number of ovarioles an ovary contains is determined during larval development through the morphogenesis of terminal filaments (TFs), each of which is composed of seven to ten terminal filament cells (TFCs) (Godt and Laski, 1995; King et al., 1968). Differentiation of the TFs begin during mid-third larval instar and complete the TF stacks, along with the cap cells at the base of these stacks, are formed by late third larval instar (Xie et al., 2002). Since there are more PGCs than needed for the formation of adult ovarioles, each of which contains two to three GSCs, the extra PGCs differentiate, bypassing the stem cell stage (King, 1970; Bhat and Schedl, 1997). These germ cells thus fuel the first wave of egg production, immediately following eclosion. Only the PGCs anchored to TFs and cap cells by DE-cadherin (Song and Xie, 2002) remain undifferentiated and become GSCs (Zhu and Xie, 2003).

18
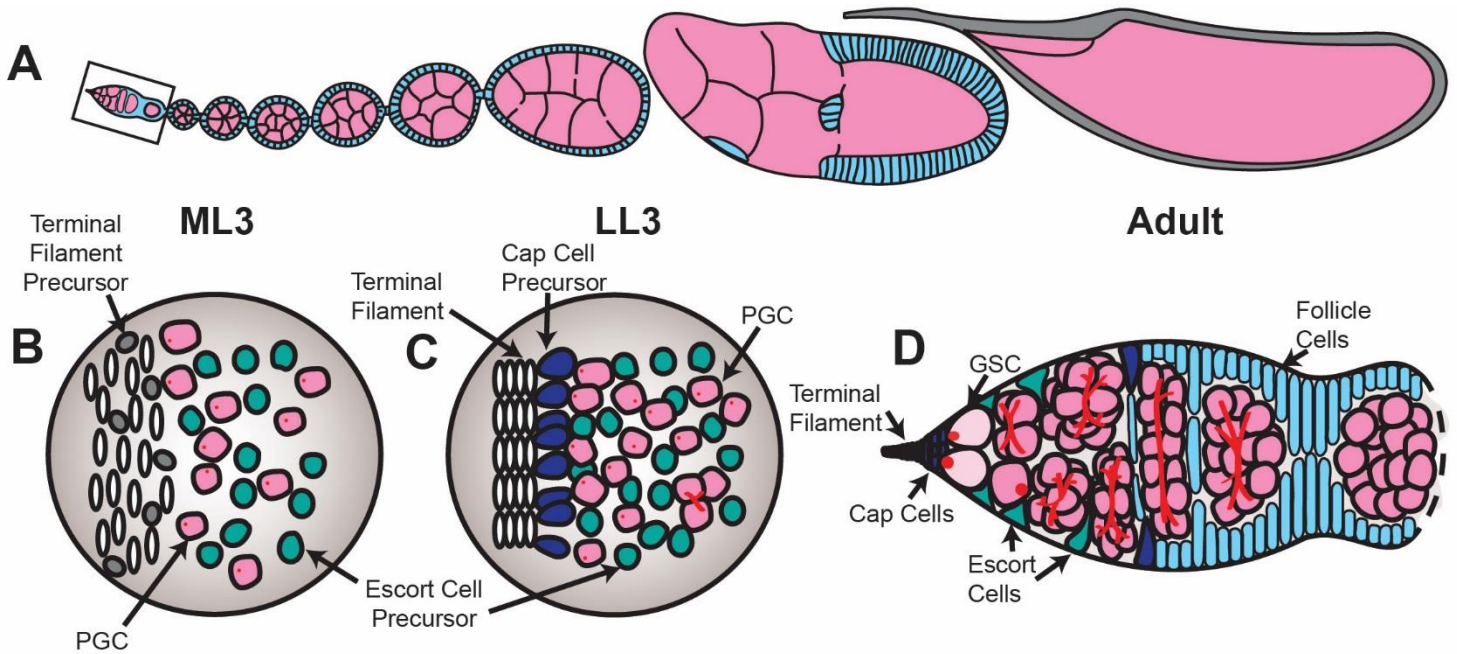
Figure 1. The *Drosophila* ovary as a model for establishment of GSCs in their somatic cell niche. (A) Schematic of *Drosophila* ovariole. Germ cells in pink and somatic cells in blue. Germarium boxed. (B) Schematic of Mid Larval 3 (ML3) ovary (144 hours AEL). Terminal filament precursors in dark grey, primordial germ cells (PGCs) in pink with their fusomes in red, and escort cell precursors or intermingled cells in green. (C) Schematic of Late Larval 3 (LL3) ovary. Terminal filament stacks in white, cap cell precursors in navy blue, PGCs in pink with their fusomes in red, and escort cell precursors or intermingled cells in green. (D) Schematic of the adult germaria (boxed in A). Terminal filament and cap cells in navy blue, GSCs in light pink, escort cells in green, differentiated germ cells (cystobloast, 2-, 4-, 8-, and 16- cell cysts) in dark pink, follicle stem cells in navy blue with their progeny, the follicle cells, in blue.

The *Drosophila* ovary is very responsive to steroid hormones. The predominant steroid hormone in *Drosophila* is ecdysone, which is structurally similar to the human sex steroid hormone estrogen (Mangelsdorf et al., 1995). Ecdysone has been widely implicated in a variety of cellular processes during *Drosophila* oogenesis (Belles and Piulachs, 2015 and Ables and Drummond-Barbosa, 2017). Ecdysone is required for follicle survival (Buszczak et al., 1999), regulates border cell migration (Bai et al., 2000), and is involved in other critical steps of oogenesis, such as eggshell formation and yolk deposition (Jang et al., 2009).

Most importantly, ecdysone signaling is critical for the development and maintenance of GSCs and their surrounding niche cells. Ecdysone signaling maintains the structure of the GSC niche and allows somatic niche cells to support a normal rather than a reduced number of GSCs (Morris and Spradling, 2012). Ecdysone signaling directly stimulates GSC proliferation and directly promotes maintenance, or the ability to self-renew, of GSCs (Ables and Drummond-Barbosa, 2010). Female flies with global reductions in ecdysone (*ecdysoneless* mutants) and ecdysone signaling (*EcR* mutants) show decreased GSC proliferation and were rapid loss of GSCs from the niche, indicative of a failure to maintain GSC fate (Ables and Drummond-Barbosa, 2010). Ecdysone signaling supports the differentiation of GSC progeny, as well as their encapsulation by follicle cells (Konig et al., 2011; Ables et al., 2016).

Ecdysone is also required for *Drosophila* growth and metamorphosis at all stages of development and is patterned spatially as well as temporally, depending on the tissue type and developmental stage (Konig et al., 2011). Ecdysone induces histolysis of nearly all of larval tissues, and promotes differentiation and morphogenesis of structures composing the adult fly, including formation of the adult ovary (Baehrecke, 1996; Gancz and Gilboa, 2011). In ovary development, ecdysone receptors are required to coordinate development of niche somatic cells

and GSC precursors. At early third instar, ecdysone receptors repress precocious differentiation of niche precursor cells and PGCs. At mid-third instar, ecdysone signaling initiates PGC differentiation (Gancz and Gilboa, 2011). The ecdysone signaling cascade induces the onset of several early genes, including the nuclear hormone receptor, *ftz transcription factor 1 (ftz-f1)*.

There are two different mRNA isoforms of *ftz-f1*: *ftz-f1-RA* (short isoform) and *ftz-f1-RB* (long isoform). Both isoforms are transcribed from the same gene locus and share a common C-terminal region, but contain different N-terminal regions (Lavorgna et al., 1991, 1993) (Figure 2). *ftz-f1-RA* is considered the early isoform and is expressed in early embryos. This mRNA isoform is maternally loaded into oocytes and required for embryogenesis (King-Jones and Thummel, 2005). *ftz-f1-RB* expression is detected in late-stage embryos, larvae, prepupae, and adults (Ueda et al., 1990; Lavorgna et al., 1993; Murata et al., 1996).

Figure 2. Schematic of *ftz-f1* gene locus. Blue bolded line indicates *ftz-f1* gene span while the two lines below are representative of the two differing *ftz-f1* isoforms. Of the isoforms, black lines indicate introns; orange and grey squares indicate exons; red squares indicate regions targeted by shRNAi in f*tz-f1*[JF02738] and *ftz-f1*[KK108995]. Green arrow indicates *ftz-f1::GFP* insertion site.

Several lines of evidence demonstrate that ecdysone and *ftz-f1* work in concert to control

growth and metamorphosis (Broadus et al., 1999; Sultan et al., 2014). *ftz-f1* is also required for

multiple developmental processes and is expressed throughout development, with especially high

levels during late pupal stage immediately after ecdysteroid pulses (Sultan et al., 2014). Using an

antibody against *Bftz-f1*, staining can be found in most larval tissues 44-46 hours after egg laying

(AEL), for example the salivary gland, fat body, trachea, ring gland, epidermis, and guts, but at

this time point, staining of gonads was not detectable (Yamada et al., 2000). *ftz-f1* has been

described as a competence factor necessary for stage-specific responses to ecdysone during

Drosophila metamorphosis (Broadus et al, 1999). *ftz-f1* is stage-specifically expressed in mid-

prepupae, during the period of low ecdysone titer, and is essential for genetic and biological

responses to ecdysone that distinguish the prepupal-pupal transition. *ftz-f1* mutants pupariate

normally in response to the late larval pulse of ecdysone, but display defects related to problems

during the prepupal-pupal transition, such as adult head eversion, leg elongation, and salivary

gland cell death (Broadus et al., 1999). In male adults, when *ftz-f1* is depleted in the *Drosophila*

testis, it induces GSC loss, similar to the phenotype resulting from the knockdown of ecdysone

receptor (EcR) (Li et al, 2014). Furthermore, mosaic analysis confirmed that these two factors

are cell autonomously required for cyst stem cell maintenance (Li et al, 2014). Over-expression

of *ftz-f1* in the somatic cells of the ovary results in precocious PGC differentiation (Gancz and

Gilboa, 2011). There is a known connection between *ftz-f1* and ecdysone but it is unclear how

ftz-f1 specifically impacts female GSC establishment and function.

Although many genes, including *ftz-f1*, are thought to be downstream targets of ecdysone

signaling, it is unclear how these targets impact GSC fate and function. We hypothesize that *ftz-f1*, a downstream target of ecdysone, is necessary for proper GSC function and for the

establishment of tissue resident stem cells in their niche in *Drosophila* oogenesis. To explore the role of *ftz-f1* in ovarian stem cells, we used the *UAS-GAL4* system in combination with short hairpin RNA interference (RNAi) to reduce *ftz-f1* function specifically in germ cells or the surrounding somatic cells. We demonstrate that *ftz-f1* is intrinsically required for the establishment of the proper number of GSCs during development. Reduced *ftz-f1* function in somatic cells of late third larval instar ovaries leads to disorganization of the GSCs while reduced *ftz-f1* function in germ cells leads to a significant decrease in the average number of GSCs. Further, our results suggest that *ftz-f1* is also required in ovarian somatic cells during development for proper movement of germ cells out of the germarium in adult stages. Reduced *ftz-f1* function in ovarian somatic cells during development leads to enlarged germaria with a significant increase the average number of 16-cell cysts. Taken together, our data suggests that *ftz-f1* function during juvenile stages is critical for the establishment and development of GSCs and their progeny.

RESULTS

ftz-f1 is widely expressed in the adult and larval ovary

ftz-f1 is expressed in a variety of tissue types and required for multiple developmental processes, including adult head eversion, leg elongation, and salivary gland cell death (Broadus et al, 1999). The pattern and localization of *ftz-f1* expression in the adult and larval ovary has not been reported, despite its known connection with ecdysone in oogenesis, including its sufficing for PGC differentiation (Gancz and Gilboa, 2011). To asses *ftz-f1* localization in the adult and larval ovary, we took advantage of a reporter transgene in which the coding region of *green fluorescent protein* (*GFP*) was inserted in frame on the C-terminal end of the *ftz-f1* locus (Spokony and White, personal communication), creating a fusion protein (*ftz-f1::GFP*) (Figure 3). To identify cell type-specific expression of *ftz-f1::GFP* in the adult ovary, we performed co-immunofluorescence with anti-Hts, a fusome and follicle cell membrane marker, and anti-LamC, a marker of the nuclear envelope of cap cells. We found that *ftz-f1::GFP* is broadly expressed at varying levels throughout the adult ovary, including the germarium and follicles at each stage of oogenesis (Figure 3 A and A'). *ftz-f1* is expressed in an array of different cell types in the adult ovariole, both somatic and germline. For the germline cell populations, *ftz-f1* is highly expressed in the GSCs and their differentiated daughter cell, the cystoblast, with less expression in the 2- , 4-, 8-, and 16- cell cysts (Figure 3 B and B'). In the adult ovary, *ftz-f1* is expressed in a variety of somatic cells, including high expression in the escort cells, the follicle stem cells, and their progeny, the follicle cells (Figure 3 C and C').

We were also interested in *ftz-f1* expression at an earlier point in development, when germ cells and somatic cells coalesce to form the larval ovary. To unambiguously identify germ

cells, we co-stained larval ovaries with anti-Hts, anti-LamC, and Vasa, a germ cell marker. Like the adult ovary, the larval ovary also has *ftz-f1::GFP* expression. *ftz-f1* is highly expressed in a subset of the PGCs closer to the terminal filament stacks (Figure 3 D and D', circled and co-stained with Vasa, a germ-cell specific protein). There are a portion of the PGCs further away from the terminal filament stacks and at the posterior end of the LL3 ovary that do not seem to express *ftz-f1* (Figure 3 D and D', arrowed and co-stained with Vasa). In the LL3 developing ovary, there seems to be little expression in somatic cell populations, including terminal filament cells and precursor escort cells, called intermingled cells (Figure 3 D and D'). This somatic cell expression pattern in the LL3 ovary differs from the somatic cell expression pattern in the adult ovary, where the escort and follicle cells highly express *ftz-f1*. This suggests that there is a point in development where *ftz-f1* somatic cell expression changes. Taken together, these data suggest that *ftz-f1* is indeed expressed in germ cells and somatic cells in both the LL3 and adult ovary.
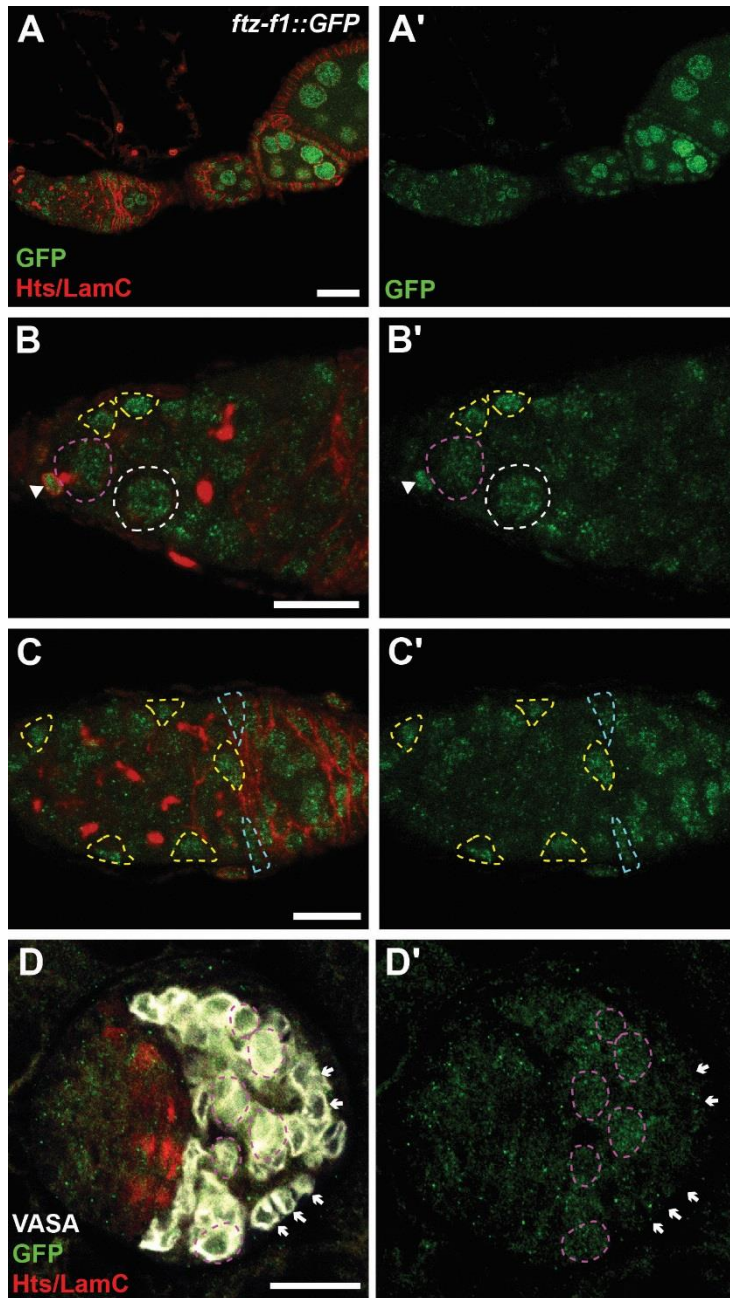
Figure 3. *ftz-f1* expression in adult and larval germ and somatic cell populations. (A and A') *ftz-f1* expression throughout the *Drosophila* ovariole. Scale bar, 10 um. (B and B') *ftz-f1* expression in a GSC circled in pink, escort cells circled in yellow, and a cystoblast circled in white. Arrow head indicates cap cell directly anterior to GSC. Scale bar, 20 um (C and C') Somatic cell populations of escort cells circled in yellow, follicle stem cells circled in blue. Adult germaria labeled with anti-GFP (green; endogenous *ftz-f1*) anti-Hts (red; fusomes and follicle cell membranes), and anti-LamC (red; nuclear envelope of cap cells). Scale bar, 20 um (D and D') *ftz-f1* expression in LL3 ovary. PGCs expressing *ftz-f1* circled in pink while PGCs not expressing *ftz-f1* are arrowed. LL3 ovary labeled with anti-GFP (green; endogenous ftz-f1), anti-Hts (red; fusomes and follicle cell membranes), and anti-Vasa (white; germ cells). Scale bar, 50 um.

ftz-f1 is intrinsically required in PGCs during development for establishment of the proper number of adult GSCs

Expression of _ftz-f1::GFP_ in adult GSCs suggested that _ftz-f1_ could be important for GSC regulation. We therefore sought to discover whether _ftz-f1_ is intrinsically required in GSCs for self-renewal. To decrease _ftz-f1_ expression in germ cells, we took advantage of the _UAS-GAL4_ system to induce tissue specific expression of a short hairpin RNA (shRNAi) targeted against the shared, common region between the two isoforms of _ftz-f1_ (Figure 2). We crossed a previously described _ftz-f1_ RNAi (Matunis et al., 2015), _ftz-f1^{JF02738}_, with _nos.NGT-Gal4_, a well-described germ cell-specific driver (Rørth, 1998). We immunostained _nos.NGT-Gal > ftz-f1^{JF02738}_ and control (carrying _nos.NGT-Gal4_ or _ftz-f1^{JF02738}_ only) ovaries with LaminC, nuclear membrane marker, and Hts, a component of the fusome marker. This staining allowed us to easily visualize and identify GSCs by their anteriorly localized fusomes (Figure 4 A and B).

