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## Regulation of Gonad Morphogenesis in *Drosophila Melanogaster* by Broad Complex, Tramtrack and Bric à Brac Transcription Factors

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LOYOLA UNIVERSITY CHICAGO

REGULATION OF GONAD MORPHOGENESIS IN *DROSOPHILA*  
*MELANOGASTER* BY BROAD COMPLEX, TRAMTRACK AND BRIC À BRAC  
TRANSCRIPTION FACTORS

A THESIS SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDACY FOR THE DEGREE OF  
MASTER OF SCIENCE

PROGRAM IN BIOLOGY

BY

DIANE SILVA

CHICAGO, ILLINOIS

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To my Mom and Dad.  
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## ABSTRACT

During embryogenesis, primordial germ cells (PGCs) and somatic gonadal precursor cells (SGPs) migrate and coalesce to form the early gonad. A failure of the SGPs and PGCs to form a gonad with the proper architecture not only affects germ cell development, but also can result in infertility. Therefore, it is critical to identify the molecular mechanisms that function within both the PGCs and SGPs to promote gonad morphogenesis. We have characterized the phenotypes of two genes, *longitudinals lacking (lola)* and *ribbon (rib)*, that are required for the coalescence and compaction of the embryonic gonad in *Drosophila melanogaster*. Both *rib* and *lola* are expressed in the SGPs and to a lesser extent in the PGCs of the developing gonad, and genetic interaction analysis suggests these proteins cooperate to regulate gonad development. This hypothesis is supported by evidence that Rib and Lola homo- and heterodimerize. Analysis of the colocalization of Rib and Lola with marks of transcriptional activation and repression on polytene chromosomes reveals that Rib and Lola colocalize with both repressive and activating marks. These results suggest that Rib and Lola are dual function transcription factors. Thus, these

studies demonstrate that Rib and Lola function cooperatively to regulate transcription of target genes and thereby promote embryonic gonad morphogenesis.

# THESIS

## REGULATION OF GONAD MORPHOGENESIS IN *DROSOPHILA* *MELANOGASTER* BY BROAD COMPLEX, TRAMTRACK AND BRIC À BRAC TRANSCRIPTION FACTORS

### **Introduction**

Organ development depends upon the migration and interaction of multiple cell types, which give structure and function to that organ. The embryonic gonad provides an excellent model to study the genes that regulate migration and cell interactions to promote organogenesis. The gonad is formed from two primary cell types, the primordial germ cells (PGCs) which give rise to the gametes, and the somatic gonadal precursors (SGPs), which are derived from the mesoderm and support development of the germ line (Brookman et al., 1992; Mahowald, 1962). PGCs are formed at the posterior end of the embryo as cellularization occurs at stage 4-5, and they remain at the posterior until gastrulation is completed at stage 7, when the midgut invaginates, passively pulling the PGCs with it (Fig. 1) (Kunwar et al., 2006; Mahowald, 1962). They begin active migration through the midgut epithelium toward the mesoderm

during germ band elongation at stage 9 (Fig. 1) (Kunwar et al., 2006; Warrior, 1994). The SGP are specified bilaterally in three clusters from the mesodermal layer of abdominal parasegments 10-12 at stage 11 (Fig. 1) (Brookman et al., 1992; Mahowald, 1962). During stage 12, the PGCs migrate bilaterally and begin to intermingle with the SGP as the germ band retracts (Fig. 1) (Boyle and DiNardo, 1995). By the end of germ band retraction at stage 13 the three SGP clusters and the PGCs have coalesced into an elongated gonad on each side of the developing embryo (Fig. 2A) (Boyle and DiNardo, 1995). SGP also begin to send out membrane extensions to ensheath the PGCs during stage 13, which persist throughout gonad development, and are critical for proper germ cell development (Decotto and Spradling, 2005; Gönczy and DiNardo, 1996; Jenkins et al., 2003). Following gonad coalescence, SGP and PGCs compact to form a spherical gonad by stage 15 of embryogenesis (Fig. 2A) (Boyle and DiNardo, 1995). Previous studies have identified many genes that are critical for PGC migration, gonad coalescence and compaction, and ensheathment. (reviewed in Jemc, 2011; Richardson and Lehmann, 2009); however, understanding of this complex process is far from complete. In this paper, we describe the role of two genes, *ribbon (rib)* and *longitudinals lacking (lola)* in the process of gonad morphogenesis.

Figure 1. *Drosophila* embryonic developmental stages and events. Schematics show gonad morphogenesis through cell movement and interactions of the PGCs (red) and the SGPs (green).

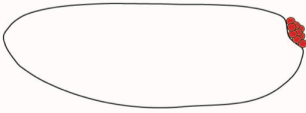
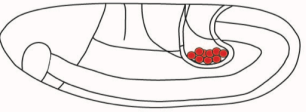
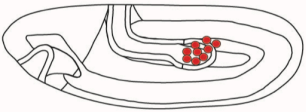


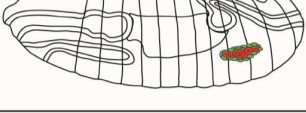
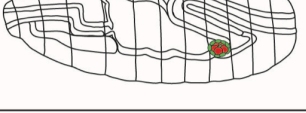

Stage	Schematic	Developmental Events
Stage 5		Cellularization of the blastoderm
Stage 8		End of gastrulation and start of rapid germ band elongation
Stage 9/10		Slow germ band elongation and formation of head features
Stage 11		End of neuroblast formation
Stage 12		Beginning of germ band retraction
Stage 13		End of germ band retraction
Stage 15		Head involution and dorsal closure; end of gonad formation
Stage 17		Differentiation

Figure 2. *lola* and *rib* exhibit defects in gonad morphogenesis. (A) Schematic and confocal images of the stages of embryonic gonad formation, at stage 12 SGP (green) clusters and PGCs (red) move toward each other and join, at stage 13 SGPs begin to ensheath the PGCs and at stage 15 compaction brings cells closer together to form a normal, spherical gonad. (B) 68-77 wild-type stage 15 gonad, used as control. (C-D) *rib*<sup>35.14/55.25</sup> stage 15 mutant embryos; stained for 68.77 *lacZ* enhancer trap, staining the somatic cells for analysis of gonad morphology (B) wild-type (C) compaction defect (D) fusion defect (E-F) *lola*<sup>46.38/22.05</sup> stage 15 mutant embryos; stained for 68-77 *lacZ* enhancer trap, staining the somatic cells for analysis of gonad morphology (E) compaction defect (F) fusion defect. (G) Quantification of gonad phenotypes in *lola* and *rib* mutant embryos.

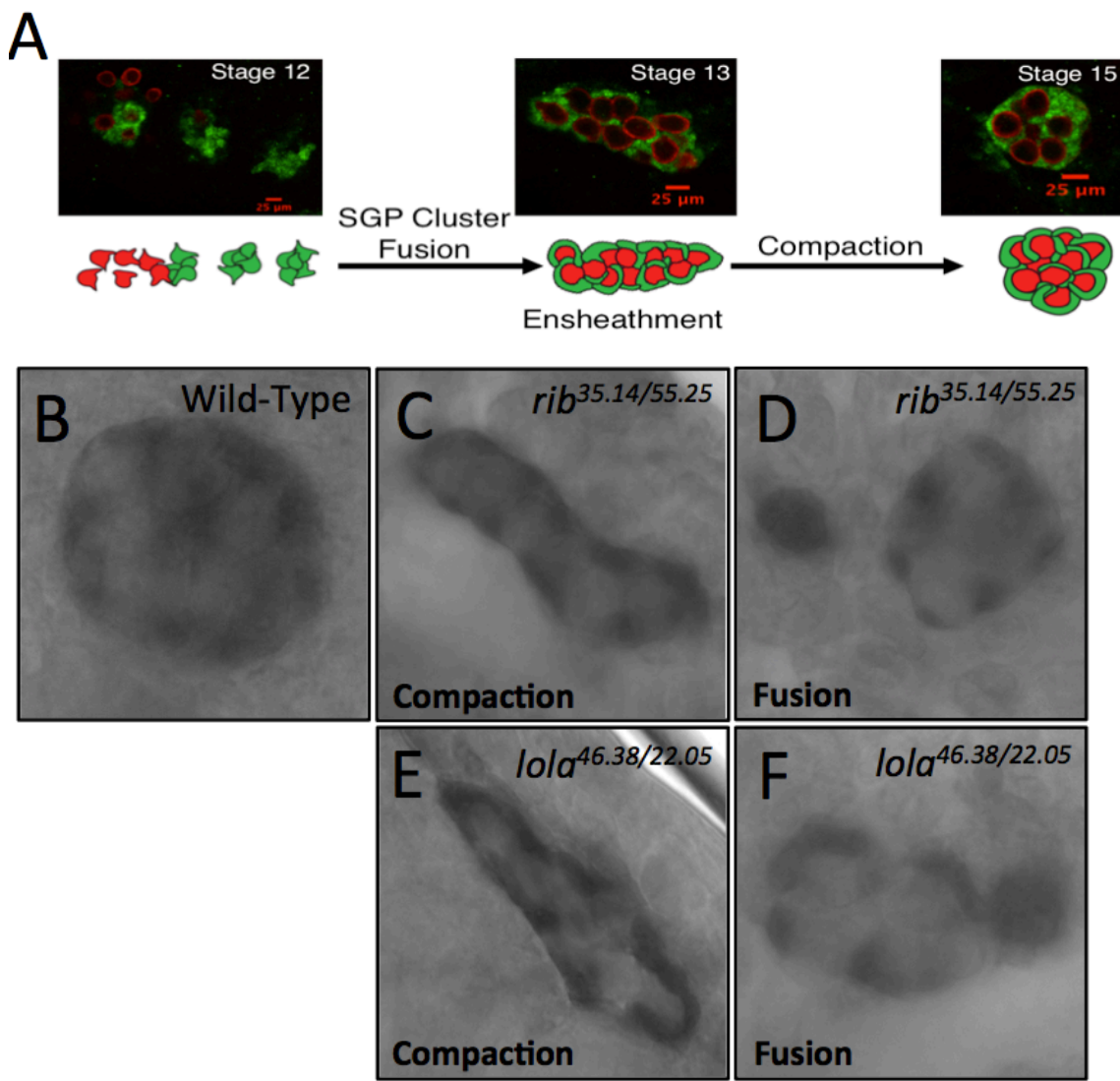
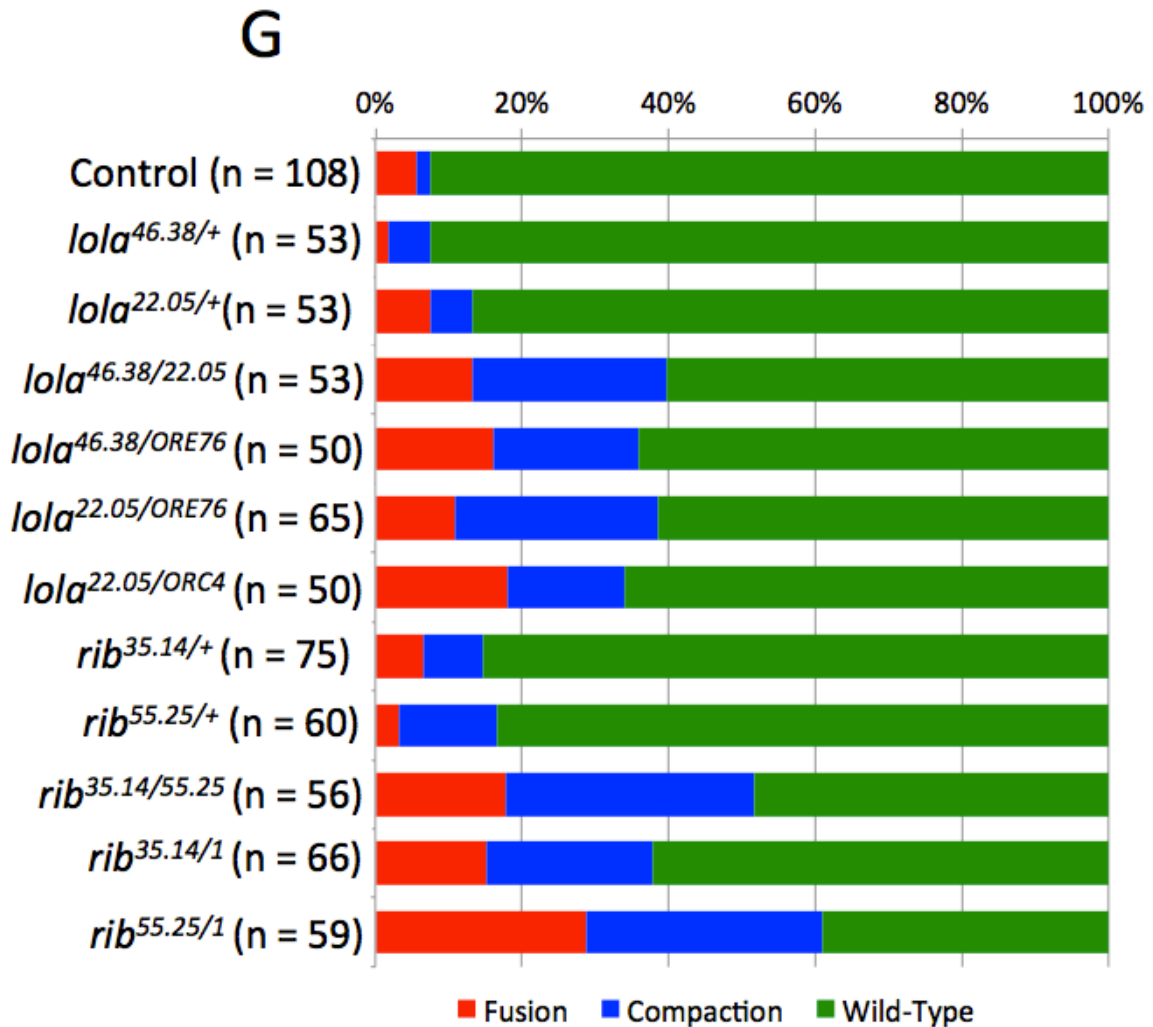




