Investigating the role of CRL5, an ubiquitin ligase, in ovarian follicle development.

by

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June 2016

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Major Department: Biology

During oogenesis, follicle cells surround maturing oocytes, which produce factors necessary for proper oocyte growth and development. Correct encapsulation of the oocyte by follicle cells is therefore essential for reproduction. While many signaling pathways have been linked to encapsulation, mechanisms of early follicle development, particularly in mammals, are not fully understood. Recent evidence demonstrates that Cullin-RING E3 ubiquitin ligases (CRLs) are necessary for oogenesis in both mammals and *Drosophila*. CRLs include a Cullin family scaffolding protein and a RING-domain protein that facilitates recruitment of ubiquitin ligases. CRLs are known to control many cellular processes; however, it is unclear how CRLs control early follicle development. In *Drosophila*, loss of *Cul5*, results in follicle death and improper encapsulation. We therefore tested whether the Cul5-containing CRL (CRL5) is required for early follicle development by analyzing loss-of-function mutants of the ligase complex. Loss of *Cul5* or the RING protein *Roc2* resulted in fused follicles, ruptured follicular epithelium, and improper encapsulation. The encapsulation phenotypes are not due to an over proliferation of germ cell; in fact, *Cul5* mutants display a disturbance of the cell cycle which causes a decrease in

germline stem cell proliferation. Genetic mosaics of *Cul5* or *Roc2* show that CRL5 is primarily required in developing follicle cells for cyst encapsulation. CRL5 mutant follicle cells display mislocalization of the polarity protein Bazooka and decreased Stat expression. Data also suggest that *Cul5* may mediate signaling between the follicle cells and the underlying cyst. Results suggest that CRL5 controls early follicle development by regulating early follicle cell polarity and specification. Our studies highlight the role of CRLs in early follicle development, and may lead towards a better understanding of the cellular and mechanical processes that control follicle formation.

Investigating the role of CRL5, an ubiquitin ligase, in ovarian follicle development.

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 Biology

by

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LIST OF SYMBOLS OR ABBREVIATIONS

GSC	Germline stem cell
CB	Cystoblast
NC	Nurse cell
OO	Oocyte
TF	Terminal filament
FSC	Follicle stem cell
CRL5	Cullin-RING ligase 5
Cul5	Cullin 5
Roc2	RING domain-containing 2
gus	Gustavus
CDK	Cyclin dependent kinase
Baz	Bazooka
Flp-FRT	Flippase recognition target
JAK-STAT	Janus kinase-signal transducers and activators of transcription
VACM-1	Vasopressin-activated Ca(2+)–mobilizing receptor
Bam	Bag of marbles
Dpp	Decapentaplegic
BMP	Bone morphogenic protein

CHAPTER 1

Thesis Proposal: Finding the function of *Cul5*, an ubiquitin ligase, in *Drosophila* oogenesis.

Introduction

When extracting a certain cell from its tissue microenvironment, the cell is unable to communicate or receive signals from the surrounding cells and other tissues causing it to possibly behave differently (Ye, Wu et al. 2014). This communication is vital in accurately understanding how a certain cell works. Unfortunately, most techniques used to study cells individually have to take that cell out of its microenvironment which usually disrupts normal cell function and communication. Advancements made in technology and developments made focusing on cell behavior and communication, it is easier to study cellular function without having to displace the cell.

Through the use of the model organism, *Drosophila melanogaster*, I will be better able to control some of the variables that can drastically change a cell's behavior *in vivo*, as well as examine two separate stem cell populations in their native environment.

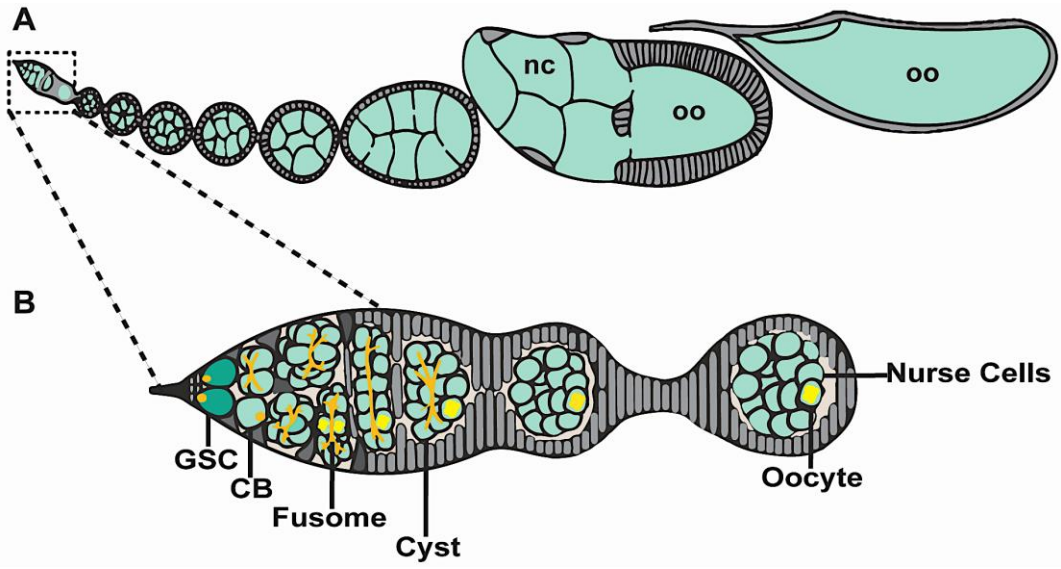

Figure 1. Germline populations in the *Drosophila* ovary. A) Cross-section of one ovariole with maturing follicles, containing nurse cells (nc) and one oocyte (oo), from anterior (boxed) to posterior. B) The anterior tip of the ovariole is called the germarium. All germaria contain germline stem cells (GSCs), its initial daughter cell, a cystoblast (CB), and subsequent 16-cell cysts. Each maturing cyst contains one specified oocyte and 15 supporting nurse cells.

In particular, *Drosophila* oogenesis is an excellent system to use for developmental biology research. The ovary is composed of 14-16 ovarioles, which are strings of ever-increasing mature eggs, providing a unique glimpse of all developmental stages (Figure 1) (Hudson and Cooley 2014). The anterior tip of each ovariole is called the germarium, which houses the two stem cell populations that give rise to all cells of the ovary. The germline stem cells (GSC) give rise to all of the germ cells and the follicle stem cells (FSC) give rise to all of the somatic cells. *Drosophila* has been widely studied, and offers a vast amount of genetic tools and information that can be easily accessed (Spradling 1993, Ables, Laws et al. 2012). Further, genetic background and physical environment can be easily controlled in *Drosophila*, in stark contrast to human studies (Roote and Prokop 2013).

Background

Cullins are an evolutionarily conserved family of proteins that regulate several cellular processes (Bosu and Kipreos 2008). They are an integral part in the Cullin-RING ubiquitin Ligase (CRL) complex. An enzymatic core, which contains a Cullin family member, is bound at each terminal end by specific substrates through the help of adaptor proteins or domains (Deshaies and Petroski 2005). Together, the CRL family of E3 ubiquitin ligases is responsible for regulating cellular processes by marking proteins for cellular degradation with ubiquitin. All of the CRLs share the same similar architecture; each possess the same catalytic core but differ in their substrate specificity. This diverse structure allows them to affect many different molecular pathways. CRLs specifically have been implicated in disturbances of DNA replication and the cell cycle (Sarikas,

Hartmann et al. 2011). Loss of *Cul1* in *C. elegans* and *Drosophila* disrupts the cell cycle (Kipreos, Lander et al. 1996, Koepp, Schaefer et al. 2001), *Cul3* deletions interrupt the meiosis/mitosis transition in *C. elegans* and the cell cycle in mice (Singer, Gurian-West et al. 1999, Pintard, Kurz et al. 2003), and loss of *Cul4* inhibits DNA replication in *C. elegans* and cell cycle disruption in *Drosophila* (Zhong, Feng et al. 2003, Higa, Yang et al. 2005).

In humans, the Cullin family consists of *Cul1, 2, 3, 4a* and *4b, 5, 6, 7, 8* and *9*, with *Cul1* through *Cul5* conserved in *Drosophila* (Chen, Sui et al. 2015*).* Some studies consider *Cul2* and *Cul5* to be homologous with one another (Deshaies and Petroski 2005). However, their C and N-terminals interact with different proteins. The structure for Cullin RING Ligase 5 or CRL5 involves Cullin5, a scaffolding protein that specifically interacts with the RING domain protein, Regulator of Cullins 2 or Roc2, on the C-terminal and the N-terminal binds to a known substrate adaptor, Gustavus or gus (Figure 2) (Reynolds, Simms et al. 2008, Kugler, Lem et al. 2010).

Cul5 is found in many species and is very closely related to its human homolog, Vasopressin-activated Ca(2+)–mobilizing receptor, VACM-1 (Sarikas, Hartmann et al. 2011). Most importantly VACM-1 has been identified in several types of cancer cells and molecular pathways surrounding metastasis formation (Chen, Sui et al. 2015). Several studies involving T47D breast cancer cells have reported that an over-expression of Cul5 leads to decreased cellular proliferation (Lewis, Willis et al. 2011). Other reports have implicated *Cul5* in cell cycle misregulations leading to lung adenocarcinoma (Singhal, Amin et al. 2003).

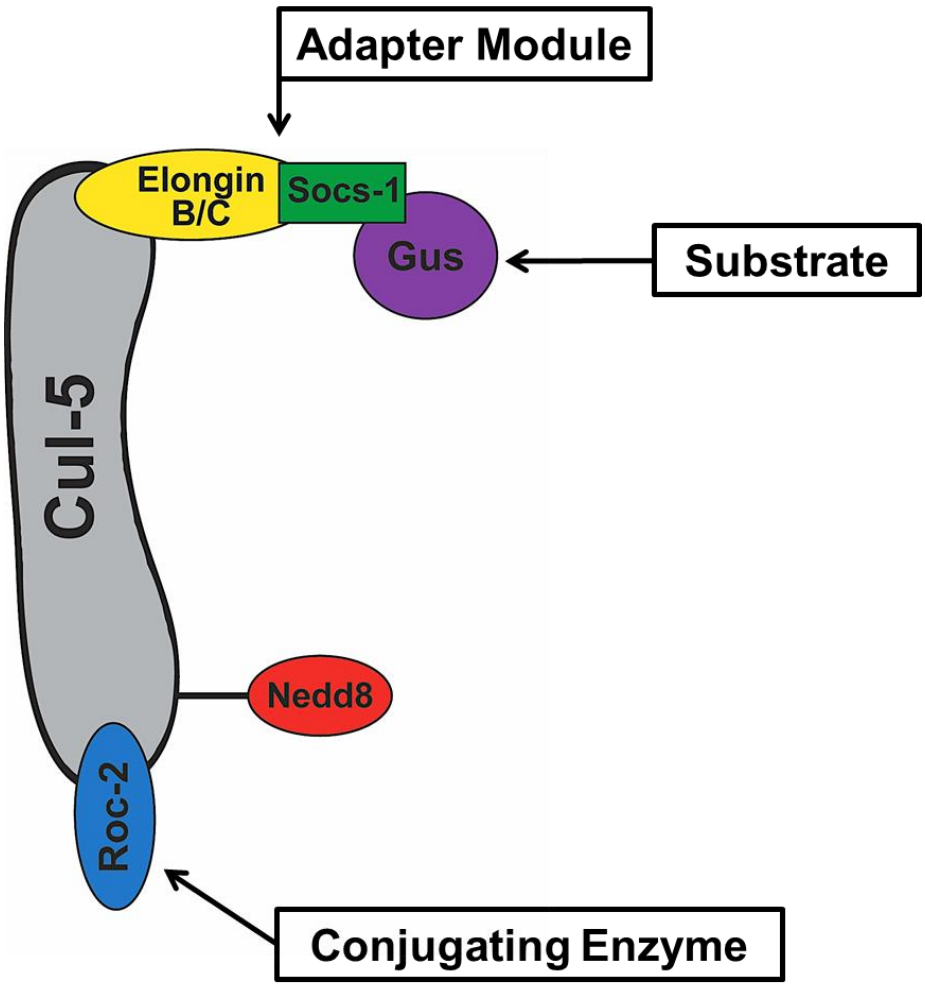

Figure 2. Cullin-RING Ligase 5 (CRL5) architecture. Cullin 5 (Cul5), a scaffolding protein, has an adaptor module composed of Elongin B and C, as well as a SOCS-box, SOCS-1. This adaptor module is able to bind various substrates, including Gustavus (gus). The CRL5 complex also consists of a conjugating enzyme, Regulator of Cullins 2 (Roc2), and a neddylation site, Nedd8.

Research done specifically in *Drosophila* has only yielded two studies that analyzed *Cul5* (Ayyub, Sen et al. 2005, Kugler, Lem et al. 2010). From these experiments, *Cul5* has been implicated in the process of oogenesis and proper cell cycle progression. When compared to its family members, *Cul5* is understudied and its role in cell cycle and cell signaling is incompletely described. Since *Cul5* is well conserved in both humans and *Drosophila melanogaster*, I will investigate the role of *Cul5* in *Drosophila* oogenesis. With its involvement in ubiquitination, a mutation in *Cul5* may have a wide impact on stem cell maintenance and proliferation.

Objectives

I hypothesize that *Cul5* is required for ovarian germline stem cell function. To test my hypothesis, I am assessing the ovarian phenotype of genetic mutants harboring loss-of-function mutations in *Cul5*, *in vivo*.

Specific Aim I: Is *Cul5* required for germline stem cell (GSC) maintenance?

Stem cells are fundamental for the creation and development of every organ in the human body. Most stem cell populations differentiate and are lost after the human body reaches maturity (Hsu and Fuchs 2012). However, there are still several populations left in the adult body, specifically in areas that go through high rates of replication, like the lining of the intestine, the skin, and mammary glands. A stem cell has two rudimentary functions: generate new daughter cells and remain in an undifferentiated state (Nakada, Levi et al. 2011). Surrounding stem cells are supporting cells, which comprise the niche.

Interactions between these cell populations are imperative for normal stem cell function. Knowing exactly how these populations communicate will provide further insight into normal cell function and development, which is essential for possible future medicinal applications.

Methods

To test the involvement of Cul5 in germline stem cell maintenance, I will examine the phenotype of two different *Cul5* mutant alleles, as well as mutants for the other integral parts of CRL5, *Roc2* and *gus*. The fly stocks were obtained from Bloomington Stock Center and each possess different P-element insertions. P-elements are transposons that are inserted in various areas of the gene of interest, resulting in interruption of gene expression (Roote and Prokop 2013). Genetic mating in *Drosophila* require selection of female virgins so as not to include any unknown genetic background, mature females can hold onto sperm for up to two weeks. The males are chosen with a specific deficient background for each mutant. The deficient fly stock is selected by their location on the gene locus, specifically chosen to have a deletion in the area of your gene of interest, in order to limit the effects of potential background mutations. Female virgins harboring a loss for $Cul5^{EY21463}$, the null mutant allele, will be bred with their corresponding deficient male, Df(3R)BSC806. The hypomorph mutant allele, $Cul5^{EY00051}$, will also be crossed to the same deficient male.

When the progeny hatch or eclose, they will be fed on yeast paste for multiple time points; 2 days, one week, two weeks, and three weeks. They will then be dissected and the ovaries stained with somatic and germ cell immunofluorescence markers, which

allows for clear imaging of germline stem cells (Figure 3) (Appendix A). 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 the control and sibling mutant germaria and computing the average per germaria.

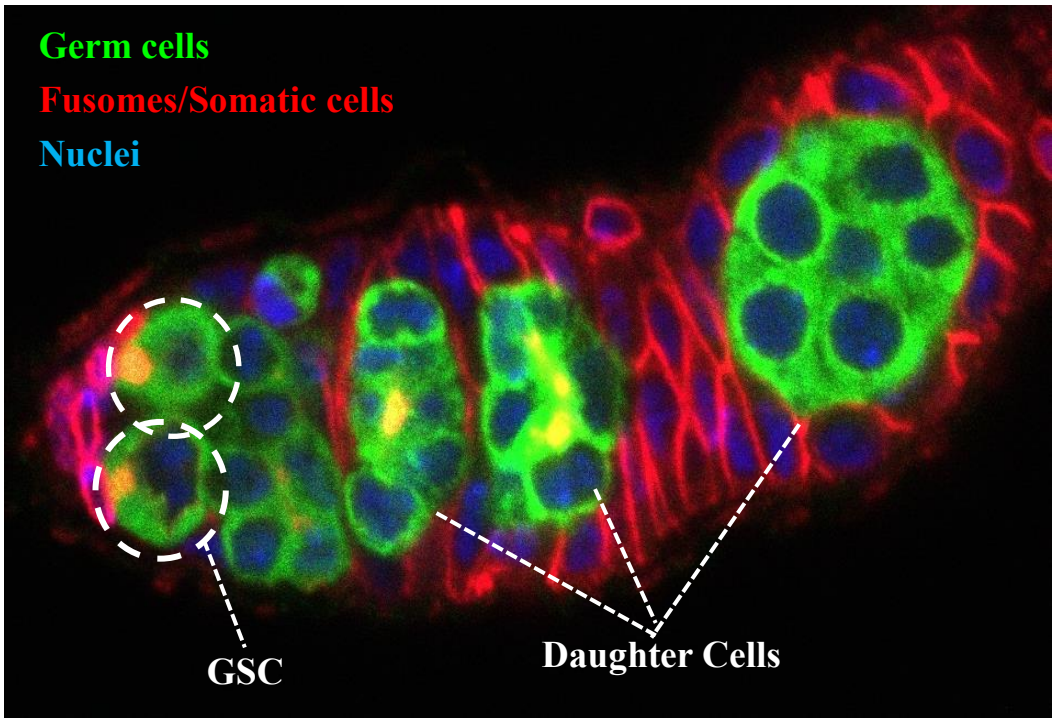

Figure 3. Immunostaining of *Drosophila* ovariole. The germ cells (green) are visible due to staining with a Vasa antibody. The fusomes and somatic cells (red) are marked with a 1B1/LamC antibody, and all nuclei (blue) are marked via DAPI. Staining with these antibodies allows for a clear picture of each specific cell type *in vivo*.

