Investigation of the Role *ribbon* Plays in Adult Gonad Function and Morphology in *Drosophila melanogaster*

BY

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THESIS

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For my family and my dearest Cheryl. I love you all.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BTB	Bric-á-brac, Tramtrack, and Broad complex (BTB) domain
chinmo	*chronologically inappropriate morphogenesis*
CySC	Cyst Stem Cell
DE-cad	*Drosophila* E-Cadherin
dsx	*doublesex*
dsx^{F}	$\text{doublesex}^{\text{Female}}$
dsx^{M}	$\text{doublesex}^{\text{Male}}$
EGFR	Epidermal growth factor receptor
FGF	Fibroblast Growth Factor
Gal4/UAS	Gal4/Upstream Activation Sequence
Gal80^{TS}	Gal80 Temperature Sensitive.
GSC	Germline Stem Cell
HH	Hedgehog
JAK/STAT Transcription proteins	Janus kinase/Signal Transducer and Activator of
MAPK	Mitogen Activated Protein Kinase
msSGP	Male Specific Somatic Gonadal Precursor Cell
nos	*nanos*
PGC	Primordial Germ Cells
PHF7	Plant Homeodomain Finger protein 7

PS	Parasegment

LIST OF ABBREVIATIONS (Continued)

PSQ	Pipsqueak domain
rib	*ribbon* (gene)
RIB	Ribbon (protein)
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
SEV	Sevenless
SGP	Somatic Gonadal Precursor Cell
SXL	Sex lethal
TGFβ	Transforming growth factor beta
tj	*traffic jam*
tra	*transformer*
WG	Wingless
WT	Wild Type

SUMMARY

A study was conducted to elucidate the role for *ribbon* in the maintenance of function and morphology in adult gonads in *Drosophila melanogaster*. Previous work showed *rib*'s importance in proper gonad development, but no one has looked into why it is continuously expressed in adult gonads. To study its potential role, *rib*'s expression levels were modulated in this study. The Gal4 – UAS system was used to overexpress *rib* throughout development. In conjunction with the Gal80 temperature sensitive repressor, the Gal4-UAS system was also used to overexpress *rib* in adult gonad cells. *rib* knockdown was performed by using the Gal4-UAS system to express an RNAi targeting *rib* transcripts. For all components of the study, the drivers Nanos and Traffic Jam were used to target gene expression alterations to germ and somatic cells in the gonads, respectively.

rib overexpression both throughout development and in adult germ and somatic cells influenced gonad morphology. In many cases, subjects were rendered unable to fully form testes and ovaries. *rib* knockdown had no effect on either male or female gonad development and morphology when targeted to either somatic or germ cells. In summary this study successfully demonstrated that rib may play a role in maintaining proper gonad morphology and function. Future studies will look into the efficacy of the RNAi line used. They will also focus on mechanism by which *rib* is affecting gonad morphology when overexpressed.

CHAPTER 1:

Introduction

Homeostasis within organisms is dependent on the maintenance and proper regulation of stem cell populations. Stem cells possess the ability to divide and give rise to both undifferentiated stem cells and differentiated daughter cells. These populations of cells are maintained through cell-cell adhesion and regulatory signaling mediated by the stem cell microenvironment, also known as the niche. Knowledge of these signaling pathways is important for understanding the precise mechanisms in place to ensure the proper growth and function of stem cells. Failures in these mechanisms for example can result in unchecked proliferation leading to cancer, as well as failed stem cell production leading to infertility and/or improper organ function. *Drosophila melanogaster,* and in particular its gonads have served as wonderful models for the study of stem cell development and function (as reviewed in Jemc, 2011; Greenspan et. al., 2016).

In the *Drosophila* embryo two types of cells come together to form functional gonads, the Primordial Germ Cells (PGCs) and Somatic Gonadal Precursor Cells (SGPs). Please see figure 1 for a schematic summary of embryonic gonadal development. During the 4^{th} and 5^{th} stages of embryogenesis the PGCs are derived from cytoplasm that has been deposited in the

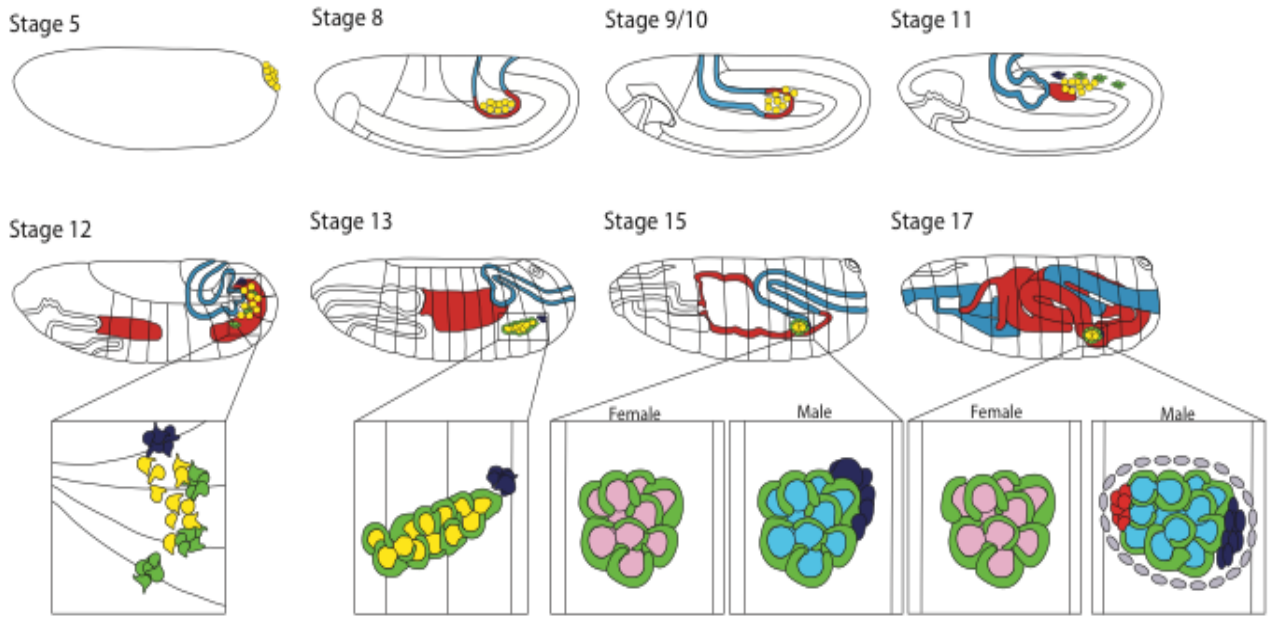

Figure 1. Schematic of Embryonic Developmental Stages and Events. Used with permission form the author, this schematic shows the different stages of embryonic development in *Drosophila melanogaster*. In stages 12-17 we can see the coalescence and compaction of primordial germ cells (PGCs; yellow) and somatic gonadal precursor cells (SGPS; green). SGPs (green) ensheath female (pink) and male (blue) PGCs. (Jemc, 2011).

embryo form a maternal source, and is called the germ plasm (Mahowald, 1962; Snee and Macdonald, 2004). The germ plasm is rich in RNAs, proteins, and the PGS will form in this region of the embryo (Jaglarz and Howard, 1995; Kunwar *et. al.*, 2008). During stage 7 of embryonic development, the posterior end of the midgut (where PGCs are localized) moves into, or invaginates into the embryo interior. This carries with it the PGCs, which no longer exhibit their cytoplasmic extensions; rather, they are more spherical in nature (Kunwar *et. al.,* 2008). During stage 9 of embryonic development, the PGCs assume an amoeboid shape and make their way towards the mesoderm (Kunwar *et. al.*, 2008; Warrior, 1994). Once embryonic development reaches stage 11, the SGPs can be found in three bilateral groupings, which are located in parasegments (PS) 10-12 in the mesoderm (Brookman *et al.,* 1992). It is of note that another group of SGPs classified as male-specific SGPs (msSGPs) develops in PS 13. Interestingly these cells occur in both males and females, but only persist in males (De Falco *et al*., 2003).

During stage 12 of embryonic development, the PGCs migrate in the direction of SGPs (Boyle and DiNardo, 1995). By stage 13, the PGCs and SGPs will gather into two groups, which can be found bilaterally. Additionally, the PGCs once again assume a rounded shape (Kunwar *et al.*, 2008). SGPs ensheath PGCs throughout embryonic development (Decotto and Spradling, 2005; Gonczy and DiNardo, 1996; Jenkins *et al*., 2003). During embryonic stage 14, the msSGPs will combine with the gonad in males (DeFalco *et al*, 2003). At stage 15 of development, the gonad undergoes processes during which it compacts into a rounded structure. Its final location is near PS 10 (Boyle and DiNardo, 1995). From this point forward gonad development becomes sexually dimorphic.

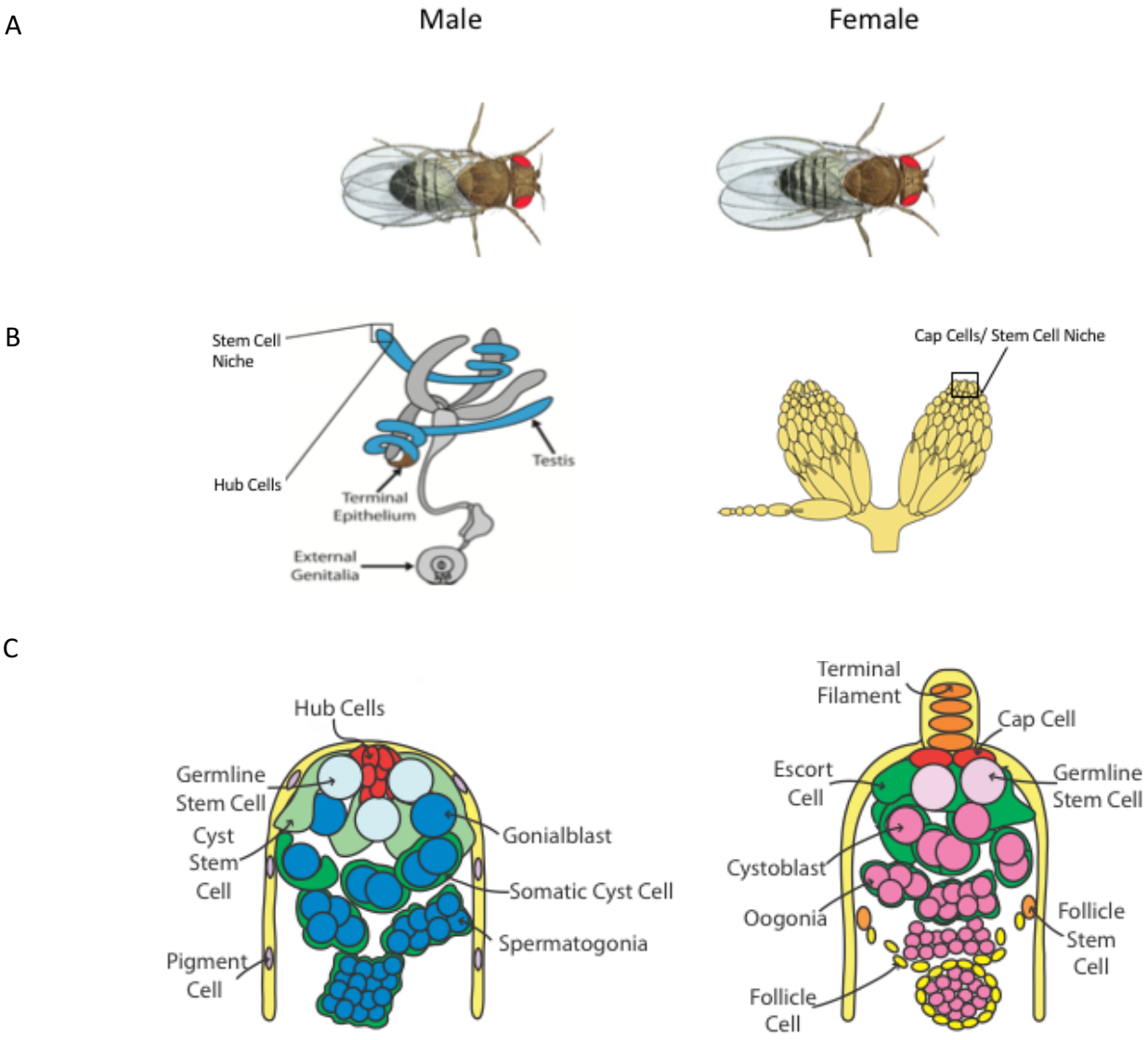

Figure 2. Schematic representation of Adult *Drosophila melanogaster* Gonads. A. These are images of adult male and female examples of *Drosophila melanogaster*. B. Here we see the depiction of an adult testis and ovary. The testes are a pair of blunt-ended tubes coiled around an accessory gland. The ovaries are a pair of aggregates of substructures known as ovarioles, with egg chambers in varying states of development. C. These schematics show a closer view of the testis apical end and the ovary germarium. Please note the stem cell niche (red) in both male (Hub Cells) and female (Cap Cells) releases signaling molecules necessary for the proper maintenance of stem cell function, spermatogenesis and oogenesis in their respective gonads. With permission from author, (Jemc, 2011).