Figure 2. *lola* and *rib* exhibit defects in gonad morphogenesis. Continued.

Molecularly, Rib and Lola belong to the BTB/POZ (Broad Complex, Tramtrack and Bric à Brac/Pox Virus and Zinc finger) family of proteins. These proteins include a conserved BTB domain, which functions in protein-protein interactions (Fig. 3A) (Bardwell and Treisman, 1994; Bradley and Andrew, 2001; Godt et al., 1993; Shim et al., 2001; Zollman et al., 1994). The BTB domain is

located at the amino-terminus where it mediates homo- and heterodimerization, as well as multimerization with other BTB and non-BTB domain-containing proteins (Ahmad et al., 1998; Bardwell and Treisman, 1994; Bonchuk et al., 2011). Many BTB domain-containing proteins, including Rib and Lola, contain an N-terminal extension of the BTB domain, which plays an important role in stabilizing BTB domain interactions (Bonchuk et al., 2011). BTB domain containing proteins are found very often in proteins that also contain DNA-binding motifs, suggesting that these BTB family proteins function as transcriptional regulators (Cavarec et al., 1997; Davies et al., 2013; Siggs and Beutler, 2012; Stogios et al., 2005). In some cases, the BTB domain has been demonstrated to interact with transcriptional repressors and activators, further supporting this role (Ahmad et al., 1998; Huynh and Bardwell, 1998; Melnick et al., 2000; Melnick et al., 2002; Staller et al., 2001). BTB domains are often found in transcription factors with roles in development, carcinogenesis, and gametogenesis (Ahmad et al., 1998; Bonchuk et al., 2011; Zollman et al., 1994). In mammals, BTB domain containing proteins are involved in hematopoiesis, limb patterning, spermatogenesis, neurological development, and physiological functions (Siggs and Beutler, 2012).

Both Lola and Rib also contain DNA binding domains. The *lola* gene locus encodes at least 20 protein isoforms, generated by alternative splicing (Goeke et

al., 2003; Ohsako et al., 2003). While all protein isoforms contain a common N-terminal region, which includes the BTB domain, their carboxy-terminal domain structure varies (Goeke et al., 2003; Horiuchi et al., 2003; Ohsako et al., 2003). Of the 20 identified protein isoforms, there are only 3 that lack a zinc finger (ZF) motif at the C-terminus, which include isoforms A, G and M, where the rest contain zinc fingers with most of the isoforms containing two zinc fingers, as shown for isoforms T and K (Fig. 3B) (Goeke et al., 2003). Variability in the sequence of these zinc fingers suggests that different Lola isoforms are likely to have different DNA binding specificities (Goeke et al., 2003). In addition, yeast two-hybrid studies suggest that different Lola isoforms are capable of heterodimerization, thereby increasing the variability of potential Lola binding sites (Giot et al., 2003; Zhang et al., 2003). The presence of zinc fingers implies that Lola may function as a transcriptional regulator, and previous studies have demonstrated the ability of Lola to repress expression of the *cop* retrotransposon in the embryonic central nervous system (Cavarec et al., 1997). While Lola is hypothesized to function primarily as a transcriptional repressor, expression of *cop* appears reduced in the embryonic gonad of *lola* mutants, suggesting the different Lola isoforms may function to regulate transcriptional targets in opposing fashion, depending on the cofactors with which they interact (Cavarec et al., 1997).

Unlike, *lola*, the *rib* gene locus only encodes at least 3 protein isoforms. Rib also has a DNA binding motif located C-terminal to the BTB domain, that is referred to as the Pipsqueak (PSQ) motif (Fig. 3B) (Bradley and Andrew, 2001; Shim et al., 2001). The PSQ motif is a 50-amino acid sequence that binds to GAGAG consensus sequence repeats (Horowitz and Berg, 1996; Lehmann et al., 1998). While Rib has been hypothesized to function as a transcriptional regulator, no direct transcriptional targets have been identified at this time. Thus, although Rib and Lola contain DNA binding domains, little is known about the direct targets for these genes and the mechanisms by which they function to regulate transcription.

Figure 3. Domain sequence and structure of Lola and Rib proteins. (A) Alignment of BTB proteins from *Drosophila melanogaster*, *Homo sapiens*, and *Mus musculus*. (B) Molecular structure of Lola and the two isoforms used in the study, isoforms K and T, and Rib. Structures show: BTB domains, zinc finger DNA binding domains (ZF), pipsqueak DNA binding motif (PSQ), and the corresponding mutations found in alleles used in this study.

A

```

Dm Lola  QIISGPPGKANNHHCSTFVSVFDLTLLNETLVDCTFAAEGKFLKAKIKVWASACSPYRATLLOEQY--
Dm Rib  QIISGPPGKANNHHCSTFVSVFDLHHEVGSYDCSIVVDDIQFQNRVWAAANSPLYRQHLKDVDP--
Dm Ttk  QIISGPPGKANNHHCSTFVSVFDQLLHAETFTDVTAVEGQHLKAKIKVWASACSPYRNTLQVSHP--
Hs PLZF  QIISGPPGKANNHHCSTFVSVFDLTLCDVIVMVDSDQEFHAKIKVWASACTSKMRELFH---
Hs Bcl6  LNLRLSRDILLTDVIVMVDSDQEFHAKIKVWASACTSKMRELFH---
Hs APM-1 LNEQRHDGLLCDVLLVVDSDQEFYRIRKIKVWASACSKMRELFH---
Mm RC/BTB2 LKREFDNPDTADIKFLVDSDGKVIYRIRKIKVWASAKIRCEHRSLSLED---

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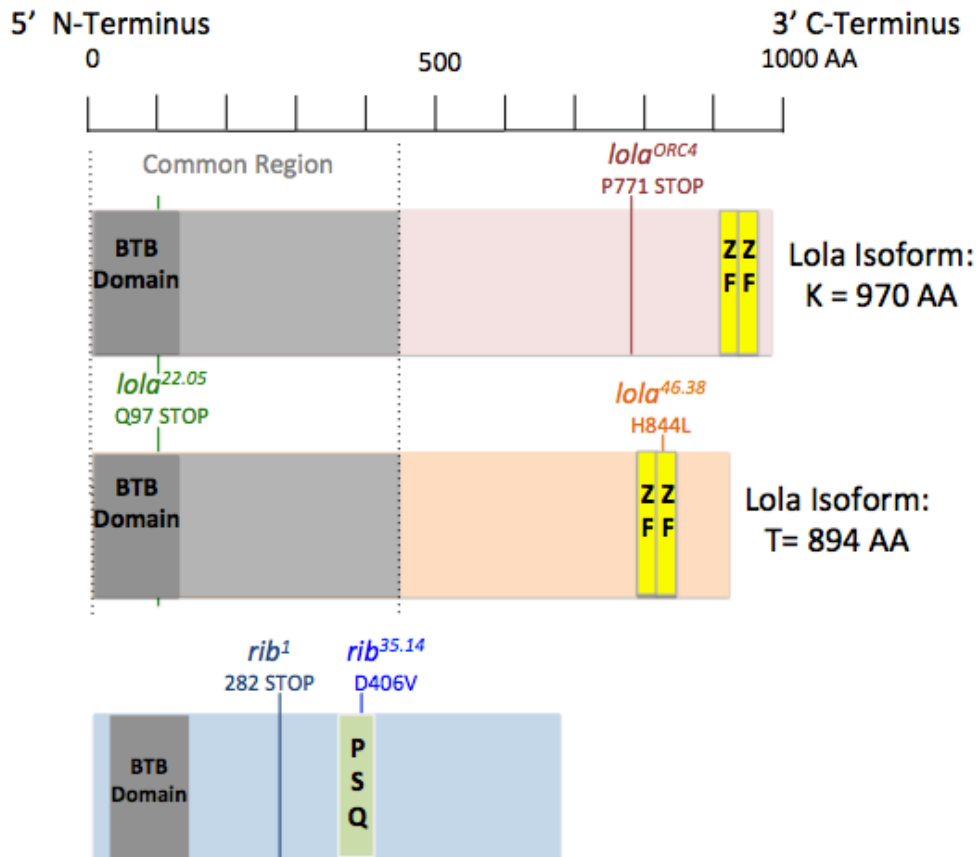
  

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Dm Lola  DKHPIFIKDKYQELRAMDYMYRGEVNISSODQLAAIKAAESIQKIGISDNRRTGGGVAPK
Dm Rib  QDHCSIIPGKKGFEIAAIDQYMYRGETTWTKSOEPEERRTAKERQYKGIYDNLKMFNHASM
Dm Ttk  EKHPVIVIKDVPYSDMKSTIKRMYRGEVSDQERITAFIRVRESRREKGIQTEVNDKPS
Hs PLZF  RNSQHYTDFVSPKTFQOIKRMYRGEVSDQERITAFIRVRESRREKGIQTEVNDKPS
Hs Bcl6  NLSVINLDPENPEGFCIIRKMYRGEVSDQERITAFIRVRESRREKGIQTEVNDKPS
Hs APM-1 SOPYVYEIDFQPEALAAIKRMYRGEVSDQERITAFIRVRESRREKGIQTEVNDKPS
Mm RC/BTB2 SEDDIVVEMSEFSYPVFRVFDSDQERITAFIRVRESRREKGIQTEVNDKPS