Anticipated Results

If *Cul5* is required for GSC maintenance, then in the mutant, a decrease in the total number of GSC per germaria will be observed. Future experiments will then be directed toward the signaling pathway that is being affected. Dpp and Bam signaling, which have been implicated in germline stem cell maintenance (Xie and Spradling 1998, Wang, Li et al. 2008), will be tested via immunofluorescence.

Specific Aim II: Is *Cul5* required for germline stem cell (GSC) proliferation?

Although maintenance and proliferation are clearly interconnected, there are several signaling pathways that can have an effect on one or the other (Ables and Drummond-Barbosa 2013). In order to get a complete picture of the effect of Cul5 on germline stem cells, testing both fundamental processes is crucial.

Methods

To assess whether *Cul5* is required for GSC proliferation, the same genetic crosses will be performed using both the null and hypomorph *Cul5* alleles. After eclosion, the progeny will be fed on wet yeast for one week, dissected, and stained using antibodies that mark specific stages of the cell cycle. EdU is a thymidine analog that denotes cells currently going through S phase (Appendix K). Quantification will be carried out in the same manner as mentioned previously with the addition of counting which GSCs are EdU positive or negative. Incorporating the EdU stain will give a

complete picture of GSC divisions during oogenesis, allowing for a better understanding of Cul5's possible effect on GSC proliferation.

Anticipated Results

If Cul5 is also required for GSC proliferation, there will be a decrease in the number of positive EdU GSC. Future experiments will then analyze specific parts of the cell cycle. A key part in the regulation of the cell cycle is the oscillations of different cyclins (Zielke, Korzelius et al. 2014). Cyclins are responsible for regulating the cell cycle by initiating and suppressing each phase (Wolgemuth, Manterola et al. 2013), and cullins have been identified as key modulators of these cyclins via ubiquitination (Deshaies and Petroski 2005). If *Cul5* mutants exhibit a decrease in proliferation, it suggests that Cul5 may be involved in ubiquitination of one of the cell cycle cyclins.

Specific Aim III: Complete characterization of *Cul5* mutants in oogenesis.

A previous study examining the role of *Cul5* in *Drosophila* oogenesis found several different phenotypes (Kugler, Lem et al. 2010). Although the interpretation and quantification of phenotypes were not fully described, I have observed some of the same phenotypes. Most of the ovairoles exhibit cell death, follicle fusions, and loss of pre-vitellogenic stages (Figure 4), along with improper encapsulation previously thought to be the result of over-proliferation (Kugler, Lem et al. 2010). Most of these phenotypes can not solely be attributed to the germline stem cells; defects in the follicle stem cells or their daughters may also be responsible for the observed mutant phenotypes. I hypothesize that *Cul5* has an affect, either similar or different, on the germline and somatic cells.

Methods

The same genetic crosses previously described will be used to fully characterize *Cul5* phenotypes, as well as utilize other mutant alleles to get a complete assesment of the CRL5 complex. All images were stained with germline and somatic cell markers and imaged using the same settings used in the maintenance and proliferation experiments (See Appendices). Quantification for each different phenotype will be counted and calculated as a percent of the total ovarioles imaged.

Germline stem cells and their daughters are influenced by a variety of intrinsic and extrinsic signals, some coming from the niche and some from the surrounding supporting cells (Ables, Laws et al. 2012). Previous chararcterization of *Cul5* mutants in *Drosophila* observe defects consistant with alterations of somatic cell behavior in the germaria (Kugler, Lem et al. 2010). By generating a clonal genetic mosaic, I will be able to ascertain which population of cells, germ or somatic, that can be attributed to the observed phenotypes. *Cul5*, *Cul2*, and *Roc2* clones will be created by the *FLP-FRT*, Flippase Recognition Target, system which utilizes a heat shock protein to induce recombination (Huang, Sahai-Hernandez et al. 2014).

Pursuit of these aims will elucidate how the loss of *Cul5* impacts stem cell functionality and developmental processes. The information obtained from these experiments may be utilized in the cancer stem cell theory and in the creation of new regenerative therapies.

Summary

From previous literature, Cullins have been implicated in germline stem cell (GSC) proliferation and maintenance, the motive for my research on *Cul5*. Preliminary data from my work has shown *Cul5* is needed for proper GSC proliferation but not maintenance, two aspects of GSC function that were originally thought to be controlled similarly. Precursory examination of ovary morphology in *Cul5* mutants has highlighted various phenotypes ranging from improper encapsulation to follicle death.

The preliminary data that was collected for my thesis proposal has led to the creation of two main stories involving Cul5 function. The first story orginates from specific aim one and two of my proposal, *Cul5*'s effect on GSC maintenance and proliferation. The data collected for these two specific aims is detailed and analyzed in Chapter 2. The second story stems from the third specific aim of my proposal which sought to fully characterize the *Cul5* mutant phenotype. Experiments done to further understand the phenotypes can be found in Chapter 3.

CHAPTER 2

Cul5, an E3 ubiquitin ligase, is necessary for germline stem cell proliferation in *Drosophila* oogenesis.

Introduction

During development, most cells cease cell division upon terminal differentiation. Maintenance and repair after injury of many adult tissues is therefore accomplished by the activity of tissue resident stem cells. Tissue resident stem cells are found in areas of high cell turnover, such as the epidermis and in the crypts of the intestinal epithelium (Barker, Bartfeld et al. 2010). These populations constantly proliferate in order to replace the terminally differentiated skin or intestinal cells that are shed every day. Regulation of stem cell activity must therefore be tightly regulated to maintain tissue homeostasis.

All stem cells have two main functions, self-renewal and proliferation (Xie 2013). The adult stem cells must constantly remain in an undifferentiated and proliferative state, from which all cell types can arise. In order to maintain the stem cell fate, the cell must communicate to not only the surrounding cells but also through long-distance signals from other tissues. Intrinsic cues originate from the neighboring support cells located in their niche (Li and Xie 2005). Extrinsic cues are long distance signals, such as steroid hormones, that originate from a variety of tissues and locations (Ables, Laws et al. 2012). Although much research has been dedicated to understanding the complex signaling patterns that regulate stem cell function, the molecular mechanisms that coordinate stem cell self-renewal and proliferation remain unclear. Understanding how stem cells function *in vivo* could lead to vital improvements in cancer treatments, regenerative therapies, and preventative medicine.

Drosophila melanogaster provides an elegant system for the study of stem cell function *in vivo*. They are easy to genetically manipulate, have a short lifespan, and are the focus of over a hundred years of dedicated research (Spradling 1993). *Drosophila*

oogenesis provides one of the best models in which to understand and study tissue-resident stem cell function. Each female fly contains two ovaries that have 14-16 ovarioles in each (Figure 1A) (King 1970). At the very anterior tip of each ovariole, are the germline stem cells (GSCs), that are always attached to the adjacent cap cells, which along with terminal filament cells comprise the GSC niche (Figure 1B). Another feature that characterizes the maturing germ cells is the fusome, which is an extension of the endoplasmic reticulum and is thought to help establish polarity in germ cells (de Cuevas and Spradling 1998). Unique to *Drosophila* oogenesis is the linear progression of maturing germline cysts (King 1970). This morphology allows not only for the study of the GSC but also its daughter cells as they mature, essentially creating a single snapshot of development.

Many proteins are known to propagate the signaling cascades that regulate stem cell function. One group of proteins, Cullin RING ligases (CRLs), are known to promote stem cells progression through the cell cycle and subsequent creation of daughter cells. CRLs have been implicated in the degradation of Cyclins, which are fundamental for the progression of the cell cycle (Follette and O'Farrell 1997). The family contains CRL-1, 2, 3, 4a and 4b, 5, 6, 7, 8, and 9, with CRL 1-5 conserved in *Drosophila* (Sarikas, Hartmann et al. 2011). Although much is known about this family of proteins and the processes they control, one family member, CRL5, has yet to be fully characterized. In this study, we fully characterize and quantify the GSC phenotype in loss-of-function alleles for CRL5. We also show that CRL5 is only needed for proliferation, not maintenance of the GSC, where the change in the cell cycle is likely due to altered communication between the GSC and the somatic niche.

RESULTS

CRL5 is not required for GSC maintenance.

Stem cell fate is regulated by the repression and induction of integrated signaling cascades. For example, *Decapentaplegic* (Dpp), the *Drosophila* homolog of Bone Morphogenetic Protein (BMP), is secreted from the niche and received by GSCs, where it represses the expression of the differentiation factor *bag of marbles* (bam) (Xie 2013). These cascades have various initiators and repressors acting together to maintain stem cell homeostasis. CRLs are known to regulate germline stem cell (GSC) function, however the role of CRL5 on adult ovarian GSC function has yet to be characterized (Ayyub, Sen et al. 2005, Kugler, Lem et al. 2010, Kugler, Woo et al. 2010, Ayyub 2011).

In this study we analyzed two previously published *Cul5* mutants, a null allele, $Cul\text{-}5^{EY21463}$, and a hypomorphic allele $Cul\text{-}5^{EY00051}$ (Kugler 2010, Ayyub 2008). They were previously reported to have functions in ovarian cyst and neuromuscular junction development. Since no studies have investigated GSC function in the ovary, we tested the function of *Cul5* mutant stem cells at time points of one week and three weeks, to determine if there was a compounding phenotype. By using the unique characteristic of fusome morphology as a marker for GSC we were able to quantify the number of GSCs present in each ovariole of both *Cul5* mutant alleles at each time point (Figure 4A-D).

Both *Cul-5* mutant alleles and a double *Cul-5* mutant were crossed over a deficiency to ensure the loss of Cul-5 protein expression. GSC numbers were counted at both time points, one week and three weeks, and found the average number of GSCs per ovariole. Each cross was maintained on a rich yeast diet at 25°C for the duration of the time points, which promotes an optimal environment (Roote and Prokop 2013). After

dissection, the tissues were stained with cell membrane markers, 1B1 and Lamin C, and a nuclear membrane marker, DAPI (Appendix A). Normally, GSC number decreases as the fly ages, but *Cul5* mutants are not statistically different from the sibling controls when comparing the one week to three week time points (Figure 4E) (Kao, Tseng et al. 2015). The data suggests that CRL5 is not required for GSC maintenance.

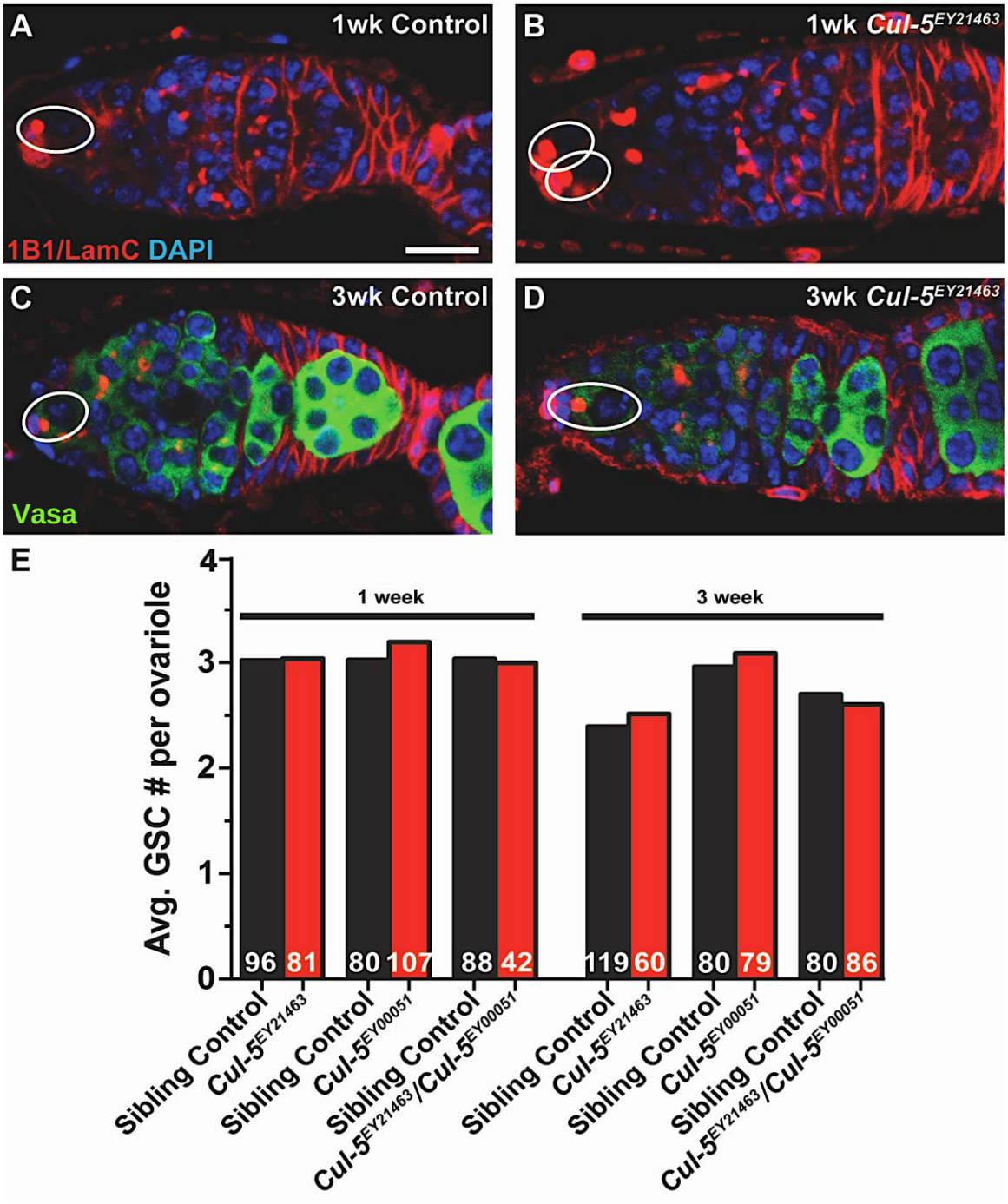

Figure 4. *Cul5* has no effect on GSC maintenance in the *Drosophila* ovary. A) 1 week control and (B) *Cul5*^{EY21463} mutant germaria with GSCs (circled) juxtaposed to the cap cells. C) 3 week control and (D) *Cul5*^{EY21463} mutant germaria with GSCs still in the correct position, next to the cap cells. 1B1/ LamC (red) marks the lateral membranes of both germline and somatic cell populations with DAPI (blue) highlighting nuclei, and Vasa (green), which labels all of the germ cells (only in 3 week samples). E) Average number of GSC per ovariole with the total number of ovarioles counted inside each bar. Statistics calculated using a chi-square test, showing no significant difference between the sibling controls and mutants at both 1 week and 3 weeks. Scale bar is 10µm.

CRL5 is required for GSC proliferation.

An important role of the GSC is to continuously proliferate without differentiation (Xie 2013). Many signaling pathways work toward this common goal. Control of GSC proliferation is also regulated by the canonical cell cycle. Cyclin-dependent kinases (CDK) are primarily responsible for the oscillations of the cell cycle (Swanson, Meserve et al. 2015). CDKs are upregulated and downregulated in order to progress the stem cell through each phase of the cell cycle. A mechanism through which this occurs is by the ubiquitination of cyclins, which regulate CDK activity, through E3 ubiquitin ligases. Of the CRL family members, CRL1 and CRL4 have been implicated in the regulation and progression of the cell cycle in *Drosophila* (Dealy, Nguyen et al. 1999, Higa, Yang et al. 2006, Swanson, Meserve et al. 2015).

To understand whether CRL5 has a role in GSC proliferation, we used a thymidine analog, EdU, to visualize GSCs undergoing S phase. Control and mutant ovaries were stained for EdU using AlexaFluor-594 via Click-It chemistry (Appendix K) (Invitrogen). In a control germarium, a GSC (circled) is EdU positive, signifying its proliferative capacity (Figure 5A). However, when *Cul5* mutant germaria were analyzed there was a significant decrease in EdU positive GSCs, thus suggesting CRL5 is necessary for GSC proliferation (Figure 5B). Data was quantified by counting the number of positive GSCs per germarium and was used in a chi-square test to calculate the percentage of EdU positive GSCs per germarium (Figure 5C).