When dissected five days after eclosion, _nos.NGT-Gal> ftz-f1^{JF02738}_ flies have a significant decrease in average number of GSCs per germaria compared to controls. We reasoned that if _ftz-f1_ was required for GSC self-renewal, then the number of _ftz-f1_ mutant GSCS should decrease faster than the rate at which normal GSCs are lost during aging. We therefore dissected these germ cell specific _ftz-f1^{JF02738}_ flies at two later time points: eight and twelve days after eclosion (Figure 4 C). _nos.NGT-Gal4 > ftz-f1^{JF02738}_ mutant females had a consistent and significant decrease in the average number of GSCs per germaria compared to controls across all three time points examined (Figure 4 C). Interestingly, these mutant flies showed no further reduction in GSC number with aging and no other obvious phenotypic abnormalities. Day five, eight, and twelve all had a similar significant decrease, suggesting that the decreased average number of GSCs in the adult ovary is not due to a loss of GSC self-renewal. Rather, these data

suggests that *ftz-f1* is intrinsically required in germ cells during development for establishment of

the proper number of GSCs.
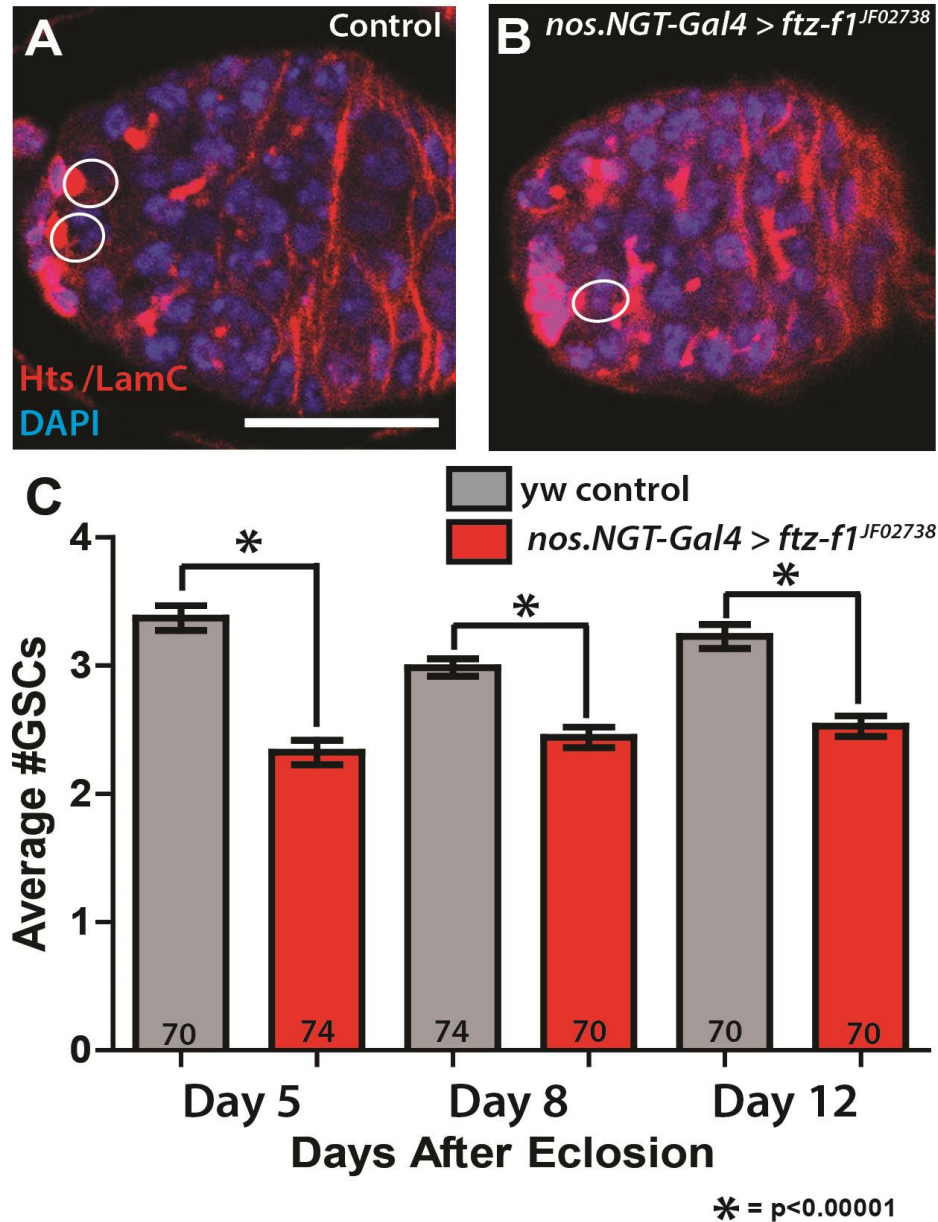
Figure 4. *ftz-f1* is intrinsically required during development for establishment of the proper number of adult GSCs. (A and B) *nos.NGT-Gal4 > yw* control (A) *nos.NGT-Gal4 > ftz-f1*[JF02738] mutant (B) germaria labeled with anti-Hts (red; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells), and DAPI (blue; DNA) GSCs circled in white. Scale bar, 20 um. (C) Average number of GSCs per germarium in both *nos.NGT-Gal4 > yw* controls *nos.NGT-Gal4 > ftz-f1*[JF02738] mutants over three time points, 5, 8, and 12 days after eclosion. Error bars, mean +/- SEM. * p < 0.00001; Student's two tailed *T*-test.
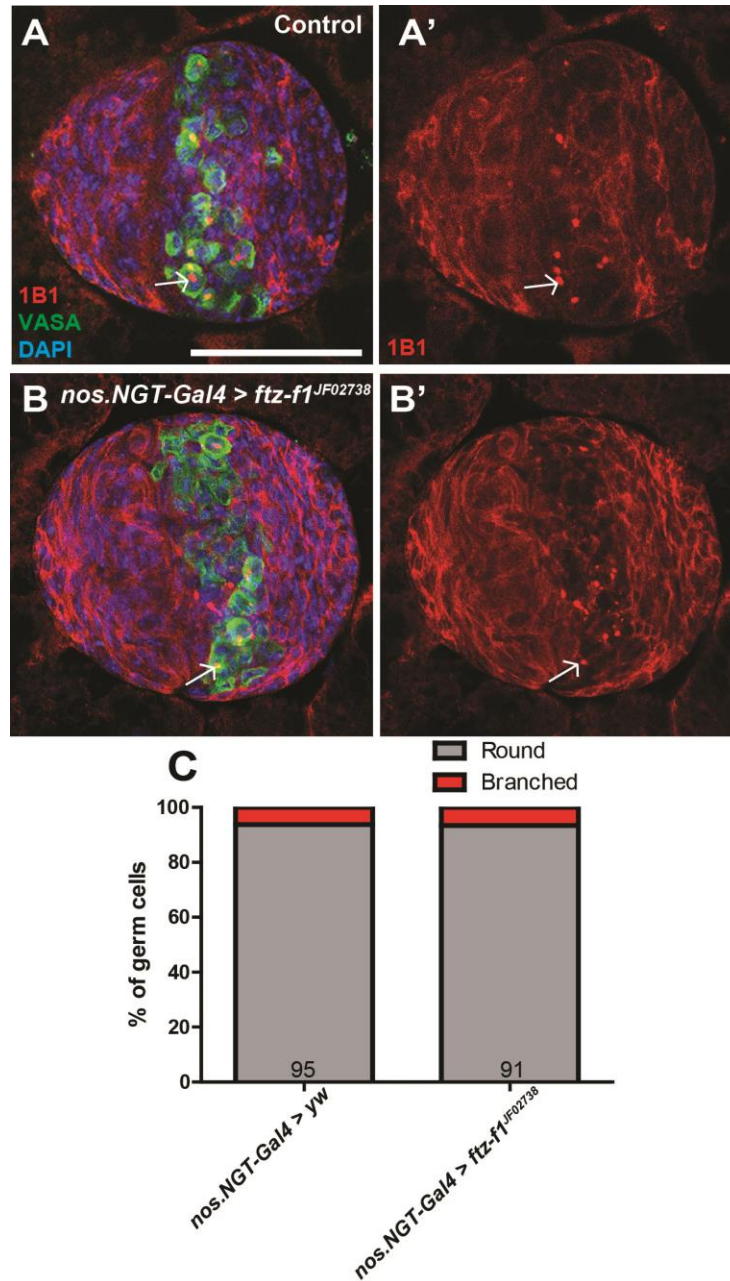
Reduction of *ftz-f1* does not induce precocious PGC differentiation

One possible explanation for the significant decrease in average number of GSCs per germaria in *nos.NGT-Gal4>ftz-f1^{JF02738}* mutant females is precocious PGC differentiation. In order for growth and development of varying tissues and organs, differentiation and proliferation are tightly regulated. The molecular mechanisms, however, can be independent of the regulation necessary in the same adult tissue type. During the development of the *Drosophila* ovary, ecdysone receptors are required to coordinate the development of the niche and GSC precursors (Gancz and Gilboa, 2011). At early third instar, ecdysone receptors repress precocious differentiation of both somatic cell precursors and PGCs. At mid-third instar, ecdysone signaling initiates PGC differentiation (Gancz and Gilboa, 2011). We tested whether *ftz-f1* is a required component of the ecdysone response cascade required for repression of precocious differentiation of PGCs. Using the same *UAS-GAL4* system to tissue specifically express *ftz-f1^{JF02738}* in germ cells (*nos.NGT- Gal4*) as in the adult ovary, we did so in the developing LL3 ovary.

We used fusome morphology to evaluate differentiation in Vasa expressing PGCs (Figure 5 A). A round fusome is indicative of undifferentiation, while an elongated fusome is indicative of differentiation (Spradling et al., 1994). This is because when a differentiated daughter cell divides to form a cystoblast, the cells remained connected due to incomplete cytokinesis. The fusome is thought to be an open tunnel, allowing for signals to be transported from one cell to another (Spradling et al., 1994). Per LL3 ovary, we counted all of the Vasa stained germ cells and tallied up fusome, stained with anti-Hts, into two categories: round or branched. There was no difference between mutant and control in regards to fusome morphology, indicating that knockdown of *ftz-f1* in the germ cells does not induce precocious differentiation (Figure 5 C).

These data suggest that the significant decrease in average number of GSCs per germaria in the adult ovary (Figure 4 C) is not due to precocious differentiation of the PGCs in the larval ovary (Figure 5 C).

Figure 5. Reduction of *ftz-f1* does not induce precocious PGC differentiation. (A and A') *nos.NGT-Gal4 > yw* control (B and B') *nos.NGT-Gal4 > ftz-f1^JF02738* mutants. (A and B) labeled with anti-Hts (red; fusomes and follicle cell membranes), anti-Vasa (white; germ cells), and DAPI (blue; DNA). (A' and B') just red layer. White arrow distinguishes rounded undifferentiated fusome example for both control and mutant. Scale bar, 50 um. (C) Frequencies germ cells containing rounded (grey) or branched (red) fusomes. Numbers in bars indicate number of germ cells analyzed.

ftz-f1 is required for the establishment of proper number of GSCs at the base of the terminal filament stacks

While imaging the _nos.NGT-Gal4>ftz-f1^{JF02738}_ female flies, we quickly began to notice a mutant phenotypic trend: the mutant larval ovary germ cells appeared disorganized. When stained for anti-engrailed (terminal filament marker), Vasa (germ cell markers), and DAPI (DNA marker), we noticed that Vasa expressing germ cells were not all located directly adjacent to the terminal filament stacks, as observed in control (carrying _nos.NGT-Gal4_ or _ftz-f1^{JF02738}_ only) ovaries (Figure 6 A and B). In order to remain undifferentiated, PGCs must be physically anchored to the TFs and cap cells via DE-cadherin (Song and Xie, 2002). Fewer PGCs in close proximity to TFs may result in fewer GSCs in the adult germarium, which was the finding displayed in Figure 4. So we quantified this phenotype by measuring the distance between the most posterior terminal filament cell in the terminal filament stack to the center of the nucleus of all the germ cells in the larval ovary. We found that there was a significant increase in TF to germ cell distance in our mutant _nos.NGT-Gal4>ftz-f1^{JF02738}_ female flies compared to controls (Figure 6 C).