```

B



Both *rib* and *lola* were previously identified in a genetic screen for mutants that affect early gonad formation (Weyers et al., 2011), and have also been shown to function in other contexts to regulate organ morphogenesis. The gene *rib* was first identified in an ethyl methanesulfonate mutagenesis screen for larval cuticle abnormalities (Nusslein-Volhard, 1984). Subsequently, *rib* was demonstrated to regulate cell migration and morphogenesis of the trachea and salivary glands (Bradley and Andrew, 2001; Jack and Myette, 1997; Shim et al., 2001). When *rib* is mutated, the tracheal cell bodies and apical surface have severe migration defects and impaired morphogenesis, while the salivary glands fail to elongate (Bradley and Andrew, 2001; Shim et al., 2001). It has been suggested that Rib functions downstream of the Mitogen Activated Protein Kinase (MAPK) signaling pathway based on the similarity of mutant phenotypes for *rib* and members of the Fibroblast Growth Factor (FGF)-MAPK signaling pathway, as well as the presence of seven MAPK consensus phosphorylation sites in Rib (Bradley and Andrew, 2001). However, the direct regulation of Rib by this pathway has not been demonstrated. Rib has also been demonstrated to interact with another BTB protein, Lola-like (Lolal), in the context of salivary gland and trachea morphogenesis (Kerman et al., 2008). In these contexts, Rib and Lolal cooperate to regulate expression of Crumbs (Crb), an apical membrane protein that functions in epithelial cell polarity and in apical membrane growth (Cheshire et

al., 2008; Kerman et al., 2008). In *rib* mutants Crb levels are significantly reduced in the epithelia (Kerman et al., 2008). Rib and Lola-like also regulate the activity of Moesin (Moe), the only Ezrin-Radixin-Moesin (ERM) family protein in *Drosophila*, which plays a role in linking the plasma membrane to the actin cytoskeleton (Kerman et al., 2008). In *rib* mutants, levels of active, phosphorylated Moe are increased; suggesting that down-regulation of Moe activity is required for salivary gland and trachea morphogenesis (Kerman et al., 2008). Given that Rib interacts with other BTB domain containing proteins, like Lola-like, it is possible that Rib may interact with other BTB family proteins in a context-dependent manner to regulate organ development.

One candidate for this interaction is Lola. Previous studies have demonstrated a critical role for Lola in *Drosophila* nervous system development. *lola* is required for axon growth in the embryonic central nervous system (CNS), and mutation of *lola* results in axon pathfinding defects along the longitudinal tracts of the CNS (Giniger et al., 1994). In addition, it is also required to prevent midline crossing of longitudinal axons (Crowner et al., 2002). In this context, Lola appears to up-regulate expression of the midline repellent protein, Slit, and its longitudinal axonal receptor Roundabout (Robo) (Crowner et al., 2002; Goeke et al., 2003). Lola is also required for embryonic gonad morphogenesis and for gametogenesis in the adult (Bass et al., 2007; Davies et al., 2013; Tripathy et al.,

2014; Weyers et al., 2011). In the *Drosophila* adult testis *lola* is required cell autonomously for the maintenance of the somatic cyst stem cells and germline stem cells, as well as regulating the transition to meiosis during male germ cell differentiation (Davies et al., 2013). *lola* has been implicated in embryonic gonad morphogenesis in two independent studies (Tripathy et al., 2014; Weyers et al., 2011).

Here, we have characterized the role of two BTB family proteins, Lola and Rib in embryonic gonad morphogenesis. Our results suggest that Rib and Lola function as dual-function transcriptional regulators to cooperatively regulate gene expression in the developing gonad.

## **Results**

### *Characterization of the role of Rib and Lola in gonad morphogenesis*

Previously, *lola* and *rib* were identified in a screen for genes that are required for embryonic gonad morphogenesis (Weyers et al., 2011). In order to further characterize the gonad phenotypes of *rib* and *lola* mutants, the 68-77 *lacZ* enhancer trap, which is expressed in the cytoplasm of the SGPs, thus it was used to mark the cytoplasm of SGPs and monitor gonad morphology (Fig. 2B) (Boyle and DiNardo, 1995; Simon et al., 1990; Warrior, 1994). By stage 13 SGP clusters have fused into an elongated, coalesced gonad, and by stage 15, PGCs and SGPs have compacted into a spherical gonad with SGPs ensheathing the PGCs (Fig.



2A, B). Therefore, we scored *rib* and *lola* embryonic gonads for a failure of SGP clusters to coalesce, referred to as fusion defects, and a failure of gonads to form a round spherical gonad, referred to as compaction defects. Immunostaining of somatic cells of the gonad in *rib* and *lola* mutants exhibited defects in gonad coalescence, such that SGP clusters often fail to coalesce into a single cluster, referred to as a fusion defect (Fig. 2D, F), and those clusters that do coalesce fail to compact into a spherical structure, referred to as a compaction defect (Fig. 2C, E). For *lola* and *rib* mutants, similar compaction and fusion defects were observed (Fig. 2C-F). Quantification of these phenotypes demonstrates that the control, a fly stock carrying the 68-77 enhancer trap, exhibits a low frequency of fusion and compaction defects of ~7% (Fig. 2G). Less than 17% of the gonads in embryos heterozygous for *lola* and *rib* mutations exhibited fusion and compaction defects. *rib* and *lola* mutants were analyzed as heteroallelic to minimize any potential contribution of second site mutations to the observed phenotype, as these mutant alleles were obtained from a mutagenesis screen. Both *rib* and *lola* heteroallelic combinations exhibited increased levels of fusion and compaction defects relative to the wild-type control and single heterozygous mutants (Fig. 2G, Table 1).

In these studies, two alleles are expected to affect all Lola protein isoforms, the *lola*<sup>ORE76</sup> allele, which is characterized as a null allele based on the

absence of Lola protein by Western blotting and a loss of all known Lola functions (Goeke et al., 2003), and the *lola*<sup>22.05</sup> allele, which encodes a protein with a Q97STOP mutation within the BTB domain (Tripathy et al., 2014). We also utilize two isoform-specific alleles: *lola*<sup>46.38</sup> encodes a H844L mutation in the second the zinc finger of Lola isoform T (Flybase designation PR/PG; Davies et al., 2013) and *lola*<sup>ORC4</sup> encodes a premature stop codon (P771STOP) in Lola isoform K (Flybase designation PI; Davies et al., 2013) (Fig. 3B). We observe defects when each isoform-specific allele is heteroallelic with a null or hypomorphic allele affecting all isoforms. With the heteroallelic mutants *lola*<sup>46.38/22.05</sup> and *lola*<sup>22.05/ORE76</sup> fusion and compaction defects were observed in just under 40% of gonads (Fig. 2G, Table 1). A small increase in the frequency of gonad defects of 4%, a total of 44% was observed in *lola*<sup>46.38/ORE76</sup> mutants (Fig. 2G, Table 1), suggesting that despite an early stop codon in *lola*<sup>22.05</sup> it may not be as strong a loss of function mutant as *lola*<sup>ORE76</sup>. Previous studies suggested that Lola isoform T was the only isoform required in gonad morphogenesis (Tripathy et al., 2014). Interestingly, *lola*<sup>22.05/ORC4</sup> mutants carrying the Lola isoform K specific mutation, exhibited gonad defects 34% of the time (Fig. 2G). Thus, all heteroallelic *lola* mutants exhibited an increase in fusion and compaction defects when compared to their heterozygous counterparts. These results also suggest that Lola isoforms other than Lola-T, namely Lola-K, also function in gonad

morphogenesis (Tripathy et al., 2014; Weyers et al., 2011).

In the case of the *ribbon* alleles, only the *rib<sup>1</sup>* allele encodes a protein with a premature stop codon (282STOP; Shim et al., 2001), while *rib<sup>35.14</sup>* encodes a protein with a D406V missense mutation, which is localized in the PSQ DNA binding motif and is likely to function as a hypomorph (Fig. 3B). While the precise mutation in the *rib<sup>55.25</sup>* allele is unknown, sequencing has revealed that it is not within the coding sequence. Examination of *rib<sup>35.14/55.25</sup>* mutants reveals the presence of fusion and compaction defects in approximately 52% of embryonic gonads (Fig. 2C, D, G, Table 1). *rib<sup>55.25/1</sup>* heteroallelic mutants exhibited the highest frequency of defects with a total of 61% (Fig. 2G, Table 1). Thus, the *rib<sup>55.25</sup>* mutant allele is likely a stronger allele than the *rib<sup>35.14</sup>* allele. Overall, these results suggest that Rib is a critical regulator of gonad coalescence and compaction.