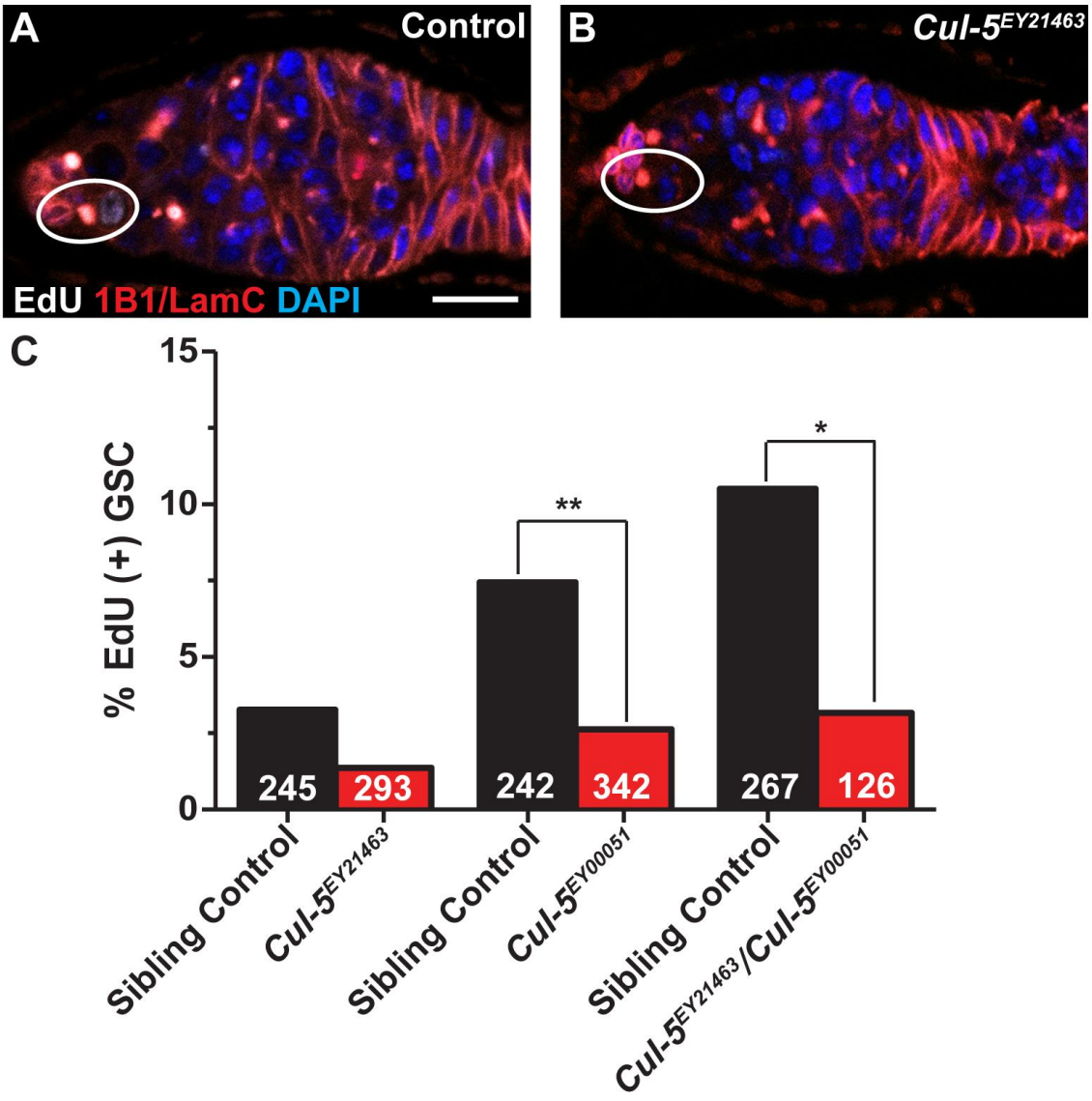

Figure 5. *Cul5* is necessary for proper proliferation of GSCs. A) Control germaria at 1 week with an EdU positive GSC (circled). B) *Cul5*^{EY21463} mutant germaria had a significant decrease in the number of EdU positive GSCs (circled). C) Data was quantified by counting the number of GSCs that were EdU positive in each germarium and calculating the percentage using the total number of germarium counted (number in bars), **p<0.005, *p<0.01. Scale bar is 10μm.

Fusome morphology indicates an altered cell cycle when CRL5 is absent.

A distinctive characteristic of stem cells in the *Drosophila* ovary is the fusome organelle. As the GSC divides the fusome morphology changes, essentially creating a map for cell cycle progression (Ables and Drummond-Barbosa 2013). Many studies have validated using fusome morphology as a marker for cell cycle phases (de Cuevas, Lilly et al. 1997, Hsu and Fuchs 2012). Traditional approaches to study the cell cycle use antibodies that tag the different phases. Two well-accepted reporters are EdU, which signifies cells going through the S phase, and phosphohistone H3 (pHH3), which signifies cells going through the M phase. When combining cell cycle markers with fusome morphology, an enhanced picture of the cell cycle is created.

To analyze cell cycle progression via fusome morphology, we recorded the shape of each fusome per GSC in each ovariole. The shape of each fusome was grouped under round, exclamation point, and elongated morphology. Previous studies have reported the round morphology as an indicator of late G2 and M phase (Ables and Drummond-Barbosa 2013). Whereas the exclamation point morphology indicates early G2 due to the abscission of the daughter from the GSC, the elongated morphology represents late G1/S phase (Figure 6A).

On average about 30% of fusomes are elongated, 10-20% are exclamation point, and around 40% are round, as seen in controls (Figure 6B-D). However, in $Cul5^{EY21463}$ and $Cul5^{EY00051}$ mutant germaria the percentage ratio shifts toward round fusomes (Figure 4B,C). Oddly, the $Cul5^{EY21463}/Cul5^{EY00051}$ double mutant presented almost the exact same percentage ratio as the sibling control, which was expected to have a worsened phenotype than that of the individual mutant alleles (Figure 6D). Taken together, this fusome

morphology data further supports our hypothesis that the loss of CRL5 alters the cell cycle by increasing the time spent in G2.

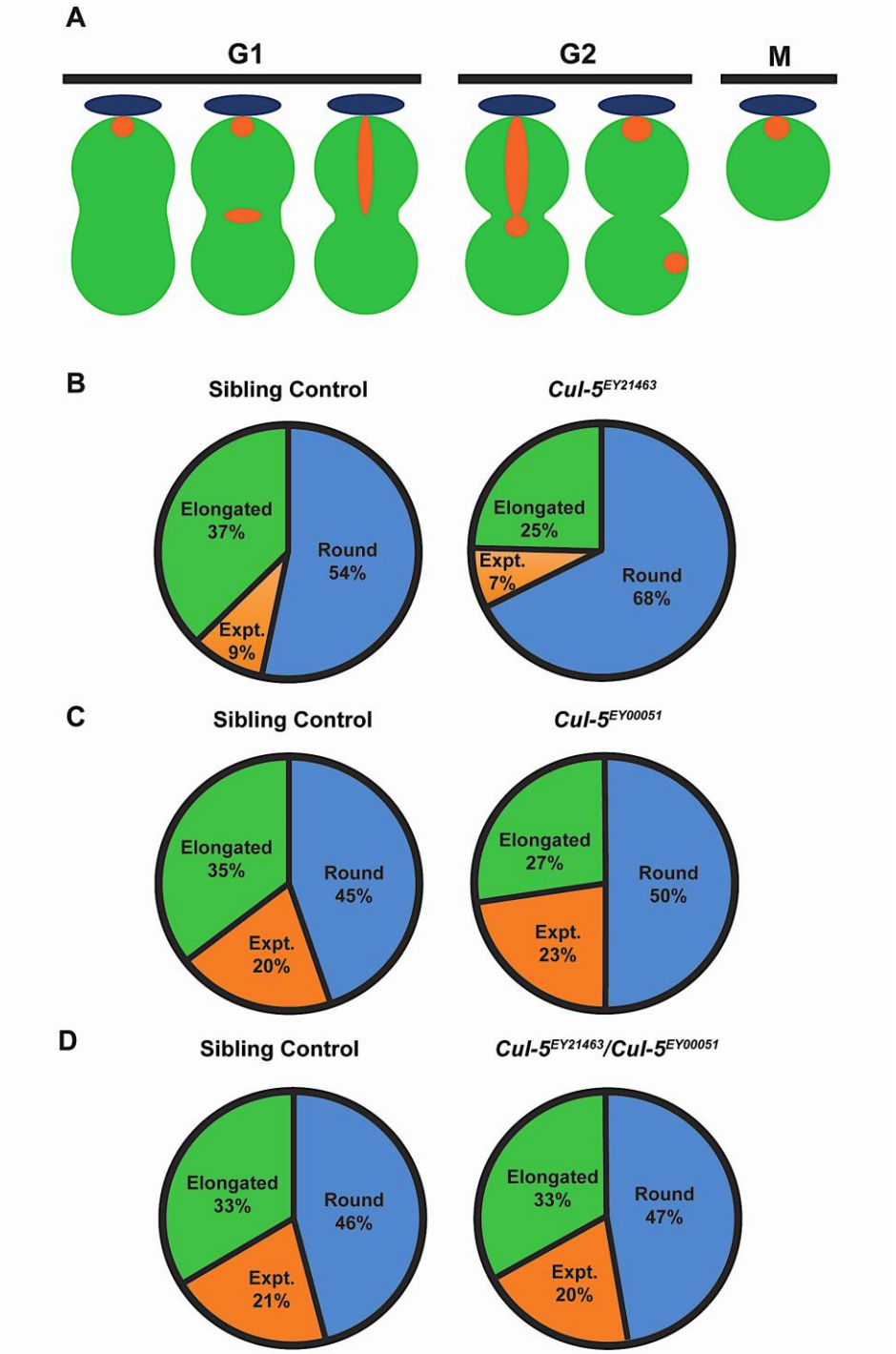

Figure 6. *Cul5* may regulate the G2 phase of the GSC cell cycle. A) Schematic of fusome morphology as the cell cycle progresses in the Drosophila ovary. B) $Cul5^{EY21463}$ mutant (n=244) and (C) $Cul5^{EY00051}$ mutant (n=340) GSCs have an increase in round and decrease in elongated fusome morphology when compared to their respective sibling controls, (B) n=288 and (C) n=244. D) The double $Cul5^{EY21463}/Cul5^{EY00051}$ mutant (n=127) GSCs has similar fusome morphologies to the sibling control (n=266).

Cell cycle alterations are likely due to decreased signals from the niche.

Many of the signals involved in self-renewal result from the contact between the cap cells and GSCs. The cap cells express the Dpp ligand that is received by a receptor complex in the GSC. The complex then phosphorylates Mothers Against Decapentaplegic (Mad), which heterodimerizes with Medea (Med) in the nucleus and represses *bam* transcription, preventing GSC differentiation.

The niche, comprised of terminal filament and cap cells, provide support for the GSCs (Liu, Zhong et al. 2015). The GSCs must remain attached to the niche in order to continue normal self-renewal and proliferation. E-cadherin is responsible for the adhesion of the GSCs to cap cells, which allows Dpp signals to transverse the cellular gap and promote self-renewal in the GSCs. Most intrinsic signals are only able to extend one cell length away (Xie 2013). So if the GSC becomes detached from the niche, the signals needed to suppress differentiation can no longer be received, resulting in GSC loss.

The βPS-Integrin is a cell adhesion protein that is highly expressed in the plasma membrane of cells, specifically the cap and terminal filament cells (Tanentzapf, Devenport et al. 2007). The bundle of cap cells utilize the Integrin protein to keep them anchored together and to the terminal filament. This anchoring is imperative for proper signals to reach the GSCs. In adult ovaries, the terminal filament cells express the Delta ligand, which binds to the Notch receptor in cap cells. From this synchronization of the Notch/Delta signaling cascade, E-cadherin and Dpp signals are able to influence the GSC self-renewal fate (Xie 2013). This suggests that loss of proper maintenance of the niche could also result in GSC loss or a decrease in proliferation.

To test whether improper niche maintenance is the cause of the decrease in proliferation within the *Cul5*^{EY21463} mutants, the tissue was stained for βPS-Integrin expression, a cell adhesion protein (Appendix E). In the control germaria, the integrin is found in the plasma membrane of the follicle cells at the junction between the extracellular matrix and the cytoskeleton, as well as high levels in the terminal filament and cap cells (Figure 7A). When we tested mutants for each integral part of the CRL5 complex, a trend in integrin expression was observed.

In *Cul5*^{EY21463}, *gus*^{f07073}, and *Roc2*^{KG07982} null mutant alleles, integrin expression is decreased in the cap cell bundle, which forms the GSC niche (Figure 7B-D). Furthermore, a significant percentage of mutant germaria had the cap cells detached from the terminal filament. Normally, the cap cells (circled) are anteriorly attached to the terminal filament cells (indicated by arrow) and posteriorly attached to the GSCs (Figure 7A). This precarious attachment allows for important cellular signals to be sent and received within a one-cell range. *Cul5*^{EY21463}, *gus*^{f07073}, and *Roc2*^{KG07982} mutants exhibited a loss or decrease of integrin expression in the cap cell bundle, as well as cap cell bundle movement (Figure 7B`-D`). Each of the improper integrin expression phenotypes were counted and quantified, resulting in a significant decrease, via chi square, in each CRL5 mutant (Figure 7E). The graph illustrates the percent of ovarioles with a loss of integrin expression over the total ovarioles counted (number in bars). The lack of maintenance of the GSC niche may be the cause of the decrease in proliferation seen in *Cul5*^{EY21463} mutants. If the cap cells move or are unable to retain the bundle formation, the GSCs may not be able to receive the proliferative signals expressed by the cap cells because they are no longer within the one-cell range.

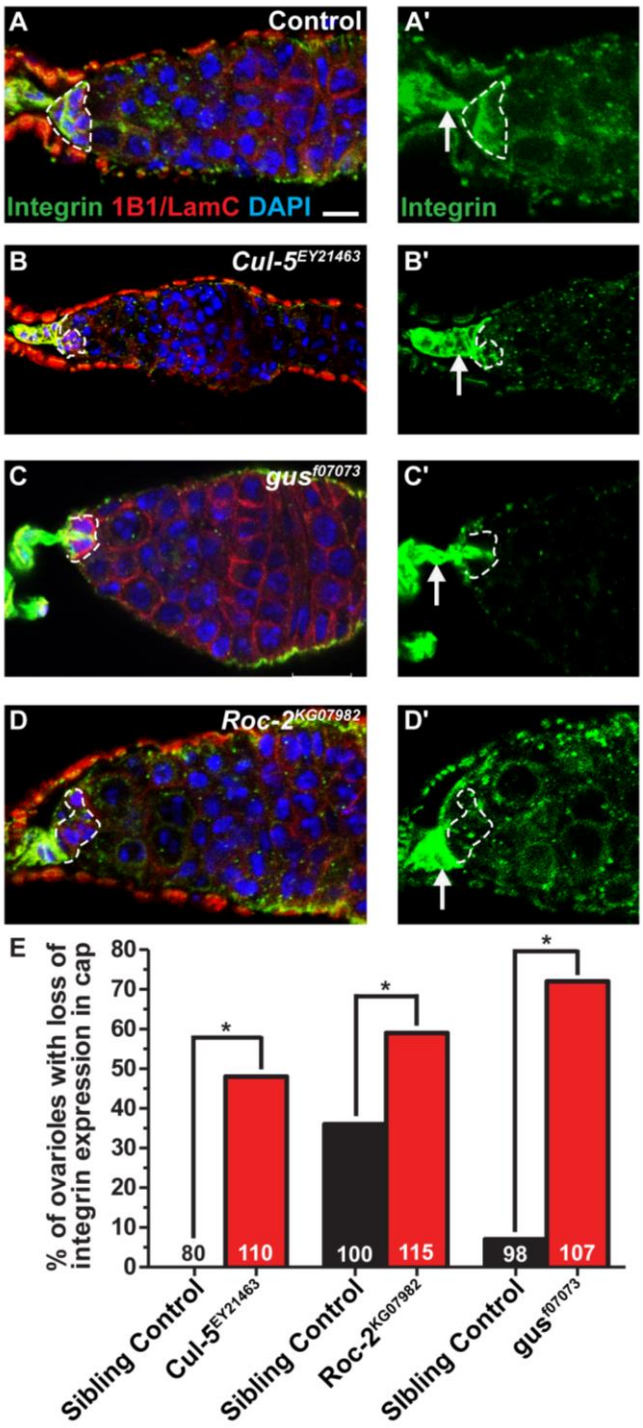

Figure 7. Loss of integrin expression is likely the cause of the cap cell bundle detachment. A) Control germarium with high expression levels of βPS-integrin (green) in the terminal filament and attached cap cell bundle. (A'-D') Inset of (A-D) with just integrin expression. B) *Cul5*^{EY21463} mutant germaria shows high levels of Integrin expression in the terminal filament but a decrease in expression in the detaching cap cell bundle (outlined in white). C) *gus*^{f07073} and *(D) Roc2*^{KG07982} mutant germaria also exhibit the same cap cell phenotype. E) Significant increase in the loss of integrin expression within the cap cell bundle of all three CRL5 mutants, *p<0.001. Within each bar are the total number of ovarioles counted. Scale bar is 10µm.

Discussion

In this study we find that *Cul5* is not required for GSC maintenance, but is necessary for proper GSC proliferation. We demonstrate through EdU incorporation that *Cul5* mutants enter or are in S phase significantly less than the Control. A common hindrance to using EdU as a cell cycle marker is its incorporation penetrance. Cells are maintained for one hour in order to stain for EdU. Unfortunately, not all cells within the germaria are dividing during that one hour time span, resulting in a 10-12% success rate for EdU incorporation. Normally, over 300 GSCs are analyzed to get a clear representation of the cell cycle. For our study, the data presented would be further enhanced with a higher number of GSCs analyzed. However, this data is also validated by fusome morphology where we show *Cul5* mutants have more fusomes with a round morphology, indicating either a longer G2 phase or shorter S phase.

Further experiments can be done to fully analyze the proliferation phenotype. Stained tissue samples have been created but not yet analyzed. *Roc2* and *gus* mutants have been stained with EdU, to determine whether they show a decrease in proliferation. *Cul5* genetic mosaic clones have been stained with EdU, to test the proliferation rates *in vivo* on a cell-by-cell basis. *Cul5* clones have been stained for Cyclin B, a known regulator of the G2 to M phase transition (Hsu, LaFever et al. 2008). *In vitro* studies using live imaging could be used to clarify CRL5's role on GSC proliferation.

In the study of integrin expression, we also showed that CRL5 is required for cap cell adhesion to the terminal filament cells. To address the cap cell movement, E-cadherin (Ecad) function, a protein vital for GSC anchorage to the niche, should be tested to determine whether the GSCs are still attached to the moving cap cells. The data could

be used to further analyze the decrease in GSC proliferation and the signaling pathways that are likely affected by the loss of CRL5.

CHAPTER 3

CRL5, an E3 ubiquitin ligase, is required for polarization and specification of precursor follicle cells via the JAK-STAT pathway.