At the end of embryonic gonad formation, important players activate to establish the stem cell niches necessary for proper gonad function in adult flies. In males the stem cell niche is called the hub, and is first established at the 17th stage of embryogenesis. Its creation is heavily reliant on the establishment of competent SGPs via Notch signaling (Kitadate and Kobayashi, 2010; Okegbe and Dinardo, 2011). Receptor tyrosine kinases such as Epidermal growth factor receptor (EGFR) and Sevenless (SEV) play a role in regulating which SGPs are recruited to create the hub (Kitadate and Kobayashi, 2010; Kitadate *et al*., 2007). In addition to this Janus kinase/Signal Transducer and Activator of Transcription proteins (JAK/STAT) signaling is key for the development of the hub. It is expressed in germ cells which are located anteriorly near where the hub will be (Le Bras and Van Doren, 2006; Sheng *et al*., 2009). Studies have implicated STAT in the establishment of GSCs next to the hub as well as the expression of *Drosophila* E-Cadherin and *Drosophila* N-Cadherin which are necessary for hub-GSC interactions, and for holding the hub together (Le Bras and Van Doren, 2006; Sheng *et al*. 2009).

Female stem cell niche formation is reliant on similar players as in male niche formation, but with a couple of nuances. As with males, EGFR signaling is important for coordinating proliferation to ensure an appropriate number of PGCs for the establishment of cap cells, the female niche (Gilboa and Lehmann 2006). Notch signaling is expressed as well between the 3rd larval stage and early pupal development. This is the time when cap cells are specified (Song et al., 2007). Unlike in male niche formation, females utilize Transforming growth factor beta (TGFβ) signaling to help coordinate the mitosis of germ cells while the female stem cell niche is formed during larval development (reviewed in Jemc, 2011). Additionally, Wingless (WG present at the 16th stage of embryogenesis) and Hedgehog (HH expressed at the 3rd larval stage) also help to regulate PGC mitosis (Sato et al., 2008, 2010). Among cell signaling, cell adhesion molecules

are also important for establishing the stem cell niche. Among them, *DE-cad* is important for maintaining interactions between cap cells and GSCs (Song *et al.,* 2002).

The adult *Drosophila* testis can be described as a tubular structure with a stem cell niche located at the apical end. Each male develops a pair of these structures (Figure 2). Within the adult testes, proper organ function is ensured by a delicate balance of two primary populations of stem cells, the Germline Stem Cells (GSCs) and the somatic Cyst Stem Cells (CySCs), which are attached to 10-15 packed somatic cells that form the hub, or stem cell niche of the testis. The hub cells maintain these stem cell populations by cadherin-based adhesion and cell signaling. GSCs typically divide to produce a daughter GSC and a gonialblast. The gonialblasts undergo four rounds of cell division with incomplete cytokinesis, resulting in 16 spermatogonia, surrounded by two somatic cyst cells (Hardy et. al. 1979). Spermatogonia mature into spermatocytes, which undergo meiosis to yield 64 haploid spermatids, that mature into sperm (as reviewed in Greenspan *et. al.*, 2015).

Adult *Drosophila* ovaries are round in structure, with each fly having two ovaries that house 12-24 sub-structures known as ovarioles. Each ovariole contains a chain of egg chambers at different developmental stages. At the anterior end of the ovariole is the germarium (Figure 2). The GSCs are anchored at the anterior end of the germarium by the cap cells, which serve as the GSC niche. GSCs will divide asymmetrically, producing a daughter GSC and cystoblast. As with testes, the cystoblasts will go through 4 sets of division with incomplete cytokinesis, resulting in 16 oogonia, which are surrounded by a pair of escort cells (King 1970, and Spradling 1993). Only one of these cells will become an oocyte, while the remaining 15 become nurse cells, supplying RNAs, proteins, and cytoplasm to the mature egg (as reviewed in Greenspan *et. al.*, 2015).

Figure 3: Representation of the Protein Structure of Ribbon. Ribbon is a protein comprised of 661 residues. It has a Bric-á-brac, Tramtrack, and Broad complex (BTB) domain, which facilitates protein-protein interactions, as well as a Pipsqueak (PSQ) domain for DNA binding, and is believed to be a transcription factor. Schematic adapted from Silva D, 2016.

It is interesting to note that many genes that have been shown to have a function in gonadal development have also been shown to play a role in organ homeostasis in adulthood (reviewed in Jemc 2011). A mutagenesis screen revealed that *rib* is also required for gonad morphogenesis in *Drosophila melanogaster* (Weyers et. al., 2011). *ribbon* (*rib*) encodes a protein containing a Bric-á-brac, Tramtrack, and Broad complex (BTB) domain, which

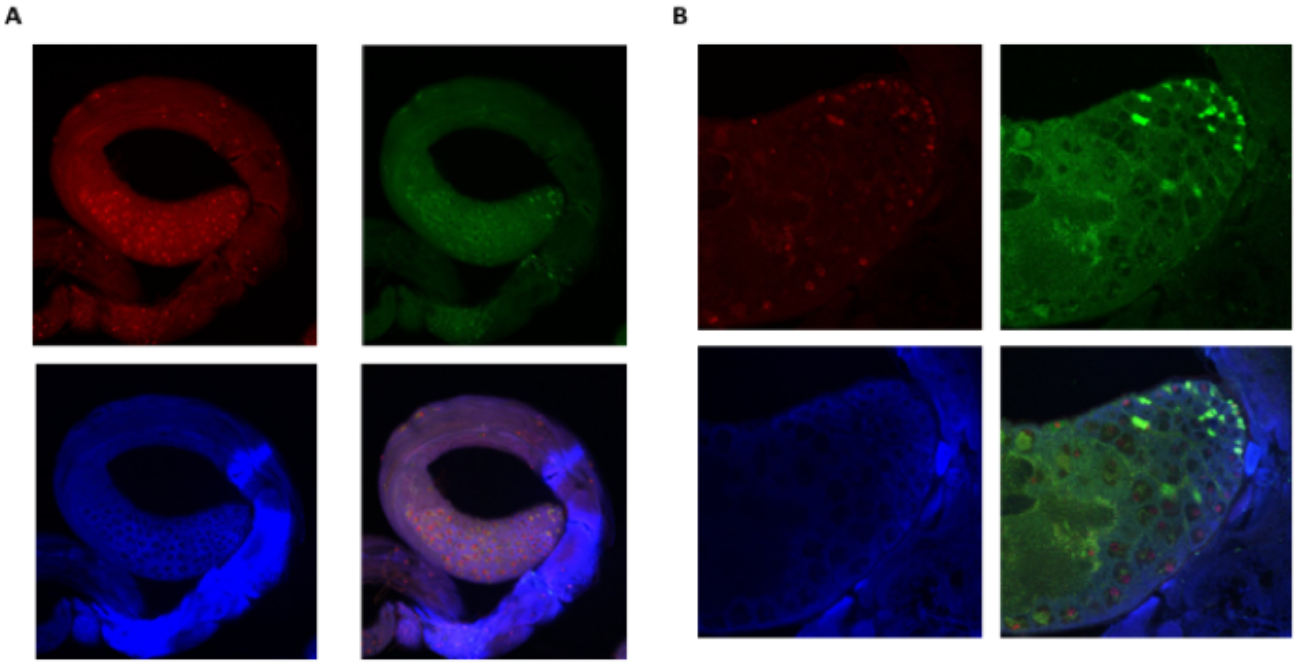

Figure 4. RIB is expressed in adult Drosophila testes. (A) The adult testis has a spiral formation. The yellow cells indicate RIB expression in the somatic cells. Germ cells are blue (anti-VASA); somatic cells are green (anti-Traffic Jam) and RIB is in red. (B) Magnified view of the adult testis. The cells that are yellow indicate RIB expression in the somatic cells. Germ cells are blue (anti-VASA); somatic cells are green (anti-TJ) and RIB is in red. RIB is present in somatic cells and germ cells. This data was compiled form work done previously (A. Droste and J. Jemc, unpublished).

facilitates protein-protein interactions, as well as a Pipsqueak domain for DNA binding (Figure 3). Previous work has demonstrated that RIB regulates the formation of the trachea and salivary glands in *Drosophila melanogaster* (Bradley and Andrew, 2001; Shim et. al., 2001; Loganathan et. al., 2016). In these contexts, it has been shown that *rib* encodes a transcription factor responsible for regulating changes in cell shape required for epithelial migration and tissue morphogenesis. Interestingly, others have pondered the possibility that RIB functions downstream of Mitogen Activated Protein Kinase (MAPK) signaling pathway. This assertion stems from the discovery of seven MAPK consensus phosphorylation sites in RIB. Additionally, studies have demonstrated a similarity between mutant phenotypes for *rib*, and members of the Fibroblast Growth Factor (FGF) – MAPK signaling pathway (Bradley and Andrew, 2001). While there is no mammalian homolog for *rib*, it's potential link to the highly-conserved MAPK signaling pathway makes any work to elucidate its role in stem cell function in *Drosophila* potentially relevant to mammalian development.

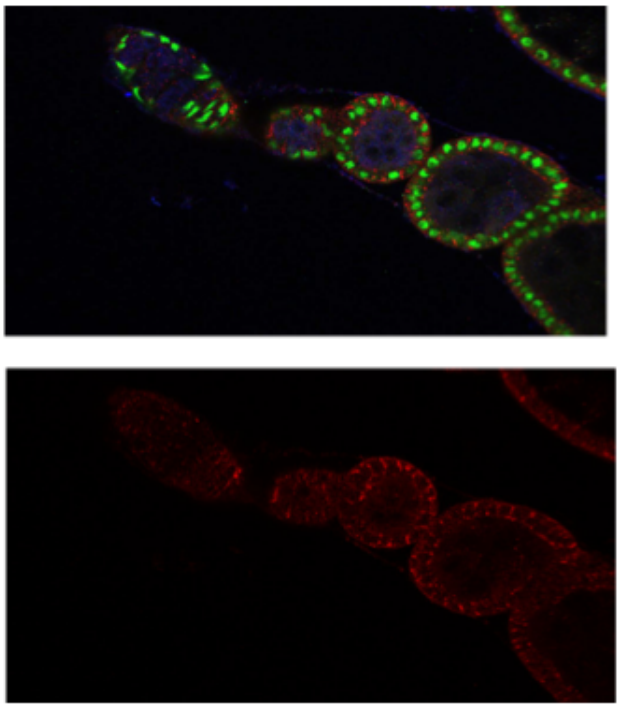

Figure 5. RIB is expressed in adult Drosophila ovaries. RIB does not appear nuclear in the ovary. In the top image, germ cells are blue (anti-VASA), somatic follicle cells are green (anti-Traffic Jam), and RIB is in red. The bottom image shows RIB alone. (A. Droste and J. Jemc, unpublished).

Further work in our lab revealed that RIB works to promote the coalescence of somatic gonadal cells during embryonic development (Silva et. al, 2016). Interestingly, *rib* continues to be expressed and localizes to the nucleus of germ cells and somatic cells in the adult testes, while there is only faint immunostaining for *rib* at the cell periphery in adult ovaries (Figures 4 & 5, A. Droste and J. Jemc, unpublished data). Although *rib* is expressed in the adult gonad, the role of *rib* in germ cells or somatic cells in the adult gonad is unclear.

RIB's difference in localization in adult testes compared to ovaries may yield some clue as to its function. In conjunction with the observation that RIB is chiefly found in adult testes, it is possible that RIB is serving some role in the maintenance of adult gonad morphology in *Drosophila*. It has been established in the literature that sexual dimorphism, and subsequently gonad morphology, is something that must be continuously maintained in adults (Ma *et al*., 2014). As an example, *chronologically inappropriate morphogenesis* (*chinmo*) is a transcription factor necessary for the maintenance of sex differences in adult *Drosophila*. In its absence, somatic cyst stem cells in an adult testis begin to produce daughter cells that resemble those found in an adult ovary (Ma *et al.*, 2014). Additionally, it has been shown that an ectopic expression of *chinmo* was able to push adult *Drosophila* ovarian somatic cells to adopt a male identity (Ma et al., 2016). Given the balance of signaling factors that work in concert to maintain proper adult gonad morphology and function during gametogenesis in *Drosophila*, it is important to explore what if any role RIB may be playing in upholding that delicate balance. To that end this project aims to explore the role of RIB in gametogenesis in *Drosophila* gonads. It is hypothesized that RIB plays a role in gametogenesis in adult testes and ovaries. The structure of this study is as follows.

Hypothesis:
rib plays a critical role in gametogenesis in *Drosophila melanogaster*.

Aim 1: To examine the effects of *rib* overexpression on the maintenance of the adult gonad.

Aim 1a: Determine the effect of *rib* overexpression on gonad development. *rib* was overexpressed using the Gal4/UAS system in the germ cells and somatic cells of the gonad. Gonads were dissected at 1-3 days and 8-10 days post-eclosion and immunostained to detect any changes in both germline cells and somatic cells via epi-fluorescent microscopy.

Aim 1b: Examine the effects of *rib* overexpression on adult gonad morphology. *rib* was overexpressed using the Gal4/UAS system in the presence of $Gal80^{TS}$, allowing overexpression to be limited to adulthood based on a temperature shift. Gonads were dissected at 1-3 days and 8-10 days post-eclosure and immunostained to detect any changes in both germline cells and somatic cells of adult testes and ovaries via epi-fluorescent microscopy.

Aim 2: Establish the effects of *rib* knockdown on proper development and function. *rib* was knocked down by RNAi (Gal4/UAS) specifically in the germ cells and somatic cells of the gonad. Gonads were dissected at 1-3 days and 8-10 days post-eclosion and immunostained to detect changes in both germline cells and somatic cells of adult testes and ovaries via confocal microscopy.