BMP (Bone Morphogenetic Protein) signaling is a major GSC-maintaining pathway in female flies. _Dpp_ (a BMP-like ligand) acts nonautonomously over the short range from the stem cell niche to the GSCs in order to repress the transcription of _bag of marbles (bam)_ (Song et al., 2004; Chen and McKearin, 2003). _bam_ expression is sufficient to drive differentiation of GSCs or cystoblasts, even in the presence of high levels of _Dpp_ (Chen and McKearin, 2003). _Mothers against Dpp (Mad)_ is a transcriptional repressor of _bam_ and binds to transcriptional silencer elements in the _bam_ promotor (Song et al., 2004; Chen and McKearin, 2003). pMAD, a marker of undifferentiated PGCs and GSCs, is only expressed in a subset of the PGCs that directly

adjacent to the TF fated to become the adult GSCs (Kai and Spradling, 2001; Gilboa et al., 2003; Gilboa and Lehmann, 2004). Since we saw a significant increase in the TF to germ cell distance was observed in the larval ovary, we hypothesized that there may be fewer undifferentiated germ cells (PGCs expressing pMAD) in *nos.NGT-Gal> ftz-f1^{JF02738}* larva, because they are too far away to make the crucial DE-cadherin physical attachment. Typically, only the PGCs that are directly adjacent to the TFs express pMAD (Gilboa and Lethmann, 2004). We immunostained *nos.NGT-Gal> ftz-f1^{JF02738}* and control larval ovaries with anti-Vasa, pMAD, anti-engrailed, and DAPI. This staining allowed us to visualize which Vasa- containing germ cells were also expressing pMAD and thus, are more likely to be maintained as GSCs (Figure 6 D and E). Expression in pMAD intensity in control larval ovary germ cells was more pronounced compared to mutant larval ovary germ cells (Figure 6 D and E). Taken together, these data suggest that *ftz-f1* expression in PGCs promotes recruitment of PGCs to the undifferentiated pre-GSC fate. This indicates that *ftz-f1* makes PGCs competent to respond to BMP signaling.
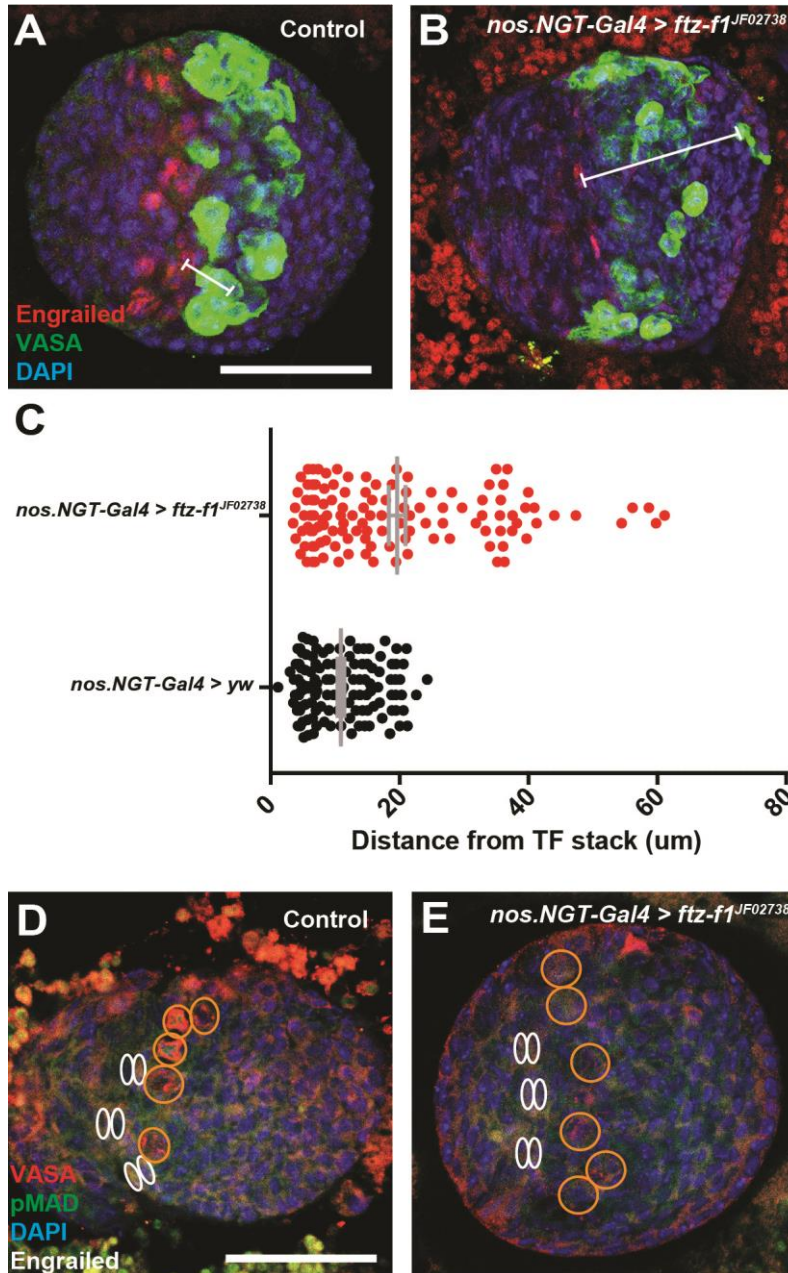
Figure 6. *ftz-f1* is required for the establishment of proper number of GSCs at the base of the terminal filament stacks. (A) *nos.NGT-Gal4 > yw* control (B) *nos.NGT-Gal4 > ftz-f1^{JF02738}* mutant. (A and B) labeled with anti- engrailed (red; terminal filaments), anti-Vasa (green; germ cells), and DAPI (blue; DNA). White lines indicate example distance measured from most anterior terminal filament cell to nuclei of the germ cell. Scale bar, 50 um. (C) Quantification of distance from the terminal filament (TF) stack in um for both control (grey) and mutant (red). Each dot is indicative of the distance from 1 germ cell to its closest terminal filament stack. (D) *nos.NGT-Gal4 > yw* control (E) *nos.NGT-Gal4 > ftz-f1^{JF02738}* mutant. (D and E) labeled with anti-Vasa (red; germ cells), pMAD (green, undifferentiated germ cells), DAPI (blue; DNA), and anti-Engrailed (white, terminal filament cells). Example terminal filament cells circled in white and example germ cells circled in orange. Scale bar, 50 um.

Reduction of *ftz-f1* in somatic cells results in pupal lethality

Because of *ftz-f1*'s high expression in adult ovary escort and follicle somatic cell populations (Figure 3 B and C), we were interested in *ftz-f1*'s role in somatic cells and its indirect role in germ cell function. In order to confine *ftz-f1* knockdown to specific somatic cell populations, we screened through a variety of available adult ovary somatic cell drivers including *bric-a-brac 1* (*bab1*), *C587*, and *traffic jam* (*tj*) (Bolívar et al, 2006; Kai et al., 2005; Li et al., 2003) in order to clarify their expression patterns and determine their suitability as tissue specific tools. Using the *UAS-GAL4* system, we crossed various somatic cell *Gal4* drivers to a *UAS-GFP* to visualize expression. We performed co-immunofluorescence with anti-Hts (fusome and follicle cell membrane marker), anti-LamC (marker of the nuclear envelope of cap cells), and DAPI in order to characterize driver specific expression in adult germaria. *Bab1-Gal4* expression in the adult ovary was specific to the cap cells and terminal filament cells (Figure 7 A and A'). *C587-Gal4* expression was specific to escort cells and early follicle cells (Figure 7 B and B'), although typically *C587-Gal4* expression did not typically contain all escort cells and follicle cells, and varied from germarium to germarium (data not shown). *Tj-Gal4* expression was specific to all follicle cells (Figure 7 C and C'). With these differing expression patterns, we were able to use the specific expression patterns of these somatic cell drivers as tools in order to investigate *ftz-f1*'s role in specific cell types of the adult ovary.
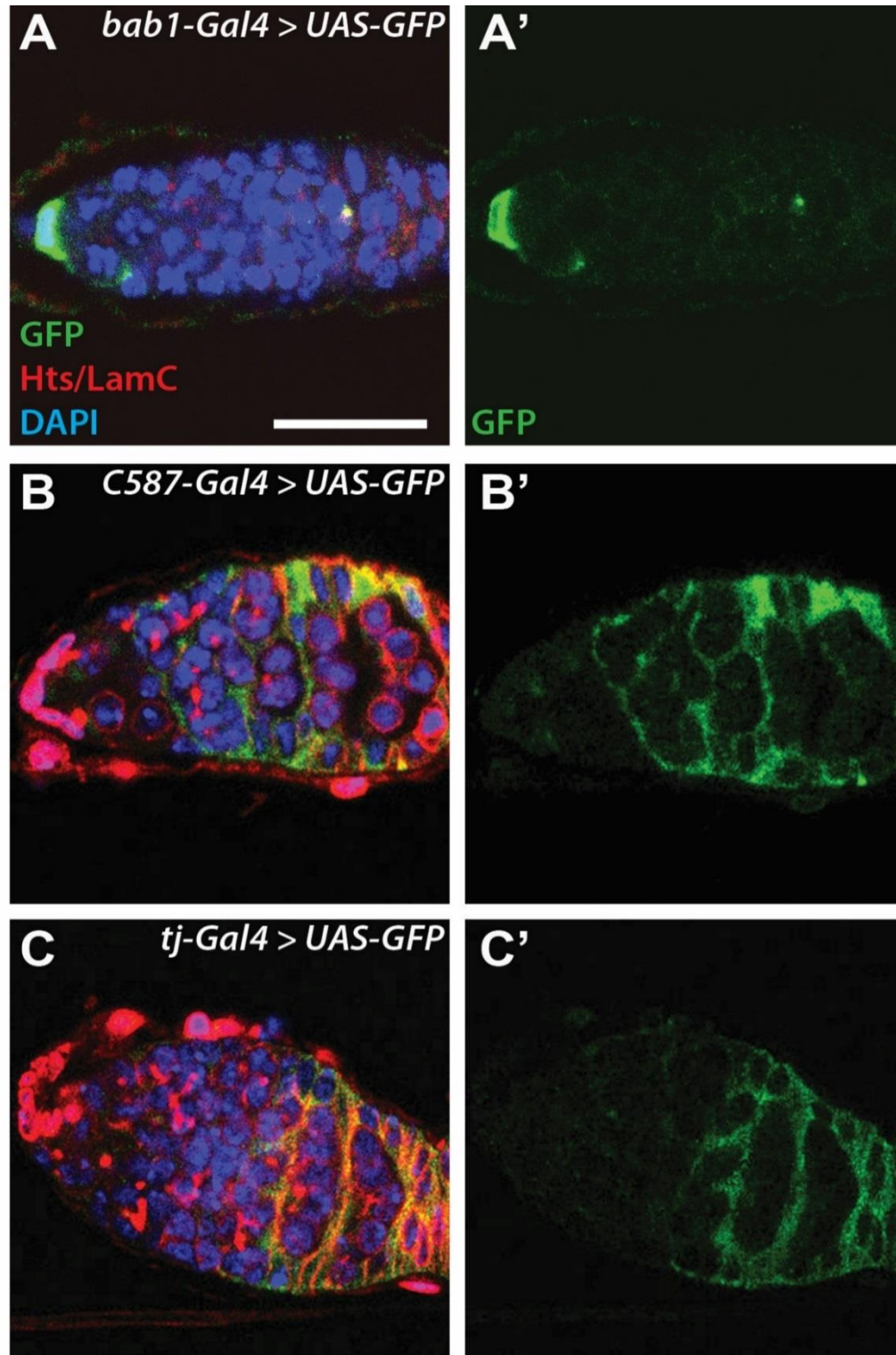
Figure 7. Adult ovary somatic cell driver expression. (A and A') *bab1-Gal4* expression (B and B') *C587-Gal4* expression (C and C') *tj-Gal4* expression. Germaria labeled with GFP (green; somatic cell driver expression), anti-Hts (red; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells), and DAPI (blue; DNA). Scale bar, 20 um.

Just as we wanted to specifically pinpoint certain somatic cell types, we would like to identify specific developmental time points. This will allow us to evaluate both temporal and spatial aspects of *ftz-f1* to tease apart its specific role in the germaria, and particularly its indirect effect on GSCs. In order to knockdown *ftz-f1* function, or switch on the RNAi, at varying time points in development, we turned to the temperature sensitive *Gal4/Gal80^{ts}* system. This temperature sensitive system also serves as a way to bypass the extreme lethality that occurs when *ftz-f1* is depleted from egg laying (Table 1). This system allows us to use environmental temperature switches, from restrictive (29°C) to permissive temperature (18°C) in order to mediate RNAi expression. We turned on the RNAi, or effectively knocked down *ftz-f1* function at three time points: egg laying, LL3, and eclosion (Figure 8 A) hoping to elucidate when exactly *ftz-f1* function is needed during development. This *Gal80^{ts}* system was verified by comparing mutants with no *Gal80^{ts}* in the background (*C587-Gal4)* to mutants with *Gal80^{ts}* in the background (*C587-Gal80^{ts})*. When RNAi was turned on and remained on all throughout development and into adulthood, these two mutants had the same phenotype, validating the *Gal80^{ts}* system (Figure 8 C-F).

To identify the role of *ftz-f1* in adult flies, *C587-Gal80^{ts} > ftz-f1^{JF02738}* females were switched from the permissive temperature (19°C) to the restrictive temperature (29°C) 1-2 days after eclosion (DAE) and then maintained at that temperature until dissection. This made it so that *ftz-f1* knockdown in escort cells only occurred in adult flies. We did not see any significant any phenotypic differences (Figure 8 G), and no significant difference in average number of GSCs or 16 cell-cysts per germarium (data not shown) between control and mutant in *C587-Gal80^{ts} > ftz-f1^{JF02738}* females. These *C587-Gal80^{ts} > ftz-f1^{JF02738}* and another *ftz-f1* RNAi, crossed with the same escort cell driver, *C587-Gal80^{ts} > ftz-f1^{KK108995}* females also had a high

38

percentage of surviving flies when RNAi was turned on 1-2 DAE and then dissected 8 days after eclosion, 90% and 87% respectively (Table 1).

C587-Gal4 it is not only expressed in escort cells of the adult female germaria, but also in other tissues in the fly (A. Armstrong, personal communication). When *ftz-f1* expression was knocked down early in development (before eclosion) a large percentage of the *C587-Gal80^{ts} > ftz-f1^{JF0273}* and *C587-Gal80^{ts} > ftz-f1^{KK108995}* mutant flies failed to eclose, suggesting that C587 is also expressed in a tissue critical for development (Table 1). When *ftz-f1* RNAi was turned on at late larval stage three, 144 hours after egg laying, we observed a small percentage of surviving mutant flies (Table 1). There were 6% of *C587-Gal80^{ts} > ftz-f1^{JF0273}* adult flies surviving at eclosion and 5% of *C587-Gal80^{ts} > ftz-f1^{KK108995}* when *ftz-f1* RNAi was turned on at LL3 (Table 1). Mutants with reduced ftz-f1 function in somatic cells at LL3 have similar, but less severe phenotypic abnormalities, as when *ftz-f1* RNAi is turned on at egg laying (Figure 8 G).

As mentioned previously, knock down of *ftz-f1* expression at egg laying, resulted in a mutant phenotype the same as *C587-Gal4*, with no *Gal80^{ts}* in the background (explored more in Figure 9 and 10). This experiment yielded an even smaller percentage of surviving mutant flies. There were 4.72% of *C587-Gal80^{ts} > ftz-f1^{JF0273}* and 3% of *C587-Gal80^{ts} > ftz-f1^{KK108995}* flies surviving at eclosion when *ftz-f1* RNAi was turned on at egg laying (Table 1). Taken together, *ftz-f1* function in the escort cells is most important during development, some time prior to LL3. We cannot rule out, however, a potential role for *ftz-f1* in somatic cells during pupal development. C587 also seemed to be expressed in other tissue in the fly, creating significant lethality.

Just as we spatial and temporally adjusted *ftz-f1* function to certain developmental time points and only in the escort cells, we did the same with two other somatic cell drivers, *bab1* and

tj. Using the somatic cell driver *bab1*, we were able to specifically knockdown *ftz-f1* function in the terminal filament and cap cells (Figure 8 H and I). Unfortunately, this induced 100% lethality when turned on any earlier than eclosion, and even surprisingly created significant lethality when turned on 1-2 DAE, with extreme phenotypic abnormalities throughout the fly, including the ovary (Figure 8 H and I). There were 16% of *bab1-Gal80^{ts} > ftz-f1^{JF0273}* and 13% of *bab1-Gal80^{ts} > ftz-f1^{KK108995}* flies surviving at eclosion when *ftz-f1* RNAi is turned on 1-2DAE and 0% flies surviving when turned on at egg laying or at LL3 (Table 1). These findings suggest *bab-1* expression is clearly not limited to cap and follicle cells during development.

We next looked at the follicle cell driver *tj*. At this point, we only have follicle cell specific knockdown at one developmental time point: egg laying (Figure 8 D). When *ftz-f1* is knocked down in follicle cells at egg laying, similar to the other 2 somatic cell drivers, we get some lethality. There are 10.6% of *tj-Gal4 > ftz-f1^{JF02738}* flies surviving at eclosion (Table 1).