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This chapter has been formatted according to the requirements necessary for submission to the scientific journal *Development.*

KEYWORDS

Drosophila, oogenesis, polarity, follicle cells, encapsulation, folliculogenesis

SUMMARY STATEMENT

CRL5 is required for proper follicle encapsulation through precursor follicle cell specification and polarization.

ABSTRACT

During oogenesis, follicle cells surround maturing oocytes, which produce factors necessary for proper oocyte growth and development. Correct encapsulation of the oocyte by follicle cells is therefore essential for reproduction. While many signaling pathways have been linked to encapsulation, mechanisms of early follicle development, particularly in mammals, are not fully understood. Recent evidence demonstrates that Cullin-RING E3 ubiquitin ligases (CRLs) are necessary for oogenesis in both mammals and *Drosophila*. CRLs include a Cullin family scaffolding protein and a RING-domain protein that facilitates recruitment of ubiquitin ligases. CRLs are known to control many cell processes; however, it is unclear how CRLs control early follicle development. In *Drosophila*, loss of *Cul5*, results in follicle death and improper encapsulation. We therefore tested whether the Cul5-containing CRL (CRL5) is required for early follicle development by analyzing loss-of-function mutants of the ligase complex. Loss of *Cul5* or the RING protein *Roc2* resulted in fused follicles, ruptured follicular epithelium, and improper encapsulation. Genetic mosaics of *Cul5* or *Roc2* show that CRL5 is primarily required in developing follicle cells for cyst encapsulation. CRL5 mutant follicle cells

display mislocalization of the polarity protein Bazooka and decreased Stat expression. Data also suggest that *Cul5* may mediate signaling between the follicle cells and the underlying cyst. Results suggest that CRL5 controls early follicle development by regulating early follicle cell polarity and specification. Our studies highlight the role of CRLs in early follicle development, and may lead towards a better understanding and treatment of infertility.

INTRODUCTION

Infertility in the United States has fallen since 1982, from 8.5% to 6% of women being labeled ‘infertile’. While the rate of infertility is decreasing, that 6% still means about 1.5 million women cannot conceive and/or carry a child to term (CDC). Research dedicated to treating infertility has grown drastically over the past decade, but there are still vague areas that need to be addressed, especially in the early developmental processes of follicle formation.

In the mammalian fetal ovary, a mass of germ cells, or cysts, undergo selection processes that break down the cyst and specify one of those germ cells as an oocyte (Grive 2015). The oocyte is then surrounded or encapsulated by a monolayer of follicle cells, creating a primordial follicle, which will mature and develop a multilayered follicular epithelium. Much is known about the maturation processes and developmental regulations that occur in the mammalian adult ovary, but very little is known about the initial encapsulation process to create the first primordial follicle.

Despite the differences in mammalian and *Drosophila* reproductive strategies, *Drosophila melanogaster* is emerging as an elegant model for early follicle development. The adult ovary has similar morphology to the mammalian primordial follicle; one oocyte and a monolayer of follicle cells (King 1970). *Drosophila* ovaries also contain many stages with this same morphology, which mature in a linear progression, making it easier to follow a generation as it develops. The adult ovary is composed of 14-16 ovarioles, seen as a cross-section in Figure 8A. At the anterior tip of each ovariole is the germarium (Figure 8B), where follicle encapsulation is initiated. The germarium contains two main types of cells, the germline and the soma. These two populations have to work

synchronously to maintain proper oogenesis (King 1970). The germline stem cells (GSCs) proliferate and create daughter cystoblasts, which mature into 16-cell cysts. The progression of this 16-cell cyst down the germarium is highly dependent on the communication between the germline and somatic cells (de Cuevas, Lilly et al. 1997). As the cyst matures, multiple points of communication between the germline and the soma are essential for proper encapsulation by a monolayer of follicle cells (Roth 2001).

Escort cells navigate the maturing cysts out of the germarium, which then become encapsulated at region 3 by the developing follicle cells (Morris and Spradling 2011). The precursor follicle cells also undergo specification into mature stalk and polar cells, as well as acquiring apical polarity at region 3 (Horne-Badovinac and Bilder 2005, Franz and Riechmann 2010). The newly specified polar cells take their positions at the anterior and posterior ends and connect to the newly specified stalk cells, which string together and separate each encapsulated follicle. When this process is disrupted, several defects can occur. The egg cannot survive until maturity, the follicle layer may not fully form, or more than one 16-cell cyst can be encapsulated together.

Cullin RING E3 ubiquitin Ligases (CRLs), are highly conserved and are required for oogenesis in mammals and *Drosophila* (Ayyub 2011, Yu, Zhang et al. 2013). CRLs have been implicated in many different cellular processes, such as the cell cycle, DNA replication, transcription, signal transduction, and development (Petroski and Deshaies 2005). CRLs have a unique structure that allows them to function in many diverse pathways, including those that are known to regulate oogenesis. Each CRL is composed of a scaffolding protein from the Cullin family, a RING-domain containing protein that

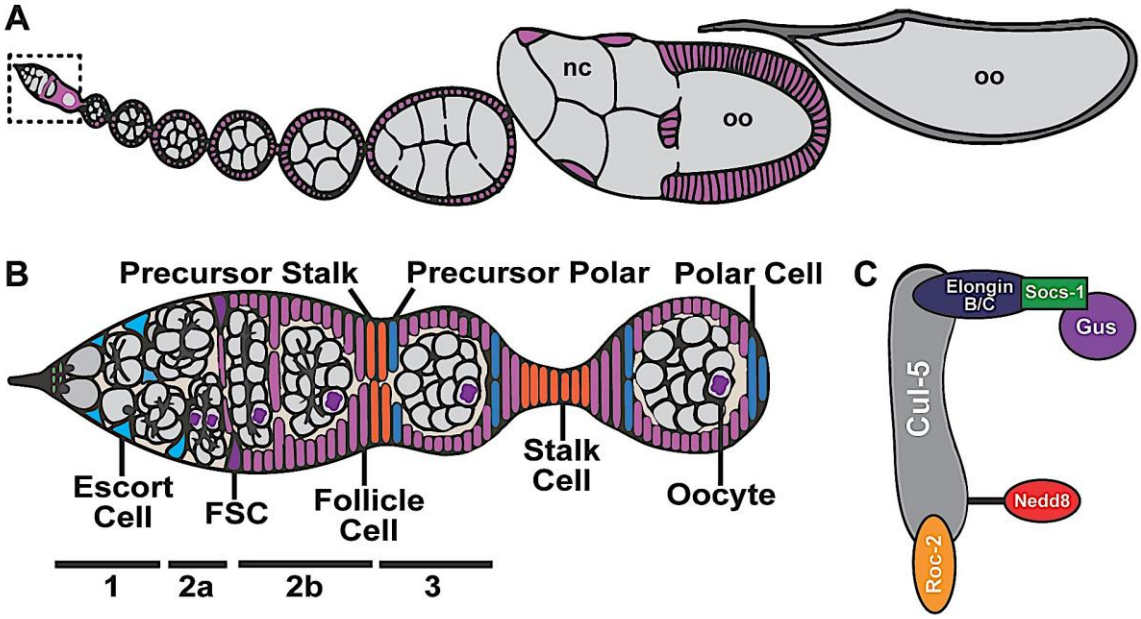

Figure 8. Somatic populations in the *Drosophila* ovary. A) Cross-section schematic of one ovariole, (boxed) is the anterior tip of the ovariole, called the germarium (B), which is divided into different developmental regions. C) CRL5, a scaffolding protein, has several integral parts that all participate in its E3 ubiquitin ligase function.

facilitates recruitment of ubiquitin ligases, and several adapters for recruitment of different substrates (Figure 8C). The Cul5-containing CRL (henceforth referred to as CRL5) is the least characterized out of the entire CRL family. Recently, a Cul5 knockout mouse has been generated but has yet to be characterized (Zhou, Wei et al. 2013). Although the mice are expected to have embryonic lethality, little is known about the function of Cul5 *in vivo* (UCDavis KOMP Repository) .

In this study, we find that CRL5 is specifically required for the encapsulation of the germline cyst by the early follicle cells. CRL5 mutant phenotypes included defects in ovariole morphology and early encapsulation. Further investigation into the improper encapsulation phenotype revealed problems with precursor follicle cell polarity in region 3, improper positioning of the oocyte, and misspecification of precursor follicle cells. Disruption of Bazooka expression and decreased STAT expression in region 3 in CRL5 mutants, implicates CRL5 in the regulation of the JAK-STAT pathway. Taken together, our results suggest that CRL5 regulates the JAK-STAT pathway in order to properly specify and polarize the precursor follicle cells.

RESULTS

CRL5 is required for proper follicle formation

Some members of the CRL family have been associated with ovarian development; however, CRL5 has yet to be characterized in mammals, and more information is needed to fully characterize its role in *Drosophila* oogenesis. The multi-subunit CRL5 is encoded by several genes (Lamsoul, Uttenweiler-Joseph et al. 2016). *Cullin 5* (*Cul5*) encodes a scaffolding protein that is critical for CRL5 function. RING

domain-containing *Regulator of cullins 2* (*Roc2*) encodes the conjugating enzyme that is crucial for binding to the E2 enzyme. Gustavus (*gus*) encodes a SOCS-box protein which enables binding of CRL5 to various substrates (Kugler, Woo et al. 2010). Roc2 is highly specific to Cul5, whereas other substrate adaptors are able to bind Cul5 in place of gus (Reynolds, Simms et al. 2008).

Previous analysis of strong hypomorphic *Cul2* and *Cul5* mutant alleles revealed multiple abnormalities in cell fate specification, synapse formation, and neuromuscular junction development (Ayyub 2005, 2011). Null *Cul5* mutant alleles were analyzed and reported irregularities in follicle development (Kugler et al 2010). Indeed, abnormalities in ovary morphology are immediately obvious upon dissection of *Cul5* mutant females (Figure 9A-B). *Cul5* mutant ovaries have multiple mature stage 14 eggs and a reduction in the number of pre-vitellogenic stages (Figure 9B).

To further examine the role of CRL5 in follicle formation, we stained control and mutant ovaries for Vasa, a germ cell marker, Hts(1B1)/LamC that stains the lateral membrane, and DAPI which stains nuclei (Figure 9A'-D') (Appendix A). Control ovarioles have a clear liner progression of maturing follicles that are separated by "strings" of stalk cells (Figure 9A′). In contrast, $Cul5^{EY21463}$ mutant ovarioles display missing middle stages, disorganized encapsulation at the posterior end of the germarium, and follicle death after stage 8 (Figure 9B′). We quantified the penetrance of ovarian phenotypes by scoring ovarioles for improper ovariole morphology. Multiple variations of this phenotype were observed, including fused follicles, follicle death, and improper encapsulation. If any or a combination of these phenotypes were present, the ovariole was counted as having improper ovariole morphology. In both *Cul5* mutant and double

mutant alleles, there are significant defects in the percent of ovarioles with improper morphology that increases over time (Figure 9E).

Since Cul5 is a scaffolding protein for the CRL5 complex, it was imperative to determine whether all or part of the complex could be attributed to the defects seen in oogenesis. We hypothesized that since Roc2 and Cul5 are exclusive binding partners (Reynolds 2008) we should observe the same ovariole defects as observed in the *Cul5* global mutants. When crossed over a deficiency, *Roc2* mutants have a more severe phenotype than what is seen in the *Cul5* mutants (Figure 9D, D′). Intriguingly, $Roc2^{KG07982}$ mutant ovarioles display complete disorganization and follicle death shortly after exiting the germarium. Since, the nature of Roc2 is the conjugating enzyme and active site of the complex, a loss-of-function *Roc2* mutant should have a worsened phenotype.

Along with the analysis of *Roc2*, *gus*, which encodes a substrate adaptor for CRL5, was also crossed over a deficiency (Figure 9C, C′). gus^{f07073} mutant ovarioles have similar disorganization at region 3, and follicle death after stage 10. However, the resulting phenotypes were less severe than both the Cul5 and Roc2 mutants. In $Roc2^{KG07982}$ mutant ovaries, the improper morphology worsens whereas gus^{f07073} mutant ovaries exhibit relatively normal morphology (Figure 9A-D). Again, the CRL family is known to have multiple substrate adaptors compatible for each Cullin protein, possibly explaining the decrease in severity of the phenotype.

Further investigation into each phenotype via immunostaining exposed a variety of cellular defects consistent with previous reports (Kugler 2010, Ayyub 2011). All CRL5 mutant alleles exhibited various morphologies, particularly in the germarium and

subsequent follicle stages, including: follicles with greater or less than 16 germ cells, fused follicles, lack of a constriction point, missing middle stages, double layered stalks, follicles with an incomplete follicle layer, and disorganization at region 3 in the germarium (Figure 10 A-F). If any of the CRL5 mutants exhibited one or multiple variations of the phenotypes shown in Figure 10, they were considered to have early improper encapsulation (Figure 11E). Taken together, these results suggest that CRL5 is required for proper oogenesis.

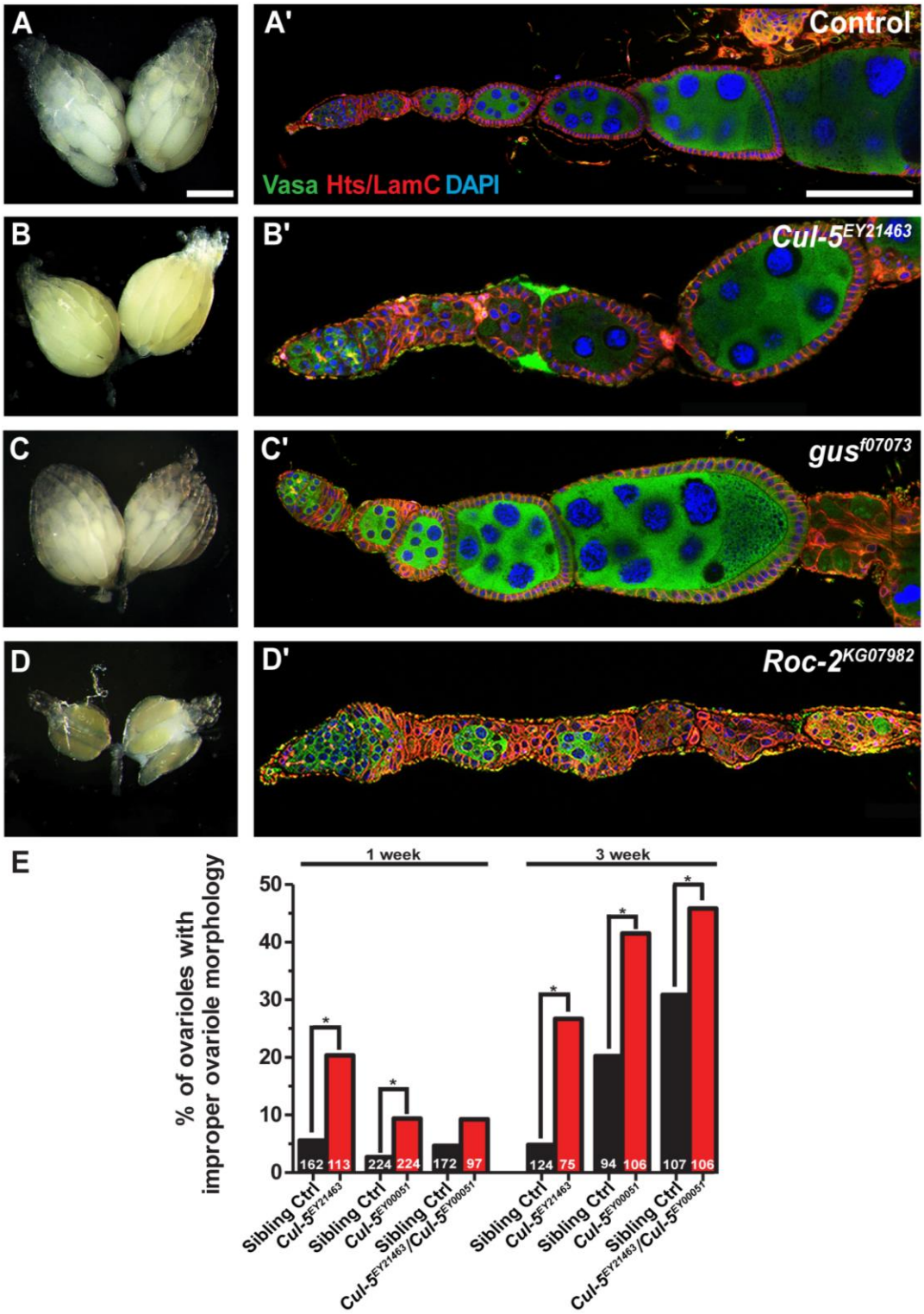

Figure 9. Loss of CRL5 results in aberrant ovariole morphology. Ovarioles were stained with Vasa (green), Hts/LamC (red), and DAPI (blue). A) Sibling control ovary contains translucent pre-vitellogenic stages which progress into a mature, yolk-containing egg. (A-D) Scale bar represents 0.5mm, (A'-D') Scale bar represents 10 µm. E) Quantification of percent of ovarioles with improper morphology at 1 and 3 weeks, via chi-square *$p<0.05$. The total number of ovarioles quantified are within each bar.