CHAPTER 2:

Results:

Aim 1a: Determine the effect of *rib* overexpression on gonad development.

rib is required for gonad morphogenesis in *Drosophila melanogaster* (Weyers et. al., 2011). Work in our lab revealed that *rib* works to promote the coalescence of somatic gonadal cells during embryonic development (Silva et. al, 2016). Of note, *rib* continues to be expressed and localizes to the nucleus of germ cells and somatic cells in the adult testes, while there is only faint immunostaining for *rib* at the cell periphery in adult ovaries (Figures 4&5). Although *rib* is expressed in the adult gonad, its role in germ cells or somatic cells in the adult gonad is unclear. To that end this project aimed to explore the role of *rib* in gametogenesis in *Drosophila* gonads. It was hypothesized that *rib*'s presence in adult gonads would play a role in gametogenesis in adult testes and ovaries.

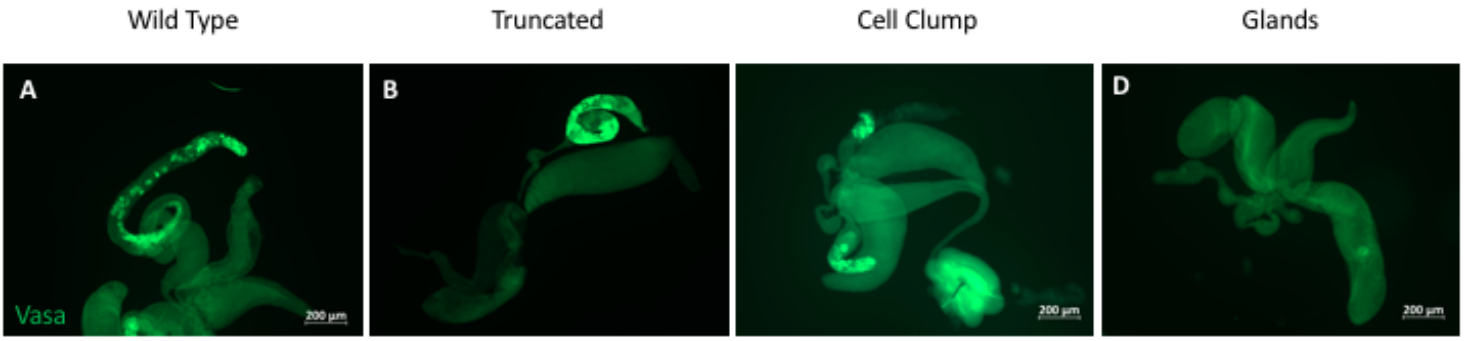

Figure 6. Representative images of male testes phenotypes. Scale Bar = 200µm. This figure shows representative images of each phenotype scored for male samples. **A.** Wild Type (WT) testes assumed the normal spiral shape. **B.** Truncated testes do retain some of the spiral shape, but are much smaller in size. **C.** Cell clumps are an amorphous group of cells, which stain positive for the germ cell marker, Vasa. **D.** A sample is scored as Gland when testes have either not developed, or they are so delicate that they are destroyed during dissection. All samples in this image have been immunostained with a marker of germ cells, (anti-Vasa).

We took a three-fold approach towards this project; 1. overexpression of *rib* throughout development and adulthood of male and female *Drosophila* to determine its effect(s) on gonad development, 2. restriction of *rib* overexpression to development post eclosion using the Gal4/UAS; $Gal80^{ts}$ system to determine the effect on adult gonads, 3. knockdown of *rib* via RNAi to elucidate which cells require *rib* for development.

Male gonad morphology was scored into one of four categories: WT describing normal and full development of the testes; Truncated denoting a phenotype in which the testes develop incompletely while retaining some ultrastructure; Cell Clump in which only an amorphous group of cells staining positive for VASA are observed; and Glands in which the phenotype is so strong that testes are not detected, (Figure 6). All testes were counted individually. Female images were analyzed by quantification of the number of ovarioles per ovary. As with the testes, all ovaries were counted individually.

Given previous work that suggests *rib* is important for proper gonad development, our first aim in this project was to investigate to what extent expression levels of the gene impacted development. Manipulations of *rib* expression for the varying parts of this project were carried out using the Gal4/UAS system first described by Brand and Perrimon in 1993. In this system Gal4 is targeted to cells/tissue of interest by using drivers. When crossed to a male containing an upstream activation sequence (UAS) which has Gal4 binding sites in its promoter linked to a gene of interest, it is possible to see the expression of the target gene in cells/tissues where Gal4 is readily expressed (Brand and Perrimon, 1993).

rib was first overexpressed in germ and somatic cells using the Gal4/UAS system with the drivers *nanos* (*nos*-Gal4) and traffic jam (*tj*-Gal4) drivers respectively to reveal its overall effects on gonad development. When overexpression was targeted to male germ cells, the results

were dramatic. Control flies in which *nos*-Gal4 is crossed to white^{1118} (w^{1118}) flies exhibited WT testes structure with a coiled, blind-ended tubular structure that stains positive for both germ and

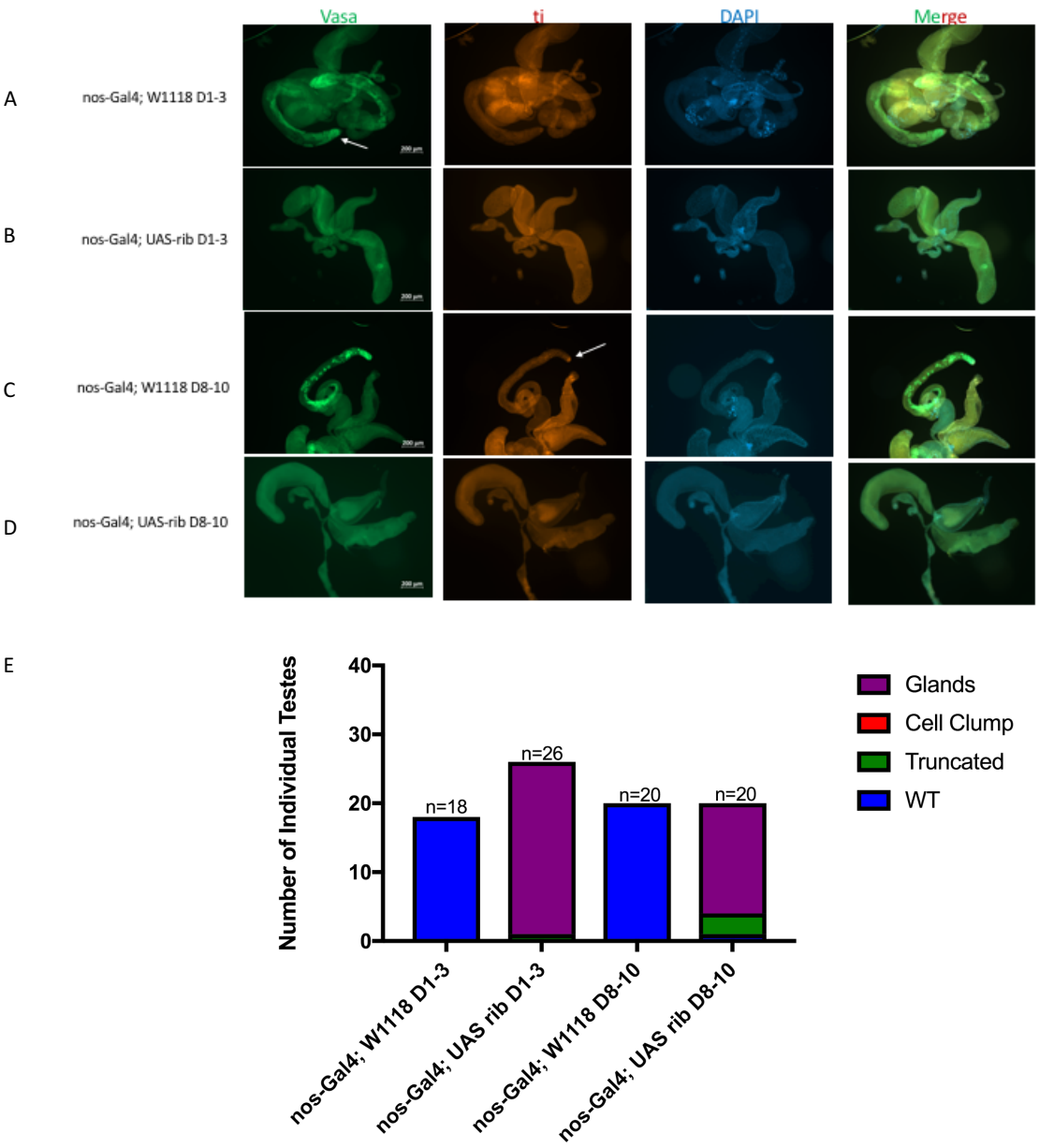

Figure 7. Overexpression of *rib* in germ cells alters testes formation. Scale Bar = 200µm. Male samples A-D immunostained with antibodies for Vasa for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*nos*-Gal4; w^{1118}) retain their wild type structure at 1-3 days post-eclosion. **B.** *nos*-Gal4; UAS-*rib* males do not form stable testes at D1-3. **C.** Control flies at D8-10. **D.** *nos*-Gal4; UAS-*rib* flies do not form stable testes at 8-10 days post-eclosion. **E.** Graph showing that mutants only yield accessory glands during dissection.

somatic cells via antibodies for VASA and TJ at both 1-3 and 8-10 days post eclosion (Figure 7A, C&E). When comparing male controls to age appropriate *nos*-Gal4; UAS-*rib* overexpression flies we see an overall lack of testes. That is to say that when the flies were dissected, only accessory glands are found reliably. In a couple of cases truncated testes were found, but this is more the exception to the rule (Figure 7B&D). Phenotypic scoring mirrors these results (Figure 7E).

Male samples in which *rib* overexpression was targeted to somatic cells also showed notable phenotypic differences relative to their controls. We see that for both 1-3 and 8-10 days post eclosion that control flies (*tj*-Gal4; w^{1118}) exhibited WT testis morphology (Figure 8A, C&E). Males that have undergone somatic cell overexpression (*tj*-Gal4; UAS-*rib*) at both time points only form clusters of cells which stain positive for germ and somatic cell biomarkers (Figure 8B&D). More rarely we see that these flies sometimes form no detectable testes, or they only form truncated testes (Figure 8E).

Next *rib* overexpression was targeted to germ cells in females. Control flies (*nos*-Gal4; w^{1118}) formed ovaries with normal morphology, immunostained positive for both germ and somatic cells, and had a WT ovariole count at both 1-3 and 8-10 days post eclosion (Figure 9A, D&E). In particular, WT ovary morphology for this project consisted of a grouping of ovarioles (chains of ova at varying stages of development stemming from the germarium). In *rib* overexpression females (*nos*-Gal4; UAS-*rib*) for both time points, ovaries show normal morphology relative to their controls (Figure 9B&D). When quantifying the number of ovarioles per ovary, there is a statistically significant ($p < 0.0001$) decrease in ovariole number as a result of *rib* overexpression relative to the control for 1-3 days post eclosion. This difference, however, is lost in the later time point (Figure 9E).

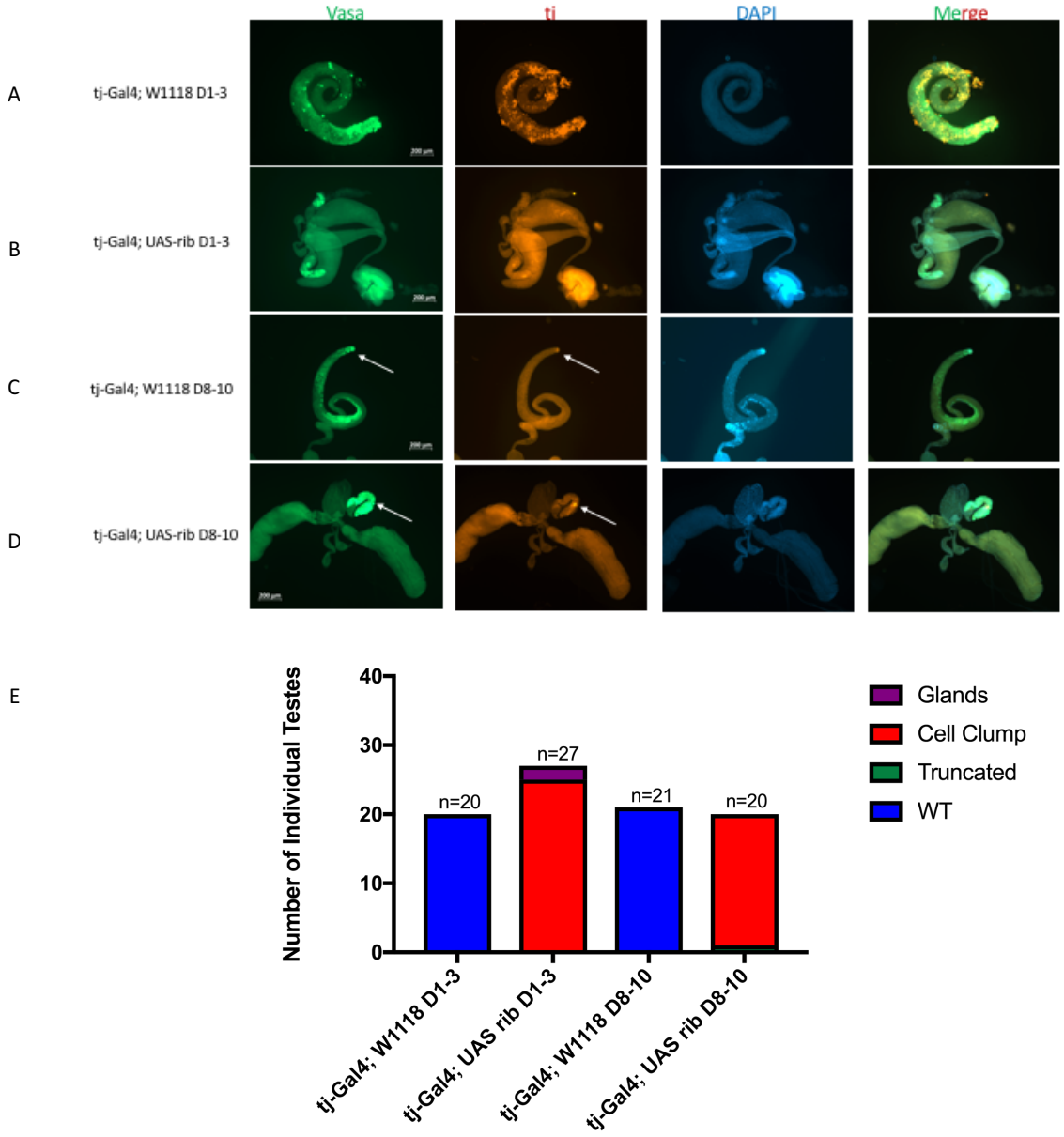

Figure 8. Overexpression of *rib* in somatic cells disrupts testes formation. Scale Bar = 200µm. Male samples A-D immunostained with antibodies for VASA for germ cells, Traffic Jam (*tj*) for somatic cells, and DAPI for nuclei. **A.** Control flies (*tj*-Gal4; w^{1118}) retain their wild type structure at 1-3 days post-eclosion. **B.** *tj*-Gal4; UAS-*rib* males form truncates testes and cell clumps at D1-3 that stain positive for VASA and TJ. **C.** Control flies at D8-10. **D.** *tj*-Gal4; UAS-*rib* flies exclusively form cell clumps that stain positive for VASA and TJ at 8-10 days post-eclosion. **E.** Graph showing that mutants only yield mainly cell clumps at both time points.