Taken together, with the *Gal4/Gal80^{ts}* system, we were able to effectively tease apart when and where *ftz-f1* expression is needed in the somatic cells of the adult ovary. On top of allowing us to have temporal and spatial control of *ftz-f1* expression, these experiments also support our confidence in both *ftz-f1* RNAi drivers *ftz-f1^{JF0273}* and *ftz-f1^{KK108995}*since we observed similar lethality and ovary phenotype results.
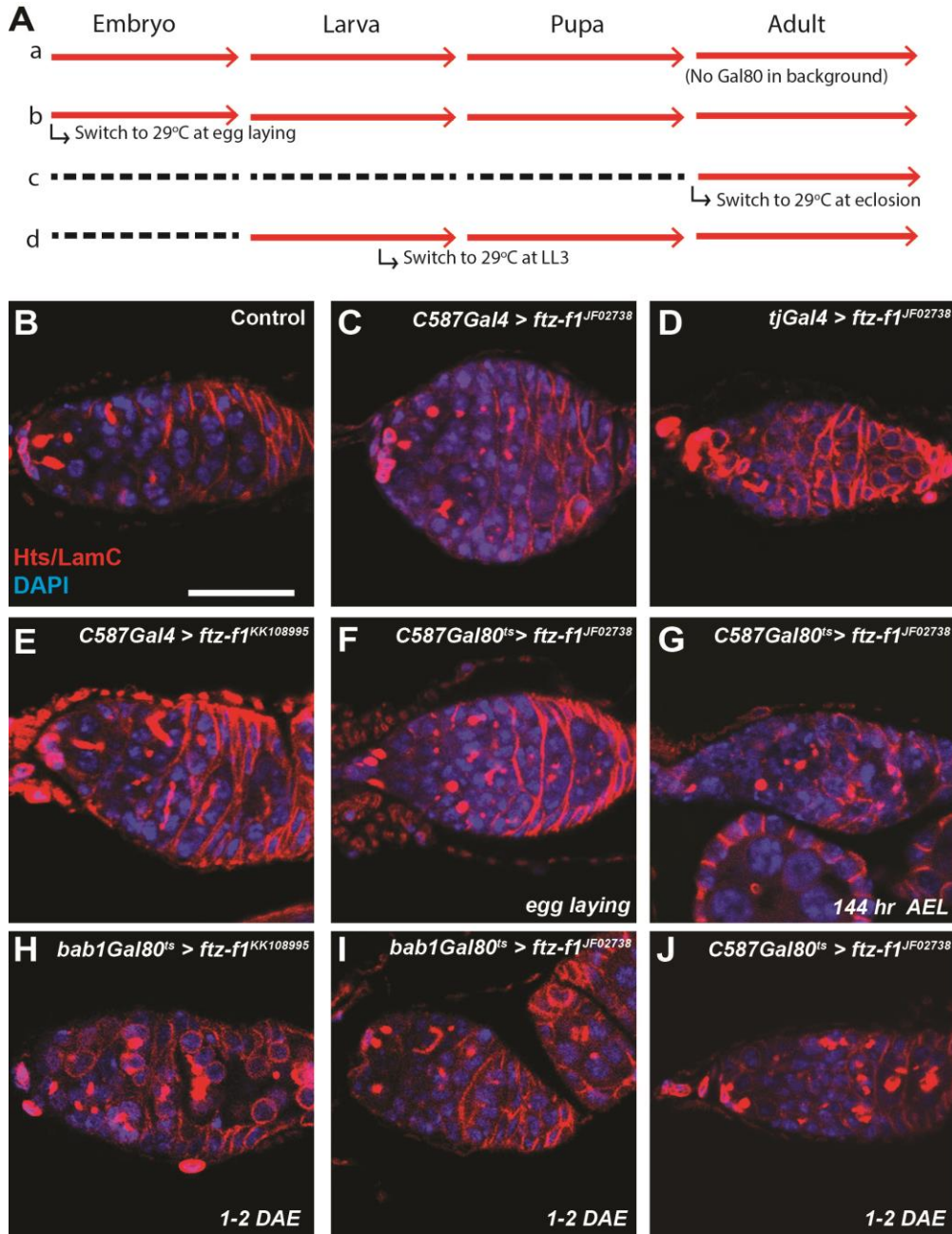
Figure 8. *ftz-f1* RNAi turned on at varying time points during development. (A) Schematic depicting when during development ftz-f1 expression was knocked down via RNAi. (Aa) positive control with no Gal80ts in background (Ab) ftz-f1 expression knocked down at egg laying (Ac) ftz-f1 expression knocked down 1-2 DAE eclosion (Ad) ftz-f1 expression knocked down at 144 hours AEL (LL3). Example images from the time points displayed in A.(B) yw control (C) C587-Gal4>*ftz-f1^JF02738* (D) *tj-Gal4> ftz-f1^JF02738* (E) *C587-Gal4> ftz-f1^KK108995* (F) *C587-Gal80^ts> ftz-f1^JF0273* expression knocked down at egg laying (G) *C587-Gal80^ts> ftz-f1^JF02738* expression knocked down 144 hours AEL (LL3) (H) *bab1-Gal80^ts> ftz-f1^JF02738*expression knocked down 1-2 DAE (I) *bab1-Gal80^ts> ftz-f1^KK108995* expression knocked down 1-2 DAE (J) *C587-Gal80^ts> ftz-f1^JF02738* expression knocked down 1-2 DAE. All germaria labeled with anti-Hts (red; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells), and DAPI (blue; DNA). Scale bar, 20 um.

Table 1. *ftz-f1*[JF02738] and *ftz-f1*[KK108995] lethality in varying somatic cell populations reduced at different developmental times points

Adult somatic cell population targeted[b]	Gal4 driver	% mutant adults surviving at eclosion[a]					
		ftz-f1[JF02738] on at egg laying (b)[c]	*ftz-f1*[KK108995] on at egg laying (b)[c]	*ftz-f1*[JF02738] on at LL3 (d)[d]	*ftz-f1*[KK108995] on at LL3 (b)[d]	*ftz-f1*[JF02738] on at eclosion (c)[e]	*ftz-f1*[KK108995] on at eclosion (c)[e]
Terminal filament and cap cells	*bab1-Gal4*	0%	0%	0%	0%	16%	13%
Escort cells	*C587-Gal4*	4.72%	3%	6%	5%	90%	87%
Follicle cells	*tj-Gal4*	10.6%	N/A	14%	N/A	N/A	N/A

[a] Mutant flies were dissected, stained, and imaged 8 days after eclosion. All flies were density matched in unpopulated vials with the same amount of food and temperature. Total number of flies examined includes all flies surviving until pupariation, averaging 300-400 flies between 5-10 vials per RNAi (JF02738 and KK108995) per developmental time point.

[b] Adult somatic cell populations targeted using *Bab1*, *C587*, and *Tj Gal4* drivers. Expression of drivers is shown in Fig. 6 via *UAS-GFP*. Adult somatic cell populations may no directly coincide with larval and pupal somatic cell populations, and may result in overlap in the somatic cell population targeted.

[c] *ftz-f1*[JF02738] and *ftz-f1*[KK108995] on at egg laying by taking advanantage of the *Gal4/Gal80*[ts] system and raising parental flies in restrictive temperature (29°C) and during egg laying. Progeny were then maintained in restrictive temperature until eclosion. *Gal80*[ts] flies at restrictive temperature starting at egg laying had same phenotype as *Gal4* mutant flies.

[d] *ftz-f1*[JF02738] and *ftz-f1*[KK108995] on at LL3 by taking advanantage of the *Gal4/Gal80*[ts] system and raising mutant flies at permissive temperature (18°C) then switching mutant flies to restrictive temperature at LL3 (about 114 hours after egg laying). After LL3 temperature switch, mutant flies were then raised until eclosion in restrictive temperature.

[e] *ftz-f1*[JF02738] and *ftz-f1*[KK108995] on at eclosion by taking advanantage of the *Gal4/Gal80*[ts] system and raising mutant flies at permissive temperature (18°C) then switching mutant flies to restrictive temperature at eclosion, so that RNAi is on only in adult stages, bypassing development.

Depletion of *ftz-f1* in escort cells or follicle cells does not influence GSC establishment or self-renewal.

We next wanted know the role of *ftz-f1* in escort cells and how its function indirectly affects GSCs in the adult ovary. Escort cells exhibit extensive interactions with differentiated germ cells (Morris and Spradling, 2011; Xie et al., 2011). They have been shown to wrap around germ cells in the anterior half of the germarium and support differentiation of GSC daughters (Schultz et al., 2002; Decotto and Spradling, 2005). The long cellular processes of the escort cells appear to be involved in passing the differentiated germline cysts posteriorly (Morris and Spradling, 2011).

To decrease *ftz-f1* expression in escort cells, we used the *UAS-GAL4* system to induce tissue specific expression of two different previously characterized RNAi lines, *ftz-f1^JF02738^* and *ftz-f1^KK108995^*. We tested to see if knock down in escort cells affects average number of GSCs per germarium. We dissected eight days after eclosion and performed co-immunoflourescence and stained with anti-Hts, a fusome and follicle cell membrane marker, anti-LamC, a marker of the nuclear envelope of cap cells, and DAPI in order to easily visualize GSCs by their anteriorly localized fusomes (Figure 9 A). We found that *C587-Gal4 > ftz-f1^JF02738^* and *C587-Gal4 > ftz-f1^KK108995^* females do not have a significant change in average number of GSCs per germarium compared to controls (carrying only *C587-Gal4*, *ftz-f1^KK108995^* or *ftz-f1^JF02738^* only) (Figure 9 B, C, and G). Interestingly, there is a slight increase in average number of GSCs per germarium in mutant females compared to controls, but this did not achieve statistical significance (*C587-Gal4 > ftz-f1^JF02738^* with a p-value of 0.0032 and *C587-Gal4 > ftz-f1^KK108995^* with a p-value of 0.32). These findings suggest *ftz-f1* function in escort cells does not significantly influence average number of GSCs per germarium.

Using the *UAS-GAL4* system and two *ftz-f1* RNAi lines previously mentioned, we tested to see if knocked down of *ftz-f1* expression in another somatic cell population in the germarium, the follicle cells, using *tj-Gal4,* and measured the effects on average number of GSCs per germarium. In addition to labeling follicle cells in the adult germarium, *traffic jam* is also expressed in the intermingle cells of the larval ovary. The soma-to-germ cell communication between intermingled cells and GSCs influences GSC establishment (Gancz et al., 2011). We tested to see if knock down of *ftz-f1* in *traffic jam* expressing somatic cells affects average number of GSCs per germarium.

Similar to experiments using the escort cell driver *C587-Gal4*, we dissected eight days after eclosion and performed co-immunoflourescence and stained with anti-Hts, anti-LamC, and DAPI to easily distinguish and quantify GCSs (Figure 9 D). Similar to *ftz-f1* knockdown in escort cells, we found that *tj-Gal4 > ftz-f1^{JF02738}* and *tj-Gal4 > ftz-f1^{KK108995}* females do not have a significant change in average number of GSCs per germarium compared to controls (carrying only *tj-Gal4*, *ftz-f1^{KK108995}* or *ftz-f1^{JF02738}* only) (Figure 9 E and F). There also seems to a slight increase in average number of GSCs, though not significant (Figure 9 G). These findings suggest *ftz-f1* in *traffic jam* expressing somatic cells does not significantly affect estblishment of GSCs.

In order to understand *ftz-f1*'s role in escort and follcile cells of the ovary over time and devlopment, we dissected these escort cell specific *ftz-f1^{JF02738}* flies also at two other time points: three days earlier at 5 DAE and four days later at 12 DAE (Figure 9 H). *C587-Gal4 > ftz-f1^{JF02738}* mutant females had no significant change in average number of GSCs per germarium over the three time points, compared to controls (Figure 9 H). Taken together, *ftz-f1* expression in escort and follicle cell populations do not significantly impact average number of GSCs per germarium at 5, 8, and 12 DAE.
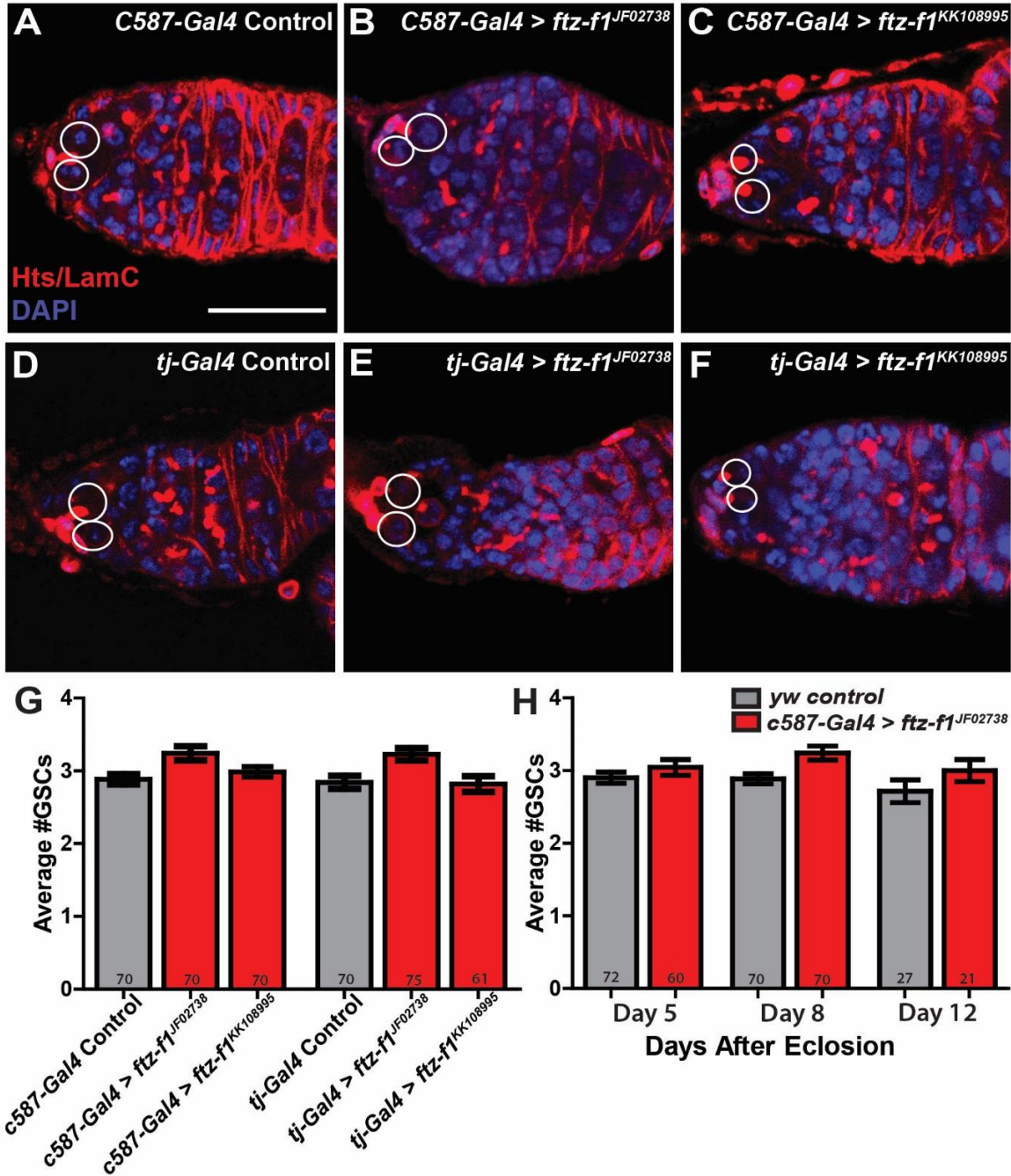
Figure 9. *ftz-f1* knockdown in escort cells or follicle cells and its role in GSCs. (A-C) *C587-Gal4 > yw* control (B) *C587-Gal4 > ftz-f1^{JF02738}* mutant (C) *C587-Gal4 > ftz-f1^{KK108995}* mutant. (G first 3) No significant change in average number of GSCs per germaria when dissected 8 DAE. (A-C) *tj-Gal4 > yw* control (B) *tj-Gal4 > ftz-f1^{JF02738}* mutant (C) *tj-Gal4 > ftz-f1^{KK108995}* mutant. (G last 3) No significant change in average number of GSCs per germaria when dissected 8 DAE. All germaria labeled with anti-Hts (red; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells), and DAPI (blue; DNA) GSCs circled in white. Scale bar, 20 um. (H) Average number of GSCs per germarium in both *C587-Gal4 > yw* controls *C587-Gal4 > ftz-f1^{JF02738}* mutants over three time points, 5, 8, and 12 days after eclosion. Error bars, mean +/- SEM. * p < 0.00001; Student's two tailed *T*-test.

45

ftz-f1 knockdown in escort cells or follicle cells creates an enlarged germarium phenotype with a significantly higher average number of 16-cell cysts per germaria.

Escort cells are thought to "escort" cysts out of the germarium via their dynamic protrusions (Schultz et al., 2002; Decotto and Spradling, 2005; Morris and Spradling, 2011). Once germline cysts pass through region 2a of the germarium, they lose their connections to escort cells and become surrounded by the follicle cells (Decotto and Spradling, 2005). Follicle cells encapsulate the passing cysts as they exit the germarium (Margolis and Spradling, 1995; Forbes et al., 1996; Zhang and Kalderon, 2000). Ecdysone signaling is necessary for proper escort cell function and cyst encapsulation (Konig, 2011; Morris and Spradling, 2012; Ables et al., 2016). We hypothesized that knock down of *ftz-f1* expression in either the escort or follicle cells may impair cyst encapsulation, resulting in to exit the germarium. Interestingly, we noticed that ftz-f1 somatic cell knockdown resulted in an enlarged germaria phenotype (Figure 10 B-F). This prompted us to investigate whether knockdown of *ftz-f1* function in either escort or follicle cells may affect cyst movement out of the germarium.