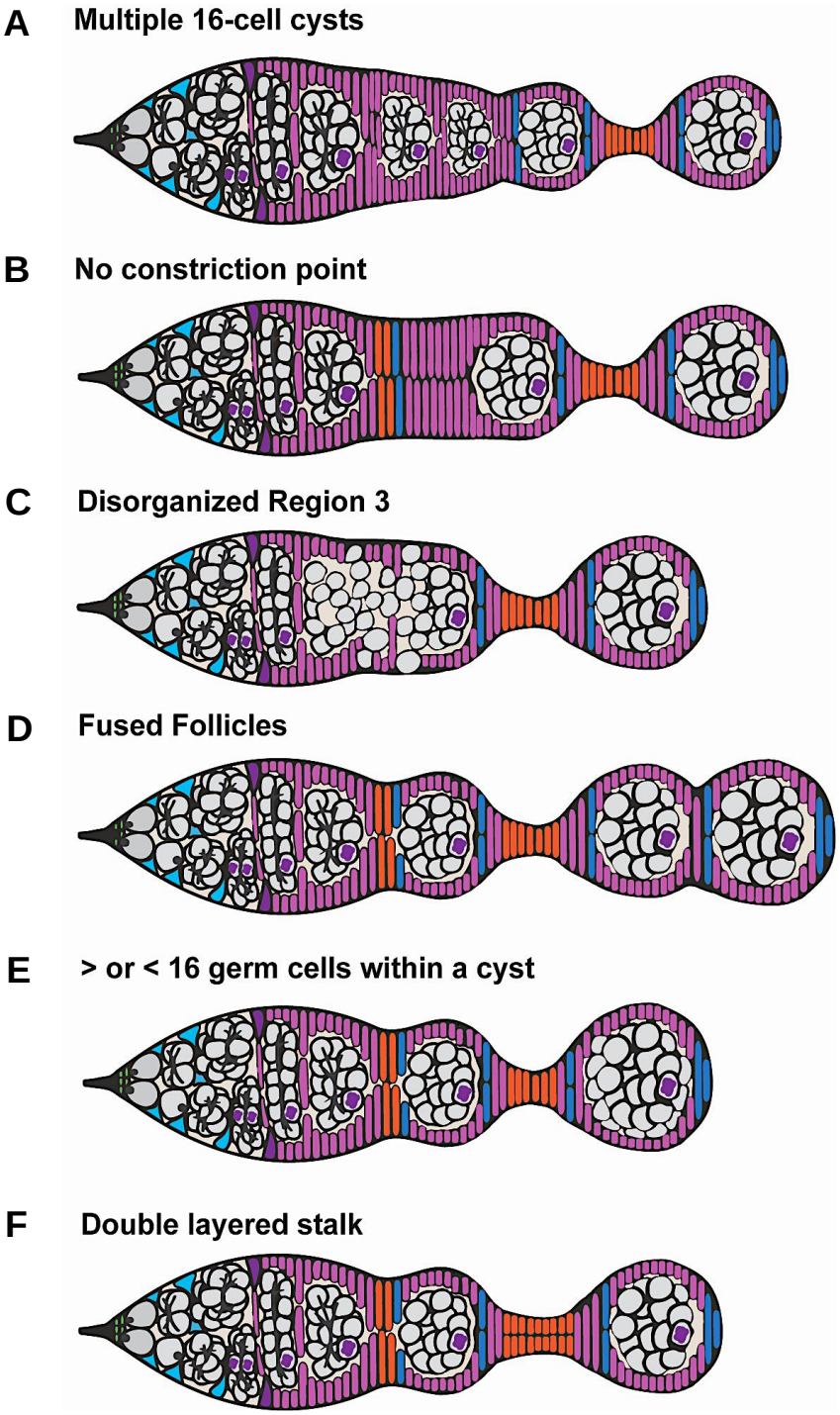

Figure 10. Various encapsulation phenotypes can be attributed to the loss of CRL5. Improper encapsulation of maturing follicles comes in various forms. Schematics represent all phenotypes that were counted in the quantification of early improper encapsulation due to the loss of CRL5.

Alterations in the basement membrane highlight errors at the constriction point

In the germarium of each ovariole, integrated communication between the germline and the soma is fundamental in the encapsulation of the first follicle. Not only is communication vital, but so is the transformation and movement of the extracellular matrix (Horne-Badovinac 2014). During the initial encapsulation event, the basement membrane is constricted, allowing the maturing follicle to exit the germarium (Pearson, Zurita et al. 2016). Immunostaining via β-PS Integrin, a cell adhesion protein, clearly highlights the junction between the extracellular matrix and the cytoskeleton. (Appendix E). In the control, a defined constriction point at the region 2b/3 border is visible and the subsequent follicle has successfully exited the germarium (Figure 11A). However, this clearly definitive landmark is lost in the *Cul5* and *Roc2* mutant alleles. $Cul5^{EY21463}$ mutants exhibit various constriction point morphologies. Some appear to have an elongated or nonexistent constriction point (Figure 11B). The same phenotype was seen in the $Roc2^{KG07982}$ mutants; however, the phenotype is significantly more penetrant, as most germaria lack a constriction point (Figure 11C, E). Again, the gus^{f07073} mutant germaria exhibit a mild case of this phenotype (Figure 11D, E). If any of the CRL5 mutants exhibited one or multiple variations of the phenotypes shown in Figure 10, they were considered to have early improper encapsulation.

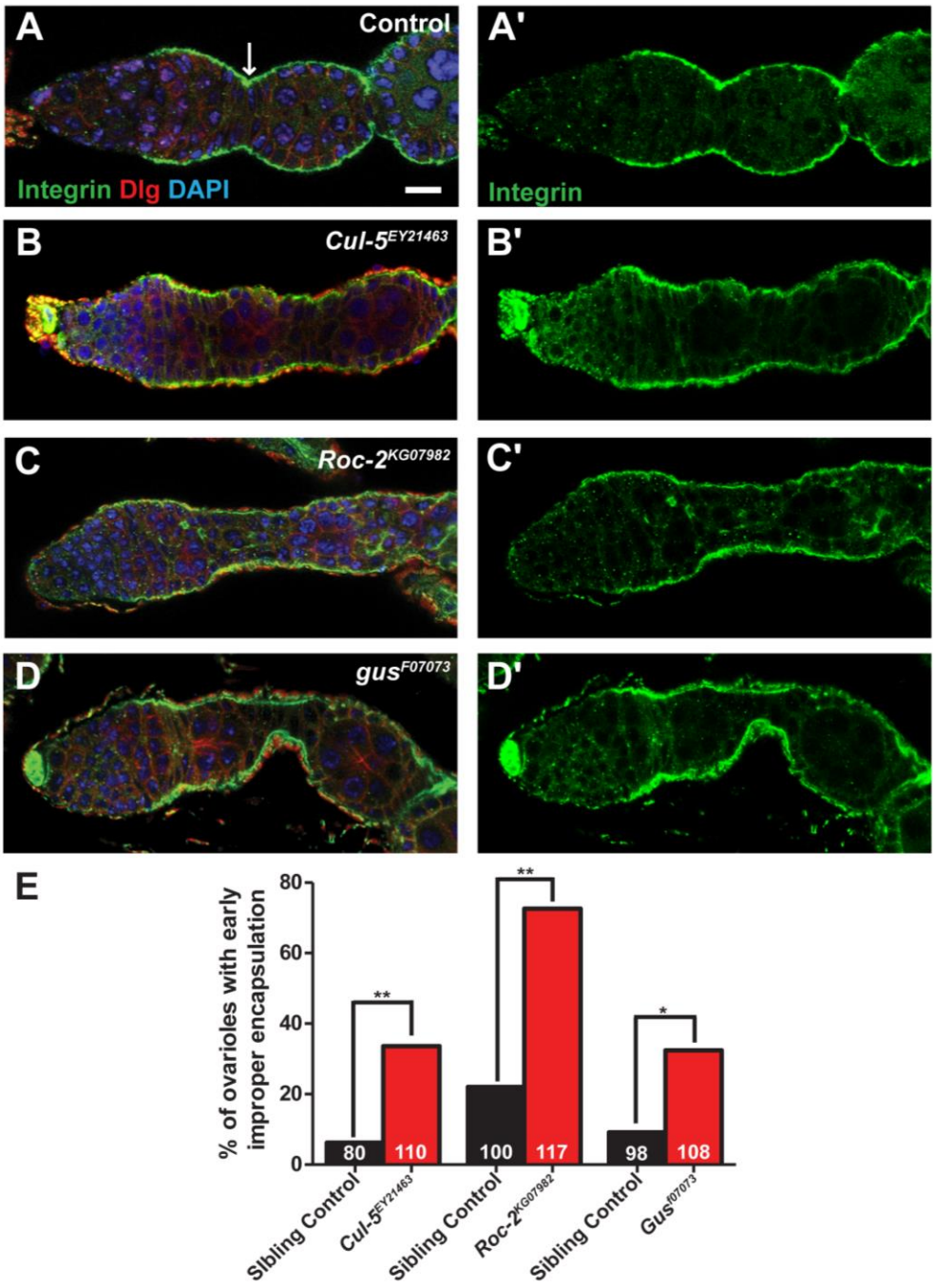

Figure 11. Improper encapsulation phenotype originates at region 3 in the germarium. Tissue stained with β-PS Integrin (green), Dlg (red), and DAPI (blue). A) In control germaria, a prominent constriction point is located at the region 2b/3 border, indicated by the white arrow. B) In *Cul5* mutants, the defined constriction point is lost. C) The phenotype worsens in the *Roc2* mutant germaria, and continues throughout the ovariole, never acquiring proper structure morphology. D) The *gus* mutant phenotype, presents the same lack of constriction but at a lesser degree. E) Percent of ovarioles with early improper encapsulation, total number of ovarioles counted are within each bar (*$p < 0.05$, **$p < 0.001$). Scale bar represents 10µm.

CRL5 is primarily required in early somatic cells for proper cyst encapsulation

Encapsulation is dependent on proper communication between the germline and the soma (Roth 2001). To determine whether CRL5 was required in the germ cells, the soma, or both, we generated *Cul5* mutant genetic mosaic clones using the Flippase recombination target (FLP;FRT) system (Golic and Lindquist 1989). Wild type cells are tagged with a green fluorescent protein (GFP), whereas mitotically induced mutant cells are negatively labeled (Margolis and Spradling 1995). This system allowed us to test the function of CRL5 *in vivo* on a cell-by-cell basis.

In control clones, where all cells are wildtype, the germaria have a prominent constriction point, all follicles are encapsulated correctly with a monolayer of follicle cells, and the linearly maturing follicles are separated by stalk cells (Figure 12A,C,E) (Appendix H). When analyzing $Cul5^{EY21463}$ clones, the ovarioles that only had negatively labeled mutant germline cells displayed no morphological changes (Figure 12B). However, $Cul5^{EY21463}$ clone ovarioles with only negatively labeled mutant somatic cells presented the previously seen encapsulation defects (Figure 12D). When $Cul5^{EY21463}$ clone ovarioles with both negatively labeled germline and somatic mutant cells the encapsulation phenotype worsened (Figure 12F). This preliminary data suggests that CRL5 is required primarily in the somatic cell population for proper follicle encapsulation.

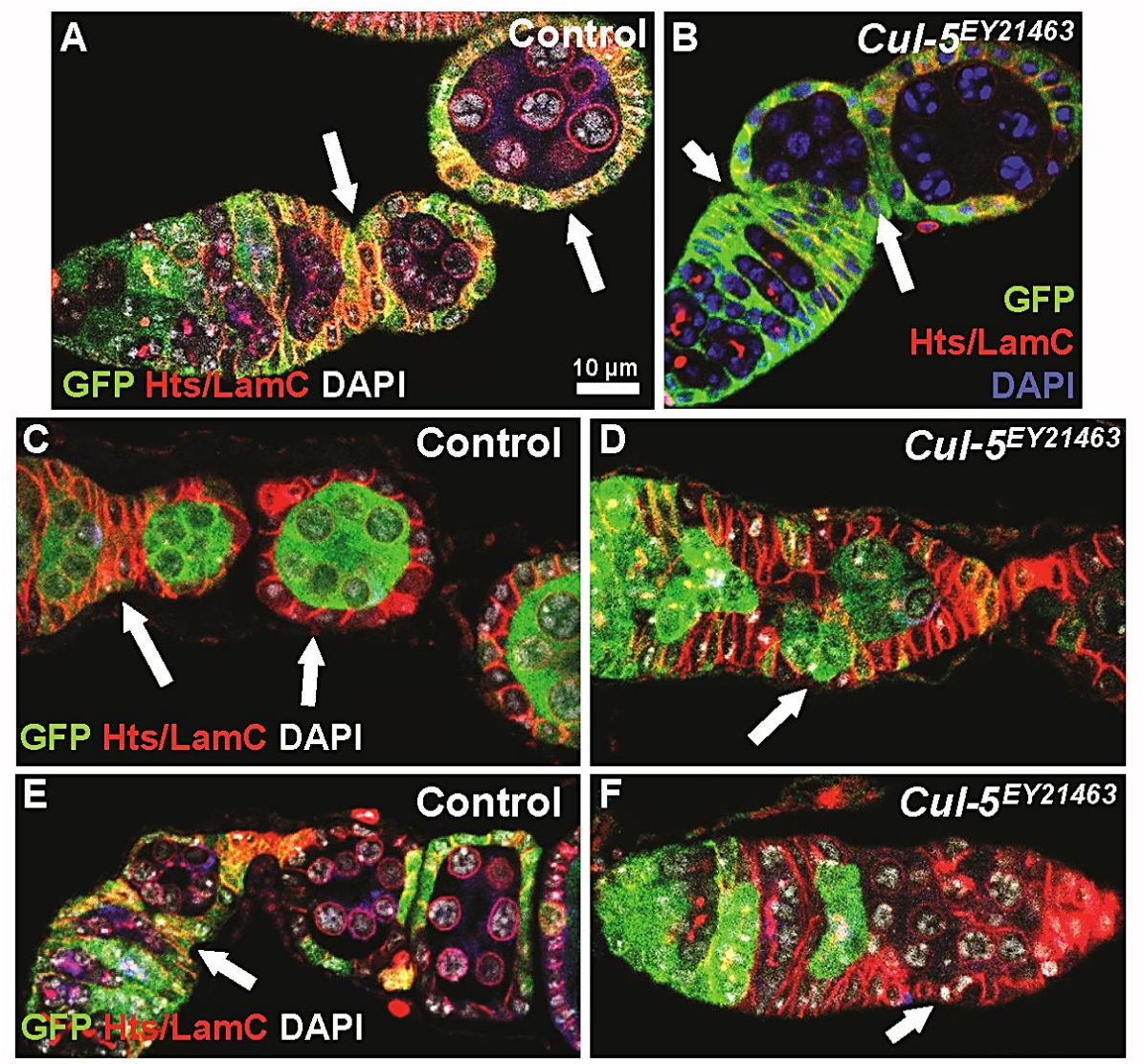

Figure 12. CRL5 is required in the somatic cells for proper follicle encapsulation. A) Control clones with only germ cells that are GFP negative have clear a constriction point (arrow) and the subsequent follicles (arrow) are properly encapsulated. B) *Cul5* mutant clones with only mutant germ cells still have a clear constriction point and proper encapsulation of maturing follicles. C) Control clones with only somatic cells that are GFP negative have a clear constriction point and proper follicle encapsulation. D) *Cul5* mutant clones that only have mutant somatic cells have lost their constriction point and have improper encapsulation (arrow). E) Both germline and somatic cells are GFP negative in the control clones, and still have a clear constriction point and proper encapsulation. F) The phenotype seen in (D) worsens when *Cul5* mutant clones have both mutant cell populations.

Escort cell morphology and signaling are unaffected by the loss of CRL5

Somatic populations are numerous in *Drosophila* ovaries; a subset of these populations is needed in the germarium to properly move the maturing germ cells. The escort cells are one of these populations. Escort cells are stationary cells with "arm-like" extensions that protract and encircle the maturing cyst (Morris and Spradling 2011). These extensions protract, push and retract to enable the movement of the cyst from the anterior to posterior end, eventually leaving the germarium. The observed phenotypes in the CRL5 mutants may be attributed to the germ cell cysts not being effectively transferred from the anterior to posterior region.

Escort cells can be recognized by the expression of Failed Axon Connection (Fax). Escort cells are triangularly shaped and are located on the basement membrane in the anterior tip of the germarium. In control ovarioles, the escort cells including their long cytoplasmic extensions, are highlighted by immunostaining against Fax (Figure 13A) (Appendix C). In both $Cul5^{EY21463}$ and $Roc2^{KG07982}$ mutant ovarioles, the escort cells and their cytoplasmic extensions exhibit normal morphology (Figure 13B,C).

Escort cells communicate with the germline cysts in order to facilitate their movement down the germarium, via the MAPK pathway (König and Shcherbata 2015). To determine whether escort cell signaling was altered in the absence of CRL5, we performed immunostaining using an antibody that specifically labels active phosphorylated MAPK (Appendix D). In the control ovarioles, the escort cells and their cytoplasmic extensions express high levels of pMAPK, suggesting that the signaling pathway is active (Figure 13D). In both $Cul5^{EY21463}$ and $Roc2^{KG07982}$ mutant ovarioles, the escort cells also brightly express the activated pMAPK (Figure 13 E,F). This data

indicates that CRL5 is not necessary for proper escort cell morphology or function. This data also suggests that the escort cells are not the cause of the improper encapsulation phenotype observed in CRL5 mutants.