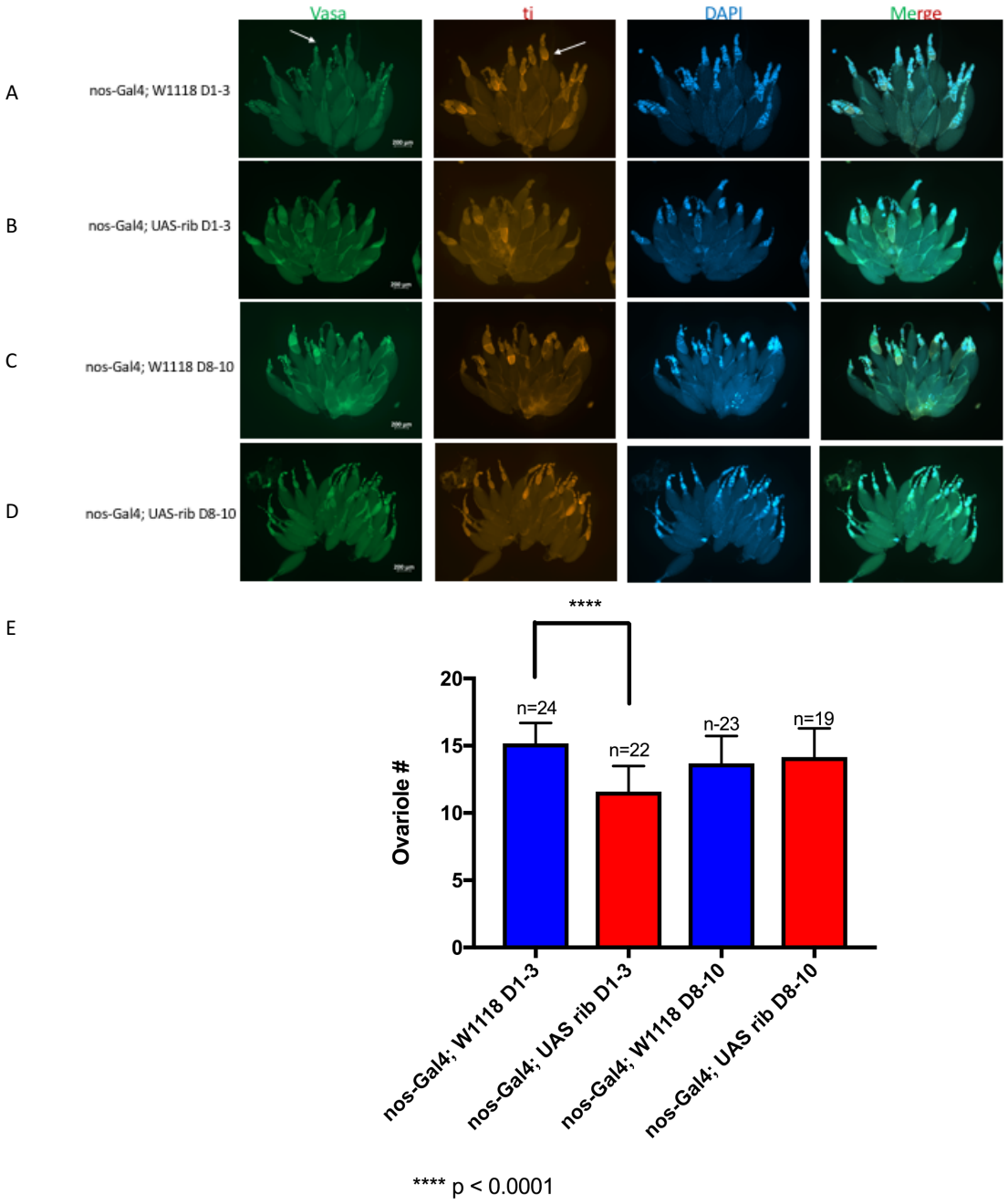

Figure 9. *rib* overexpression in germ cells may decrease ovariole number in day 1-3 female flies. Scale Bar = 200µm. Female samples A-D immunostained antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*nos*-Gal4; w^{1118}) show their wild type structure at 1-3 days post-eclosion. **B.** *nos*-Gal4; UAS-*rib* ovaries show normal morphology at D1-3. **C.** Control flies at D8-10. **D.** *nos*-Gal4; UAS-*rib* ovaries show a normal morphology at 8-10 days post-eclosion. **E.** *nos*-Gal4; UAS-*rib* females show a significant decrease in ovariole number at Day 1-3, but not at Day 8-10.

Controls for female somatic cell *rib* overexpression (*tj*-Gal4; w^{1118}) were able to form ovaries at both 1-3 and 8-10 days post eclosion that have normal morphology, immunostain positive for germ and somatic cells, and have a WT ovariole count (Figure 10A, C& E). Female samples in which *rib* overexpression was targeted to somatic cells (*tj*-Gal4; UAS-*rib*) saw inhibited ovary development to the point that no structure was achieved at both 1-3 and 8-10 days post eclosion. Instead they only developed sacs of cells which immunostain positively for markers of germ and somatic cells (Figure 10B&D). Ovariole count for these samples was not possible to achieve as mutants form no ovarioles (Figure 10).

Conclusion:

In summary, the data support the hypothesis that overexpression of *rib* affects gonad development. The effects are seen in males where overexpression is targeted to both the germ and somatic cells, and in females where overexpression is targeted to somatic cells, with some effect on ovariole count when targeted to early stage female germ cells. We see that increased levels of *rib* in germ and somatic cells appear to be detrimental to testes development.

It is possible that an increase of *rib* in germ cells could result in a failure to maintain germline stem cells and premature differentiation of germ cells. This leads to reduced gonad formation. *rib* overexpression in somatic cells may also affect hub formation or cyst stem cell maintenance and cell differentiation. With female samples we see that *rib* overexpression in somatic cells causes the improper development of ovaries. This may result from interference with proper somatic gonadal cell development as well as the establishment of niche structures.

CHAPTER 3:

Aim 1b: Examine the effects of *rib* overexpression on adult gonad morphology.

In our lab we have seen data which demonstrate the immunofluorescent detection of RIB in adult *Drosophila* testes, and its peripheral presence in adult ovaries, pointing to some potential role in adult gonad maintenance. As such we hypothesized that this protein's overexpression would impact adult gonad maintenance (Figures 4 & 5). *rib* was overexpressed in adult germ and somatic cells using the Gal4/UAS system with the drivers *nos*-Gal4 and *tj*-Gal4 drivers respectively. The Gal80^{ts} repressor was implemented under the control of the tubulin promoter as a temperature sensitive inhibitor if Gal4 allowing us to ensure overexpression only occurred post eclosion, or when desired.

Control flies for *rib* overexpression targeted to germ and somatic male cells (*nos*-Gal4; Gal80^{ts}; w^{1118} and *tj*-Gal4; Gal80^{ts}; w^{1118} respectively) were able to produce testes with the WT blunt ended tubular morphology that immunostained positive for germ and somatic cells at 1-3 Days post eclosion (Figure 11A, C&E). In males in which adult germ cells were targeted (*nos*-Gal4; Gal80^{ts}; UAS-*rib*), they were unable to form testes at 1-3 days post eclosion relative to its appropriate control (Figure 11A, B&E). Only glands were able to be retrieved. In some cases a truncated structure was affixed to the accessory glands, but staining for germ, somatic, and nuclei was inconclusive.

When temperature restricted *rib* overexpression was targeted to adult somatic cells, males (*tj*-Gal4; Gal80^{ts}; UAS-*rib*) were able to form full testes with a WT morphology. These testes had normal staining patterns for germ and somatic cell lines in developed gonads (VASA and TJ respectively). For the most part testes from both the control and mutant Day 1-3 testes were classified as WT in terms of morphology and immunostaining for germ and somatic cells.

(Figure 11C, D&E). Of note data were only able to be collected for the Day 1-3 time point for both adult germ and somatic cell targeted *rib* overexpression samples due to a high mortality seen in both male and female samples. In many cases, flies would die within the 1-3 days post eclosion time point.

Female control samples for adult germ and somatic cells targeted for *rib* overexpression (*nos*-Gal4; Gal80^{ts}; w^{1118} and *tj*-Gal4; Gal80^{ts}; w^{1118} respectively) retain WT morphology, immunostaining for VASA and TJ, and WT ovariole count (Figure 12A, C&E). Female *nos*-Gal4; Gal80^{ts}; UAS-*rib* flies produced ovaries with a WT morphology, and immunostaining pattern for VASA and TJ. Of note, there was a significant decrease ($p = 0.0068$) in the ovariole count in these flies (Figure 12E). Females targeted for *rib* overexpression in adult somatic cells (*tj*-Gal4; Gal80^{ts}; UAS-*rib*) exhibited quite a unique phenotype relative to their control counterparts at Day 1-3 post eclosion. Ovaries from these flies formed structures that seemed to mimic the ovarioles and egg chambers of WT ovaries.

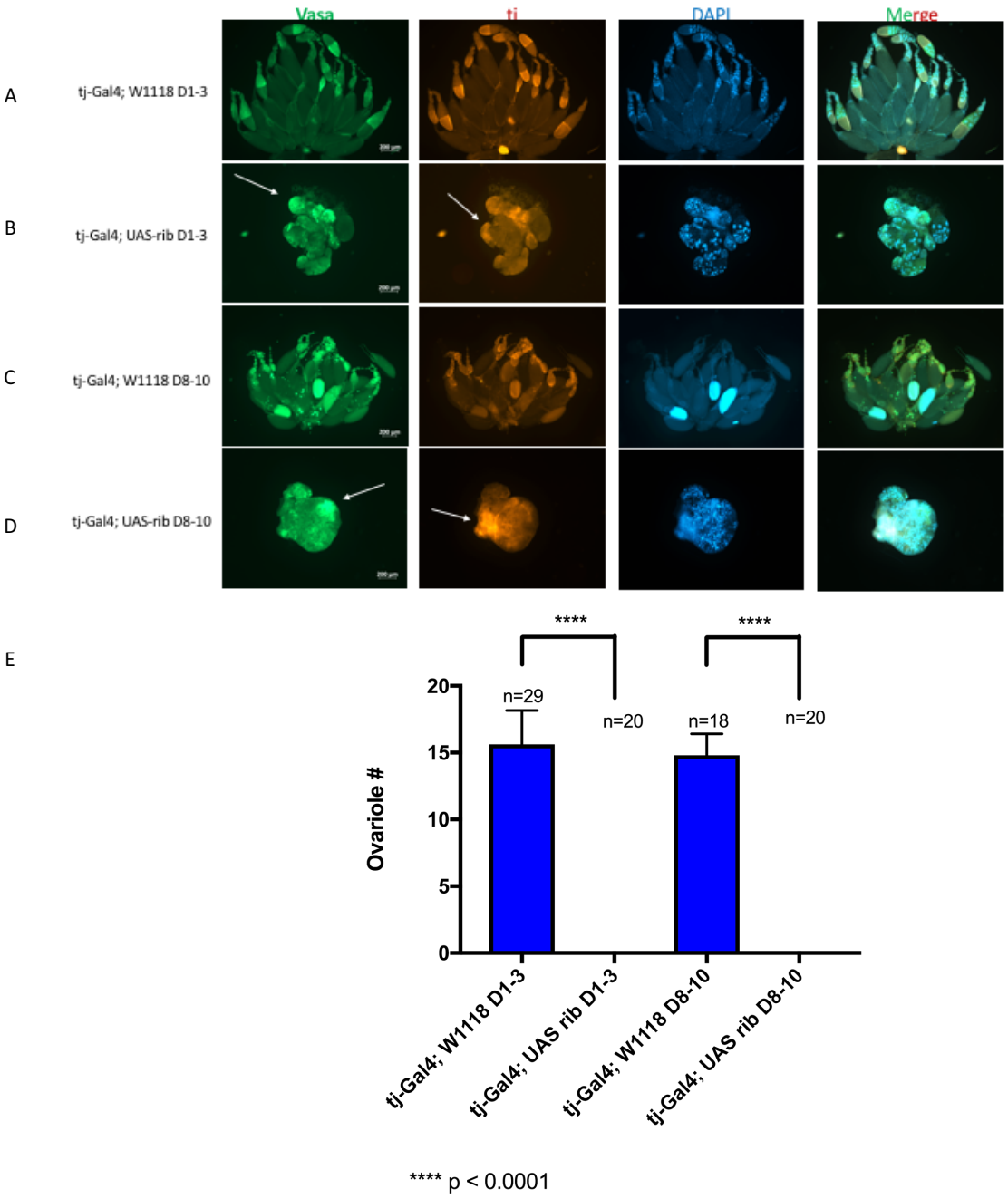

Figure 10. *rib* overexpression in somatic cells impedes ovary development. Scale Bar = 200µm. Female samples A-D immunostained with antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*tj*-Gal4; w^{1118}) show their wild type structure at 1-3 days post-eclosion. **B.** *tj*-Gal4; UAS-*rib* female flies for cell sacks that stain positive for VASA and TJ, but never form antibodies at D1-3. **C.** Control flies at D8-10. **D.** This yields the same phenotype as in B. **E.** *rib* overexpression in somatic cells significantly decreases the ovariole/ovary ratio at both 1-3 and 8-10 days post-eclosion.