To decrease *ftz-f1* expression in escort cells, we used the *UAS-GAL4* system to induce tissue specific expression of two different previously characterized RNAi lines, *ftz-f1^JF02738* and *ftz-f1^KK108995*. We tested to see if knock down in escort cells affects average number of 16-cell cysts per germarium. We dissected flies eight days after eclosion and performed co-immunoflourescence for anti-Hts (a fusome and follicle cell membrane marker) anti-LamC (a marker of the nuclear envelope of cap cells) and DAPI in order to easily visualize the 16-cell cysts by their branched fusome, with nodes connecting 16 germ cells (Figure 10 A). We found that *C587-Gal4 > ftz-f1^JF02738* and *C587-Gal4 > ftz-f1^KK108995* females have a significant increase in the average number of 16-cell cysts per germarium as compared to controls (carrying *C587-*

Gal4, *ftz-f1^{KK108995}* or *ftz-f1^{JF02738}* only) (Figure 10 B, C, and G). In addition, we hypothesize that there may be an increase in 2-, 4-, and 8- cell cysts but have yet to quantify those cyst numbers. These findings suggest that loss of *ftz-f1* function in escort cells creates an enlarged germarium phenotype with a significant increase in average number of 16-cell cysts.

We then tested to see whether knock down of *ftz-f1* expression in another somatic cell population in the germarium, the follicle cells (*tj-Gal4*), affects average number of 16-cell cysts per germarium. Similar to the escort cell driver *C587-Gal4*, we dissected eight days after eclosion and performed co-immunoflourescence with anti-Hts, anti-LamC, and DAPI to distinguish and quantify 16- cell cysts (Figure 10 D). Similar to the findings following *ftz-f1* knockdown in escort cells, we found that *tj-Gal4 > ftz-f1^{JF02738}* and *C587-Gal4 > ftz-f1^{KK108995}* females have a significant increase in average number of 16-cell cysts per germarium compared to controls (carrying only *tj-Gal4*, *ftz-f1^{KK108995}* or *ftz-f1^{JF02738}* only) (Figure 10 E and F). Phenotypically, *tj-Gal4 > ftz-f1^{JF02738}* and *tj-Gal4 > ftz-f1^{KK108995}* ovaries are more disorganized than the previous escort cell mutants. These findings suggest disruption of *ftz-f1* expression in the follicle cells creates a disorganized enlarged germarium with a significantly increase in the average number of 16-cell cysts per germarium compared to controls.

In order to understand *ftz-f1*'s role in the somatic cells of the ovary over time with aging, we also dissected escort cell specific *ftz-f1^{JF02738}* flies also at two additional time points: three days earlier at 5 DAE and four days later at 12 DAE (Figure 10 H). *C587-Gal4 > ftz-f1^{JF02738}* mutant females had a significant increase in average number of 16-cell cysts per germarium over the three time points, compared to controls (Figure 10 H). Taken together, *ftz-f1* expression in escort and follicle cell populations contributes to proper 16-cell cyst number at 5, 8, and 12 DAE.
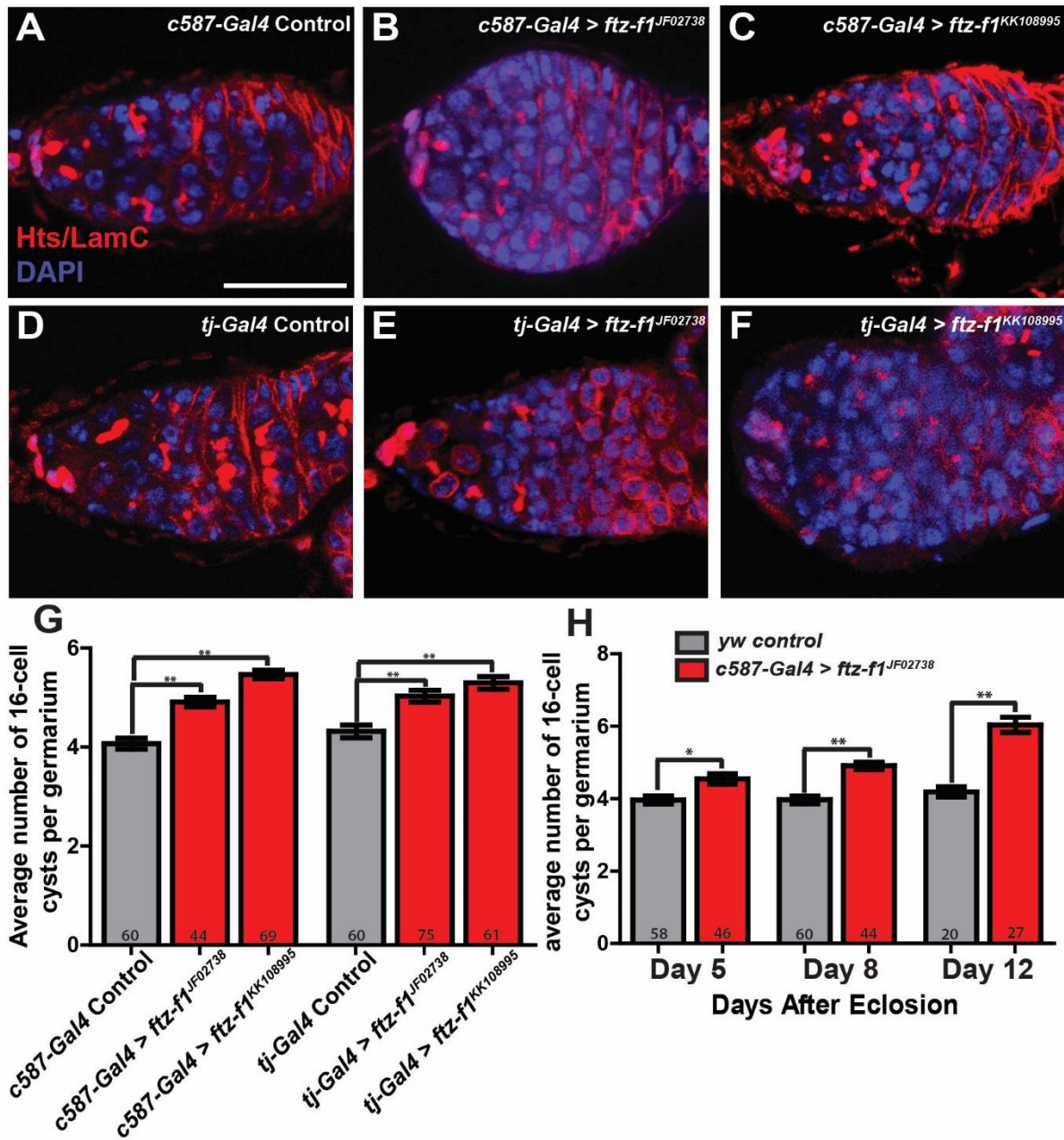
Figure 10. *ftz-f1* knockdown in escort cells or follicle cells creates an enlarged germarium phenotype with a significantly higher average number of 16-cell cysts per germaria. *ftz-f1* knockdown in escort cells or follicle cells and its role in 16-cell cysts. (A-C) *C587-Gal4 > yw* control (B) *C587-Gal4 > ftz-f1^{JF02738}* mutant (C) *C587-Gal4 > ftz-f1^{KK108995}* mutant. (G first 3) Significant increase in average number of 16-cell cysts per germaria when dissected 8 DAE. (D-F) *tj-Gal4 > yw* control (E) *tj-Gal4 > ftz-f1^{JF02738}* mutant (F) *tj-Gal4 > ftz-f1^{KK108995}* mutant. (G last 3) Significant increase in average number of 16-cell cysts per germaria when dissected 8 DAE. All germaria labeled with anti-Hts (red; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells), and DAPI (blue; DNA) Scale bar, 20 um. (H) Average number of 16-cell cysts per germarium in both *C587-Gal4 > yw* controls *C587-Gal4 > ftz-f1^{JF02738}* mutants over three time points, 5, 8, and 12 days after eclosion. Signifcant increase in average number of 16-cell cysts per germaria over time. Error bars, mean +/- SEM. * p < 0.00001; Student's two tailed *T*-test.

DISCUSSION

Although *ftz-f1* has been shown to be a key node in the regulation of ecdysone signaling in other tissue types, it has been unclear how *ftz-f1* impacts GSC fate and function. Our study adds to a growing body of literature demonstrating that the ecdysone induced factors critical for morphogenesis and developmental timing in other tissue types are also integral for germline stem cell establishment and follicle development. While it remains unclear how *ftz-f1* contributes mechanistically to the establishment of GSCs and progression of differentiating germ cells out of the germarium, our studies highlight its critical role for both actions. Given the level of structural and function conservation between *Drosophila* and mammalian hormonal signaling pathways and relation to tissue resident stem cells, we propose that similar connections may exist among mammalian subtypes. Further studies will be necessary to fully elucidate the intricate molecular networks needed to achieve such important biological regulation.

In this study, we demonstrated that *ftz-f1* is expressed in both the adult and developing larval ovary, particularly in the germ cells of the larval ovary. *ftz-f1::GFP* is not expressed in the germ cells that are further away from the terminal filament stacks (Figure 3 D and D'). This *ftz-f1::GFP* expression pattern and our *nos.NGT-Gal > ftz-f1^{JF02738}* findings, showing that *ftz-f1* is required for the establishment of the proper number of GSCs, gives us reason to believe that in the larval ovary, *ftz-f1* acts as a competence factor, permitting PGCs closest to the terminal filament stacks to establish as undifferentiated GSCs by providing the ability to respond to BMP signaling (Figure 11 A and B). This finding is consistent with *ftz-f1*'s role in metamorphosis, as a competence factor for stage-specific responses to ecdysone (Broadus et al., 1999).

Similar to our results, reduction of ecdysone signaling components (*ftz-f1*) in PGCs did not induce PGC differentiation in Gilboa et al., 2011. To which repression of *ftz-f1* in the somatic cells, using *tj-Gal4*, of the larval ovary resulted in precocious PGC differentiation (Gilboa et al., 2011). This suggested that ecdysone receptors in the somatic cells of the larval ovary are required non-autonomously to repress precocious PGC differentiation (Gilboa et al., 2011). Though reduction of *ftz-f1* in PGCs did not induce precocious PGC differentiation, it did result in a significant increase in PGC distance from the terminal filament stacks, decreased BMP signaling, and also a significant decrease in the average number of GSCs in adults (Figure 4 and 6). Our data, along with Gilboa et al., 2011 suggest *ftz-f1* has functional roles both autonomously and non-autonomously in the developing larval ovary.

Our investigation of *ftz-f1*'s indirect role in somatic cells, was complicated by several, unanticipated interactions. When screening through available adult somatic cell drivers, all of which are previously published in germline stem cell related studies, we quickly realized that all of our chosen drivers (*bab-1*, *C587*, and *traffic jam*) are expressed in cell types outside of the ovary. Because *ftz-f1* is expressed broadly throughout the developing and adult fly (Broadus et al., 1999), we encountered significant pupal lethality upon reduction of *ftz-f1* in somatic cells. It is possibile that reduction of *ftz-f1* in cell types outside the ovary, but still under the control of our drivers, may contribute to phenotypic abnormalities in the germarium. Incidentally, *C587* and *traffic jam* expression overlap in early follicle cells, but we see slightly different phenotypes (Figure 9 and 10), which could suggest independent roles for *ftz-f1* in escort and follicle cells. *tj-Gal4> ftz-f1^{JF02738}* and *tj-Gal4> ftz-f1^{KK108995}* have greater disorganization of germ cells, and resemble knockdown of EcR in developing escort/follicle cells (Konig et al., 2011). *C587-Gal4>*

ftz-f1^{JF02738} and *C587-Gal4> ftz-f1^{KK108995}* seemed to be more bulbous with a more significant increase in 16- cell cysts.

Future experiments will investigate why *ftz-f1* mutant escort cells fail to "hand off" cysts for encapsulation. This enlarged germarium phenotype and failure to "hand off" could be due to a variety of mechanistic problems including but not limited to: incorrect escort cell signaling, problems with differentiation/specification of the oocyte, and not enough/misplacement/incorrect shape or size of the escort or follicle cells (Figure 11 C and D). Investigating these possibilities will include pMAPK, orb, and traffic jam antibody staining. pMAPK staining will tell us if the escort cells are signaling correctly. Orb staining is an oocyte marker and will allow us to see if there is a problem with differentiation and specification. Traffic jam antibody will tell us if there are enough escort cell and follicle cells and if they are shaped correctly. These three different types of staining with allow us to see how *ftz-f1* is creating this problem and the mechanism that's behind it.
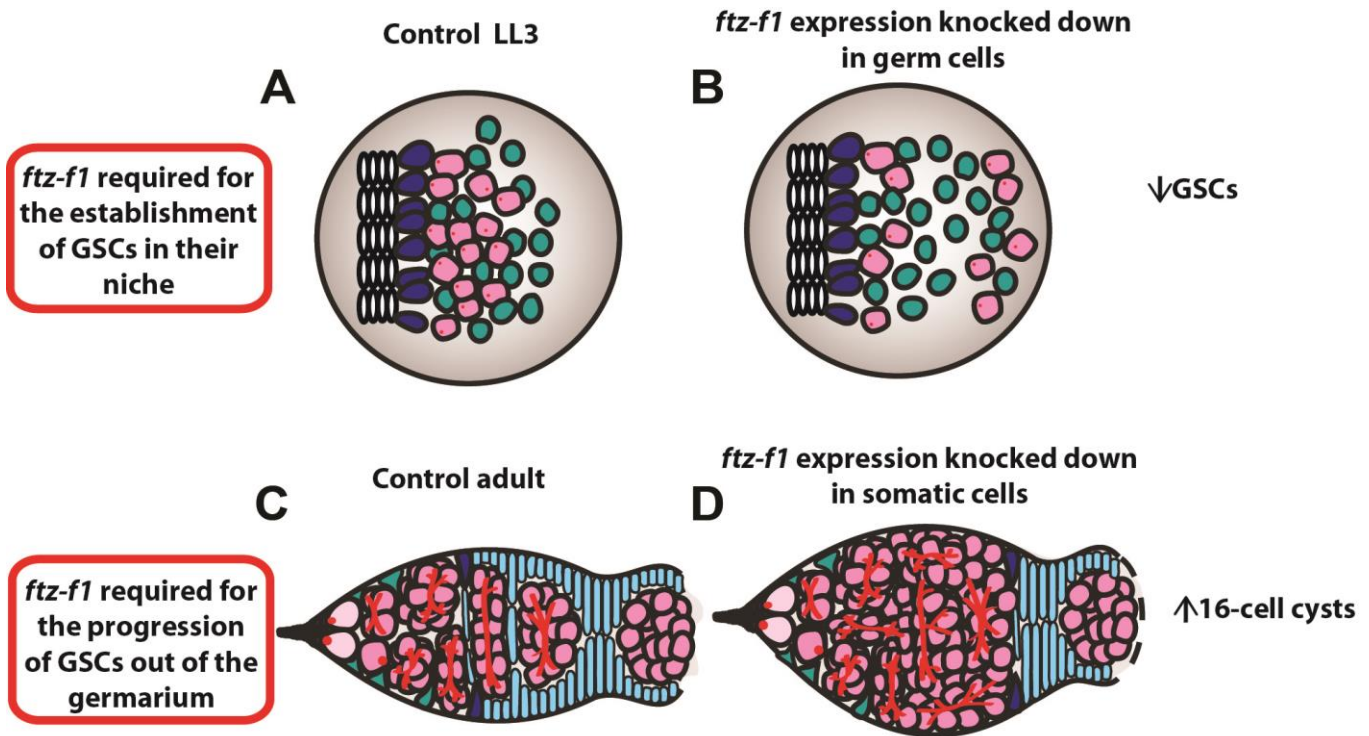
Figure 11. Conceptual diagram of *ftz-f1* role in the establishment of GSCs and their progression out of the germaria. (A) Schematic of a control LL3 ovary. (B) *ftz-f1* expression knocked down in germ cells. *Nos.NGT-Gal4 > ftz-f1^{JF02738}* mutant LL3 ovary with a decrease in germ cells directly adjacent to the TF stacks. Also containing a subset of germ cells further away from the TF stacks. (A and B) TF stacks in white, cap cells in navy blue, germ cells in pink with their fusomes in red, and intermingled cells (precursor escort cells) in green. (C) Schematic of a control adult germarium. (D) *ftz-f1* expression knocked down in either escort or follicle cells. *C587-Gal4 > ftz-f1^{JF0273}* or *tj-Gal4 > ftz-f1^{JF0273}* mutant adult germaria with enlarged germaria phenotype containing a significant increase in average number of 16-cell cysts. (C and D) GSCs in light pink, differentiated cysts in dark pink with fusomes in red, escort cells in green, FSC in navy blue, and follicle cells in light blue.