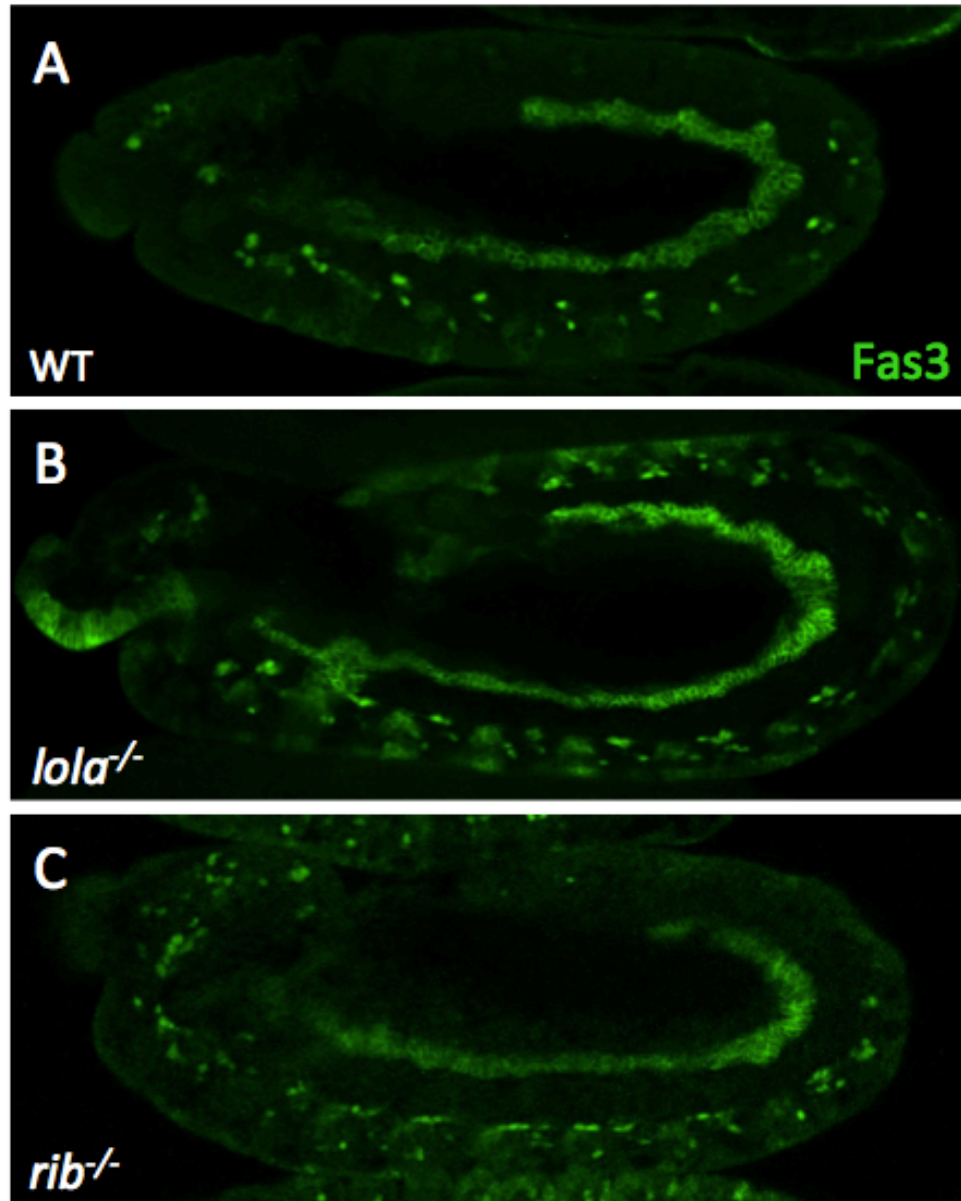
#### *Mesoderm specification is normal in rib and lola mutants*

The defects in gonad fusion and compaction observed in *lola* and *rib* mutants suggest that these genes are required in the mesodermally-derived SGP. Therefore, it was necessary to eliminate the possibility that the gonad defects were due to a more generalized defect in mesoderm development. In order to look at mesoderm development, stage 12 embryos were immunostained with anti-Fasciclin III to examine visceral mesoderm development (Fig. 4).

Mesoderm development was indistinguishable from controls for both *lola* and *rib*

mutant embryos (Fig. 4). This data suggests that the gonad defects observed in *rib* and *lola* mutant embryos are due to a specific requirement of the function of these genes in the SGPs.

Figure 4. Mesoderm develops normally in *rib* and *lola* mutants. (A) Oregon R, (B) *lola*<sup>46.38/22.05</sup>, and (C) *rib*<sup>35.14/55.25</sup> stage 12 embryos immunostained for the visceral mesodermal marker Fasciclin III.



*lola and rib expression in developing gonads*

The fusion and compaction defects observed in the gonad suggest that these proteins function in the SGPs during gonad morphogenesis. Therefore, immunohistochemistry for Rib and Lola proteins was performed to determine where Rib and Lola are expressed in the developing gonad. Antibody staining for both Lola and Rib demonstrated that both proteins are expressed in the SGPs and to a lesser extent in the PGCs, as well as being present in the surrounding mesodermal cells (Fig. 5). Given the similar phenotypes observed in *rib* and *lola* mutants and similar expression patterns in the embryonic gonad, the colocalization of both of these proteins was examined. Immunohistochemistry analysis reveals the colocalization of Lola and Rib in SGPs (Fig. 5C-C''), suggesting that Lola and Rib may function in the same pathway to regulate gonad morphogenesis.

In order to examine the possibility that these proteins may regulate expression of each other, we examined *rib* expression in *lola* mutants and *lola* expression in *rib* mutants (Fig. 5D-G''). No difference in Lola expression was observed in *rib* homozygotes when compared to heterozygous sibling controls (Fig. 5D-E''). Similarly, Rib expression remained unchanged in *lola* mutants relative to sibling controls (Fig. 5F-G''). These results suggest that Rib and Lola are not regulating each other, but rather may cooperate to regulate gene

expression during embryonic gonad development.

Figure 5. Expression of Rib and Lola in the embryonic gonad. (A) Expression of Lola in an Oregon-R stage 15 gonad. Anti-Lola (green), anti-Tj marks SGP (red), anti-Vasa marks PGCs (blue). (A') Lola alone. (A'') Tj alone. (B) Expression of Rib in a stage 15 gonad. Anti-Rib (red), anti-Tj marks SGP (green), anti-Vasa marks PGCs (blue). (B') Rib alone. (B'') Tj alone. Posterior to the right; (C-C'') Colocalization of *rib* and *lola* in Oregon-R stage 15 gonad, posterior to the right. Scale bar: 10 $\mu$ m. (C) Anti-Rib (green), (C') Anti-Lola (red), (C'') Merge of Rib and Lola expression in a stage 15 gonad, where there are areas of high colocalization between Rib and Lola (arrows) (D-E'') *lola* expression in *rib*<sup>35.14/55.25</sup> mutant stage 15 gonads, posterior to the right. (D-D'') *rib*<sup>+/+</sup> gonad. Anti-Lola (green) and anti-Tj marks SGP (red). (E-E'') *rib*<sup>-/-</sup> mutant stage 15 gonad. Anti-Lola (green) and anti-Tj marks SGP (red). (F-G'') *rib* expression in *lola*<sup>46.38/22.05</sup> mutant stage 15 gonads, posterior to the right. (F-F'') *lola*<sup>+/+</sup> gonad. Anti-Rib (green) and anti-Tj marks SGP (red). (G-G'') *lola*<sup>-/-</sup> gonad. Anti-Rib (green) and anti-Tj marks SGP (red).

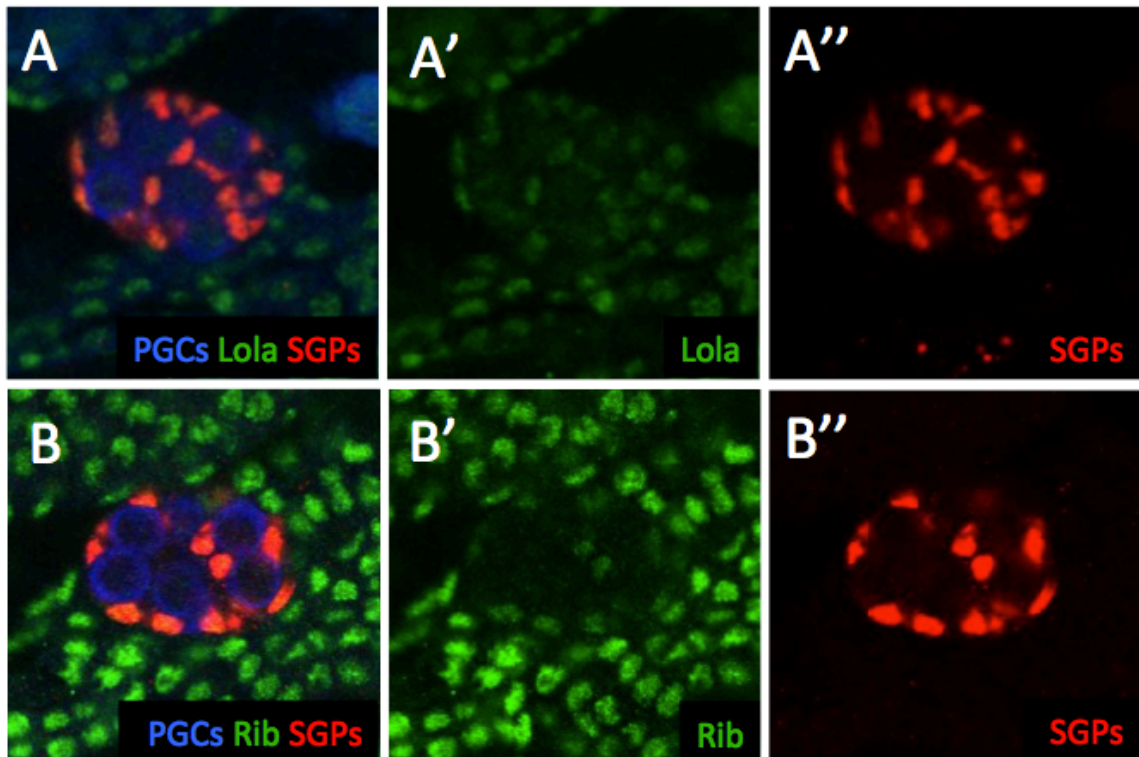


Figure 5. Expression of Rib and Lola in the embryonic gonad. Continued.