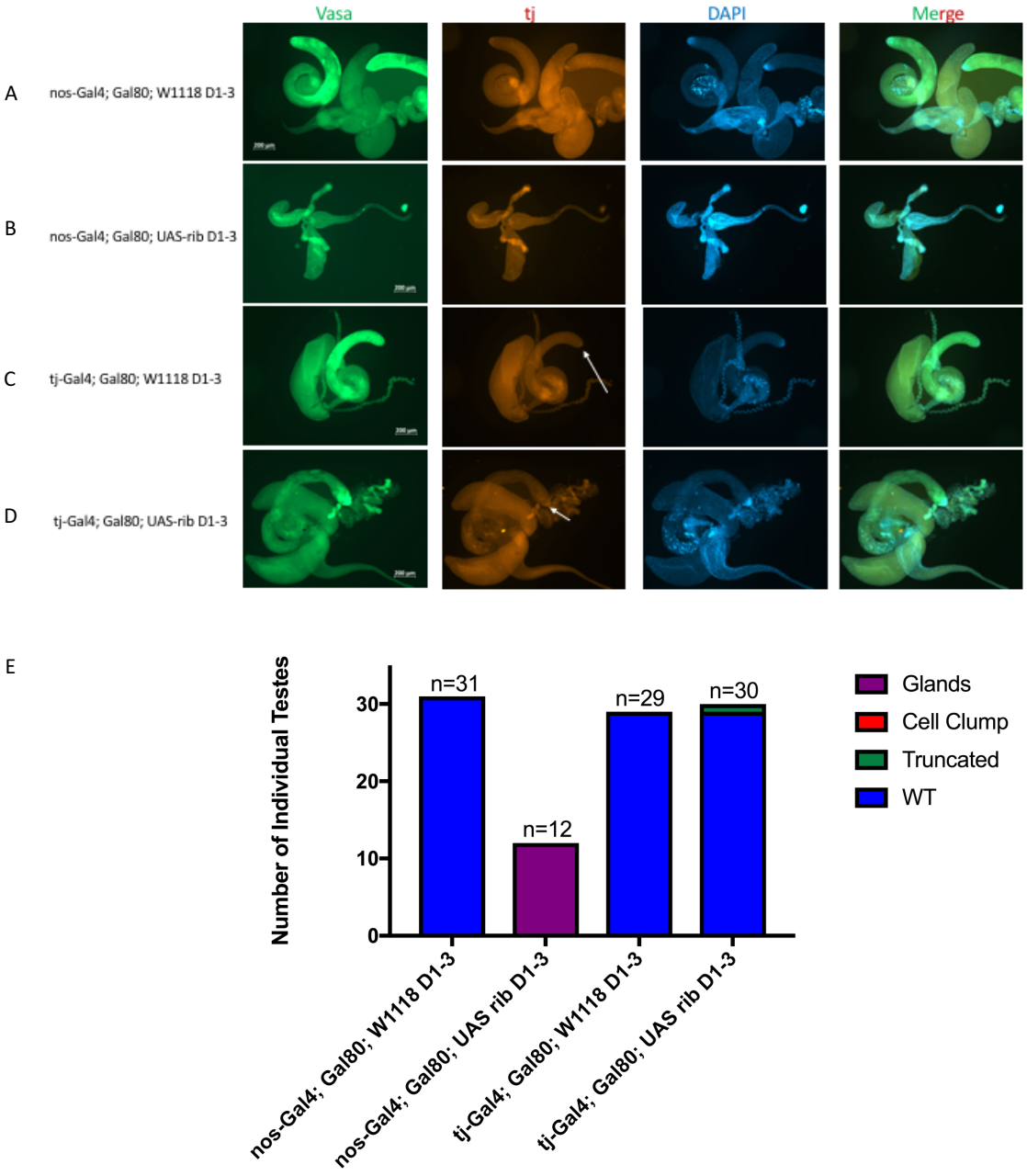

Figure 11. *rib* overexpression in adult germ and somatic cells impedes testis development. Scale Bar = 200µm. Samples A-D immunostained antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*nos*-Gal4; Gal80^{ts}; w^{1118}) show their wild type structure at 1-3 days post-eclosion. **B.** *nos*-Gal4; Gal80^{ts}; UAS-*rib* male flies fail to form stable testes by morphology at D1-3. **C.** Control flies (*tj*-Gal4; Gal80*ts*; w^{1118}) for adult overexpression targeted to the somatic cells at D1-3. **D.** *tj*-Gal4; Gal80*ts*; UAS-*rib* males form testis that appear to have normal morphology. **E.** This graph shows that all male *nos*-Gal4; Gal80*ts*; UAS-*rib* males were found to only have accessory glands, and no gonads. All other groups formed WT coiled testis.

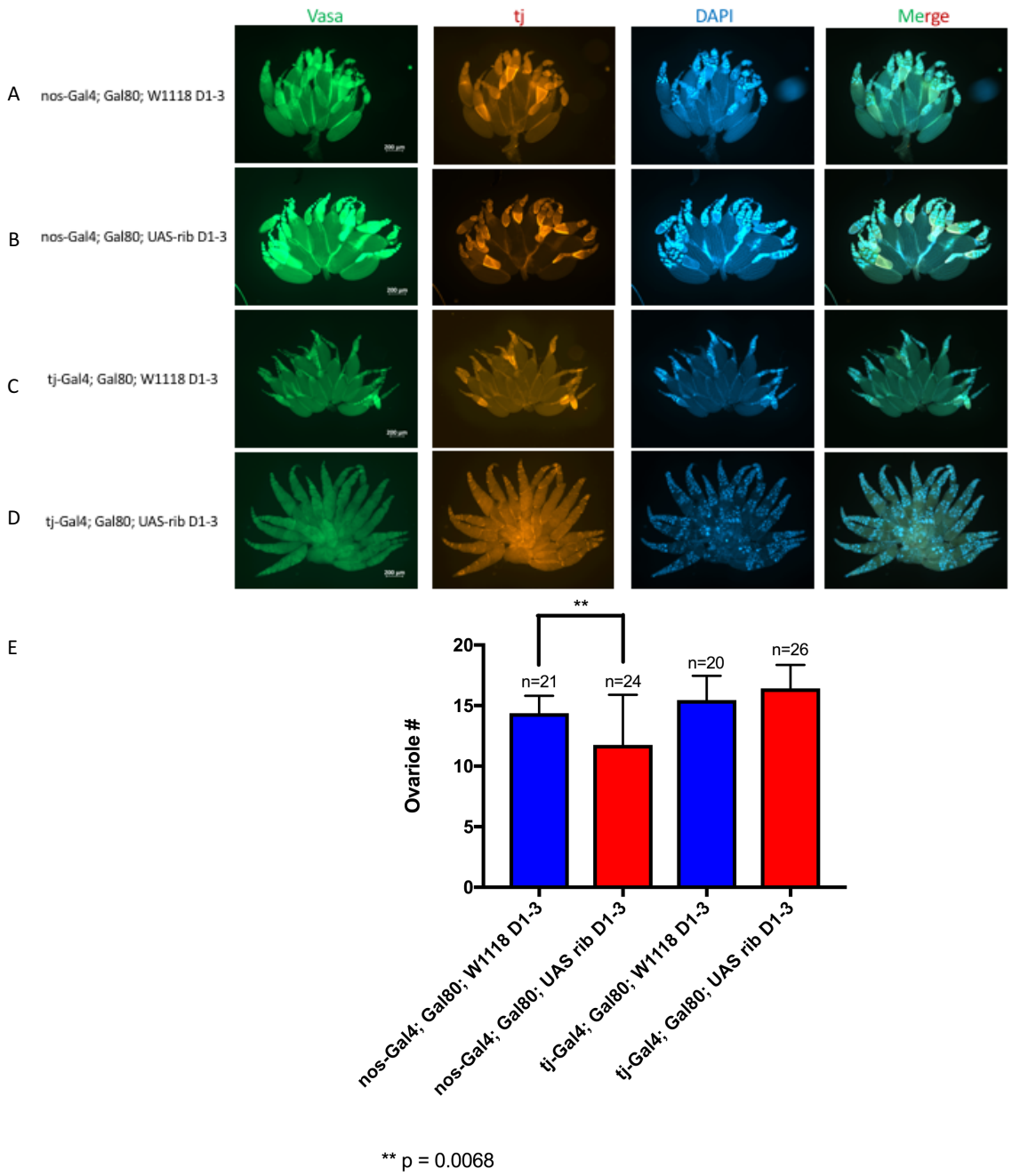

Figure 12. *rib* overexpression in adult germ and somatic cells alters ovary development. Scale Bar = 200µm. Samples A-D immunostained antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*nos*-Gal4; Gal80*ts*; w^{1118}) show their wild type structure at 1-3 days post-eclosion. **B.** *nos*-Gal4; Gal80*ts*; UAS-*rib* female flies form morphologically WT ovaries with normal staining of both germ and somatic gonadal cells at D1-3. **C.** Control flies (*tj*-Gal4; Gal80*ts*; w^{1118}) for adult *rib* overexpression targeted to somatic cells shows a normal morphology. **D.** Adult overexpression in somatic cells yields a new phenotype in which mutant females assume a palm-like morphology. **E.** This graph shows that there is a decrease in ovariole number when *rib* is overexpressed in adult germ cells.

Staining for germ and somatic cells was seen, but gonads from this genotype resembled a palm tree by morphology, and did not appear to be functioning gonads, irrespective of ovariole count analysis (Figure 12C, D&E).

Conclusion:

The data here support the hypothesis that *rib* overexpression in adult *Drosophila* males and females would impact gonad maintenance. Specifically, we see this in *nos*-Gal4; Gal80^{ts}; UAS-*rib* males which are unable to form proper testes and in females which do see a decrease of ovariole count, as well as in *tj*-Gal4; Gal80^{ts}; UAS-*rib* females which create ovaries lacking proper morphology. The continued interference with testes development indicates that the issue is occurring between larval stages and adulthood. The morphology seen in ovaries where *rib* overexpression was targeted to somatic cells points to interference with proper germ cells development, and needs to be explored further.

CHAPTER 4:

Aim 2: Establish the effects of *rib* knockdown on proper development and function.

Having seen that *rib* overexpression has such an impact on gonad development and adult gonad maintenance we next hypothesized that if *ribbon* was knocked down by use of RNAi we would also observe deleterious effects on gonad development. Germ cells were targeted using the *nos*-Gal4 driver crossed to males expressing UAS-*rib* RNAi. Control flies in this group (*nos*-Gal4; w^{1118}) formed WT testes which stained positive for germ and somatic cells (Figure 13A, C&E) at both 1-3 and 8-10 days post eclosion. Knocking down *rib* in male germ cells (*nos*-Gal4; UAS-*rib* RNAi) did not impact the ability to form testes with complete morphology and positive immunostaining for germ and somatic cells at either 1-3 or 8-10 days post eclosion (Figure 13B, D&E). We observed the production of testes that were slightly wider looking at 1-3 days post eclosion, but this variation subsided by day 8-10 (Figure 13B). Ultimately, most all of the testes from this genotype were scored as WT irrespective of time point (Figure 13E).

rib knockdown was next targeted to somatic cells by using a *tj*-Gal4 driver in a female crossed to a male carrying UAS-*rib* RNAi. Here again we see that mutant (*tj*-Ga4; UAS-*rib* RNAi) testes dissected at both 1-3 days and 8-10 days post eclosion exhibit a WT morphology and positive staining for germ and somatic cells, much like their control (*tj*-Gal4; w^{1118}) counterparts at both time points (Figure 14A-D). Of note, all samples had their phenotypes scored as WT (Figure 14E).