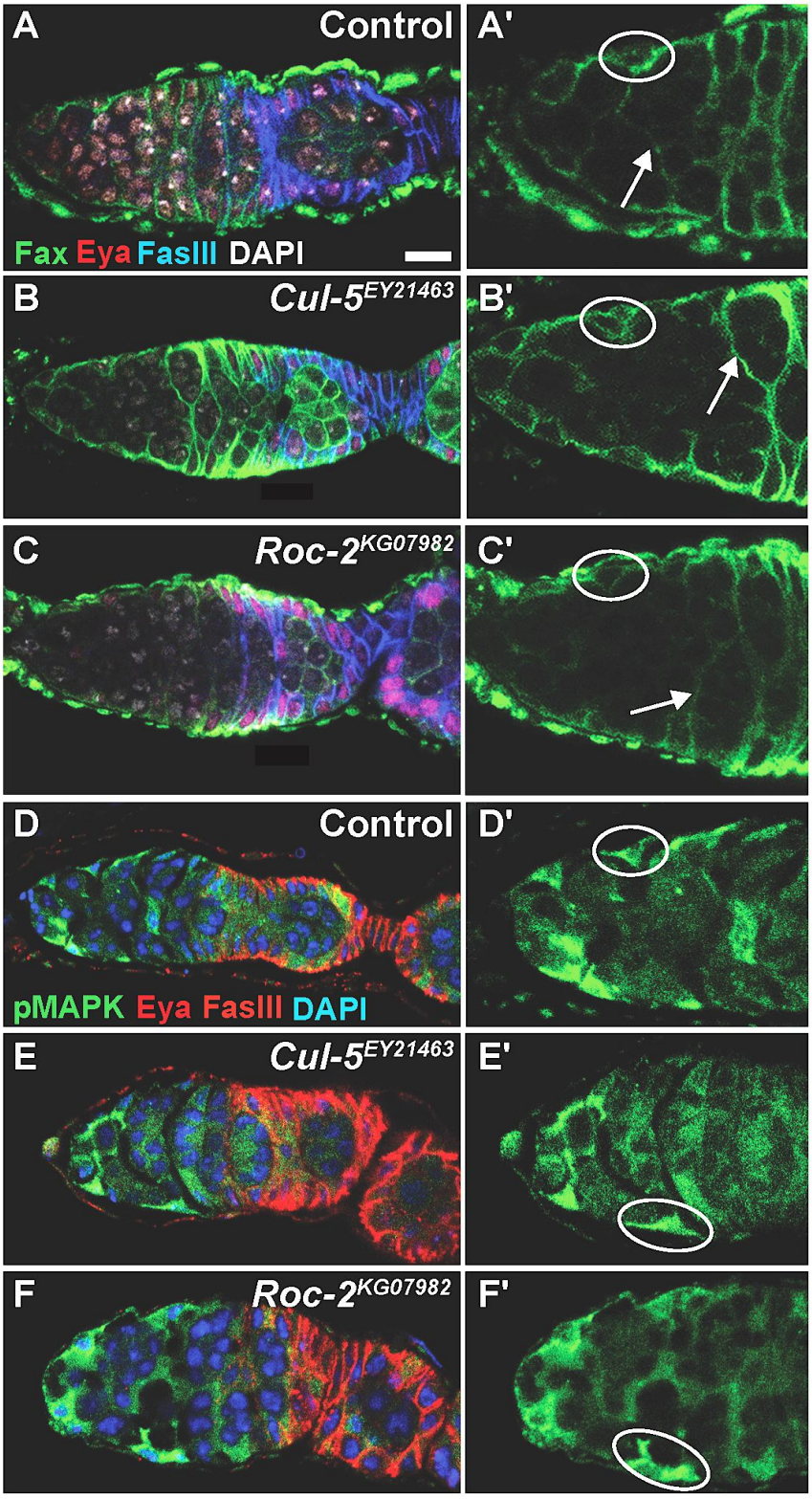

Figure 13. Escort cell morphology and signaling are not affected by the absence of CRL5. (A-C) Tissue stained with Fax (green), Eya (red), FasIII (blue) and DAPI (white). (D-F) Tissue stained with pMAPK (green), Eya and FasIII (red) and DAPI (blue). (A`-C`) Escort cells (circled) and their cytoplasmic extensions (arrow) are present and morphologically resemble wildtype cells. (D`-F`) Escort cells brightly expressing activated phosphorylated MAPK signaling demonstrate expression similar to wildtype. Scale bar represents 10µm.

Precursor follicle cells undergo specification and polarization at region 3

Since the escort cells were found not to be the cause of the early encapsulation phenotype, the subsequent somatic cell populations were tested. In region 3 of the germarium, the precursor follicle cells are fated into mature polar and stalk cells (Wu, Tanwar et al. 2008). Two polar cells are aligned on each anterior and posterior pole. Mature polar cells are responsible for the alignment of the anterior-posterior axis, help to organize the different domains of each follicle, and in later stage follicles provide an entry point for sperm. The precursor follicle cells are located at the region 2b/3 border, where the constriction point occurs. This population simultaneously undergoes two significant events during encapsulation; specification and polarization (Horne-Badovinac and Bilder 2005). The precursor population is specified by two integral pathways, Notch/Delta and JAK-STAT, through communication between the germline and the somatic cells (Assa-Kunik, Torres et al. 2007). The precursor follicle cells also acquire apical polarity at this region (Franz and Riechmann 2010). Once specified, wild type polar cells exhibit lateral expression patterns of Fasciclin III (Fas3), an adhesion molecule (Wu, Tanwar et al. 2008). This protein is highly expressed throughout oogenesis and is a good marker for mature polar cell morphology.

In control ovarioles, the polar cells are clearly identified by their round shape and unique high LaminC protein expression levels (Horne-Badovinac and Bilder 2005). Conversely, stalk cells have a more elongated morphology that eventually intercalates into a single linear arrangement separating each maturing follicle (Figure 14A, B).

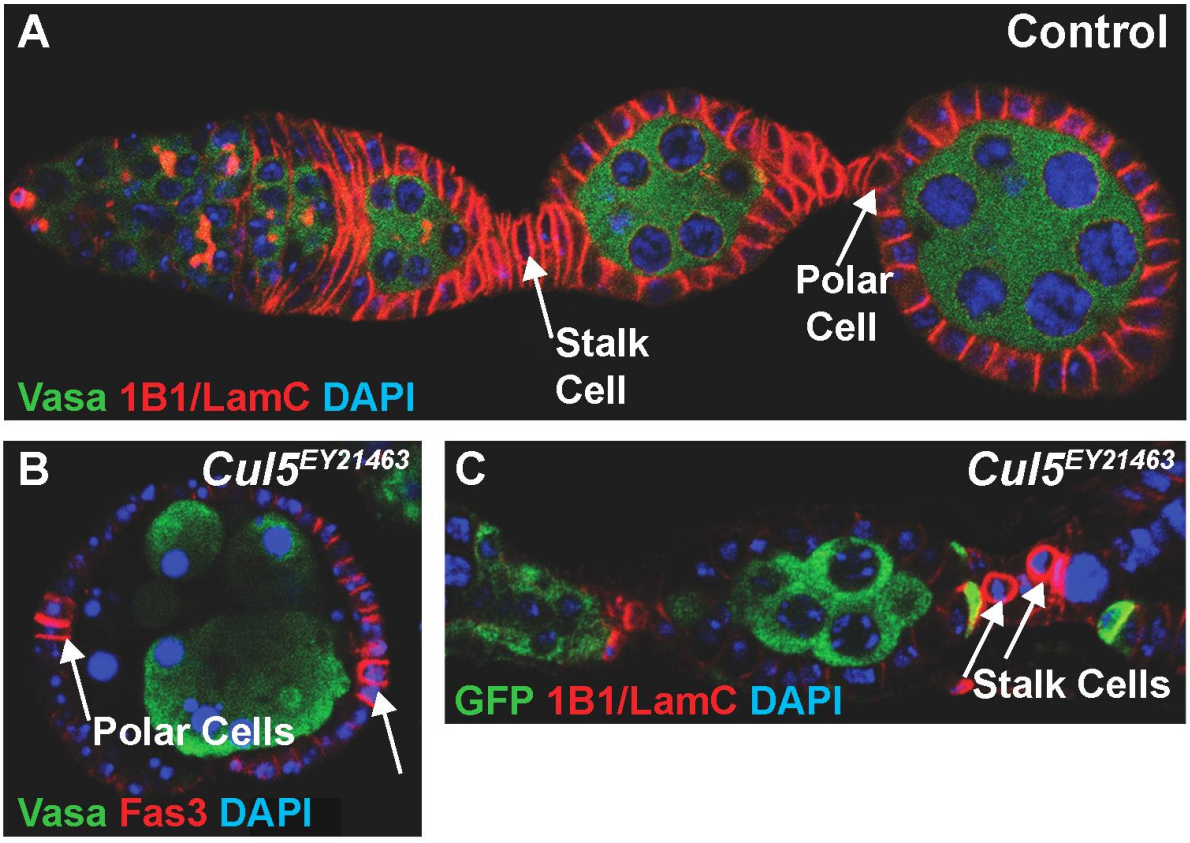

Figure 14. Loss of CRL5 in precursor follicle cells disrupts specification. A) In a control ovariole, the stalk cells separate each maturing follicle and the polar cells establish the anterior-posterior axis. B) In *Cul5* mutants, the polar cells (arrows) have correct morphology; 2 circular cells on each pole and bright immunofluorescence expression. C) *Cul5* mutant stalk cells (arrows) are possibly incorrectly specified. Normally, stalk cells have an elongated cell morphology and display normal levels of immunofluorescence, here they resemble polar cells.

In *Cul5*^{EY21463} global null mutant alleles, the polar cells appear to be correctly specified. Immunostaining with Fas3 clearly highlighted two mature polar cells on each axis that exhibit the typical round morphology (Figure 14C) (Appendix L). Conversely, the stalk cells possess polar-cell like qualities (Figure 14D). In *Cul5*^{EY21463} mutants, the stalk cells present a more round than elongated morphology and brightly express Lamin C, another polar cell quality (Appendix A) (Wu, Tanwar et al. 2008). Along with the change in individual morphology, the overall morphology of the stalk is either absent or minimal, eventually leading to fusions of maturing follicles. From this preliminary data, CRL5 is needed for proper precursor follicle cell specification that is vital for normal follicle development.

Another monumental event at region 3 is the acquisition of apical polarity in the precursor follicle cells. In the germarium, apical polarity is not yet established until region 3 (Franz and Riechmann 2010, Kronen, Schoenfelder et al. 2014). This establishment can be seen by immunostaining for the known apical marker, Bazooka. Bazooka is a scaffolding protein, known to interact in cell-to-cell adheren junctions (Kronen, Schoenfelder et al. 2014). In the germaria prior to region 3, Bazooka is sporadically expressed throughout. As the cyst matures and is encapsulated by the follicle cells, the defined apical border is localized between the germline cyst and the monolayer of follicle cells (Franz and Riechmann 2010). This expression pattern continues throughout oogenesis. Previous studies have focused on Bazooka expression at the junction between germline and somatic cells (Tanentzapf, Devenport et al. 2007, Franz and Riechmann 2010). When tested in CRL5 mutants this particular Bazooka expression

pattern was unaltered (Figure 15A, B) (Appendix B). However, there is an alternate Bazooka expression pattern in the germaria, at region 3, that is rarely mentioned.

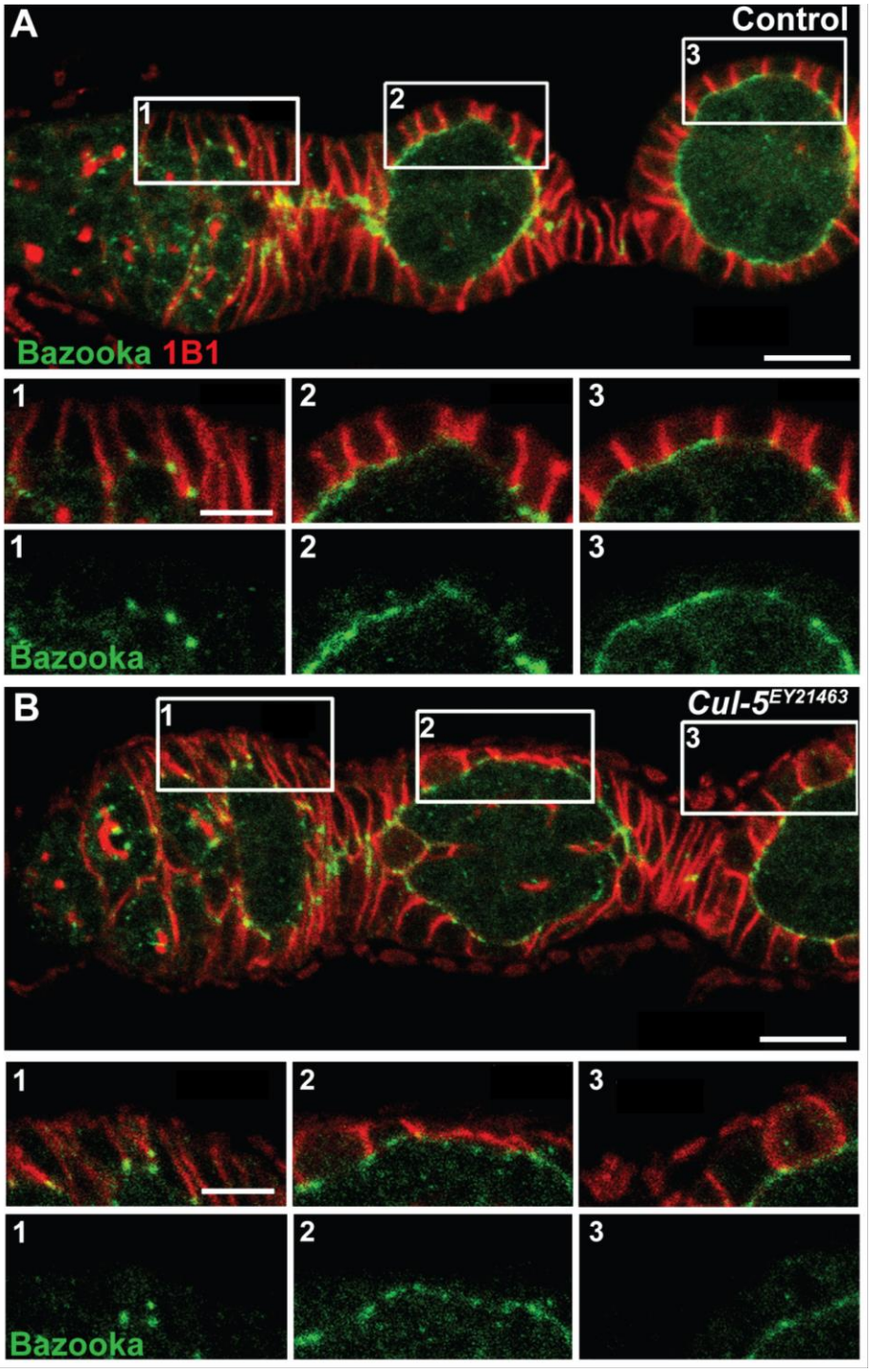

Figure 15. Bazooka expression is not disrupted at the apical border between germ and somatic cells. A) Control ovarioles express Bazooka (green) at the apical border of the germ and soma. Inset 1 is prior to the acquisition of apical polarity and by inset 2 and 3, Bazooka is established at the border. B) Cul5 mutant ovarioles exhibit similar Bazooka expression at this particular apical border. Scale bars represent 10µm.

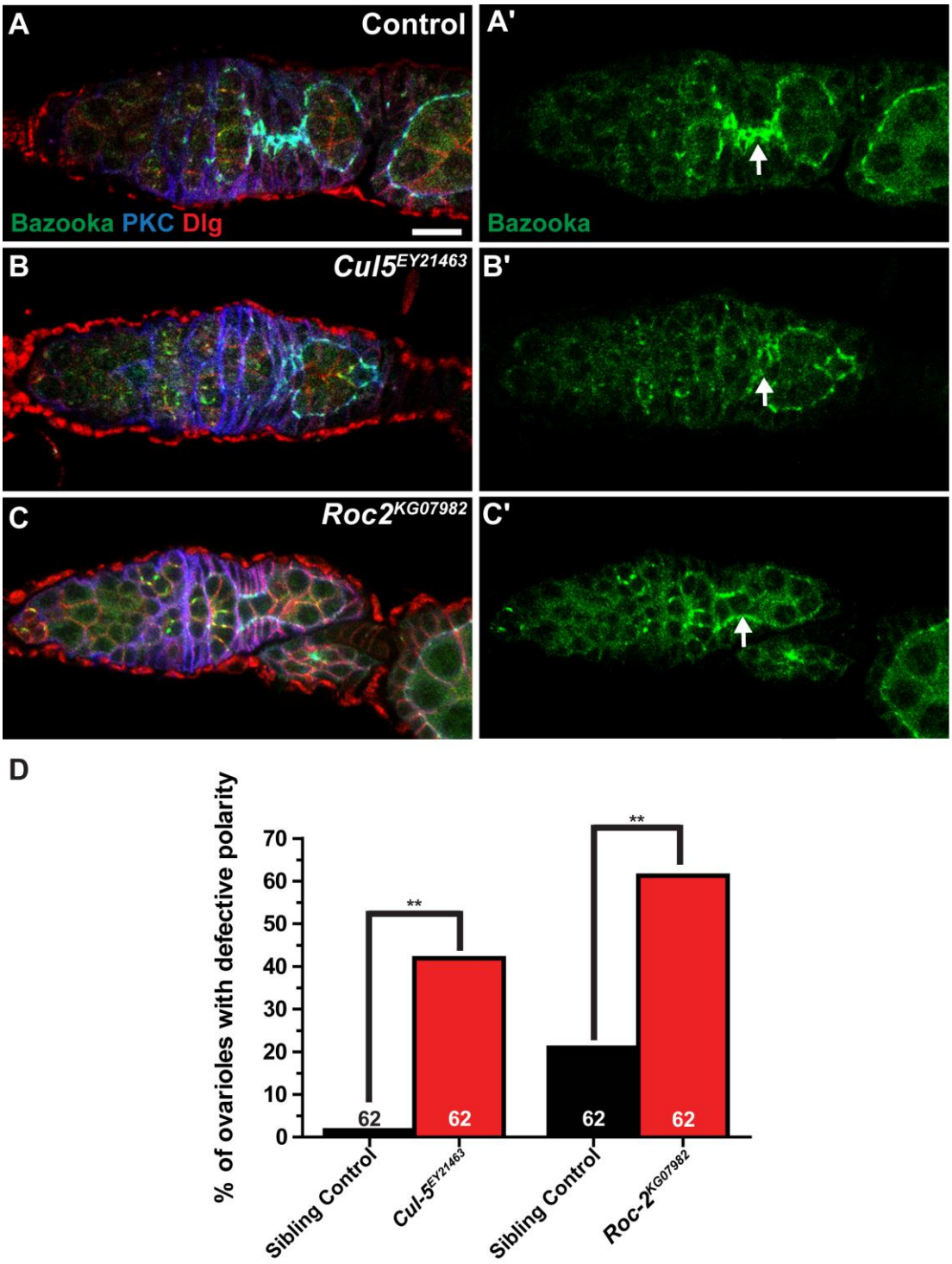

Figure 16. CRL5 is required for proper acquisition of apical polarity. A) In control ovarioles, prefollicle cells turn inward, intercalate, and establish apical polarity creating a linear expression pattern of Bazooka at the region 2b/3 border (A`)(arrow). B) In *Cul5* mutant ovarioles, this polarization process is disrupted resulting in sporadic Bazooka expression (B`). C) *Roc2* mutant ovarioles also exhibit the same disruption in Bazooka expression (C`). Both *Cul5* and *Roc2* mutant phenotypes display sporadic expression, multiple lines of expression, or no expression of Bazooka. D) All three variations of the phenotype were counted in the quantification of percent of ovarioles with defective apical polarity, via-chi-square, **p< 0.0001. The total number of ovarioles counted are within each bar. Scale bar represents 10µm.