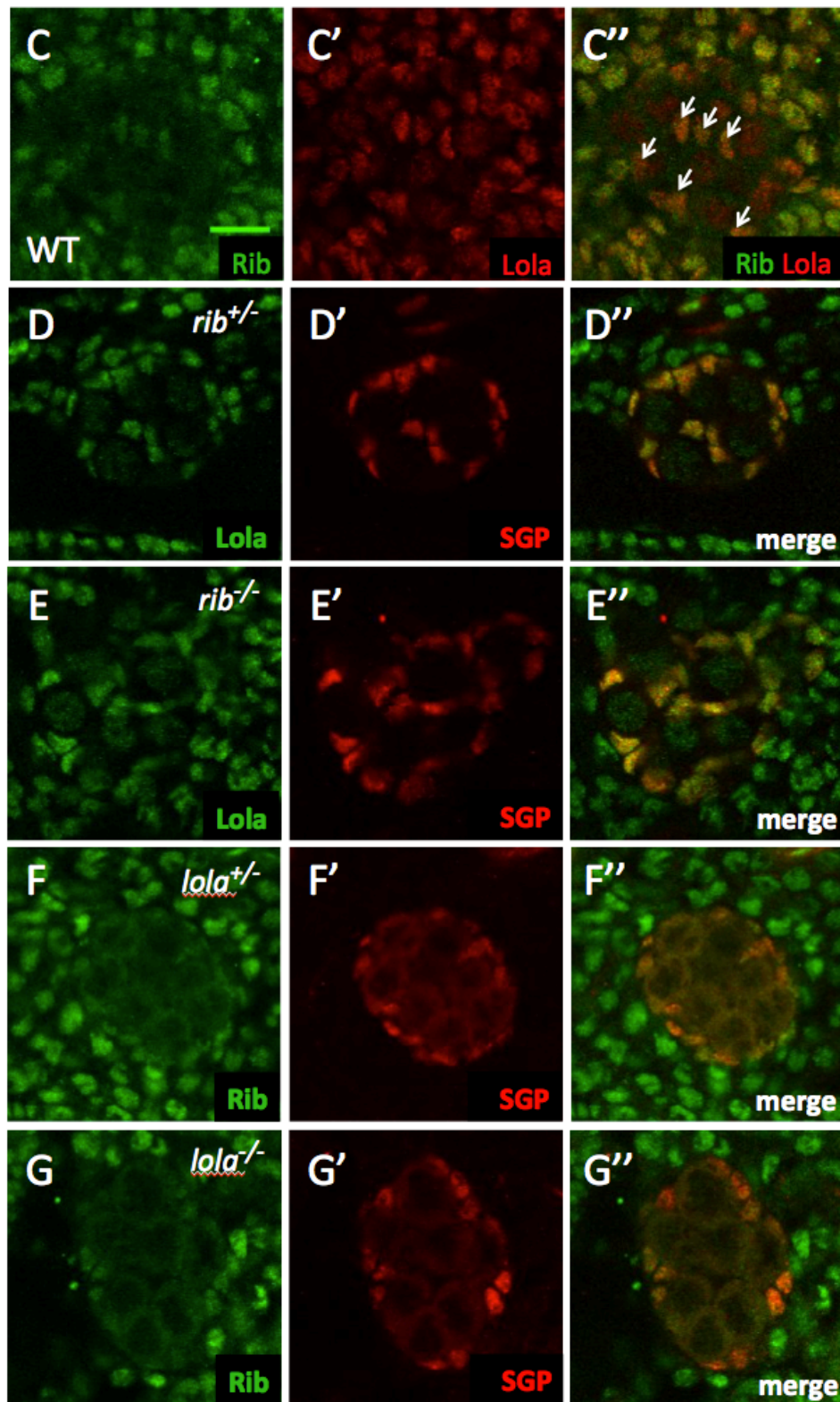




Table 1. Percentage of gonad defects for all stage 15 genotype embryos

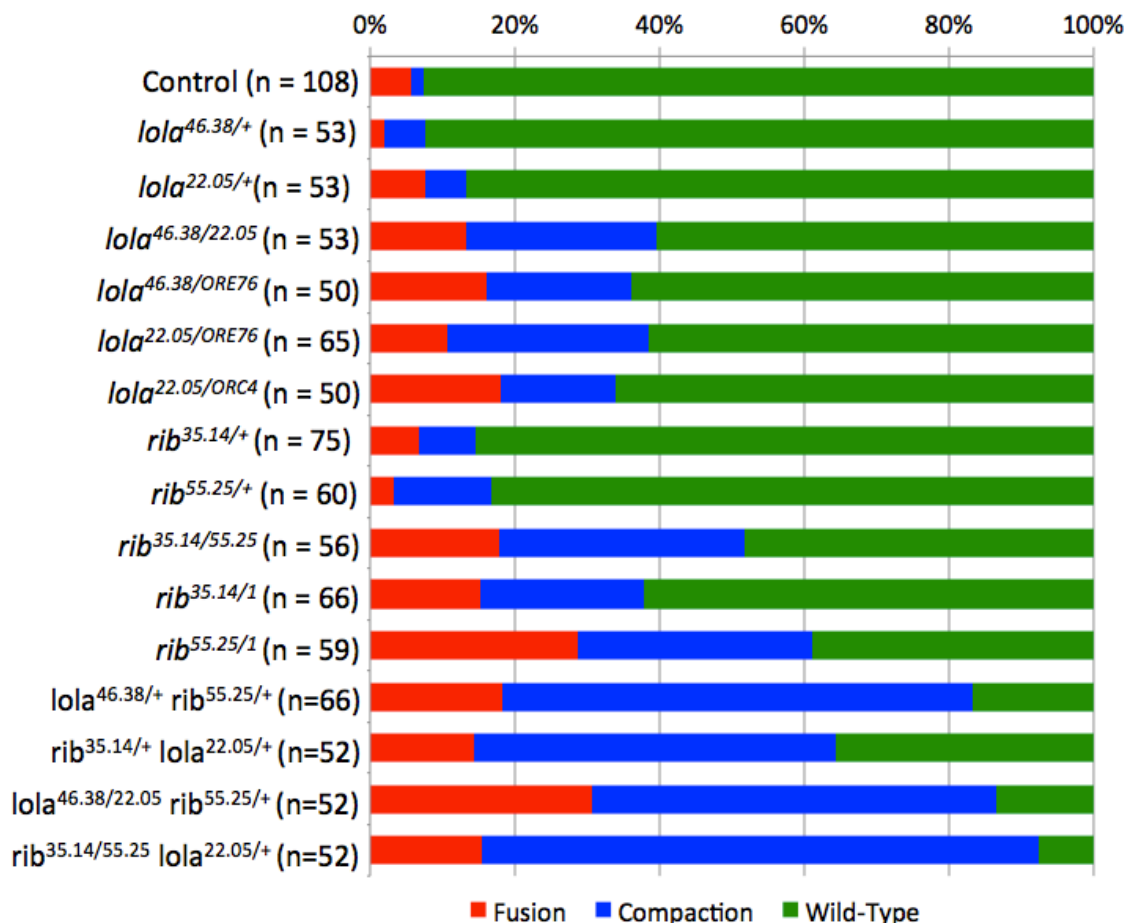
Genotype	Fusion %	Compaction %	WT %	N
Control 68-77 #5	5.6	1.9	92.6	108
<i>lola</i> <sup>46.38/+</sup>	1.9	5.7	92.5	53
<i>rib</i> <sup>35.14/+</sup>	6.7	8.0	85.3	75
<i>lola</i> <sup>22.05/+</sup>	7.5	5.7	86.8	53
<i>rib</i> <sup>55.25/+</sup>	3.3	13.3	83.3	60
<i>lola</i> <sup>46.38/22.05</sup>	13.2	26.4	60.4	53
<i>rib</i> <sup>35.14/55.25</sup>	17.9	33.9	48.2	56
<i>lola</i> <sup>46.38/ORE76</sup>	16.0	28.0	56.0	50
<i>lola</i> <sup>22.05/ORE76</sup>	10.8	27.7	61.5	65
<i>lola</i> <sup>22.05/ORC4</sup>	18.0	16.0	66.0	50
<i>rib</i> <sup>35.14/1</sup>	15.2	22.7	62.1	66
<i>rib</i> <sup>55.25/1</sup>	28.8	32.2	39.0	59
<i>lola</i> <sup>46.38/+</sup> <i>rib</i> <sup>55.25/+</sup>	18.2	65.2	16.7	66
<i>rib</i> <sup>35.14/+</sup> <i>lola</i> <sup>22.05/+</sup>	14.3	50.0	35.7	56
<i>lola</i> <sup>46.38/22.05</sup> <i>rib</i> <sup>55.25/+</sup>	30.8	55.8	13.5	52
<i>rib</i> <sup>35.14/55.25</sup> <i>lola</i> <sup>22.05/+</sup>	15.4	76.9	7.7	52

*rib* and *lola* genetically interact

With the observations that both *rib* and *lola* exhibit similar mutant defects and are both expressed in the SGPs, we explored the possibility these proteins may cooperate to regulate gonad morphogenesis through genetic interaction studies. Gonad development was examined in embryos heterozygous for both *rib* and *lola* alleles. Stage 15 embryonic gonads were scored as wild-type, or as having fusion or compaction defects. The frequency of defects in double

heterozygotes was compared to 68-77 control embryos, as well as embryos heterozygous for either *rib* or *lola* (*lola*<sup>46.38/+</sup>, *rib*<sup>35.14/+</sup>, *lola*<sup>22.05/+</sup>, and *rib*<sup>55.25/+</sup>) (Fig. 6, Table 1). The percentage of defective gonads ranged from 8-17% in the controls (Fig. 6, Table 1). In the case of embryos heterozygous for both *rib* and *lola* (*lola*<sup>46.38/+</sup> *rib*<sup>55.25/+</sup>, *rib*<sup>35.14/+</sup> *lola*<sup>22.05/+</sup>), the frequency of gonad defects increased significantly to between 65-85% (Fig. 6, Table 1), demonstrating a synergistic effect and suggesting that *rib* and *lola* cooperate to regulate gonad morphogenesis. In addition, mutants for *rib* or *lola* were examined that were also heterozygous for *lola* or *rib*, respectively (*rib*<sup>35.14/55.25</sup> *lola*<sup>22.05/+</sup>, *lola*<sup>46.38/22.05</sup> *rib*<sup>55.25/+</sup>). These mutants showed a dramatic increase in gonad defects relative to *rib* and *lola* mutants alone with more than 85% of the gonads exhibiting fusion and compactions, compared to ~50% in *rib* and ~40% in *lola* heteroallelic combinations (Fig. 6, Table 1). These results suggest that Rib and Lola function cooperatively in the same pathway or in parallel pathways to regulate embryonic gonad development.

Figure 6. *rib* and *lola* genetically interact. Graph of phenotypic penetrance's for each group for observed stage 15 embryonic gonads; each bar represents the percentage of gonads within that group showing the indicated phenotype. Every group shows gonad phenotypes of fusion (red), compaction (blue) and wild-type (green). Gonads were scored by staining somatic cells of the gonad for the 68-77 *lacZ* enhancer trap, using a fluorescently-marked balancer chromosome for genotyping.