When looking at female samples where *rib* knock down was targeted to germ cells, one can see that ovary development remains unperturbed in control flies (*nos*-Gal4; w^{1118}). They exhibit normal morphology and immunostaining for germ and somatic cells. One can note the presence of intact germaria, as well as egg chambers containing ova in various stages of

development as well in *rib* knock-down females (*nos*-Gal4; UAS-*rib* RNAi) as well as WT immunostaining (Figure 15A-D). When looking at the ovariole number of ovaries from both control and mutant flies, an ANOVA analysis with Tukey's post hoc test reveals no significant difference in the number of ovarioles counted. This also occurs irrespective of whether the samples were dissected at 1-3 or 8-10 days post eclosion (Figure 15E).

Controls for *rib* knockdown targeted to female somatic cells (*tj*-Gal4; w^{1118}) produce ovaries with WT morphology, immunostaining for germ and somatic cells, and ovariole count across both time points (Figure 16A, C&E). There is no observable difference in ovary morphology or ovariole number between *tj*-Gal4; UAS-*rib* RNAi females and their control counterparts across both time points (Figure 16).

Conclusion:

rib knockdown resulted in almost no variation from controls whether targeted to germ or somatic cells in males and females across both time points. Given RIB's established role in the ensuring proper gonad development, it was anticipated that it's knockdown would severely impact gonad morphology (Silva *et al.*, 2016). It is possible that the RNAi line used was not effective.

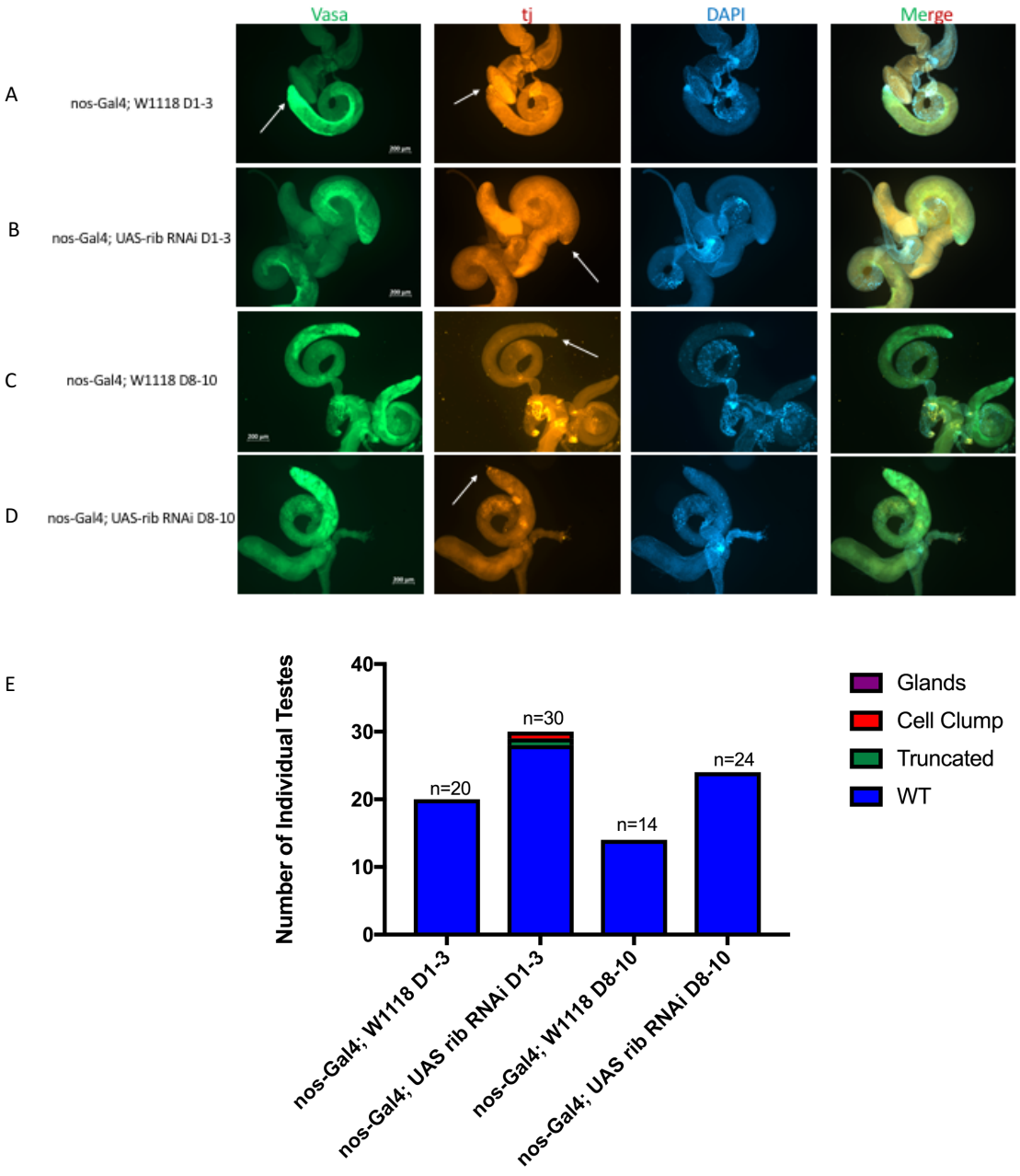

Figure 13. RNAi knockdown of *rib* in germ and somatic cells does little to alter testes development. Scale Bar = 200µm. Male samples A-D immunostained with antibodies with VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*nos*-Gal4; w1118) retain their wild type structure at 1-3 days post-eclosion. **B.** *nos*-Gal4; UAS-*rib*-RNAi testes retain a Wild Type (WT) Morphology at D1-3. **C.** Control flies at D8-10. **D.** *nos*-Gal4; UAS-*rib*-RNAi testes retain a WT morphology at 8-10 days post-eclosion. **E.** Graph demonstrating the occurrence of each of 4 phenotypes. All variants show a WT morphology irrespective of genotype.

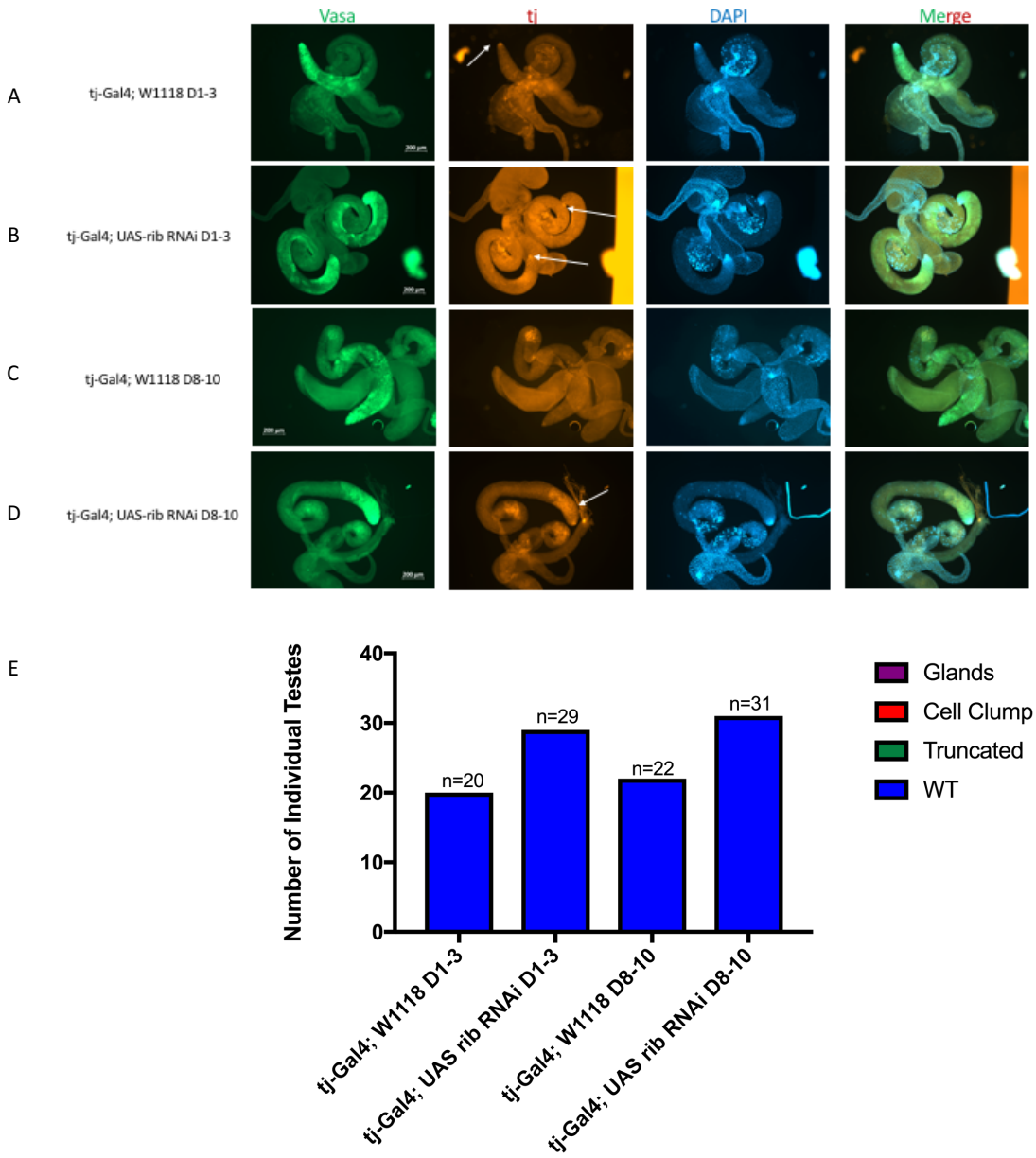

Figure 14. RNAi knockdown of *rib* in somatic cells does not alter testes structure. Scale Bar = 200µm. Male samples A-D immunostained with antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*tj*-Gal4; w1118) retain their wild type structure at 1-3 days post-eclosion. **B.** *tj*-Gal4; UAS-*rib*-RNAi testes retain a Wild Type (WT) Morphology at D1-3. **C.** Control flies at D8-10. **D.** *tj*-Gal4; UAS-*rib*-RNAi testes retain a WT morphology at 8-10 days post-eclosion. **E.** Graph demonstrating the occurrence of each of 4 phenotypes. All variants show a WT morphology irrespective of genotype.

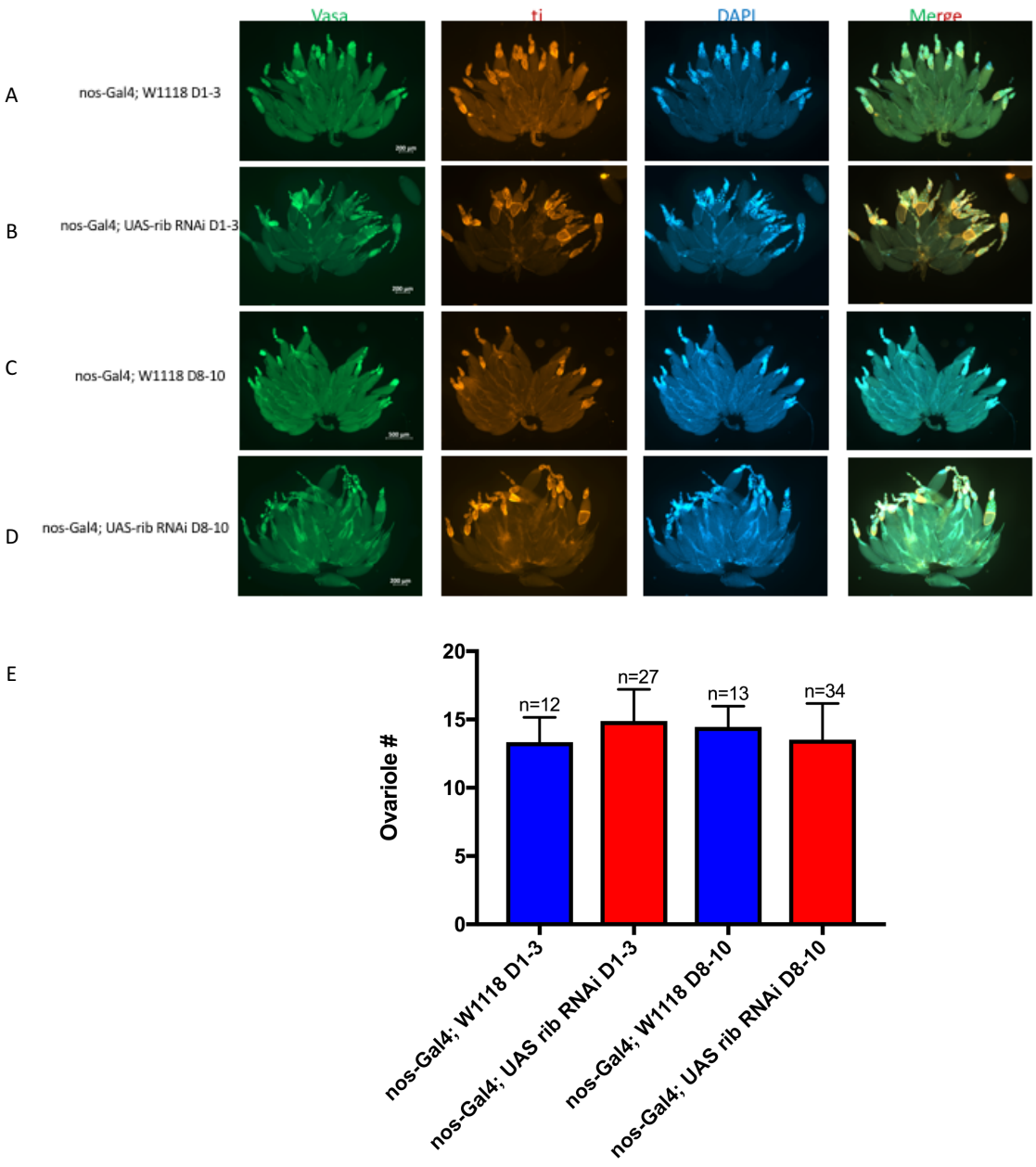

Figure 15. RNAi knockdown of *rib* in germ cells does not alter ovary structure. Scale Bar = 200µm. Female samples A-D immunostained with antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*nos*-Gal4; w1118) show their wild type structure at 1-3 days post-eclosion. **B.** *nos*-Gal4; UAS-*rib*-RNAi ovaries retain a Wild Type (WT) Morphology at D1-3. **C.** Control flies at D8-10. **D.** *nos*-Gal4; UAS-*rib*-RNAi ovaries retain a WT morphology at 8-10 days post-eclosion. **E.** Graph showing the average number of ovarioles per ovary. Ovariole count remains unchanged.