In previous studies, at region 3, while the precursor follicle cells are specified, they also move and intercalate together caused by the procuring of apical polarity (Horne-Badovinac and Bilder 2005). This alignment of follicle cells exhibits a clear linear Bazooka expression pattern (Figure 16A). CRL5 mutants demonstrate misregulation of this process. In both *Cul5* and *Roc2* null mutant alleles, Bazooka expression is disrupted only at the apical border of the precursor follicle cells and presents various expression patterns ranging from sporadic expression, multiple lines of expression, or none at all (Figure 16B, C) (Appendix B). These phenotypes were used to quantify the mis-expression of Bazooka by calculating the percent of ovarioles with defective polarity (Figure 16D). This preliminary data suggests that the absence of CRL5 is likely interrupting polar and stalk specification and the acquisition of apical polarity in the precursor follicle cells.

CRL5 is necessary for proper JAK-STAT signaling in precursor follicle cells.

Coordination of the germline and somatic cells are essential for proper formation of the maturing follicle, particularly in the precursor follicle cells. Incorrect specification and polarization of these integrated cells can result in improper encapsulation. The germline cysts express a *Delta* ligand, which binds to the Notch receptor in the adjacent precursor follicle cell population, specifying four of them as polar cells (Assa-Kunik, Torres et al. 2007). The newly specified polar cells in turn express the *Unpaired* ligand, which activates the JAK-STAT pathway, specifying the remaining precursor follicle cells as stalk cells. Also, the Notch receptor located in the new polar cells determines the number and size of the newly fated stalk cells.

To understand the molecular mechanisms by which CRL5 controls precursor follicle cell specification and polarization, we first tested whether Notch/Delta proteins were correctly expressed in the soma and germline, respectively. In *Cul-5*^{EY21463} genetic mosaic clones, ovarioles were stained with a concentrated Delta antibody (Appendix J). The control ovarioles ubiquitously express Delta, with higher levels in the germline as previously reported (Figure 17A)(Assa-Kunik, Torres et al. 2007). The same expression pattern is seen in an improperly encapsulated *Cul-5*^{EY21463} mutant clone that has both germline and somatic cells negatively labeled (Figure 17B). A Notch antibody was used to test for Notch signaling in genetic mosaics (Appendix I). Control ovarioles clearly express Notch at the apical border between the germline and somatic cells (Figure 17C). In *Cul-5*^{EY21463} mutant clones with improper encapsulation defects, the same expression pattern is seen even when follicles are completely mutant (Figure 17D). This preliminary data suggests that there is no change to the Notch/Delta pathway in the absence of CRL5. This hypothesis is further supported by the preliminary data showing normal polar cell morphology.

Next, we tested the JAK-STAT pathway by using a STAT antibody in genetic mosaics (Appendix G). Comparing wild type cells that are positively labeled to negatively labeled cells, *in vivo*, delivers an internal control that can accurately assess changes in protein expression. Control ovarioles highly express STAT in the follicle cells beginning at region 2. When analyzing the positively and negatively labeled cells in the control germarium, there is no change in STAT expression (Figure 17E). However, when comparing GFP positive wildtype cell to negatively labeled *Cul-5*^{EY21463} mutant cells, there is a decrease in STAT expression (Figure 17F). Taken together with the previous

mis-specified stalk cell data, this preliminary data suggests that there is a change in the JAK-STAT pathway in the absence of CRL5. Interestingly, Bazooka has also been implicated as a possible downstream target of the JAK-STAT pathway (Bina, Wright et al. 2010). This suggests that the absence of CRL5 is likely inhibiting the JAK-STAT pathway and thus misregulating Bazooka expression (Figure 18).

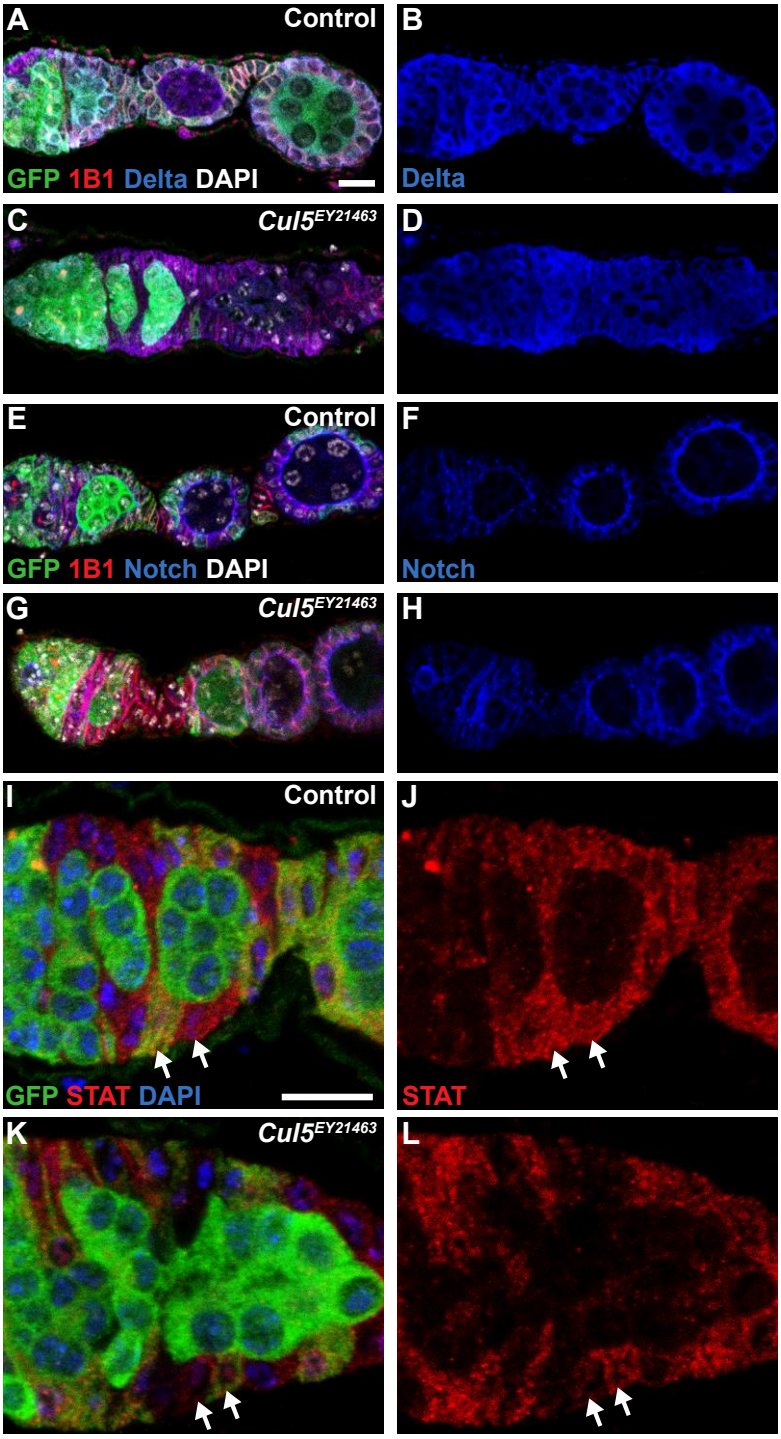

Figure 17. CRL5 is likely upstream of the JAK-STAT pathway, but not Notch/Delta. A) Control clones ubiquitously express Delta (B). C) *Cul5* clones with both mutant germline and soma do not display a reduction in Delta expression (D). E) Control clones express Notch (F) at the apical border between the germ and soma. G) *Cul5* clones with both mutant germ and soma still have normal Notch expression patterns (H). I) Control clones highly express STAT (J) in regions 2 and 3 of the germaria. K) *Cul5* clones with mutant somatic cells have a decrease in STAT expression (L). *In vivo* comparison between two adjacent cells, one wildtype and one mutant (arrows), clearly indicates the misregulation of STAT expression when *Cul5* is absent. Scale bar represents 10µm.

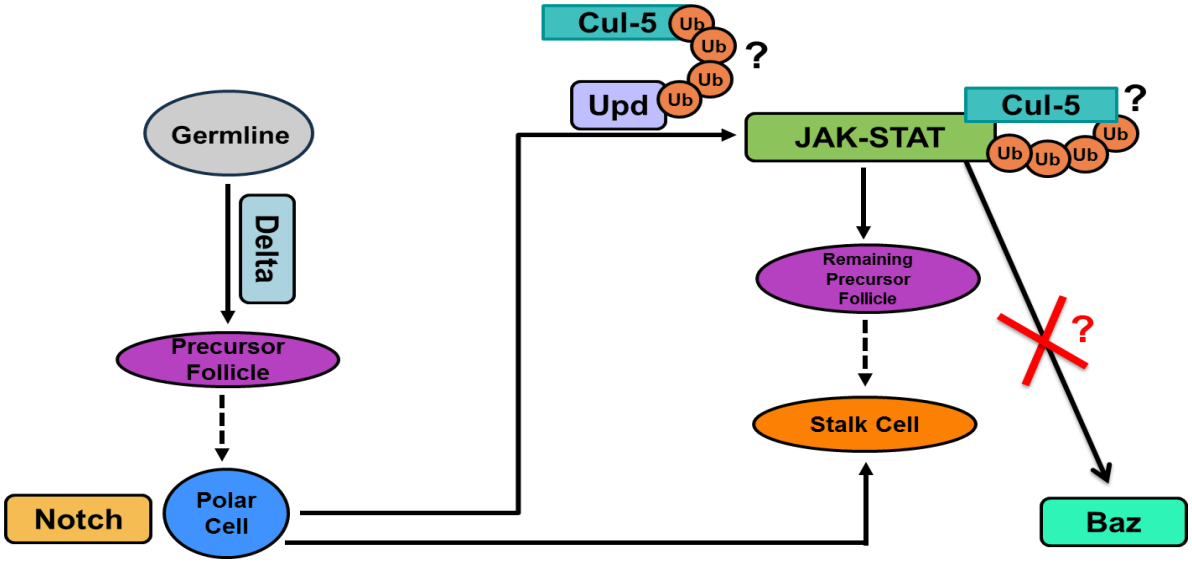

Figure 18. The absence of CRL5 likely interrupts polar and stalk specification and the acquisition of apical polarity in the precursor follicle cells. Precursor follicle cells are fated through communication between two pathways, Notch/Delta and JAK-STAT. Preliminary data suggests CRL5 is not needed for proper Notch/Delta signaling but is necessary for proper JAK-STAT signaling. Further supported by the misregulation of Bazooka expression, which is a possible downstream target of the JAK-STAT pathway.

DISCUSSION

Previous studies did an initial characterization of the *Cul5* mutant phenotype, which implicated its role in follicle development (Ayyub, Sen et al. 2005, Kugler, Lem et al. 2010). In this study we further characterized the CRL5 phenotype by analyzing *Cul5*, *Roc2*, and *gus* mutants, which demonstrated that CRL5 is required for *Drosophila* oogenesis. The absence of CRL5 leads to a lack of constriction point in the germaria and misspecification and polarization of the precursor follicle cells. This phenotype is likely due to a decrease of JAK-STAT signaling, which leads to various other phenotypes in the later stages of oogenesis.

The original study that implicated *Cul5* in improper follicle encapsulation noted the possibility of the phenotype being due to an over proliferation of germ cells,

insinuating that the follicle stem cells (FSC) could not produce enough follicle cells to properly encapsulate the maturing germline cysts (Kugler, Lem et al. 2010). Through our analysis of GSC proliferation, the previous hypothesis is incorrect. *Cul5* mutant GSCs have decreased levels of proliferation, thus eliminating GSC proliferation as the cause of the improper encapsulation. However, FSC proliferation has not been directly tested and would need to be analyzed to fully understand the improper encapsulation phenotype.

Further experiments are also needed to analyze the effect of CRL5 on the implicated signaling pathways, Notch/Delta and JAK-STAT. *Cul5* genetic mosaic clones have been crossed into a Notch reporter line, which will provide a more complete study of CRL5's effect on Notch signaling. The same experiment will be done for JAK-STAT signaling and another possible target, Wnt signaling.

MATERIALS AND METHODS

Drosophila strains and culture

All *Drosophila* stocks were kept on standard medium (Genesee Scientific, Nutri-Fly-MF) that contains Quaker yellow cornmeal, Agar Type II, light molasses, and inactive yeast, at 22ºC-25ºC. All experiments were done using a nutrient-rich diet containing the Nutri-Fly-MF medium with added wet yeast paste. *Cul-5^{EY21463}* (Bloomington Drosophila Stock Center (BLM) #22482), *Cul-5^{EY00051}/TM3 Sb Ser* (BLM#16535), *Df(3R)BSC806/TM6 Sb Ser* (BLM#27378), *Roc-2^{KG07982}* (BLM#15124), *Df(2R)BSC259/cyo* (BLM#23159), *gus^{f07073}* (gift from P. Lasko), *Df(2R)Nap14/cyo* (BLM#4308), *FRT82BCul-5$^{EY21463(4)/}$TM3 Sb*, *FRT82B/ TM3 Sb*.

Tissue Preparation, Immunofluorescence, and Microscopy

All tissue was collected as previously described in (Ables and Drummond-Barbosa 2010). The following primary antibodies were incubated in blocking solution overnight 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), guinea pig anti-Fax (a gift from Allan Spradling; 1:1000), mouse anti-Eya (DSHB 10H6; 1:1000), mouse anti-Dlg (DSHB 4F3; 1:50), rabbit anti-pMAPK (Cell Signaling #4370; 1:50), mouse anti-Fas III (DSHB 7G10; 1:50), rabbit anti-αPKC (Santa Cruz SC-216; 1:100), mouse anti-βPS Integrin (DSHB CF.6G11; 1:100), guinea pig anti-Baz (a gift from Yukiko Yamashita; 1:500), rabbit anti-bib (a gift from Yun Nung Jan; 1:1000), rabbit anti-STAT (a gift from Steven Hou; 1:1000), mouse anti-Orb (DSHB 4H8; 1:500), mouse anti-Notch (DSHB C17.9C6; 1:50), mouse anti-Delta (DSHB C594.9B; 1:100), and mouse anti-ubiquitin (ENZO LifeScience; 1:100). AlexaFluor 488-, 568-, or 633-conjugated goat species-specific secondary antibodies (1:200; Molecular Probes/Invitrogen) were incubated 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.

Generation of Genetically Mosaic Germaria

Genetic mosaics were generated by *FLP/FRT*-mediated mitotic recombination (Xu and Rubin, 1993) as described in (LaFever and Drummond-Barbosa, 2005). In short, 2-3 days after eclosion females were collected and heat shocked for one hour at 37ºC

twice a day for two consecutive days. Ovaries were dissected 10 days after the last heat shock (AHS). GFP labeled wild-type FRT chromosomes were used as control clones.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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CHAPTER 4

Thesis Summary: the role of CRL5 in *Drosophila* oogenesis

From previous literature, Cullins have been implicated in germline stem cell (GSC) proliferation and maintenance, the motive for my research on *Cul5*. Preliminary data from my work has shown *Cul5* is necessary for proper GSC proliferation but not maintenance, two aspects of GSC function that were originally thought to be controlled similarly. Closer analysis of the decrease in proliferation rates indicated a possible lenthening of the G2 phase or shortening of the S phase, via examination of fusome morphology. One possible explanation for the alteration in the cell cycle is improper maintenance of the GSC niche, which is composed of cap cells and terminal filament cells that send vital signals within a one cell range. Through analysis of the cell adhesion protein, βPS-Integrin, CRL5 mutants exhibit a detachment of cap cells from the normal point of anchorage. With cap cell detachment and movement, the mutant GSCs may not be able to receive the vital signals that control differentiation and maintenance. More studies are planned and need to be completed to fully determine Cul5's function on the cell cycle in GSCs and niche maintenance.

A previous study had noted morphological changes in the ovaries of mutant *Cul5* flies. The initial characterization of the *Cul5* ovarian phenotype noticed multiple follicle defects. My research sought to further understand and quantify these phenotypes. Closer examination of the mutant ovarioles revealed the improper ovary morphology is due to improper encapsulation, which originates at region 3 in the germarium. In that particular region of the germaria, multiple cell populations are undergoing dynamic changes to their structure and polarity. Through the generation of *Cul5* genetic mosaics, I was able to analyze each cell populations' function and morphology *in vivo*.

These experiments determined the precursor follicle cells as the possible cause of the encapsulation phenotypes. In loss of function CRL5 mutants, the precursor follicle cell polarity and specification is misregulated. This disruption may be stemming from improper signaling of the Notch/Delta pathway or the JAK-STAT pathway. Preliminary data suggests that the JAK-STAT pathway is disrupted when *Cul5* is absent, resulting in downstream effects, such as improper Bazooka expression. Further experiments need to be completed in order to determine exactly how CRL5 regulates JAK-STAT signaling to control follicle cell specification and polarity.