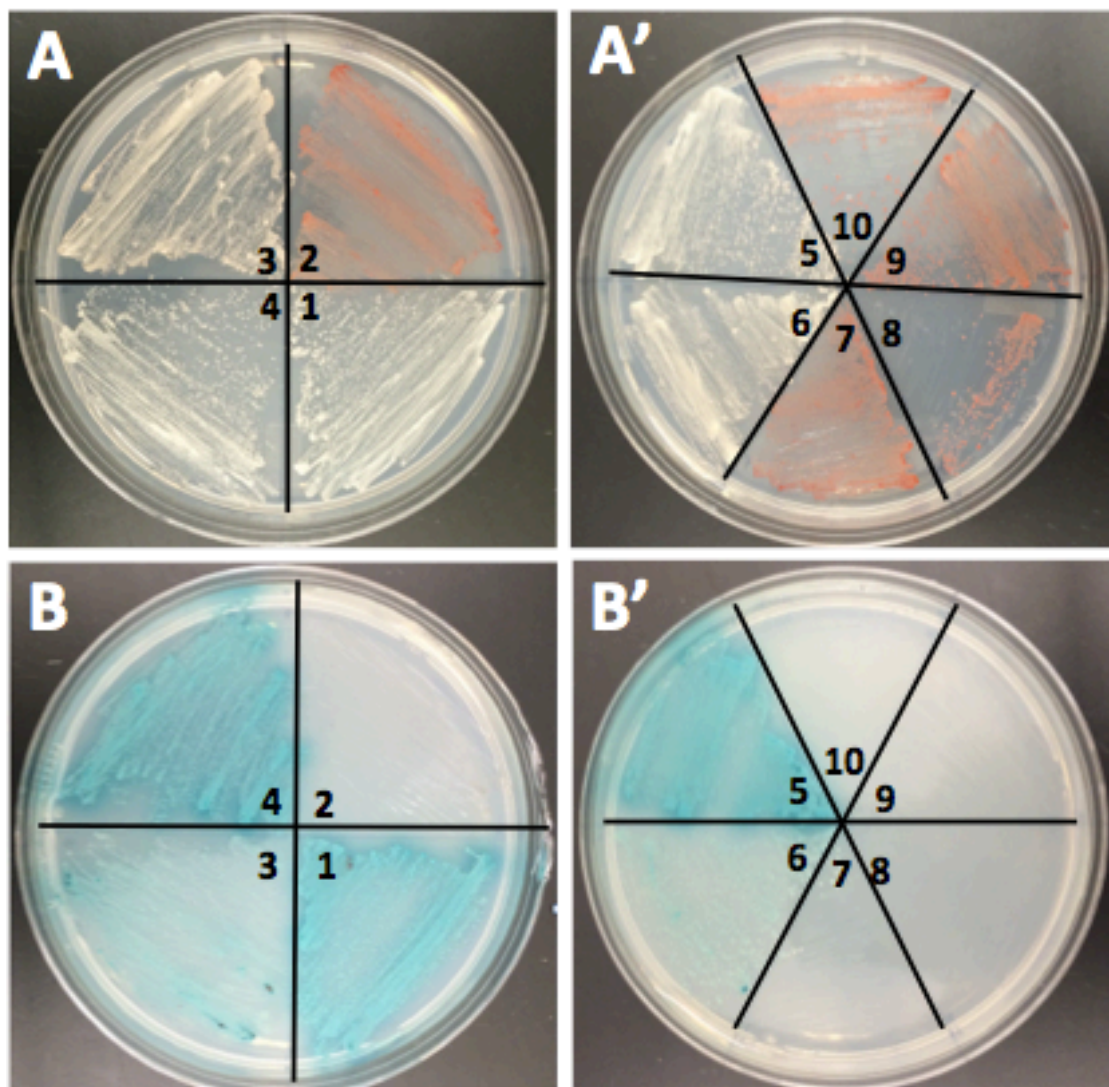
#### *Rib and Lola physically interact via their BTB domains*

Given the observations that *rib* and *lola* show a strong genetic interaction, co-localize in the SGPs, and both contain a BTB domain, we hypothesized that

these proteins physically interact to regulate embryonic gonad development. Therefore, we tested the ability of the BTB domains of these proteins to interact physically by performing a yeast two-hybrid assay. The Rib and Lola BTB domains were each fused to the yeast GAL4 DNA binding domain (BD) and the GAL4 activation domain (AD). Positive and negative controls, as well as strains expressing BD-RIB and AD-Lola, AD-Rib and BD-Lola, AD-Rib and BD-Rib, and AD-Lola and BD-Lola were successfully mated (Fig. 7A). The ability of AD and BD fusion proteins were tested using four reporters under the control of GAL4 upstream activating sequences: His3, Ade2, *LacZ*, and the *AUR1-C* gene, which confers resistance to Aureobasidin A. Mated yeast were grown on a quadruple dropout plate (QDO), lacking Trp, Leu, His, and Ade, and supplemented with X- $\alpha$ -Gal and Aureobasidin A, referred to as QDO/X/A plates. The positive control (Fig. 7A, B; 1) exhibited activation of all reporters, while the negative control (Fig. 7A, B; 2) failed to grow on the QDO/X/A plates (Fig. 7B). Lola and Rib-BD fusions were mated to the GAL4-AD (Fig. 7A, B; 7-10) alone and failed to activate reporters, demonstrating that Rib and Lola BTB domains do not autoactivate reporters (Fig. 5B; 7-10). Additionally, Lola and Rib-AD fusions mated to GAL4-BD also failed to activate reporters, demonstrating that the GAL4-BD does not nonspecifically bind to the Lola or Rib BTB domains (Fig. 7B; 3-6). Mating of BD-

Lola with AD-Rib (#5) and BD-Rib with AD-Lola (#6) resulted in activation of all reporters (Fig. 7B). We also tested the ability of the Rib and Lola BTB domains to form homodimers, and observed a more robust interaction for Lola than for Rib (Fig. 7B). These results demonstrate that Rib and Lola BTB domains are capable of homo- and heterodimerization, and suggest that Rib and Lola may form a complex to cooperatively regulate gene expression during gonad morphogenesis.

Figure 7. Rib and Lola physically interact via their BTB domains. Yeast two-hybrid assay for physical interaction of Rib and Lola. (A-A') Double dropout (DDO) SD medium without Trp and Leu; (B-B') Quadruple dropout (QDO)/X-alpha-gal, SD medium without Trp, Leu, His, Ade and with X-alpha-gal and Aureobasidin A. 1. BD-53 & AD-T (positive control); 2. BD-Lam & AD-T (negative control); 3. BD-RIB & AD-RIB; 4. BD-LOLA & AD-LOLA; 5. BD-LOLA & AD-RIB; 6. BD-RIB & AD-LOLA; 7. BD-RIB & AD alone; 8. BD-LOLA & AD alone 9. BD alone & AD-RIB; 10. BD alone & AD- LOLA.



*rib and lola are transcriptional activators and repressors*

Previous studies have suggested that Rib and Lola function as transcriptional repressors (Cavarec et al., 1997; Kerman et al., 2008). However, few potential targets have been identified, and it remains unclear if they function solely as transcriptional repressors or may also function in transcriptional activation. In order to determine if Rib and Lola function as transcriptional activators and/or repressors, we examined the localization of the Rib and Lola protein with marks of transcriptional activation and repression on polytene chromosomes. First, Rib and Lola expression was examined in combination with immunostaining for H3K27me3, a mark of transcriptional repression. Results demonstrated that Lola colocalized at H3K27me3 on numerous sites on the chromosome, as noted in Figure 8 where yellow indicates colocalization of green (Lola) and red (H3K27me3) signals (Fig. 8A, A'). As the Rib antibody did not exhibit strong staining on polytene chromosomes in our hands, we expressed 3x-HA tagged Rib in salivary glands using Forkhead-Gal4. We observed colocalization of 3x-HA-Rib and HK3K27me3 at numerous sites on polytene chromosomes (Figure 8C, C'). While Lola and Rib colocalize with H3K27me3 at a number of loci on polytene chromosomes, the presence of Lola and Rib at other, distinct, sites suggests that Rib and Lola may also localize at sites of

transcriptional activation. In order to determine if Lola and Rib may function in transcriptional activation, we examined Rib and Lola colocalization with sites of RNA Polymerase II phosphoserine 5 (PolIIser5). Lola and Rib also colocalized with PolIIser5 at many sites on polytene chromosomes (Fig 8B, B', D, D'). These results suggest that Rib and Lola function may not be limited to transcriptional repression, but rather that these proteins have dual functions as transcriptional activators and repressors.

## **Materials and Methods**

### *Fly strains and genetics*

For this work the following fly stocks were used: *lola*<sup>46.38</sup>, *lola*<sup>22.05</sup>, *rib*<sup>35.14</sup>, *rib*<sup>55.25</sup> (Weyers et al., 2011), *lola*<sup>ORE76</sup>, *lola*<sup>ORC4</sup> (Giniger et al., 1994), *rib*<sup>1</sup> (Bradley and Andrew, 2001), Forkhead-Gal4 (Henderson and Andrew, 2000), 68-77-*lacZ* (Simon et al., 1990). Balancer chromosomes carrying a GFP or YFP transgene were used for genotyping. Stocks not specified in this section are from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN, USA).



Figure 8. Colocalization of Rib and Lola with transcriptional activator and repressor markers. Immunofluorescence staining of polytene chromosomes. (A-A') Oregon-R polytene chromosomes stained with anti-Lola (green) and anti-H3K27me3 (red), with the merge showing areas of colocalization. Scale bar: 10 $\mu$ m. (A') Zoomed image of colocalization (arrows) between Lola and the transcriptional repressor marker H3K27me3. (B-B') Oregon-R polytene chromosomes stained with anti-Lola (green) and anti-PolIIser5 (red), with the merge showing areas of colocalization. Scale bar: 10 $\mu$ m. (B') Zoomed image of colocalization (arrows) between Lola and a marker for transcriptional activation, PolIIser5. (C-C') Forkhead-Gal4; UAS-3x-HA-Rib polytene chromosomes stained with anti-HA (green) and anti-H3K27me3 (red), with the merge showing areas of colocalization. Scale bar: 10 $\mu$ m. (C') Zoomed image of colocalization (arrows) between HA-Rib and the transcriptional repressor marker H3K27me3. (D-D') Forkhead-Gal4; UAS-3x-HA-Rib polytene chromosomes stained with anti-HA (green) and anti-PolIIser5 (red), with the merge showing areas of colocalization. Scale bar: 10 $\mu$ m. (D') Zoomed image of colocalization (arrows) between HA-Rib and a marker for transcriptional activation, PolIIser5.