MATERIALS AND METHODS

Drosophila strains and culture

All *Drosophila* stocks were maintained on standard cornmeal/molasses/yeast medium (Genesee Scientific, Nutri-Fly-MF) at 22ºC-25ºC and supplemented with yeast paste. RNAi lines y1 v1; P{TRiP.JF02738}attP2 (*ftz-f1^{JF02738}*) (Matunis et al., 2014) (Harvard Medical School) and P{KK108995}VIE-260B (*ftz-f1^{KK108995}*) (KK) were used to knock down *ftz-f1* expression. The following Gal4 fly stocks were used: c587-Gal4 (Kai and Spradling, 2003), UAS-Dcr2.D(1),w;nosGal4(II)NGT (Brand and Perrimon, 1993), Hh-Gal4 (II)/ TM6B (Xie et al., 2015), w;bab1-Gal4(48e)/TM3 (Gonzalez-Reyes et al., 2006), and Tj-Gal4 (II) (Matunis et al., 2014). All driver expression was confirmed using reporters: w*; (UASp-tubGFP/TM3sb), y[1] w[*]; P{w[+mC]=UAS-mCD8::GFP.L}LL5, P{UAS-mCD8::GFP.L}2, y[1] w[1118]; P{w[+mC]=GMR-UAS-lacZ}3, or y[1] w[1118]; P{w[+mC]=GMR-UAS-lacZ}2. Flies were collected one to two days after eclosion and maintained on standard medium supplemented with wet yeast paste for 5 to 12 days prior to ovary dissection.

Temperature-sensitive regulation of Gal4 activity

To promote Gal4 expression specifically during points in development, the *Gal4/Gal80^{ts}* system (McGuire et al., 2003) was used. Flies bearing a *tubPGal80^{ts}* construct were raised at 18 ºC and then shifted to 29ºC as varying time points (egg laying, LL3, and eclosion) to induce expression of the UAS constructs as described (Helms et al., 2017). The following *Gal80^{ts}* fly stocks were used: TjGal4/cyo; tubGal80[ts]/MKRS (*tj-Gal80^{ts}*) (E. Matunis), w;tubPGal80[ts];

bab1-gal4/tm6b (*bab1-Gal80^{ts}*), and hu,C587-Gal4;tubPGal80[ts] (*C587-Gal80^{ts}*) (D. Drummond-Barbosa).

Adult Immunofluorescence and microscopy

Adult ovaries were prepared for immunofluorescence microscopy as described (Ables and Drummond-Barbosa, 2010). In summary, ovaries were dissected and ovarioles were teased apart in Grace's medium without additives (Lonza) and fixed in 5.3% formaldehyde in Graces medium for 13 minutes at room temperature. Samples were washed with 0.1% Triton X-100, and blocked for three 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 diluted in blocking solution over night at 4ºC: mouse anti-Hts (1B1) (DSHB 7H9; 1:10), mouse anti-LamC (DSHB LC28.26; 1:100), chicken anti-GFP (Abcam #13970; 1:2000), mouse anti-hts-RC (DSHB; 1:1), rabbit anti-pMAPK (Cell Signaling #4370; 1:50), guinea pig anti-TJ-G6 (D. Godt; 1:5000), and mouse anti-Orb (DSHB 4H8; 1:500) . To detect anti-pMAPK staining, adult ovaries were dissected in Grace's medium and Pierce Phosphatase Inhibitor (1 tablet: 10 ml Grace's Media).

AlexaFluor 488-, 568-, or 633-conjugated goat species-specific secondary antibodies (1:200; Molecular Probes/Invitrogen) were incubated in the dark at room temperature for two hours in blocking solution and counterstained with 0.5µg/mL DAPI (1:1000 in PBS; Sigma) to visualize nuclei. Samples were mounted in 90% glycerol containing 20.0 µg/mL N-propyl gallate (Sigma). Images were taken using a Zeiss LSM700 confocal microscope using ZEN Black 2012 software.

Larval Immunofluorescence and microscopy

Larval ovaries were prepared for immunofluorescence microscopy as described (Matsuoka, 2013). Briefly, LL3 larvae were sorted by sex and fat bodies (with the ovary attached) were dissected out of larvae in Ringer's medium (1.3 M NaCl, 50 mM KCl, 20 mM CaCl$_2$, and 100 mM Hepes pH 6.9). Sample was fixed (1 volume Buffer B (100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ pH 6.8, 450 mM KCl, 150 mM NaCl, and 20 mM MgCl$_2$), 1 volume 37% formaldehyde, and 4 volumes ddH$_2$O) for 10 minutes at room temperature. Sample was washed extensively with 0.1% Triton X-100 and then blocked for 30 minutes in blocking solution (5% normal goat serum/0.1% Triton-X in PBS). The following primary antibodies were diluted in blocking solution over night at 4ºC: rabbit anti-vasa (DSHB; 1:1000), mouse anti-Hts (1B1) (DSHB 7H9; 1:100), mouse anti-Engrailed (DHSB; 1:20), and rabbit anti-pMAD (abcam anti-Smad3; 1:200). When primary antibody was removed, samples were then washed extensively with 0.1% Triton X-100 and blocked for 30 minutes at room temperature again. Samples were then incubated in AlexaFluor 488-, 568-, or 633- conjugated goat species-specific secondary antibodies (1:200; Molecular Probes/Invitrogen) for 3.5 hours at room temperature in the dark. Samples were washed extensively then counterstained with 0.5µg/mL DAPI (1:1000 in PBS; Sigma). Gentle agitation was used throughout the fixing, blocking, and staining protocol. Fat body was then dissected away from ovary and mounted onto a coverslip. Images were taken using a Zeiss LSM700 confocal microscope using ZEN Black 2012 software.

Larval and adult germ cell analyses

Images were analyzed using Zeiss ZEN Blue 2012 software. In LL3 ovaries, PGCs were identified based on their Vasa (germ cell marker) expression, germ cell distance was measure from nucleus of most posterior terminal filament cell to the nucleus of the Vasa containing germ cell, and precocious differentiation was determined by branching of the fusome. Round fusome is indicative of undifferentiation when an elongation or branched fusome is indicative of differentiation. In the adult ovary, GSCs were identified based on their juxtaposition to cap cells and fusome morphology and position, as described previously (Deng and Lin, 1997) along with co-immunofluorescence of Vasa. All graphs were created using Prism 5. Statistical analysis of germline stem cell, cysts number, distance from TFs, rounded vs. branched fusomes, and so on was performed using Prism 5 (GraphPad Software, Inc.).

Competing interests

The authors declare no competing or financial interests.

Author contributions

H. E. B. and E. T. A. designed and performed the experiments and co-wrote the manuscript.

References

Ables, E., Drummond-Barbosa, D. (2010). The Steroid Hormone Ecdysone Functions with Intrinsic Chromatin Remodeling Factors to Control Female Germline Stem Cells in Drosophila, Cell Stem Cell, Volume 7, Issue 5, 5 November 2010, Pages 581-592, ISSN 1934-5909

Ables, E., Laws E., and Drummond-Barbosa, D. (2012). In vivo control of adult stem cells by a dynamic physiological environment: diet-dependent systemic factors in Drosophila and beyond. Wiley Interdisciplinary Reviews Developmental Biology, 1: 657-674

Alberts B, Johnson A, Lewis J, et al. (2002) Molecular Biology of the Cell. 4th edition. New York: Garland Science. Primordial Germ Cells and Sex Determination in Mammals. http://www.ncbi.nlm.nih.gov/books/NBK26940/

Baehrecke EH. (1996). Ecdysone signaling cascade and regulation of Drosophila Metamorphosis. Arch Insect Biochem Physiology 33(3-4): 231-44

Bai, J., Uehara, Y., and Montell, D.J. (2000). Regulation of invasive cell behavior by Taiman, a Drosophila protein related to AIB1, a steroid receptor co-activator amplified in breast cancer. Cell 103, 1047–1058.

Beat, M. & Klug, J. (2000). Steroid hormone receptors: an update. Human. Reproduction. Update, 6, 225–236.

Belles, X., Piulachs, M. (2015). Ecdysone signalling and ovarian development in insects: from stem cells to ovarian follicle formation, Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, Volume 1849, Issue 2, Pages 181-186, ISSN 1874-9399, http://dx.doi.org/10.1016/j.bbagrm.2014.05.025.

Bernstein, H. S. and D. Srivastava (2012). "Stem cell therapy for cardiac disease." Pediatr Res 71(4-2): 491-499.

Bhat, K. M. and Schedl, P. (1997), Establishment of stem cell identity in the Drosophila germline. Dev. Dyn., 210: 371–382.

Bolívar, J., Pearson, J., López-Onieva, L. and González-Reyes, A. (2006), Genetic dissection of a stem cell niche: The case of the Drosophila ovary. Dev. Dyn., 235: 2969–2979. doi:10.1002/dvdy.20967

Broadus, J. et al.(1999). The Drosophila βFTZ-F1 Orphan Nuclear Receptor Provides Competence for Stage-Specific Responses to the Steroid Hormone Ecdysone. Molecular Cell , Volume 3, Issue 2 , 143 - 149

Buszczak, M., et al. (1999). "Ecdysone response genes govern egg chamber development during mid-oogenesis in Drosophila." Development 126(20): 4581-4589.

Carney, G.E., and Bender, M. (2000). The Drosophila ecdysone receptor (EcR) gene is required maternally for normal oogenesis. Genetics 154, 1203–1211.

Chen D, McKearin DM. A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. Development 2003;130:1159–1170.

Chen D, McKearin D. Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. Curr. Biol 2003;13:1786–1791.

Drummond-Barbosa, D. (2008). Stem cells, their niches and the systemic environment: An aging network. Genetics 180, 1787–1797.

Evans, R. (1988). "The steroid and thyroid hormone receptor superfamily." Science 240(4854): 889-895.

Gancz, D., Lengil, T., Gilboa, L. (2011). Coordinated Regulation of Niche and Stem Cell Precursors by Hormonal Signaling. PLoS Biology, 9(11).

Gancz, D., Gilboa, L. Hormonal Control of Stem Cell Systems (2013). Annu. Rev. Cell Dev. Biol., 29, 137-162.

Gilboa, L., Lehmann, R. (2004). Repression of primordial germ cell differentiation parallels germ line stem cell maintenance. Current Biol., 14, 981-986.

Gilboa, L. Organizing Stem Cell Units in the Drosophila Ovary (2015) Curr. Opin. Genet. 32, 31-36.

Godt, D. and F. A. Laski (1995). "Mechanisms of cell rearrangement and cell recruitment in Drosophila ovary morphogenesis and the requirement of bric a brac." Development 121(1): 173-187.

Jang, A.C., Chang, Y.C., Bai, J., Montell, D. (2009). Border-cell migration requires integration of spatial and temporal signals by the BTB protein Abrupt. Nat. Cell Biol. 11(5): 569—579

Joshi, P. A., et al. (2012). "Active allies: hormones, stem cells and the niche in adult mammopoiesis." Trends in Endocrinology & Metabolism 23(6): 299-309.

Kai, T., et al. (2005). "The expression profile of purified Drosophila germline stem cells." Developmental Biology 283(2): 486-502.

Kim, J., et al. (2016). "17β-Estradiol and Agonism of G-protein-Coupled Estrogen Receptor

Enhance Hippocampal Memory via Different Cell-Signaling Mechanisms." The Journal of Neuroscience 36(11): 3309-3321.

King, R. C. (1970). Ovarian development in Drosophila melanogaster, Academic Press.

King, R. C., Aggarwal, S. K. and Aggarwal, U. (1968), The development of the female Drosophila reproductive system. J. Morphol., 124: 143–165. doi:10.1002/jmor.1051240203

König, A., Yatsenko, A. S., Weiss, M., & Shcherbata, H. R. (2011). Ecdysteroids affect Drosophila ovarian stem cell niche formation and early germline differentiation. The EMBO Journal, 30(8), 1549–1562. http://doi.org/10.1038/emboj.2011.73

Lavorgna, G., et al. (1991). "FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu." Science 252(5007): 848-851.

Lavorgna, G., Karim, F. D., Thummel, C. S., & Wu, C. (1993). Potential role for a FTZ-F1 steroid receptor superfamily member in the control of Drosophila metamorphosis. Proceedings of the National Academy of Sciences of the United States of America, 90(7), 3004–3008.

Li, M. A., et al. (2003). "The large Maf factor Traffic Jam controls gonad morphogenesis in Drosophila." Nat Cell Biol 5(11): 994-1000.

Li, Y., Ma, Q., Cherry, C. M., & Matunis, E. L. (2014). Steroid Signaling Promotes Stem Cell Maintenance in the *Drosophila* Testis. *Developmental Biology*, *394*(1), 129-141. http://doi.org/10.1016/j.ydbio.2014.07.016

Lavorgna, G., Karim, F. D., Thummel, C. S., & Wu, C. (1993). Potential role for a FTZ-F1 steroid receptor superfamily member in the control of Drosophila metamorphosis. Proceedings of the National Academy of Sciences of the United States of America, 90(7), 3004–3008.

Lawson et al., (2009). J.C. Lawson, G.L. Blatch, A.L. Edkins. Cancer stem cells in breast cancer and metastasis. Breast Cancer Research and Treatment, 118 (2009), pp. 241–254

Maimon, I., Gilboa, L. (2011). Dissection and Staining of Drosophila Larval Ovaries. J. Vis. Exp. (51), e2537, doi:10.3791/2537.

Maity B., Sheff D., Fisher R., (2013). Chapter 5 - Immunostaining: Detection of Signaling Protein Location in Tissues, Cells and Subcellular Compartments, In: P. Michael Conn, Editor(s), Methods in Cell Biology, Academic Press, Volume 113, Pages 81-105, ISSN 0091-679X, ISBN 9780124072398, http://dx.doi.org/10.1016/B978-0-12-4072398.00005-7.

Mahowald, A.P., Kambysellis, M.P. (1980). Oogenesis. Ashburner, Wright, 1978-1980 d: 141—224.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schu¨tz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995). The nuclear receptor superfamily: The second decade. Cell 83, 835–839.

Morris LX, Spradling AC. (2012). Steroid Signaling within Drosophila Ovarian Epithelial Cells Sex-Specifically Modulates Early Germ Cell Development and Meiotic Entry. PLoS ONE 7(10): e46109. doi:10.1371/journal.pone.0046109

Morrison, S. J., & Spradling, A. C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell, 132(4), 598–611. http://doi.org/10.1016/j.cell.2008.01.038

Murata, T., Kageyama, Y., Hirose, S., & Ueda, H. (1996). Regulation of the EDG84A gene by FTZ-F1 during metamorphosis in Drosophila melanogaster. Molecular and Cellular Biology, 16(11), 6509–6515.

Nakada, D., et al. (2014). "Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy." Nature 505(7484): 555-558.

Rippon, H. J. and Bishop, A. E. (2004), Embryonic stem cells. Cell Proliferation, 37: 23–34. doi: 10.1111/j.1365-2184.2004.00298.x

Rørth, P. (1998). Gal4 in the Drosophila female germline, Mechanisms of Development, Volume 78, Issues 1–2, Pages 113-118, ISSN 0925-4773, http://dx.doi.org/10.1016/S0925-4773(98)00157-9.

Schwedes, C. C., Carney G. E., (2012). Ecdysone signaling in adult Drosophila melanogaster, Journal of Insect Physiology, Volume 58, Issue 3, March 2012, Pages 293-302, ISSN 0022-1910, (http://www.sciencedirect.com/science/article/pii/S0022191012000145)

Song, X., et al. (2002). "Germline Stem Cells Anchored by Adherens Junctions in the Drosophila Ovary Niches." Science 296(5574): 1855-1857.

Song X, Wong MD, Kawase E, et al. (2004) Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. Development;131:1353–1364

Spradling, A. (1993). Developmental Genetics of Oogenesis. In: Bate, M., editor. The Development of Drosophila melanogaster. Plainview, N.Y.: Cold Spring Harbor Laboratory Press. p. 1-70

Sultan, A.-R. S., Oish, Y. and Ueda, H. (2014), Function of the nuclear receptor FTZ-F1 during

the pupal stage in Drosophila melanogaster. Development, Growth & Differentiation, 56: 245–253. doi: 10.1111/dgd.12125

Thummel, C.S. (2001). Molecular mechanisms of developmental timing in C. elegans and Drosophila. Dev. Cell, 1:453-465.

Ueda, H., S. Sonoda, J. L. Brown, M. P. Scott, and C. Wu. (1990). "A Sequence-specific DNA-binding Protein That Activates Fushi Tarazu Segmentation Gene Expression." Genes & Development. U.S. National Library of Medicine

Ul Hassan, A., Hassan, G., & Rasool, Z. (2009). Role of Stem Cells in Treatment of Neurological Disorder. International Journal of Health Sciences, 3(2), 227–233.

Wagers A., Weissman I. (2004). Plasticity of Adult Stem Cells, Cell, Volume 116, Issue 5, Pages 639-648, ISSN 0092-8674, http://dx.doi.org/10.1016/S0092-8674(04)002089 (http://www.sciencedirect.com/science/article/pii/S0092867404002089)

Xie T., et al. (2002). "Germline Stem Cells Anchored by Adherens Junctions in the Drosophila Ovary Niches." Science 296(5574): 1855-1857.

Yamada, M., et al. (2000). "Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in Drosophila melanogaster." Development 127(23): 5083-5092.