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APPENDIX A

Immunostaining Protocol: Vasa (Rat), 1B1/LamC, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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 for 30 minutes at RT on nutator in 0.5% Triton-PBS
- Block 3hrs in blocking solution, RT on nutator
- Incubate in primary (rt-Vasa) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in primary (mse-1B1/LamC) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 10 min in 0.1% PBS-triton
- Incubate in secondary (488 rat, 568 mouse) RT 1-2 hrs on nutator in **DARK** (or 4^{o} O/N)

- 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^{o}C

APPENDIX B

Immunostaining Protocol: Baz, αPKC, Dlg, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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

- .5% Triton 20 minutes on nutator (25 mL 1 X PBS: 25 mL 0.1 X PBS)
- Block 3hrs, RT on nutator
- Incubate in primary (gp-Baz) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in primary (rab-αPKC) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in primary (mse-Dlg) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 10 min in 0.1% PBS-triton
- Incubate in secondary (488 guinea pig, 568 mouse, 633 rabbit) 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^{o}C

APPENDIX C

Immunostaining Protocol: Fax, Eya, FasIII, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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

- .5% Triton 20 minutes on nutator (25 mL 1 X PBS: 25 mL 0.1 X PBS)
- Block 3hrs, RT on nutator
- Incubate in primary (gp-Fax, mse-Eya) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 10 min in 0.1% PBS-triton
- Incubate in secondary (488 guinea pig, 568 mouse) RT 1-2 hrs on nutator in **DARK**
- Remove secondary ab
- Wash 4x 20 min in 0.1% PBS-triton
- Block 30 min, RT on nutator
- Incubate in primary (mse-FasIII) 4^{o} O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 10 min in 0.1% PBS-triton
- Incubate in secondary (633 mouse) 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^{o}C

APPENDIX D

Immunostaining Protocol: pMAPK, Eya, FasIII, 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:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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 (rab-pMAPK) 4^{o} O/N on nutator

- Remove primary ab
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in primary (mse-FasIII and Eya) 4^{o} O/N on nutator

- Remove primary ab
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in secondary (488 rabbit, 568 mouse) 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^{o}C

APPENDIX E

Immunostaining Protocol: βPS-Integrin, Dlg, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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

- Remove previous wash
- Block 3hrs, RT on nutator
- Incubate in primary (mse-Integrin) 4° O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in secondary (488 mouse) RT 1-2 hrs on nutator in **DARK**
- Remove secondary ab
- Wash 4x 30 min in 0.1% PBS-triton
- Block 30 min, RT on nutator
- Incubate in primary (mse-Dlg) 4° O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in secondary (568 mouse) 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

APPENDIX F

Immunostaining Protocol: Orb, Baz, 1B1, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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% Triton-PBS
- Block 3 hours at RT on nutator in blocking solution
- Incubate in primary (mse-Orb) antibody at 4°C O/N on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS.
- Incubate in primary (gp-Baz) antibody O/N at 4°C on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (488 guinea pig, 568 mouse) for 1-2 hours at RT on nutator, DARK.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Block 30 minutes at RT on nutator in 0.5% Triton-PBS
- Incubate in primary (mse-1B1) antibody at 4^{o} O/N on nutator

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (633 mouse) for 1-2 hours at RT on nutator, DARK.
- Wash 2x 15 minutes in 0.1% Triton-PBS
- Incubate in 1:1000 DAPI in 0.1% Triton-PBS, 15 minutes at RT on nutator, DARK.
- Wash 3X 5 minutes in 0.1% Triton-PBS, DARK.
- Aspirate PBS, add 2-3 drops mounting media, store at 4°C

APPENDIX G

Immunostaining Protocol: GFP, STAT, 1B1, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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% Triton-PBS
- Block 3 hours at RT on nutator in blocking solution
- Incubate in primary (ckn-GFP) antibody at 4°C O/N on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS.
- Incubate in primary (rab-STAT) antibody O/N at 4°C on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS.
- Incubate in primary (mse-1B1) antibody O/N at 4°C on nutator.

- Remove and save primary ab for later use
- Wash 4x 10 min in 0.1% PBS-triton
- Incubate in secondary (488 chicken, 568 rabbit, 633 mouse), 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 4ºC

APPENDIX H

Immunostaining Protocol: GFP, Orb, 1B1/LamC, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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% Triton-PBS
- Block 3 hours at RT on nutator in blocking solution
- Incubate in primary (ckn-GFP) antibody at 4°C O/N on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS.
- Incubate in primary (mse-Orb) antibody O/N at 4°C on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (488 chicken, 568 mouse) for 1-2 hours at RT on nutator, DARK.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Block 30 minutes at RT on nutator in blocking solution
- Incubate in primary (mse-1B1 and LamC) antibody at 4^{o} O/N on nutator

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (633 mouse) for 1-2 hours at RT on nutator, DARK.
- Wash 2x 15 minutes in 0.1% Triton-PBS
- Incubate in 1:1000 DAPI in 0.1% Triton-PBS, 15 minutes at RT on nutator, DARK.
- Wash 3X 5 minutes in 0.1% Triton-PBS, DARK.
- Aspirate PBS, add 2-3 drops mounting media, store at 4°C

APPENDIX I

Immunostaining Protocol: GFP, Notch, 1B1, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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% Triton-PBS
- Block 3 hours at RT on nutator in blocking solution
- Incubate in primary (ckn-GFP) antibody at 4°C O/N on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS.
- Incubate in primary (mse-Notch) antibody O/N at 4°C on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (488 chicken, 568 mouse) for 1-2 hours at RT on nutator, DARK.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Block 30 minutes at RT on nutator in blocking solution
- Incubate in primary (mse-1B1) antibody at 4^{o} O/N on nutator

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (633 mouse) for 1-2 hours at RT on nutator, DARK.
- Wash 2x 15 minutes in 0.1% Triton-PBS
- Incubate in 1:1000 DAPI in 0.1% Triton-PBS, 15 minutes at RT on nutator, DARK.
- Wash 3X 5 minutes in 0.1% Triton-PBS, DARK.
- Aspirate PBS, add 2-3 drops mounting media, store at 4°C

APPENDIX J

Immunostaining Protocol: GFP, Delta, 1B1, DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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% Triton-PBS
- Block 3 hours at RT on nutator in blocking solution
- Incubate in primary (ckn-GFP) antibody at 4°C O/N on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS.
- Incubate in primary (mse-Delta) antibody O/N at 4°C on nutator.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (488 chicken, 568 mouse) for 1-2 hours at RT on nutator, DARK.

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Block 30 minutes at RT on nutator in blocking solution
- Incubate in primary (mse-1B1) antibody at 4^{o} O/N on nutator

- Wash 4x 30 minutes in 0.1% Triton-PBS
- Incubate in secondary (633 mouse) for 1-2 hours at RT on nutator, DARK.
- Wash 2x 15 minutes in 0.1% Triton-PBS
- Incubate in 1:1000 DAPI in 0.1% Triton-PBS, 15 minutes at RT on nutator, DARK.
- Wash 3X 5 minutes in 0.1% Triton-PBS, DARK.
- Aspirate PBS, add 2-3 drops mounting media, store at 4°C

APPENDIX K

Immunostaining Protocol: EdU, 1B1/LamC, DAPI

- Grab 1.5 mL tubes pre-coated with 3% BSA from 4ºC fridge.
- Make 5.3% formaldehyde (FA) fix for _____ samples
  (# of samples +1) X 300µL 16% FA
  (# of samples +1) X 600µL Grace's media

- Prepare EdU reagent: 10uL 10mM Edu-594 (in DSMO) into 490 uL R/T Grace's media – total 500 µL per sample. Leave covered in tinfoil at R/T
- Dissect ovaries in R/T Grace's, **DO NOT TEASE OVARIOLES APART**, and move to BSA-precoated tube (take BSA out of tube first!).
- Incubate ovaries in EdU solution (from above) for 1hr @ R/T on nutator in **DARK**. Ensure that all ovaries floating.
- Remove EdU and rinse 1x in R/T Grace's (1mL/sample)
- Wash 2 x 5min in R/T Grace's.
- **Tease ovarioles apart.**
- Fix in 1mL 5.3% FA for 13 min on nutator.
- Remove fix to FA waste
- Rinse with 0.1% PBS-triton (1mL/sample)
- Wash 3x10 minutes 0.1% PBS-triton
- Store in **DARK** 4°C in clean 0.1% PBS-Triton-X100

Day 2:

- Wash 20 min @ R/T in **0.5%** PBS-triton
- Block 3hrs, RT on nutator in **DARK**
  Block (store at 4º): 5% NGS, 5% BSA, 0.1% PBS-Triton-X100
  For 50 mL: 8.3mL 30% BSA
  2.5mL NGS
  5 mL 1% PBS-Triton-X100
  34.2mL (to volume) PBS
- Incubate in primary antibodies @ 4ºC for 2 nights on nutator:
  - Mouse α-1B1, 1:10
  - Mouse α-LamC, 1:100

Day 3:

- Use previous one!!Remove and save α-1B1 + α-LamC for later use (can be used 1X more)
- Wash 4x 20 min in 0.1% PBS-triton

- Incubate in secondary (not 568) 500 uL per sample RT 1-2 hrs on nutator in **DARK** (or 4^{o}C O/N)
    - Goat α-mouse-633, 1:200
    - Goat α-rabbit-488, 1:200

Day 4:

- Wash 2 x 15 min in 0.1% PBS-triton in **DARK**
- Prepare Click-It reaction cocktail. <u>Must use within 15 min of mixing!</u>:
    - In this order, add:
        - 43 µL 10x Click-it rxn buffer (stored @ 4^{o}C) X (# of samples)
        - 387 µL ddH_2O (# of samples)
        - 20 µL $CuSO_4$ (stored @ 4^{o}C) (# of samples)
        - 1.2 µL Alexa-Flour 594 azide (light sensitive; stored @ -20^{o}C) (# of samples)
        - 40 µL reaction buffer additive (stored @ -20^{o}C) (# of samples)
    - Add 500 µL of the master mix to each tube.

- Remove wash, add Click-It reaction cocktail to ovaries and incubate @ R/T for 30 min in **DARK** on nutator.
- Wash 4 x 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

APPENDIX L

Immunostaining Protocol: FasIII, Vasa (Rat), DAPI

- Precoat 1.5mL tubes with 3% BSA for each sample, leave on nutator ~1hr
- Make 5.3% formaldehyde fix:
    - (# of samples +1) X 300µL 16% FA
    - (# of samples +1) X 600µL Grace's media
- 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

- .5% Triton 20 minutes on nutator (25 mL 1 X PBS: 25 mL 0.1 X PBS)
- Block 3hrs, RT on nutator
- Incubate in primary (mse-FasIII) 4º O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 20 min in 0.1% PBS-triton
- Incubate in primary (rt-Vasa) 4º O/N on nutator

- Remove and save primary ab for later use
- Wash 4x 10 min in 0.1% PBS-triton
- Incubate in secondary (488 rabbit, 568 mouse) 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 4ºC

APPENDIX M

Table of Fly Stocks Used

Personal Stocks	Stock #	Description
Df(2R)Nap14/cyo	BLM#4308	uncovers Gse and also EcR
Df(2R)Nap1	BLM#1006	uncovers Gse and also EcR
$gus^{B70/73}$	P.Lasko	tumors over Df in wings
Df(2R)BSC259/cyo	BLM#23159	uncovers Roc-2
Roc-2^{KG07982}	BLM#15124	
EcR^{5546}		
Df(3R)BSC806/TM6 Sb Ser	BLM#27378	longer than Exe6211, uncovers Cul-5
Df(3R)Exe6211	BLM#7689	uncovers Cul-5
Cul-5^{EY21463}	BLM#22482	null
Cul-5^{EY00051}/TM3 Sb Ser	BLM#16535	hypomorph
Df(2L)BSC103	BLM#8669	uncovers Cul-2
Cul-2^{EY03074}	BLM#11176	lethal over Df, use for creating FRT recombinant
Cul-2^{EY09124}	BLM#19883	weaker allele, adults viable over Df
flped; NP4124FRT19a/FrnZc	Alana O'Reilly	
Gal180/19AFlp122/UAS-GFPnls5/cyo	Alana O'Reilly	
19A FLP122/109-30Gal4/cyo	Alana O'Reilly	
;wfwre/cyo	Todd Nystul	wg reporter
PTC GFP/cyo; HhRNAi/TM3	Todd Nystul	Hh reporter
FRT82BCul-5EY00051(4)/TM3 Sb	VH	Cul-5 clones
FRT82BCul-5EY00051(2)/TM3 Sb	VH	Cul-5 clones
FRT82BCul-52146(4)/TM3 Sb	VH	Cul-5 clones
FRT82BCul-52146(2)/TM3 Sb	VH	Cul-5 clones
FRT82BCul-52146(1)/TM3 Sb	VH	Cul-5 clones
TM3/TM6 [ry^{RK} in background]	BLM#120	
FRT82B/ TM3 Sb	K. Laws	
hsFLP;FRT82BUbiGFP/TM3 Sb	DDB stocks	generate genetic mosaics with Cul-5
neoFRT42D	BLM #1802	
hsFLP; FRT42DubiGFP/Cyo	ETA stocks	
Gla/CyO		
GbeSu(H)_m8 lacz (II)	W-M. Deng	Notch reporter
E(spl)C[D2/cyo; MKRS/TM6B	W-M. Deng	Notch reporter
sp/cyo;nT-Jacz	W-M. Deng	Notch reporter
)f[d2] wf[1.118] P{ry[+t7.2]=ey-FLP.N12 P{ry[+t7.2]=GMR-lacZ.C(38.1)}TPN1; P{ry[+t7.2]=neoFRT142D P{y[+mDint2] wf[BR.E.BR]=SUPor-P}Cul-4[KG02900] / CyO, y[+]	DGRC#111538	Cul-4 FRT
)f[d2] wf[1.118] P{ry[+t7.2]=ey-FLP.N12; P{ry[+t7.2]=neoFRT142D P{w[+mC]=EP}Roc2[EP2487] / CyO, y[+]	DGRC#114465	Roc-2 FRT
ywhsFlp;FRT82BamnZ/TM3 Sb	DDB stocks	
P{w[+mW.hs]=GawB}185Y, w[1.118]	BLM#3731	early stalk cells, follicle cells
P{w[+mW.hs]=GawB}c306, w[1.118]	BLM#3743	stalk and border cells
w[1.118]; P{w[+mW.hs]=GawB}c368	BLM#3744	stalk cells after stage 1
w[1.118] P{w[+mW.hs]=GawB}c614	BLM#6711	centripetal and posterior most follicle cells
P{w[+mW.hs]=GawB}1.12A, w[*]	BLM#7021	stalk and follicel cells at anterior and posterior poles
)f[1.w[*]; P{w[+mW.hs]=GawB}109-30/CyO	BLM#7023	stalk cells, follicle cells at poles, and precursor follicle cells
w[1.118]; P{w[+mC]=2XStat92E-GFP}6-1	BLM#26196	Two STAT92E binding sites drive expression of GFP
w[1.118]; P{w[+mC]=10XStat92E-DGFP}2/CyO	BLM#26199	Ten STAT92E binding sites drive expression of destabilized GFP
w[1.118]; P{w[+mC]=10XStat92E-DGFP}3/TM6C, Sb[1] Tb[1]	BLM#26200	Ten STAT92E binding sites drive expression of destabilized GFP
P{KK109372}VIE-260B	v108817	Cul-5 RNAi
P{KK100540}VIE-260B	v105101	Cul-2 RNAi
;wfwe/cyo;FRT82BCul5EY21463/TM3 Sb		Wingless reporter in Cul5 mosaic background
GbeSu(H)_m8 lacz (II)/cyo;FRT82BCul5EY21463/TM3 Sb		Notch reporter in Cul5 mosaic background

APPENDIX N

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
EdU	Click-IT	N/A	Cells going through S phase, Thymidine analog	Thymidine	Invitrogen
Vasa	1:1000	Rabbit	germ cells	Vasa	Paul Lasko
	1:1000	Rat	germ cells	Vasa	DSHB
Fax	1:1000	Guinea Pig	Escort cells	Failed Acon Connections	Allan Spradling
Eya	1:1000	Mouse	FSC and follicle daughters, faint expression in escort cells	Eyes Absent	DSHB-eya10H6
Dlg	1:50	Mouse	apical membrane of cells	Discs Large	DSHB-4F3
pMAPK	1:50	Rabbit	Escort cell extensions and follicle cell membranes	phosphorylated MAP Kinase	Cell Signaling #4370
FasIII	1:50	Mouse	polar and stalk cells	Fascillin 3	DSHB
αPKC	1:100	Rabbit	apical membrane of cells	Aytipical Protein Kinase C	Santa Cruz (C-20):sc-216
β-PS Integrin	1:100	Mouse	Basal membrane of follicle cells, cap cells, and terminal filament cells	Integrin	DSHB-CF.6G11
Baz	1:500	Guinea Pig	border cells	Bazooka	Yukiko Yamashita
Cyclin B	1:20	Mouse	Cyclin B activity	Cyclin B	DSHB-F2F4
CDK1/CDC-2		Rabbit	Entry into mitosis, activation of CDC2 kinase		Millipore #06-923
Bib	1:1000	Rabbit	stains stalk cells	Big Brain	Yun Nung Jan
STAT	1:1000	Rabbit	STAT expression	STAT	Steven Hou 2016
Orb	1:500	Mouse	earliest marker of oocyte formation	Orb	DSHB
Notch	1:50	Mouse	Notch expression	Notch	DSHB #C17.9C6
Delta	1:100	Mouse	Delta expression	Delta	DSHB #C594.9B
Ubiquitin	1:100	Mouse	mono- and polyubiquitinylated conjugates	Ubiquitin	ENZO lifescience #BML-PW8810