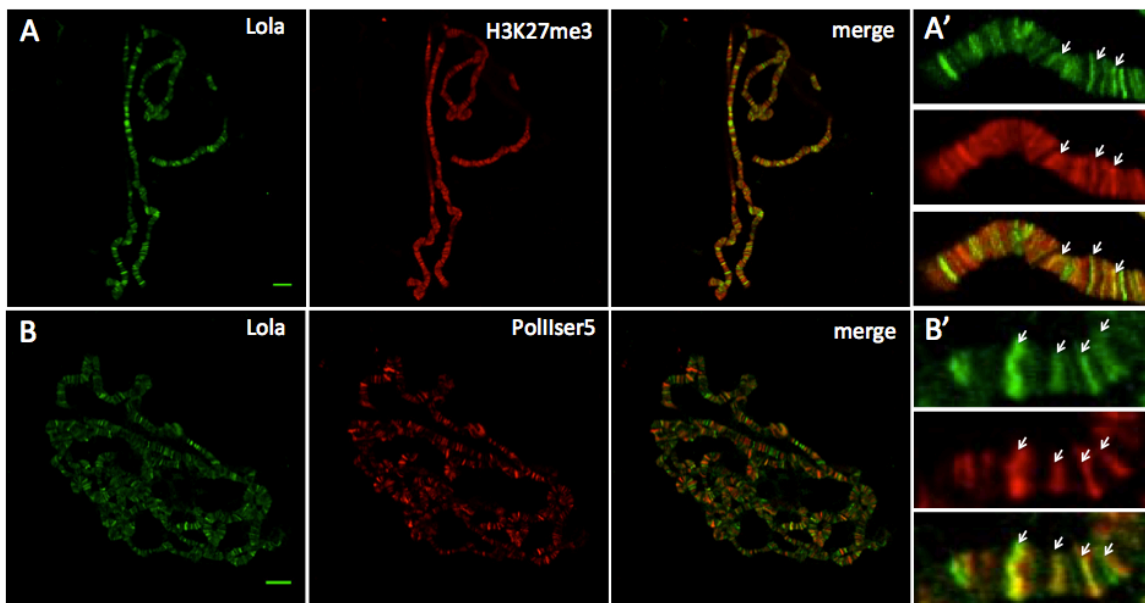
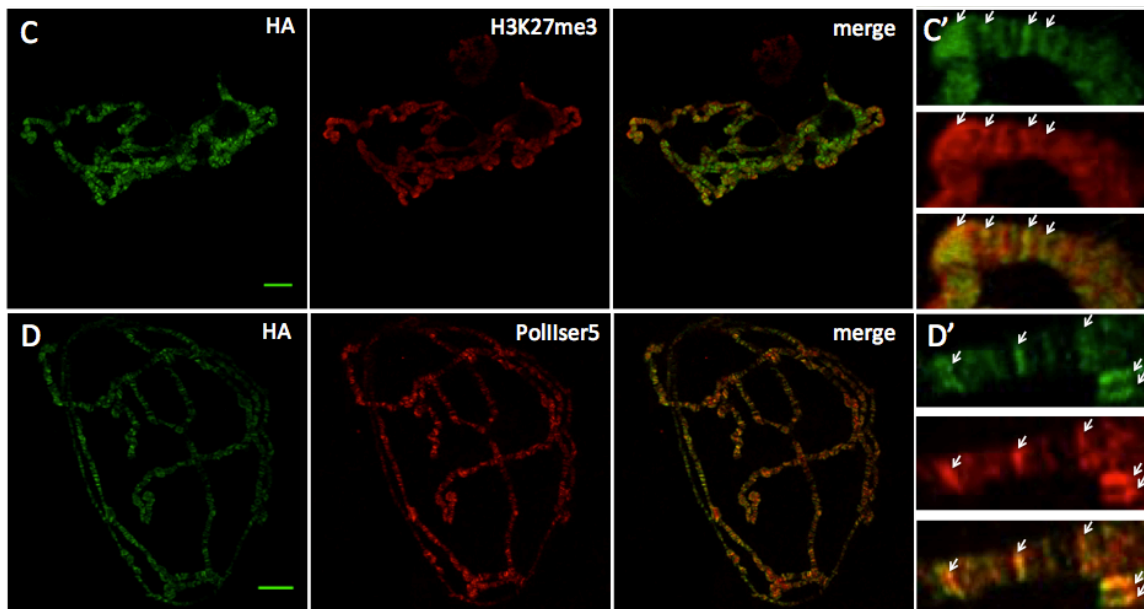


Figure 8. Colocalization of Rib and Lola with transcriptional activator and repressor markers. Continued.



*Immunohistochemistry and microscopy*

Embryo fixation and immunostaining were performed as previously described in (Jemc et al., 2012). The following primary antibodies were used (dilution, source): chick-GFP (1:1000, Abcam); rabbit anti-GFP (1:2000, Torrey Pines Biolabs); rabbit anti- $\beta$ -Galactosidase (1:10,000, Cappel); rat anti-Rib (1:50, Kerman et al., 2008); rabbit anti-Lola (1:100, Giniger et al., 1994); guinea pig anti-Traffic Jam (1:1000, Jemc et al., 2012); rabbit anti-Vasa (1:200, Santa Cruz Biotechnology); rat anti-Vasa (1:50, Developmental Studies Hybridoma Bank (DSHB)); and mouse anti-Fasciclin 3 (1:30, DSHB). Alexafluor 488, 546, and 633 conjugated secondary antibodies used at 1:500 (Invitrogen) and mounted in

DABCO for immunofluorescence microscopy. For immunohistochemical staining, biotin conjugated secondaries (Jackson ImmunoResearch) were used at 1:5000, and the stain was developed using the ABC Elite kit (Vector Labs) using DAB (3'3'-diaminobenzidine) as a substrate (Vector Labs). These embryo stains were mounted on 80% glycerol with 20% PBTween solution. Embryos were staged according to their gut morphology. Fluorescently stained embryos were imaged on an Olympus Fluoview 1000 confocal microscope equipped with 488, 561 and 633 lasers. Immunohistochemically stained embryos were imaged on a Zeiss AxioImager. Images were processed using ImageJ software.

#### *Plasmid construction*

For yeast two-hybrid analysis, DNA fragments were PCR amplified from LD16058 DNA (*rib*) and LD28033 DNA (*lola*) (Drosophila Genomics Resource Center), using the following primers: Rib-NdeI-Fwd 5'-CAT GCA TAT GGG CGG CCC AAC GGC G-3', Rib-BamHI-Rev 5'-TGC AAG GAT CCT ATG ATT GAA CTT CAT CAA GTT GTC GTA CAG AC-3', Lola-NdeI-Fwd 5'-CAT GCA TAT GGA TGA CGA TCA GCA GTT TTG TTT G-3', Lola-BamHI-Rev 5'-TGC AAG GAT CCT TAC TCC GCC GCC AGT GCG-3'. PCR fragments were cloned into the MCS of the pGADT7 AD and pGBKT7 vectors (Clontech) using NdeI and BamHI.

For the UAS-3x-HA-rib transgene, DNA fragments were PCR amplified from LD16058 DNA (*rib*), using the following primers: Rib-FL-Reverse 5'- CAA GGG ATC CGC GTT AAT CAG TCG GCC CGG GCC TGA GCG T-3", 3xHA-rib-Kozak 5'- CAA GGC GGC CGC GCC GCC ACC ATG GGA TAC CCA TAC GAT GTT CCA GAT TAC GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT GGA GGA GGC GGC CCA ACG GCG CCG -3'. PCR fragments were cloned into the pUASpB (a modified version of pUASP (Rorth, 1998) with an attB site for phiC31-mediated integration) using NotI and BamHI. Transgenic flies were generated by integration of this construct into P{CARYP} attP40 (Groth et al., 2004; Markstein et al., 2008) by phiC31 integration by BestGene Inc. (Chino Hills, CA).

#### *Yeast-two hybrid interaction*

The yeast strains Y2H-Gold and Y187 plasmid were transformed with pGADT7 and pGBKT7 vectors, respectively, by standard LiAc-TE transformation and transformants selected on SD-LEU and SD-TRP plates. The yeast two-hybrid was performed according to the Matchmaker Gold Yeast Two-Hybrid System User Manual (Clontech) with a few variations. For yeast mating, individual yeast colonies were inoculated into 5ml of YPDA in 250ml Erlenmeyer flasks in the morning. In the mid/late afternoon, yeast growth was measured by a

spectrophotometer to ensure similar culture density. The desired mating combinations were mixed with equal amounts of media in a 500ml Erlenmeyer flask and then the volume was brought up to ~10ml for incubation overnight at 30°C. After 20 hours, cultures were examined for the presence of zygotes in the media and plated on SD-Trp-Leu media to select for successful mating.

Following successful mating, colonies were streaked on quadruple dropout media (SD-Leu-Trp-His-Ade), supplemented with X- $\alpha$ -Gal and Aureobasidin A for yeast two-hybrid analysis.

#### *Salivary Gland Polytene Chromosome Squashes*

Third instar larval salivary glands were dissected in 1xPBS and fixed as follows: fix 1 (100ul 37% formaldehyde, 700ul H<sub>2</sub>O, 100ul 10xPBS and 100ul 10% Tween-20) for 1 minute, fix 2 (100ul 37% formaldehyde, 300ul H<sub>2</sub>O, 500ul glacial acetic acid and 100ul 10% Tween-20) for 2 minutes, fix 3 (550ul H<sub>2</sub>O and 450ul glacial acetic acid) for 5 minutes. After fixation the salivary glands were transferred onto a siliconized cover slip (using Sigmacote SL-2; Sigma-Aldrich) and was then flipped over onto a poly-L-lysine treated slide and squashed using the thumb and applying firm pressure down onto the salivary glands for about 50-60 seconds - 1 minute. The slides were frozen in liquid nitrogen, the cover slip was popped off immediately after freezing, and slides were transferred to 1x

PBS. The slides were then washed 2 times for 30 minutes in PBST (1xPBS and 0.1% Tween-20) and 1 time for 30 minutes in antibody dilution buffer (1xPBS, 0.1% Triton-X-100, 5% milk). Slides were incubated overnight at 4°C in a humid chamber in antibody dilution buffer containing the following antibodies: mouse anti-H3K27me3 (1:125, Millipore); mouse anti-RNA Polymerase II H14 (1:35, BioLegend), rat anti-HA (1:100, Roche Diagnostics), rabbit anti-Lola (1:100, Giniger et al., 1994). Following incubation, slides were washed three times in PBST for 15 minutes and incubated for 1 hour at 37°C in the appropriate secondary antibodies, diluted 1:400 in antibody dilution buffer. After incubation, slides were washed once again three times in PBST for 15 minutes and mounted in DABCO for viewing. Polytene chromosomes were imaged on an Olympus Fluoview 1000 equipped with 488, 561 and 633 lasers. Images were processed using ImageJ software.

## **Discussion**

*rib* and *lola* mutants exhibit defects in SGP cluster fusion and gonad compaction during embryonic gonad development (Fig. 2B-G). Consistent with the mutant phenotypes, both proteins are expressed in the SGPs; however, they are also expressed strongly in the surrounding mesoderm and weakly in the PGCs (Fig. 5A-B''). Given that the SGPs are derived from the mesoderm, it was

critical to examine the specification and early development of another mesodermally derived tissue to determine if the gonad defects observed in *rib* and *lola* mutants were due to global defects in mesoderm development. Examination of the specification and early development of the visceral mesoderm revealed that the tissue appeared normal, suggesting that the gonad defects observed in *rib* and *lola* mutants are due to a specific requirement for these proteins in the SGP (Fig. 4). While SGP cluster fusion defects result when SGPs fail to interact with each other, defects in compaction are suggestive of altered PGC-SGP interaction. Extragonadal PGCs were observed in many *rib* and *lola* mutant embryos, suggesting the Rib and Lola may function directly or indirectly to regulate the migration of PGCs and/or their ability to interact with SGPs (data not shown). Other studies have reported reduced ensheathment of PGCs by SGPs in *lola* mutants (Tripathy et al., 2014; Weyers et al., 2011), consistent with a role for Lola in regulating SGP-PGC interactions. However, these phenotypes were rescued by overexpression of *lola* in the SGPs (Tripathy et al., 2014). Identification of the molecular mechanisms through which Rib and Lola function to regulate cell-cell interactions is critical for understanding how these proteins regulate tissue morphogenesis.