Zhu, C.-H. and T. Xie (2003). "Clonal expansion of ovarian germline stem cells during niche formation in Drosophila." Development 130(12): 2579-2588.

APPENDIX A
Immunostaining Protocol: pMAPK, 1B1, LamC, DAPI

- ☐ Precoat 1.5mL tubes with 3% BSA for each sample
- ☐ Dissolve 2 phosphatase inhibitor tablets to 10mL Grace's, keep protected from light
- ☐ Make 5.3% formaldehyde fix:

  __ X 300µL 16% FA = _____ µl   for __ samples

  __ X 600µL Grace's media/phosphatase = _____ µl

- ☐ Put fix and Grace's on ice
- ☐ Dissect ovaries in **Grace's/phosphatase**, tease apart ovarioles, move to BSA-precoated tube
- ☐ Fix in 1000 µL 5.3% FA for 13 min on nutator
- ☐ Remove fix to FA waste
- ☐ Rinse with 0.1% PBS-triton
- ☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
- ☐ Wash 2 more times >10 min , last wash left O/N at 4°C

- ☐ Remove previous wash
- ☐ Block 3hrs, RT on nutator
- ☐ Incubate in primary 4° O/N on nutator

$$\frac{1}{50} = \frac{\mu l\ rab\ \alpha-pMAPK}{X\ 500\ \mu l\ Block}$$

- ☐ Remove primary ab
- ☐ Wash 4x 20 min in 0.1% PBS-triton
- ☐ Incubate in primary 4° O/N on nutator

$$\frac{1}{10} = \frac{\mu l\ mse\ \alpha-1B1}{X\ 500\ \mu l\ Block}$$

$$\frac{1}{100} = \frac{\mu l\ mse\ \alpha-LamC}{X\ 500\ \mu l\ Block}$$

- ☐ Remove primary ab
- ☐ Wash 4x 20 min in 0.1% PBS-triton
- ☐ Incubate in secondary RT 1-2 hrs on nutator in **DARK**
- ☐ Wash 2x 15 min in 0.1% PBS-triton in **DARK**
- ☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in **DARK** on nutator
- ☐ Wash 1x 5 min in 0.1% PBS-triton in **DARK**
- ☐ Aspirate off last wash, add 3 drops of mounting media, store at 4°C

$$\frac{1}{200} = \frac{\mu l\ goat\ \alpha-rab\ (488)}{X\ 500\ \mu l\ Block}$$

$$\frac{1}{200} = \frac{\mu l\ goat\ \alpha-mouse\ (568)}{X\ 500\ \mu l\ Block}$$

$$\frac{1}{1000} = \frac{\mu l\ DAPI\ (405)}{X\ 500\ \mu l\ Block}$$

APPENDIX B

Immunostaining Protocol: hts-RC, 1B1, LamC, DAPI

☐ Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr

☐ Make 5.3% formaldehyde fix:

　　__ X 300µL 16% FA =　　　　µl　　　　　　　　　　　for __samples

　　__ X 600µL Grace's media =　　　µl

☐ Put fix and Grace's on ice

☐ Dissect ovaries in Grace's, tease apart ovarioles, move to BSA-precoated tube, place on ice.

☐ Fix in 1000 µL 5.3% FA for 13 min on nutator

☐ Remove fix to FA waste

☐ Rinse with 0.1% PBS-triton

☐ Wash for 10 minutes on nutator, 0.1% PBS-triton

☐ Wash 2 more times >10 min , last wash left O/N at 4°C

☐ Block 3hrs, RT on nutator

☐ Incubate in primary 4° O/N on nutator

$$\frac{1}{1} = \frac{\text{µl mse } \alpha-\text{htsRC}}{X\ 500\ \text{µl Block}}$$

☐ Remove and save primary ab for later use

☐ Wash 4x 20 min in 0.1% PBS-triton

☐ Incubate in secondary RT 1-2 hrs on nutator in **DARK**

$$\frac{1}{200} = \frac{\text{µl } \alpha-\text{mse (488)}}{X\ 500\ \text{µl Block}}$$

☐ Wash 4x 20 min in 0.1% PBS-triton

☐ Incubate in primary 4° for 2 nights on nutator in **DARK**

$$\frac{1}{10} = \frac{\text{µl mouse } \alpha-\text{1B1}}{X\ 500\ \text{µl Block}} \qquad \frac{1}{100} = \frac{\text{µl mouse } \alpha-\text{LamC}}{X\ 500\ \text{µl Block}}$$

☐ Remove and save primary ab for later use

☐ Wash 4x 10 min in 0.1% PBS-triton

☐ Incubate in secondary RT 1-2 hrs on nutator in **DARK** (or 4° O/N)

$$\frac{1}{200} = \frac{\text{µl goat } \alpha-\text{mouse (568)}}{X\ 500\ \text{µl Block}}$$

☐ Wash 2x 15 min in 0.1% PBS-triton in **DARK**

☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in **DARK** on nutator

☐ Wash 2x 10 min in 0.1% PBS-triton in **DARK**

☐ Aspirate off last wash, add 3 drops of mounting media, store at 4°C

$$\frac{1}{1000} = \frac{\text{µl DAPI (405)}}{X\ 500\ \text{µl Block}}$$

APPENDIX C

Immunostaining Protocol: 1B1, LamC, DAPI

☐ Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
☐ Make 5.3% formaldehyde fix:

    __ X 300µL 16% FA = _____ µl   for __ samples
    __ X 600µL Grace's media = _____ µl

☐ Put fix and Grace's on ice
☐ Dissect ovaries in Grace's, tease apart ovarioles, move to BSA-precoated tube, place on ice.
☐ Fix in 1000 µL 5.3% FA for 13 min on nutator
☐ Remove fix to FA waste
☐ Rinse with 0.1% PBS-triton
☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
☐ Wash 2 more times >10 min , last wash left O/N at 4°C

☐ Block 3hrs, RT on nutator $\dfrac{1}{10} = \dfrac{\mu l\ mouse\ \alpha-1B1}{X\ 500\ \mu l\ Block}$ $\dfrac{1}{100} = \dfrac{\mu l\ mouse\ \alpha-LamC}{X\ 500\ \mu l\ Block}$

☐ Incubate in primary 4° for 2 nights on nutator
    _____uL α–1B1 + _____uL α-LamC = _____uL *ab* + _____uL block

☐ Remove and save primary ab for later use
☐ Wash 4x 20 min in 0.1% PBS-triton $\dfrac{1}{200} = \dfrac{\mu l\ goat\ \alpha-mouse\ (568)}{X\ 500\ \mu l\ Block}$

☐ Remove and save primary ab for later use
☐ Wash 4x 10 min in 0.1% PBS-triton
☐ Incubate in secondary RT 1-2 hrs on nutator in **DARK** (or 4° O/N)

                                                   $\dfrac{1}{1000} = \dfrac{\mu l\ DAPI\ (405)}{X\ 500\ \mu l\ Block}$
☐ Wash 2x 15 min in 0.1% PBS-triton in **DARK**
☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in **DARK** on nutator
☐ Wash 1x 5 min in 0.1% PBS-triton in **DARK**
☐ Aspirate off last wash, add 3 drops of mounting media, store at 4°C

APPENDIX D

Immunostaining Protocol: orb, 1B1, LamC, DAPI

☐ Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
☐ Make 5.3% formaldehyde fix:

    __ X 300µL 16% FA =   µl   for __samples

    __ X 600µL Grace's media =   µl

☐ Put fix and Grace's on ice
☐ Dissect ovaries in Grace's, tease apart ovarioles, move to BSA-precoated tube, place on ice.
☐ Fix in 1000 µL 5.3% FA for 13 min on nutator
☐ Remove fix to FA waste
☐ Rinse with 0.1% PBS-triton
☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
☐ Wash 2 more times >10 min , last wash left O/N at 4°C

☐ Wash 30 min at RT on nutator in 0.5% Trition-PBS
☐ Block 3 hours, RT on nutator in blocking solution $\frac{1}{500} = \frac{\mu l \; mouse \; \alpha-Orb}{X \; 500 \; \mu l \; Block}$
☐ Incubate in primary antibody at 4°C O/N on nutator

☐ Remove and save primary ab for later use
☐ Wash 4x 20 min in 0.1% PBS-triton

                                              $\frac{1}{200} = \frac{\mu l \; goat \; \alpha-mouse \; (488)}{X \; 500 \; \mu l \; Block}$

☐ Incubate in secondary for 1-2 hours at RT on nutator in **DARK**
☐ Wash 8x 20 min in 0.1% PBS-triton in **DARK**
☐ Block 30 minutes, RT on nutator in blocking solution in **DARK**
☐ Incubate in primary antibody for 2 nights at 4°C on nutator in **DARK**

    _____uL α−1B1 + _____uL α-LamC = _____uL *ab* + _____uL block

         $\frac{1}{10} = \frac{\mu l \; mouse \; \alpha-1B1}{X \; 500 \; \mu l \; Block}$ $\frac{1}{100} = \frac{\mu l \; mouse \; \alpha-LamC}{X \; 500 \; \mu l \; Block}$

☐ Remove and save primary ab for later use $\frac{1}{200} = \frac{\mu l \; goat \; \alpha-mouse \; (568)}{X \; 500 \; \mu l \; Block}$
☐ Wash 4x 20 min in 0.1% PBS-triton in **DARK**
☐ Incubate in secondary for 1-2 hours at RT on nutator in **DARK**
☐ Wash 4x 20 min in 0.1% PBS-triton in **DARK**
☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in **DARK** on nutator $\frac{1}{1000} = \frac{\mu l \; DAPI \; (405)}{X \; 500 \; \mu l \; Block}$
☐ Wash 2x 5 min in 0.1% PBS-triton in **DARK**
☐ Aspirate off last wash, add 3 drops of mounting media, store at 4°

APPENDIX E

Immunostaining Protocol (Larval Ovaries): 1B1, Engrailed, Vasa, DAPI

☐ Dissect larvae in 10x EBR in a glass dissection dish
- Place larvae in one well for sorting by sex. Male testes are identified as big clear ovals embedded in posterior 1/3rd, females as much smaller, clear, round spheres
- Move selected larva to another well
- Remove anterior 1/4th of the larva (head region), then "roll" out the fat body from the remaining larvae
- Remove the dark gut that is attached to the fat body
- Move fat body to 10x EBR in another dissecting dish on ice

☐ Fix sample in 1000 µL for 10 minutes at room temperature in glass dissection dish
$\underline{}$ X 166.7 µL 37% FA = µl for $\underline{}$ samples
$\underline{}$ X 166.7 µL Buffer B = µl
$\underline{}$ X 666.7 µL ddH2O = µl

☐ Remove fix solution to FA waste
☐ Rinse with 0.1% PBS-triton
☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
☐ Wash 2 more times >10 min
☐ Block for 30 minutes at room temperature.

☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{100} = \frac{\text{µl mse } \alpha-1B1}{X\ 500\ \text{µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.

$$\frac{1}{200} = \frac{\text{µl } \alpha-\text{mouse (568)}}{X\ 500\ \text{µl Block}}$$

☐ Wash 4x 20 min in 0.1% PBS-triton
☐ Stain with primary antibody overnight at 4°C in a humidified chamber in the dark.

$$\frac{1}{20} = \frac{\text{µl mse } \alpha-\text{engrailed}}{X\ 500\ \text{µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.

$$\frac{1}{200} = \frac{\text{µl } \alpha-\text{mouse (488)}}{X\ 500\ \text{µl Block}}$$

- ☐ Wash 4x 20 min in 0.1% PBS-triton
- ☐ Block 30 minutes
- ☐ Stain with primary antibody overnight at 4°C in a humidified chamber in the dark.

$$\frac{1}{1000} = \frac{\mu l \; rbt \; \alpha-VASA}{X \; 500 \; \mu l \; Block}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 3x 15 min in 0.1% PBS-triton
- ☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.

$$\frac{1}{200} = \frac{\mu l \; \alpha-rbt \; (633)}{X \; 500 \; \mu l \; Block}$$

- ☐ Wash 3x15 minutes at room temperature.
- ☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in the dark.

$$\frac{1}{1000} = \frac{\mu l \; DAPI \; (405)}{X \; 500 \; \mu l \; Block}$$

- ☐ Wash 2x10 minutes at room temperature.
- ☐ Remove wash and cover samples in mounting media
- ☐ Place ovary containing fat bodies on slide and carefully separate ovaries from fat bodies as best as you can. Also remove miscellaneous debris from slide too.

APPENDIX F

Immunostaining Protocol (Larval Ovaries): 1B1, Vasa, DAPI

☐ Dissect larvae in 10x EBR in a glass dissection dish
- Place larvae in one well for sorting by sex. Male testes are identified as big clear ovals embedded in posterior 1/3rd, females as much smaller, clear, round spheres
- Move selected larva to another well
- Remove anterior 1/4th of the larva (head region), then "roll" out the fat body from the remaining larvae
- Remove the dark gut that is attached to the fat body
- Move fat body to 10x EBR in another dissecting dish on ice

☐ Fix sample in 1000 µL for 10 minutes at room temperature in glass dissection dish

$\_\_$ X 166.7 µL 37% FA $=$ $\_\_\_$ µl   for $\_\_$samples
$\_\_$ X 166.7 µL Buffer B $=$ $\_\_\_$ µl
$\_\_$ X 666.7 µL ddH2O $=$ $\_\_\_$ µl

☐ Remove fix solution to FA waste
☐ Rinse with 0.1% PBS-triton
☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
☐ Wash 2 more times >10 min
☐ Block for 30 minutes at room temperature.

☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{100} = \frac{\text{µL mse } \alpha-1B1}{X \text{ 500 µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with primary antibody overnight at 4°C in a humidified chamber

$$\frac{1}{1000} = \frac{\text{µL rbt } \alpha-VASA}{X \text{ 500 µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Block 30 minutes
☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.

$\_\_\_\_$ µL α -mse (568) + $\_\_\_$ µL α -rbt (488) = $\_\_\_$ µL *ab* + $\_\_\_$ µL block

$$\frac{1}{200} = \frac{\text{µL } \alpha-mouse (568)}{X \text{ 500 µl Block}} \qquad \frac{1}{200} = \frac{\text{µL } \alpha-rabbit (488)}{X \text{ 500 µl Block}}$$

☐ Wash 3x15 minutes at room temperature.
☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in the dark**.**

$$\frac{1}{1000} = \frac{\text{µL DAPI (405)}}{X \text{ 500 µl Block}}$$

☐ Wash 2x10 minutes at room temperature.
☐ Remove wash and cover samples in mounting media
☐ Place ovary containing fat bodies on slide and carefully separate ovaries from fat bodies as best as you can. Also remove miscellaneous debris from slide too.

APPENDIX G
Immunostaining Protocol (Larval Ovaries): Engrailed, Vasa, DAPI

- ☐ Dissect larvae in 10x EBR in a glass dissection dish
    - Place larvae in one well for sorting by sex. Male testes are identified as big clear ovals embedded in posterior 1/3rd, females as much smaller, clear, round spheres
    - Move selected larva to another well
    - Remove anterior 1/4th of the larva (head region), then "roll" out the fat body from the remaining larvae
    - Remove the dark gut that is attached to the fat body
    - Move fat body to 10x EBR in another dissecting dish on ice

- ☐ Fix sample in 1000 µL for 10 minutes at room temperature in glass dissection dish
    - __ X 166.7 µL 37% FA = _____ µl   for __ samples
    - __ X 166.7 µL Buffer B = _____ µl
    - __ X 666.7 µL ddH2O = _____ µl

- ☐ Remove fix solution to FA waste
- ☐ Rinse with 0.1% PBS-triton
- ☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
- ☐ Wash 2 more times >10 min
- ☐ Block for 30 minutes at room temperature.

- ☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{20} = \frac{\mu L\ mse\ \alpha-engrailed}{X\ 500\ \mu l\ Block}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 3x 15 min in 0.1% PBS-triton
- ☐ Stain with primary antibody overnight at 4°C in a humidified chamber

$$\frac{1}{1000} = \frac{\mu L\ rbt\ \alpha-VASA}{X\ 500\ \mu l\ Block}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 3x 15 min in 0.1% PBS-triton
- ☐ Block 30 minutes
- ☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.
    - ____ µL α -mse (568) + ___ µL α -rbt (488) = ___ µL *ab* + ___ µL block

$$\frac{1}{200} = \frac{\mu L\ \alpha-mouse\ (568)}{X\ 500\ \mu l\ Block} \qquad \frac{1}{200} = \frac{\mu L\ \alpha-rabbit\ (488)}{X\ 500\ \mu l\ Block}$$

- ☐ Wash 3x15 minutes at room temperature.
- ☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in the dark**.**

$$\frac{1}{1000} = \frac{\mu L\ DAPI\ (405)}{X\ 500\ \mu l\ Block}$$

- Wash 2x10 minutes at room temperature.
- Remove wash and cover samples in mounting media
- Place ovary containing fat bodies on slide and carefully separate ovaries from fat bodies as best as you can. Also remove miscellaneous debris from slide too.