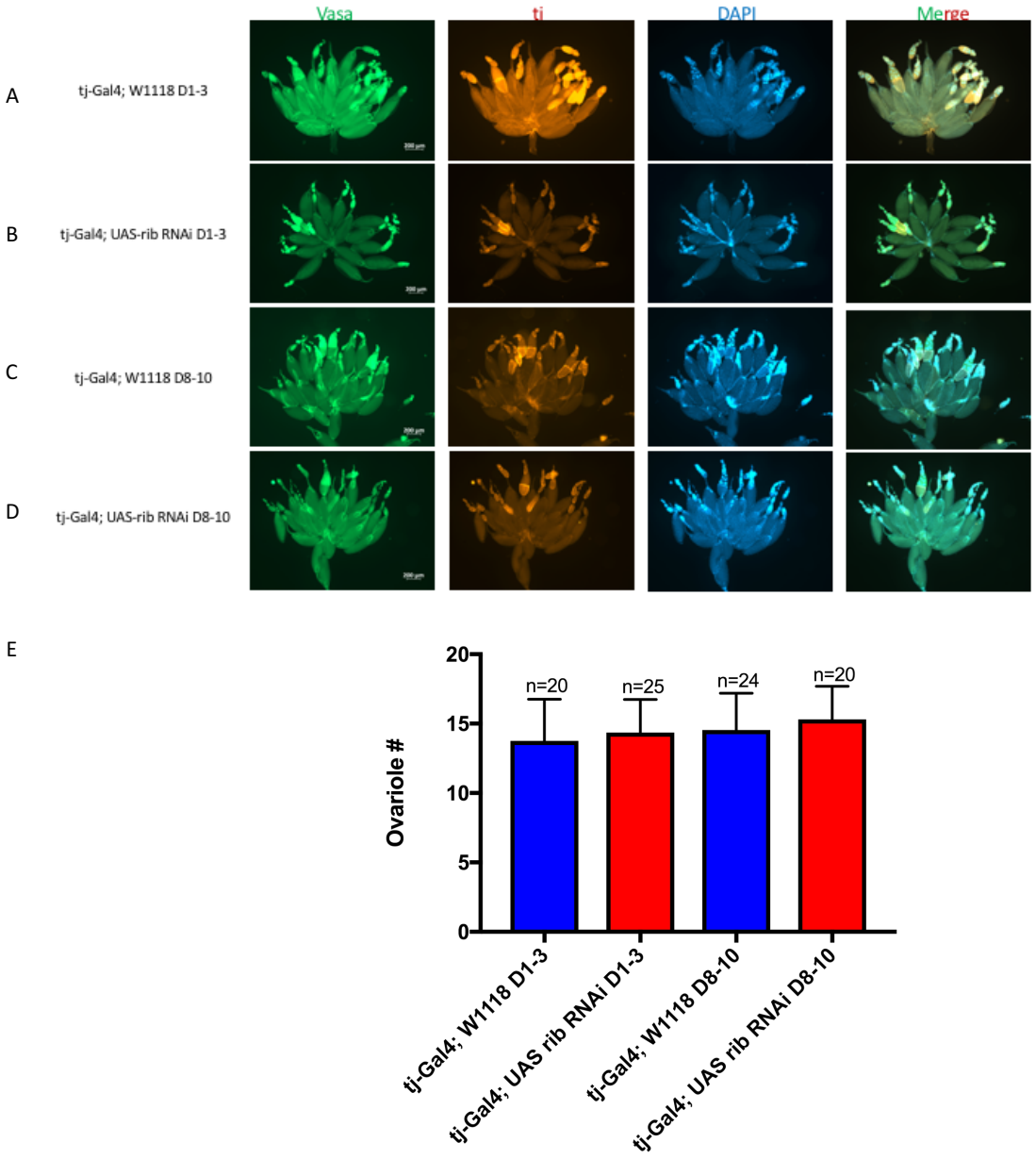

Figure 16. RNAi knockdown of *rib* in somatic cells does not alter ovary structure. Scale Bar = 200µm. Female samples A-D immunostained with antibodies for VASA for germ cells, Traffic Jam for somatic cells, and DAPI for nuclei. **A.** Control flies (*tj*-Gal4; w1118) show their wild type structure at 1-3 days post-eclosion. **B.** *tj*-Gal4; UAS-*rib*-RNAi ovaries retain a Wild Type (WT) Morphology at D1-3. **C.** Control flies at D8-10. **D.** *tj*-Gal4; UAS-*rib*-RNAi ovaries retain a WT morphology at 8-10 days post-eclosion. **E.** Graph showing the average number of ovarioles per ovary. Ovariole count remains unchanged.

CHAPTER 5:

Discussion:

The aim of this study was to elucidate what, if any role RIB plays in the establishment and maintenance of adult gonads with proper structure and function in *Drosophila melanogaster*. While previous work has shown that RIB is required for the coalescence of germ cells and somatic gonadal cells during embryogenesis, a potential role for RIB in later stages of gonad development and in tissue homeostasis in the adult has not been explored (Weyers et. al., 2011; Silva et. al, 2016). Further, work in our lab has shown that RIB can still be found via immunostaining in adult testes. Additionally, it is found in the periphery of adult ovaries (Figures 4&5). This led us to explore the role RIB plays in the maintenance of adult gonads in *Drosophila*.

We took a three-fold approach towards this project; 1. overexpression of *rib* throughout development and adulthood of male and female *Drosophila* via the Gal4/UAS-rib system to determine its effect(s) on gonad development, 2. restriction of *rib* overexpression to development post eclosion using the Gal4/UAS; Gal80^{ts} system to determine the effect on adult gonads, 3. knockdown of *rib* via RNAi to elucidate which cells require *rib* for development.

Aim 1a: Determine the effect of *rib* overexpression on gonad development.

rib overexpression throughout development yielded dramatic effects on male gonad development. When targeted to germ cells, *rib* overexpression impeded the flies' ability to develop testes, if any at all (Figure 7). Several attempts were made to capture intact testes with both a posterior and mid-line approach during dissection with only accessory glands able to be isolated. The more likely conclusion here is that overexpression leads to the formation of gonads that fail to complete development. Increased levels of RIB appear to be detrimental to testes

development. Alternatively, increased levels of RIB could result in a failure to maintain germline stem cells, and premature differentiation of germ cells, again leading to a reduced gonad.

When *rib* overexpression was targeted to somatic gonadal cells we again observe significant defects in testis morphology and development. We observed either a truncated structure or no structure at all, irrespective of time point (Figure 8). These results suggest that increased levels of RIB in somatic gonadal cells is also detrimental to gonad development. In males, somatic gonadal cells make up the male stem cell niche, known as the hub, raising the possibility that hub formation or maintenance could be affected. However, analysis of gonads overexpressing *rib* during larval stages reveal that the hub still appears to be present (U. Khan and J. Jemc Mierisch, unpublished data). It is also feasible that increased *rib* expression could affect cyst stem cell maintenance of cyst cell differentiation, both of which would adversely affect germ cell development and differentiation.

Females on the other hand seem able to form ovaries with normal morphology when *rib* overexpression is targeted to germ cells, however, quantification of ovarioles reveals a statistically significant drop in number of ovarioles per ovary at 1-3 days post-eclosion. Interestingly, this effect seems to dissipate at the later time point (8-10 days post-eclosion). It is possible that the flies may be exhibiting some form of compensatory mechanism to normalize ovariole count in the later time point, and further studies are needed to explore this possibility (Figure 9).

Overexpression of *rib* in somatic gonadal cells in females resulted in the failure to form a structured gonad at both 1-3 and 8-10 days post-eclosion (Figure 10). All we see are amorphous blobs composed of germ and somatic cells. Ovaries appear to completely lack ovariole structures, suggesting that organized niche formation failed.

Studies have demonstrated that sexual is something that must be constantly maintained

throughout adulthood. The failure of presence of certain signaling factors can result in a change of sexual identity. This brings to mind the studies in which the absence of *chinmo* in adult *Drosophila* testes results in its somatic cells assuming a female identity (Ma *et al.*, 2014). Further it has been shown that the ectopic expression of *chinmo* in ovarian somatic cells can push them to adopt a male identity (Ma et al., 2016). It is interesting to note that others have shown that mutations in the transcription factor *doublesex (dsx),* or in this case its downstream targets in male and female Drosophila resulted in the development of ovaries and testes in which the morphology was in disarray (Clough *et al.*, 2014).

dsx has been shown to be alternatively spliced in males and females, and crucial in determining sexual dimorphism in *Drosophila*. Given the similarity in phenotype to these mutants, it would be prudent to begin to look into expression levels and protein presence of the male (dsx^{M}) and female (dsx^{F}) of *dsx* using RT-PCR and Western Blot analysis, respectively. Figure 17 is a schematic representation of the cell-autonomous and cell-nonautonomous signaling responsible for sexual dimorphism in SGPs and PGCs (adapted from review by Whitworth et. al., 2012). If differences in either *dsx* alternatively spliced version are detected in SGP isolates, it would be necessary to investigate downstream target using similar assays where possible. Cell autonomous signaling which gives rise to male/female identify in PGCs such as Plant Homeodomain Finger protein 7 (PHF7) in males and sex lethal (SXL) in females (reviewed in Whitworth et al., 2012).

It is also feasible that *rib* overexpression in somatic gonadal cells, where it is not observed to be nuclear in the ovary may interfere with proper somatic gonadal cell development and the establishment of niche structures. This possibility is currently being investigated in third instar larvae, as this is the stage at which the female stem cell niche begins to form in the ovary. The data support the hypothesis that rib overexpression would affect gonad development.

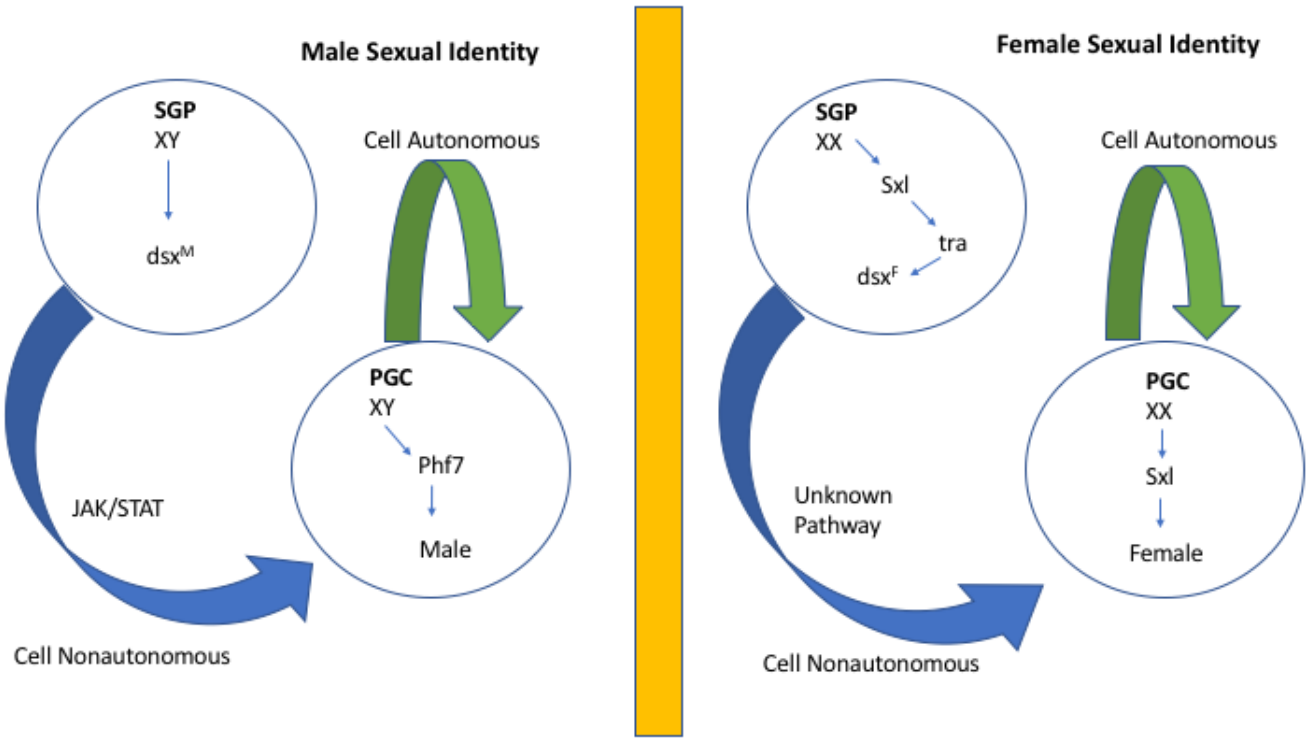

Figure 17. Signaling which Establishes Sexual Dimorphism in *Drosophila melanogaster* gonads. Here we see that in both male and female PGCs there are both cell nonautonomous signals from neighboring SGPs, and cell autonomous signals from within the PGC itself which drive the development of a male/female identity in these cells. tra = transformer. Adapted from information found in Whitworth *et al.*, 2012.