APPENDIX H
Immunostaining Protocol (Larval Ovaries): GFP, Engrailed, Vasa, DAPI

☐ Dissect larvae in 10x EBR in a glass dissection dish
- Place larvae in one well for sorting by sex. Male testes are identified as big clear ovals embedded in posterior $1/3^{rd}$, females as much smaller, clear, round spheres
- Move selected larva to another well
- Remove anterior $1/4^{th}$ of the larva (head region), then "roll" out the fat body from the remaining larvae
- Remove the dark gut that is attached to the fat body
- Move fat body to 10x EBR in another dissecting dish on ice

☐ Fix sample in 1000 µL for 10 minutes at room temperature in glass dissection dish
__ X 166.7 µL 37% FA = _____ µl   for __ samples
__ X 166.7 µL Buffer B = _____ µl
__ X 666.7 µL ddH2O = _____ µl

☐ Remove fix solution to FA waste
☐ Rinse with 0.1% PBS-triton
☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
☐ Wash 2 more times >10 min
☐ Block for 30 minutes at room temperature.
☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{2000} = \frac{\mu L\ ckn\ \alpha-GFP}{X\ 500\ \mu l\ Block}$$

☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{20} = \frac{\mu L\ mse\ \alpha-engrailed}{X\ 500\ \mu l\ Block}$$

☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with primary antibody overnight at 4°C in a humidified chamber

$$\frac{1}{1000} = \frac{\mu L\ rbt\ \alpha-VASA}{X\ 500\ \mu l\ Block}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Block 30 minutes
☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.

___ µL α -mse (568) + ___ µL α -rbt (633) + ___µL α -ckn (488) = ____ µL *ab* + ___ µL block

$$\frac{1}{200} = \frac{\mu L\ \alpha-mouse\ (568)}{X\ 500\ \mu l\ Block}$$ $$\frac{1}{200} = \frac{\mu L\ \alpha-rabbit\ (633)}{X\ 500\ \mu l\ Block}$$ $$\frac{1}{200} = \frac{\mu L\ \alpha-ckn\ (488)}{X\ 500\ \mu l\ Block}$$

☐ Wash 3x15 minutes at room temperature.
☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in the dark**.**

$$\frac{1}{1000} = \frac{\mu L\ DAPI\ (405)}{X\ 500\ \mu l\ Block}$$

☐ Wash 2x10 minutes at room temperature.
☐ Remove wash and cover samples in mounting media
☐ Place ovary containing fat bodies on slide and carefully separate ovaries from fat bodies as best as you can. Also remove miscellaneous debris from slide too.

APPENDIX I
Immunostaining Protocol: 1B1, Lamc, Traffic Jam, DAPI

- ☐ Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- ☐ Make 5.3% formaldehyde fix:
  - __ X 300µL 16% FA =   µl   for __samples
  - __ X 600µL Grace's media =   µl
- ☐ Put fix and Grace's on ice
- ☐ Dissect ovaries in Grace's, tease apart ovarioles, move to BSA-precoated tube, place on ice.
- ☐ Fix in 1000 µL 5.3% FA for 13 min on nutator
- ☐ Remove fix to FA waste
- ☐ Rinse with 0.1% PBS-triton
- ☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
- ☐ Wash 2 more times >10 min , last wash left O/N at 4°C

- ☐ Wash 30 min at RT on nutator in 0.5% Trition-PBS
- ☐ Block 3 hours, RT on nutator in blocking solution
- ☐ Incubate in primary antibody at 4°C O/N on nutator

$$\frac{1}{5000} = \frac{\mu l\ \text{guinea pig } \alpha-\text{Tj}}{X\ 500\ \mu l\ \text{Block}}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 4x 20 min in 0.1% PBS-triton

$$\frac{1}{200} = \frac{\mu l\ \text{goat } \alpha-\text{guinea pig (488)}}{X\ 500\ \mu l\ \text{Block}}$$

- ☐ Incubate in secondary for 1-2 hours at RT on nutator in **DARK**
- ☐ Wash 8x 20 min in 0.1% PBS-triton in **DARK**
- ☐ Block 30 minutes, RT on nutator in blocking solution in **DARK**
- ☐ Incubate in primary antibody for 2 nights at 4°C on nutator in **DARK**
  - _____uL α–1B1 + _____uL α-LamC = _____uL *ab* + _____uL block

$$\frac{1}{10} = \frac{\mu l\ \text{mouse } \alpha-\text{1B1}}{X\ 500\ \mu l\ \text{Block}} \qquad \frac{1}{100} = \frac{\mu l\ \text{mouse } \alpha-\text{LamC}}{X\ 500\ \mu l\ \text{Block}}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 4x 20 min in 0.1% PBS-triton in **DARK**

$$\frac{1}{200} = \frac{\mu l\ \text{goat } \alpha-\text{mouse (568)}}{X\ 500\ \mu l\ \text{Block}}$$

- ☐ Incubate in secondary for 1-2 hours at RT on nutator in **DARK**
- ☐ Wash 4x 20 min in 0.1% PBS-triton in **DARK**
- ☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in **DARK** on nutator

$$\frac{1}{1000} = \frac{\mu l\ \text{DAPI (405)}}{X\ 500\ \mu l\ \text{Block}}$$

- ☐ Wash 2x 5 min in 0.1% PBS-triton in **DARK**
- ☐ Aspirate off last wash, add 3 drops of mounting media, store at 4°C

APPENDIX J

Immunostaining Protocol (Larval Ovaries): Traffic Jam, Engrailed, Vasa, DAPI

☐ Dissect larvae in 10x EBR in a glass dissection dish
- Place larvae in one well for sorting by sex. Male testes are identified as big clear ovals embedded in posterior 1/3rd, females as much smaller, clear, round spheres
- Move selected larva to another well
- Remove anterior 1/4th of the larva (head region), then "roll" out the fat body from the remaining larvae
- Remove the dark gut that is attached to the fat body
- Move fat body to 10x EBR in another dissecting dish on ice

☐ Fix sample in 1000 µL for 10 minutes at room temperature in glass dissection dish

__ X 166.7 µL 37% FA =   µl   for __ samples
__ X 166.7 µL Buffer B =   µl
__ X 666.7 µL ddH2O =   µl

☐ Remove fix solution to FA waste
☐ Rinse with 0.1% PBS-triton
☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
☐ Wash 2 more times >10 min
☐ Block for 30 minutes at room temperature.

☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{5000} = \frac{\mu L \text{ guinea pig } \alpha-TJ}{X \text{ 500 µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{20} = \frac{\mu L \text{ mse } \alpha-\text{engrailed}}{X \text{ 500 µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Stain with primary antibody overnight at 4°C in a humidified chamber

$$\frac{1}{1000} = \frac{\mu L \text{ rbt } \alpha-\text{VASA}}{X \text{ 500 µl Block}}$$

☐ Remove and save primary ab for later use
☐ Wash 3x 15 min in 0.1% PBS-triton
☐ Block 30 minutes

☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.
____ µL α -mse (568) + ___ µL α -rbt (633) ___ µL α –guinea pig (488) = ___ µL *ab* + ___ µL block

$$\frac{1}{200} = \frac{\text{µL } \alpha-\text{mouse (568)}}{X \text{ 500 µl Block}}$$ $$\frac{1}{200} = \frac{\text{µL } \alpha-\text{rabbit (633)}}{X \text{ 500 µl Block}}$$ $$\frac{1}{200} = \frac{\text{µl goat } \alpha-\text{guinea pig (488)}}{X \text{ 500 µl Block}}$$

☐ Wash 3x15 minutes at room temperature.
☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in the dark.

$$\frac{1}{1000} = \frac{\text{µL DAPI (405)}}{X \text{ 500 µl Block}}$$

☐ Wash 2x10 minutes at room temperature.
☐ Remove wash and cover samples in mounting media
☐ Place ovary containing fat bodies on slide and carefully separate ovaries from fat bodies as best as you can. Also remove miscellaneous debris from slide too.

Immunostaining Protocol (Larval Ovaries): pMAD, Engrailed, Vasa, DAPI

- ☐ Dissect larvae in 10x EBR in a glass dissection dish
  - Place larvae in one well for sorting by sex. Male testes are identified as big clear ovals embedded in posterior 1/3rd, females as much smaller, clear, round spheres
  - Move selected larva to another well
  - Remove anterior 1/4th of the larva (head region), then "roll" out the fat body from the remaining larvae
  - Remove the dark gut that is attached to the fat body
  - Move fat body to 10x EBR in another dissecting dish on ice

- ☐ Fix sample in 1000 µL for 10 minutes at room temperature in glass dissection dish
  - __ X 166.7 µL 37% FA =   µl   for __samples
  - __ X 166.7 µL Buffer B =   µl
  - __ X 666.7 µL ddH2O =   µl

- ☐ Remove fix solution to FA waste
- ☐ Rinse with 0.1% PBS-triton
- ☐ Wash for 10 minutes on nutator, 0.1% PBS-triton
- ☐ Wash 2 more times >10 min
- ☐ Incubate in primary 4° O/N on nutator

$$\frac{1}{200} = \frac{\text{µl rabbit } \alpha-\text{pMAD}}{X\ 500\ \text{µl Block}}$$

- ☐ Remove primary
- ☐ Wash 3x 15 min in 0.1% PBS-triton
- ☐ Stain with primary antibody overnight at 4°C in a humidified chamber.

$$\frac{1}{20} = \frac{\text{µL mse } \alpha-\text{engrailed}}{X\ 500\ \text{µl Block}}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 3x 15 min in 0.1% PBS-triton
- ☐ Stain with primary antibody overnight at 4°C in a humidified chamber

$$\frac{1}{1000} = \frac{\text{µL ckn } \alpha-\text{VASA}}{X\ 500\ \text{µl Block}}$$

- ☐ Remove and save primary ab for later use
- ☐ Wash 3x 15 min in 0.1% PBS-triton
- ☐ Block 30 minutes
- ☐ Stain with secondary antibody for 3.5 hours at room temperature in the dark.

____ μL α -mse (633) + ___ μL α -rbt (488) + ___ μL α -ckn (568) = ___ μL *ab* + ___ μLblock

$$\frac{1}{200} = \frac{\text{μL α−mouse (633)}}{X \text{ 500 μl Block}}$$ $$\frac{1}{200} = \frac{\text{μL α−ckn (568)}}{X \text{ 500 μl Block}}$$ $$\frac{1}{200} = \frac{\text{μL α−rbt(488)}}{X \text{ 500 μl Block}}$$

- ☐ Wash 3x15 minutes at room temperature.
- ☐ Incubate in DAPI (1:1000 in 0.1% PBS-triton), 15 min in the dark**.**

$$\frac{1}{1000} = \frac{\text{μL DAPI (405)}}{X \text{ 500 μl Block}}$$

- ☐ Wash 2x10 minutes at room temperature.
- ☐ Remove wash and cover samples in mounting media
- ☐ Place ovary containing fat bodies on slide and carefully separate ovaries from fat bodies as best as you can. Also remove miscellaneous debris from slide too.

APPENDIX L
Table of Fly Stocks Used

#	Genotype	Date	Received From	ID#	Box	Cell	Notes
2	P{w[+mW.hs]=GawB}185Y, w[1118]	2/2/2016	Blm	3731	1	A1	Expressed in region 1 and 3 cysts
3	w[1118] P{w[+mW.hs]=GawB}cb14	2/2/2016	Blm	6711	1	A2	not expressed well in germarium
4	P{w[+mW.hs]=GawB}112A, w[*]	2/2/2016	Blm	7021	1	A3	not expressed well in germarium
5	P{w[+mW.hs]=GawB}c306, w[1118]	2/2/2016	Blm	3743	1	A4	not expressed well in germarium
6	y[1] w[*]; P{w[+mW.hs]=GawB}109-30/CyO	2/2/2016	Blm	7023	1	A5	not expressed well/select against Cyo
7	w[1118]; P{w[+mW.hs]=GawB}c368	2/2/2016	Blm	3744	1	A6	Expressed in niche sometimes
8	yw	11/28/2012	DDB stocks		1	B1	
9	c587-Gal4 (x)	3/20/2008	D. Barbosa, A. Spradling		1	B2	
10	UAS-Dcr2.D(1),w;nosGal4(II)NGT	5/10/2013	Blm	25751	1	B3	
11	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02738}attP2	42192	Blm	27659	1	B4	ftz-f1 rnai (Matunis Dev Biol. 2014 Oct 1;394(1):129-41)
12	FRT40A/cyo (#4)	7/28/2009	L.LaFever	4	1	C1	
13	w*; (UASp-tubGFP/TM3sb)	4/27/2011	Blm	7373	1	C2	
14	w[-]; FRT82B hepII[e2]/TM3	6/25/2015	W. Brook		1	C3	PTB-3
15	w[1118]; P{w[+mC]=UAS-lacZ.NZ}J312	9/9/2014	Blm	3956	1	C4	
16	Hh-Gal4 (II)/ TM6B	4/1/2011	T.Xie		1	D1	
17	w;bab1-Gal4(48e)/TM3	4/11/2008	A. Gonzalez-Reyes		1	D2	
18	Tj-Gal4 (II)	11/13/2012	E Matunis (EM Stock #30)		1	D3	same as Kyoto Stock
19	w;tubPGal80[ts]; bab1-gal4/tm6b, hu	08/03/16	Drummond-Barbosa		1	D4	
20	ptc-Gal4/CyO.Act-GFP;tubPGal80[ts]/tm6B,hu	08/03/16	Drummond-Barbosa		1	D5	
21	C587-Gal4;tubPGal80[ts]	08/03/16	Drummond-Barbosa		1	D6	
22	w;tubPGal80[ts]/sm6,Bro,cnbw;hh-gal4/tm6B	08/03/16	Drummond-Barbosa		1	D7	
23	y[1] w[*]; P{w[+mC]=UAS-mCD8:GFP.L}LL5, P{UAS-mCD8-GFP.L}2	8/17/2016		5137	1	E2	Donor: Liqun Luo, Stanford University Comments: MARCM set, GFP labels the cell surface (mouse CD8 is a transmembrane protein), highly concentrated in neuronal processes, L.L. Viable P insertion, but stock is segregating CyO, K.C. 1/00
24	Df(1)JA27/FM7c, P{w[+mC]=GAL4-Kr.C}DC1, P{w[+mC]=UAS-GFP.S65T...	8/17/2016	Bloomington	5193	1	E1	green balancer; no sn bristle phenotype and homozygous females are fertile. K.M. 6/1/01
25	y[1] w[1118]; P{w[+mC]=GMR-UAS-lacZ}3	8/17/2016	Bloomington	8409	1	E4	Expresses lacZ in the eye and under UAS control
26	y[1] w[1118]; P{w[+mC]=GMR-UAS-lacZ}2	8/17/2016	Bloomington	8531	1	E3	Expresses lacZ in the eye and under UAS control. May be segregating SM6a, K.C
27	y[1] w[67c23]; ftz-f1[ex7] P{w[+mW.hs]=FRT(w[hs])}2A P{w[+mC]=UAS-b	8/17/2016	Bloomington	64301			Expresses the beta isoform of ftz-f1 under UAS control, J.-M.D
28	ftz-f1 kk rnai		42551 VDRC	104463	9	C7	
29	TfGal4/SM6B; tubGal80[ts]/MKRS		11/13/2012 E Matunis		2	D3	
30	TfGal4/cyo; tubGal80[ts]/MKRS		11/13/2012 E Matunis		2	D4	

APPENDIX M

Table of Antibodies Used

Antibodies Used	Dilution	Species	Stains	Protein	Received From:
1B1 or Hts	1:10	Mouse	nuclear membrane in follicle and cap cells	Hu-Li Tai Shao	DSHB
LamC	1:100	Mouse	lamen of epithelial cells	Laminin	DSHB
DAPI	1:1000	N/A	nuclei	DAPI	DSHB
Vasa	1:1000	Rabbit	germ cells	Vasa	Paul Lasko
	1:1000	Rat	germ cells	Vasa	DSHB
pMAPK	1:50	Rabbit	Escort cell extensions and follicle cell membranes	phosphorylated MAP Kinase	Cell Signaling #4370
Orb	1:500	Mouse	earliest marker of oocyte formation	Orb	DSHB
pMAD	1:200	Rabbit	undifferentiated GSCs	phosphorylated MAD	abcam
Engrailed	1:20	Mouse	Terminal filament cell marker	engrailed	DHSB
Traffic Jam	1:5000	Guinea pig	Intermingled cells of larval ovary and somatic cells of adult ovary	traffic jam	D. Godt
hts-RC	1:1	Mouse	Ring canals	Hu-Li Tai Shao-RC	DHSB
GFP	1:2000	Chicken	desired cell population		abcam