Aim 1b: Examine the effects of *rib* overexpression on adult gonad morphology.

Given that *rib* overexpression throughout development was observed to adversely affect gonad development, we next limited *rib* overexpression to adult gonads to examine the role of RIB in the maintenance of gonad function in the adult. Of note, this aspect of the project only included data from 1-3 days post-eclosion as mutant progeny were so sick they typically died at 1-2 days post eclosion. When targeted to the germ cells, males were still unable to form stable gonadal structures, indicating that the effects seen previously may occur between larval stages and adulthood (Figure 11). Targeting of *rib* overexpression to male adult somatic gonadal cells did not affect testes development.

Females whose adult germ cells were targeted for *rib* overexpression were able to form ovaries with somewhat normal morphology, but saw a statistically significant drop in ovariole number relative to their control (Figure 12). When *rib* overexpression was targeted to adult somatic cells, females were unable to form ovaries with normal morphology. Instead of ovarioles with ova at various stages of development, females formed rigid extensions giving the appearance of a palm tree, and eggs were not observed at the end of each ovariole (Figure 12). While ovariole count remains unchanged, the outward appearance would suggest issues with infertility, which were seen in fly crosses. Thus, increased levels of RIB in somatic cells interferes with proper germ cell development, which again could result from altered somatic gonadal cell sexual identity. In order to further explore the effects of *rib* overexpression in the adult gonad, studies are ongoing to obtain gonads at 8-10 days post-eclosion. Thus far the data seem to support the hypothesis that *rib* overexpression does in some way alter normal gonad function.

Aim 2: Establish the effects of *rib* knockdown on proper development and function.

Next *rib* was knocked down in both germ and somatic cells. The intention of this set of experiments was to reveal which cells require *rib* to ensure proper gonadal development. Interestingly, the results for both germ cell targeted and somatic cell targeted knockdowns showed no observable change in morphology or ovariole count relative to their sex and age appropriate controls (Figures 13-16). Given that previous work has demonstrated the importance of Ribbon's presence to ensure proper development, the likely conclusion here is that the RNAi line used may not have been effective. Future experiments in the lab will revisit this by adding enhancing agents such as overexpression of Dicer. Additionally, other RNAi lines will be tested for their efficacy in knocking down Rib levels and their effects on gonad development. One further experiment proposed with this study would be to test the efficacy of the RNAi line. One method by which this could be achieved is by using a tagged RIB antibody to detect the presence of RIB in somatic and germ cells targeted for *rib* knockdown.

In conclusion, the data support the overall hypothesis that altering *rib* expression levels will impact gonad development and/or maintenance. This study has begun to demonstrate a potential regulatory role for RIB in the proper maintenance and function of adult gonads in *Drosophila melanogaster*. Further studies are needed to conclusively establish what effects *rib* knockdown is having. The mechanism through which *ribbon* exhibits this impact also remains to be elucidated. Given that *rib* has shown to code for a transcription factor, the answer may lie in identifying its downstream effectors in germ and somatic cells in Drosophila gonads. Future studies should also look into niche formation, cell differentiation and proliferation.

CHAPTER 6:

Materials and Methods

Fly Stocks and Genotypes:

The following fly stocks were utilized in this project: *tj*-Gal4 (Guy *et al*., 2007), *nanos*-Gal4 (A4-2; Van Doren lab), UAS-*rib* (13.3; Bradley and Andrew, 2001) , UAS-*rib*-RNAi (Bloomington #50682), *Sco/Cyo*; *tub-gal80^{ts}* (Bloomington #7018), *tub-gal80^{ts}; TM2/TM6B, Tb* (Bloomington #7108), *w^{1118}*, and Oregon R.

ribbon overexpression:

ribbon was overexpressed via expression of UAS-*rib* in somatic gonadal cells and germ cells using the *tj*-Gal4 and *nanos*-Gal4 drivers respectively (Figure 18). Female Gal4 flies were crossed to UAS-*rib* males at 25°C and progeny were collected and raised at 25°C. Offspring were dissected at 1-3 days or 8-10 days post-eclosion.

ribbon overexpression in the presence of Gal80^{ts}:

Temperature sensitive *ribbon* overexpression was achieved by expression of UAS-*rib* in somatic gonadal cells and germ cells using the strains carrying *tj*-Gal4; *gal80^{ts}* and *gal80^{ts}*; *nanos*-Gal4, respectively (Figure 19). Female flies carrying Gal4 were crossed to UAS-*rib* males at 18°C. Offspring were collected and raised at 29°C. Progeny were dissected at 1-3 day or 8-10 days post-eclosion.

ribbon knockdown:

ribbon was knocked down by expression of UAS-*rib*-RNAi in somatic gonadal cells and

germ cells via the *tj*-Gal4 and *nanos*-Gal4 drivers respectively (Figure 18). Female Gal4 flies were crossed to UAS-*rib*-RNAi males at 29°C and progeny were collected and raised at 29°C. Progeny were dissected at 1-3 days or 8-10 days post-eclosion.

Immunostaining of Samples:

Ovaries and testes were harvested at 1-3 days and at 8-10 days post eclosion. Harvested gonads were fixed for 30 min at room temperature on a shaker in 4% formaldehyde in 1X 1xPBS with 0.1% Triton X-100 (PBTx). Samples were rinsed twice with PBTx, and then washed twice for 10 minutes each at room temperature. All samples were blocked in 5% Normal Goat Serum (NGS) and 5% BSA in PBTx (BBTx) for 1 hour at room temperature. Primary antibodies were diluted in 5% NGS in BBTx at a final volume of 300mL per tube as follows: rabbit anti-Vasa (1:200, Santa Cruz Biotechnology), guinea pig anti-Traffic Jam (1:1000; Van Doren Lab Johns Hopkins University), mouse anti-Eya (1:25; Developmental Studies Hybridoma Bank (DSHB)), rat anti-N-cadherin (1:20; DSHB), and mouse anti-Fasciclin III (1:30; DSHB).

Samples were incubated in primary antibody overnight in 4°C. On the second day samples were rinsed 2x quickly in PBTx, and washed 2x for 10 minutes with PBTx at 25°C. Alexafluor secondary antibodies (goat anti-rabbit 488, goat anti-guinea pig 555, goat anti-mouse 555, goat anti-rat 633, and goat anti-mouse 633; Fisher Scientific) were used at a 1:500 dilution in 5% NGS in BBTx and incubated with samples overnight at 4°C. Samples were incubated with 300mL of 4′, 6-Diamidine-2′-phenylindole dihydrochloride (DAPI) at 1μg/mL at room temperature for 10 minutes. DAPI solution was removed, and samples were rinsed two times with PBTx and then washed twice with PBTx for 10 minutes at 25°C. All samples were

mounted in 70% glycerol, 2.5% 1,4-diazabicyclo[2.2.2]octane, 10mM Tris-HCl pH 7.5 (DABCO).

Imaging and Analysis of Testes and Ovary Morphology:

All samples were imaged with the Zeiss AXIO Zoom.V16 Epifluorescent Stereo Scope at 488nm, 555nm, and DAPI channel. Images were captured at 2.3X optical zoom, and were taken at varying digital zooms to ensure all gonads were completely in frame. For scoring of male gonad morphology, testes were organized into one of four categories: WT describing normal and full development of the testes; Truncated denoting a phenotype in which the testes develop incompletely while retaining some ultrastructure; Cell Clump in which only an amorphous group of cells staining positive for Vasa are observed; and Glands in which the phenotype is so strong that either no testes are detected, or their integrity is compromised to the extent that they fall apart during the dissection and staining process leaving only accessory glands. All testes were counted individually. Female images were analyzed by quantification of the number of ovarioles per ovary. As with the testes, all ovaries were counted individually.

Statistical Analysis:

For male data analysis a Chi-Square test was attempted; however, the phenotypic traits between groups were extremely different. This made it difficult for the tests to be run in males. For females with two groups, comparisons were made using an unpaired t-test with Welch's correction. For graphs with more than two groups a One-way ANOVA assuming a Gaussian Distribution was conducted. This was followed by a Tukey's Post-hoc test. All statistical analysis was done using Graphpad Prism.

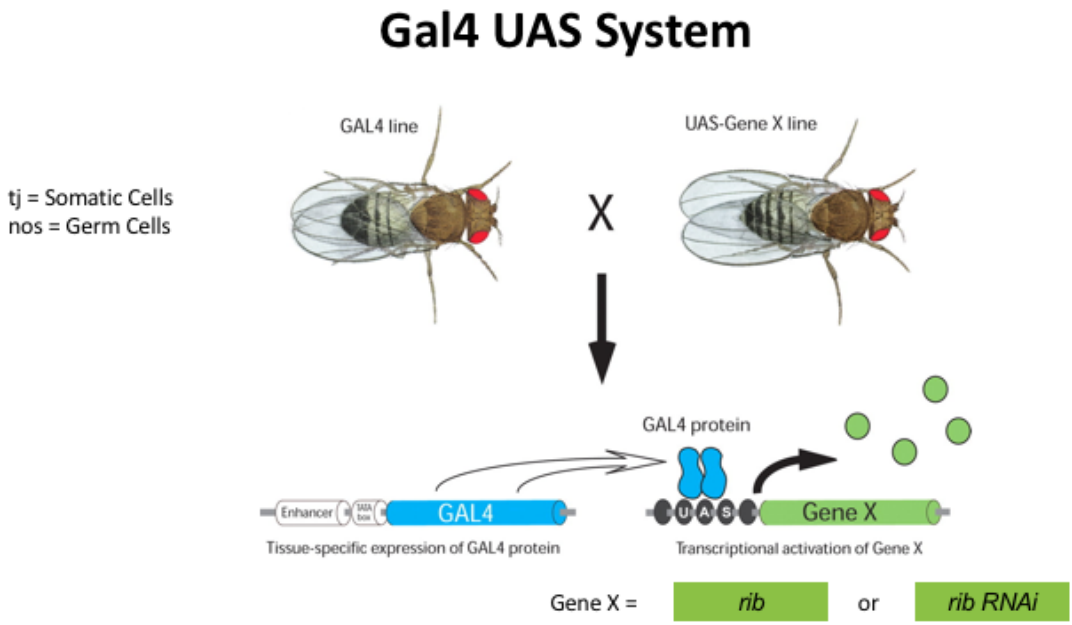

Figure 18. Schematic of the Gal4 UAS System. This schematic illustrates the method by which both rib overexpression and *rib* specific RNAi knockdown were implemented in this study. Parents, one containing the Gal4 gene enhancer linked to a driver, and one containing an Upstream Activation Sequence (UAS) linked to a gene of interest are crossed. Progeny express Gal4 in tissues specified by the drivers. Gal4 will bind a UAS, and enhance the expression of the Gene of interest.

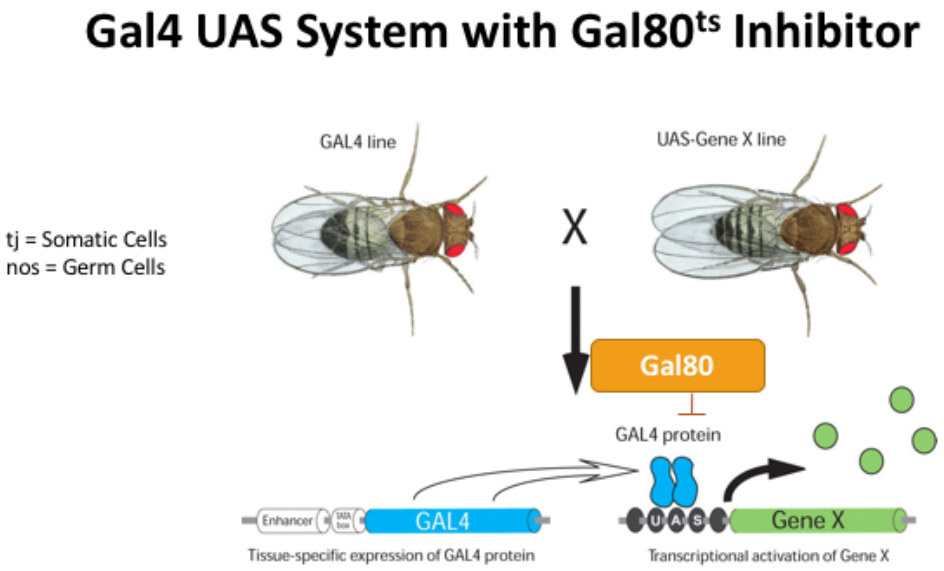

Figure 19. Schematic of the Gal4 UAS System. This schematic illustrates the method by which *rib* overexpression was carried out only in adults. Parents, one containing the Gal4 gene enhancer linked to a driver, and one containing an Upstream Activation Sequence (UAS) linked to a gene of interest are crossed. Progeny express Gal4 in tissues specified by the drivers. Gal4 will bind a UAS, and enhance the expression of the Gene of interest. The Gal80 inhibitor is temperature sensitive. At a 18°C, Gal80 inhibits Gal4 from acting as a gene enhancer. When moved to 29°C, Gal80 undergoes a conformational change, and released Gal4 to act as an enhancer.

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