Molecularly, both Lola and Rib contain an N-terminal BTB domain and unique C-terminal domains. While Rib encodes 3 protein isoforms that all contain a central PSQ DNA binding motif, alternative splicing of *lola* transcripts gives rise to at least 20 different protein isoforms with variable C-terminal domains. Of the 20 different *lola* protein isoforms identified, only 3 lack a zinc finger DNA binding domain at the C-terminus, which are isoforms A, G and M, the rest of the 17 isoforms have either one or two zinc fingers (Davies et al., 2013). The presence of these zinc fingers on these *lola* isoforms most likely increases the possibility of these isoforms having the capability of binding to DNA. Thus, if Lola has the capability of binding to DNA then it most likely must be a transcriptional activator or repressor of a group of target genes. Not surprisingly, we and others have found that different isoforms are expressed and function in different tissues (Davies et al., 2013; Goeke et al., 2003; Southall et al., 2014; Tripathy et al., 2014). In the embryo we find that Lola-T, which is specifically mutated by the *lola*<sup>46,38</sup> allele, is required for gonad morphogenesis, consistent with previous studies by Tripathy et al (Fig. 3B, 6) (Tripathy et al., 2014). However, we also observe defects in gonad morphogenesis with a Lola-K isoform-specific mutant allele, *lola*<sup>ORC4</sup> (Fig. 3B, 6). Comparison of the T and K isoforms reveals significant similarity in their ZF domains, suggesting these



isoforms may regulate transcription of a common set of genes (Goeke et al., 2003). Interestingly, we also observe expression of Lola-K and Lola-T GFP fusion proteins in the developing embryonic gonad, supporting a role for both isoforms in the gonad (data not shown; S. Elahi and J. Jemc). The ability of overexpression of *lola-T* to rescue the *lola* mutant gonad phenotype in mutants predicted to lack all Lola isoforms, suggests that different isoforms may be able to compensate for each other if they share DNA binding similarities. It remains to be determined if overexpression of *lola-K* would be sufficient to rescue the *lola* null mutant phenotype. Given the presence of the BTB domain in all Lola isoforms, it is feasible that different Lola isoforms dimerize to cooperatively regulate gonad development; however, this hypothesis remains untested.

The presence of the BTB domain in Rib and Lola suggested that these proteins may cooperate to regulate gonad morphogenesis, as has been observed for other BTB containing proteins, including Pipsqueak, Trithorax-like, Batman and Bric-a-brac, which function together to limit ovariole number in the *Drosophila* ovary (Bartoletti et al., 2012). Rib also interacts with another BTB family protein, Lola-like, to regulate salivary gland and trachea development (Kerman et al., 2008).

Genetic analysis reveals that these genes genetically interact in the context of gonad development based on phenotypic synergy (Fig. 6), suggesting that these genes function cooperatively in the same pathway or in parallel pathways to regulate gonad development. Expression analysis of Rib protein in *lola* mutants and Lola protein in *rib* mutants reveals that these proteins do not regulate expression of each other (Fig. 5D-G''), making it unlikely that they function in a stepwise fashion to promote gonad morphogenesis. The observation that Rib and Lola BTB domains physically interact in a yeast two-hybrid assay, suggests that they may form a complex to regulate gene expression and promote gonad development (Fig. 7). The synergistic effect of *lola* and *rib* mutations observed in the gonad, and their physical interaction by yeast two-hybrid analysis leads to ask if these proteins may also be interacting in other contexts, including the adult gonad, salivary gland and the nervous system. It is also feasible that Rib and Lola may cooperate with other BTB family in a context-dependent manner.

Given that Rib and Lola both contain DNA binding domains, with Rib containing a PSQ motif, and many Lola isoforms containing at least one ZF domain, both proteins have been predicted to function as transcriptional regulators (Fig. 3B) (Bradley and Andrew, 2001; Goetze et al., 2003; Shim et al.,

2001). Previous work has demonstrated that various BTB family proteins have acted as transcriptional regulators and repressors. While both Rib and Lola have been shown to regulate expression of a number of genes, their ability to directly activate to repress gene expression remains largely uncharacterized (Cavarec et al., 1997; Gates et al., 2011; Giniger et al., 1994; Kerman et al., 2008). Only in the case of the *copia* retrotransposon was Lola found to directly bind DNA to repress its expression in the embryonic central nervous system (Cavarec et al., 1997). Interestingly, Lola appears to activate expression of *copia* in the gonad, suggesting that a single Lola isoform may have dual functions or that different Lola isoforms cause divergent regulation of *copia* expression in different tissues. The transcriptional activity of Lola could also depend on its interaction with other BTB and/or non-BTB domain-containing proteins. Therefore, we examined the possibility that Rib and Lola function as dual function transcriptional regulators using polytene chromosome squashes and immunofluorescence analysis. We observe the colocalization of Rib and Lola with marks of transcriptional activation and repression, suggesting that these proteins act as dual-function transcriptional regulators. Future studies will be critical for the identification of these transcriptional targets in gonad development, in order to understand the defects in tissue morphogenesis that arise in *rib* and *lola* mutants.

Previous studies have revealed numerous putative targets of Lola in the central nervous system, including the cytoskeletal regulator *spire*, as well as members of the Slit/Robo signaling pathway (Gates et al., 2011; Giniger et al., 1994). Both *slit* and *robo* have also been implicated embryonic gonad morphogenesis, suggesting these genes might be downstream targets of Lola in the gonad as well (Weyers et al., 2011). Further studies are needed to determine if these genes are direct targets of Lola, or if they are regulated by Lola indirectly.

Rib has been demonstrated to regulate expression of *crb* and the activity of Moe in the polarized epithelium of the salivary gland and trachea (Kerman et al., 2008). It remains to be determined if Rib regulates these downstream targets in the gonad as well. Given the lack of apico-basal polarity in the SGPs and PGCs of the embryonic gonad, it is possible that Rib functions through other downstream targets to promote gonad morphogenesis. It is also feasible that Rib may differentially regulate these targets in a tissue-specific manner based on its interactions with different BTB family proteins, as Rib genetically interacts with Lola in the salivary gland and trachea and with Lola in the gonad (Kerman et al., 2008). Chromatin immunoprecipitation-sequencing studies and expression profiling experiments will be critical for the identification of Lola and Rib target

genes, and for our understanding of how BTB domain proteins cooperate to regulate gene expression.

In order to more fully understand how Rib and Lola promote tissue morphogenesis, it is also critical to characterize the molecular mechanisms regulating Rib and Lola expression and activity. Rib has been suggested to function downstream of the Mitogen Activated Protein Kinase (MAPK) signaling pathway based on the similarity of mutant phenotypes of *rib* and members of the Fibroblast Growth Factor (FGF)-MAPK signaling pathway, as well as the presence of seven MAPK consensus phosphorylation sites in Rib (Bradley and Andrew, 2001). In contrast, we know nothing about the mechanisms regulating Lola function. With little to no knowledge of signaling pathways for each of these proteins, it would be beneficial to perform a genetic screen to identify signaling pathways with which Lola and Rib may interact in. Identification of signaling pathways with which *rib* and *lola* genetically interact, would provide insight into the mechanisms regulating expression and function of these proteins and allow us to better understand how they are functioning in within a network to regulate tissue development.

Although there are no human orthologs of *lola* and *rib*, there are similar proteins to Lola in vertebrates, which include Zfp131, Miz-1, and Leukemia-

Related Factor (LRF) (Southall et al., 2014). Zfp131 exhibits the most similar expression pattern to Lola, as it is expressed in the testes, adult brain and the developing nervous system (Southall et al., 2014; Trappe et al., 2002). In contrast, Rib does not have any similar vertebrate proteins, as the BTB domain has not been observed in combination with the PSQ motif in vertebrates. However, other BTB family proteins may functionally substitute for Rib in the context of vertebrate gonad development. The characterization of the roles of Rib and Lola in embryonic gonad development, their genetic and physical interaction, and their colocalization with regions of active and inactive transcription on polytene chromosomes, suggests that cooperative regulation of gene expression by BTB family proteins may be used in a variety of developmental and disease contexts. The implication of *Drosophila* and mammalian BTB proteins in lymphocyte, skeletal, gonad and neurological development, as well as in cancer, illustrates the importance of understanding the mechanisms by which these proteins cooperate to regulate gene expression (Siggs and Beutler, 2012). Identification of the direct downstream transcriptional targets of these genes and molecular pathways in which they function is critical for understanding how these regulate development and contribute to disease.

APPENDIX A

SORTING OUT IDENTITIES: AN EDUCATIONAL PRIMER FOR USE WITH  
"NOVEL TOOLS FOR GENETIC MANIPULATION OF FOLLICLE STEM CELLS  
IN THE *DROSOPHILA* OVARY REVEAL AN INTEGRIN-DEPENDENT  
TRANSITION FROM QUIESCENCE TO PROLIFERATION"

Diane Silva and Jennifer C. Jemc

*Genetics* 2015. 13-22.

## Summary

Organisms are made up of thousands of different cell types that must migrate, proliferate, and interact with each other to yield functional organ systems and ultimately a viable organism. A characteristic that distinguishes one cell type from another is the set of genes that it expresses. An article by Hartman et al. in the April 2015 issue of GENETICS identified methods to uniquely identify different cell populations during oogenesis, providing valuable tools for future studies. This Primer article provides background information on the *Drosophila* ovary as a system in which to study stem cell regulation, mechanisms for regulating gene expression, and the techniques used by Hartman et al. to identify specific cell populations and study their function.

## Background

Proper regulation of cell survival, division, and differentiation to a specific fate is critical throughout the lifetime of an organism. In the developing embryo, a single cell eventually gives rise to all the cells composing the adult. However, not all cells are created equal—if they were, the body would not be able to function. This raises the fundamental question “What allows one cell to look and function differently from another?”



Examination of cells of different origins reveals that specific cell types express different sets of genes, allowing them to assume diverse functions. Cells begin to assume different fates based on signals received from their extracellular environment, including the cells around them. Amazingly, the molecules that control these outcomes are highly conserved from organisms like the nematode worm, composed of, 1000 cells, to more complex eukaryotes including fruit flies, mice, and humans. When signals diffuse from one cell to the next, they set into motion a series of events that frequently leads to changes in gene expression. Genes consist not only of the DNA sequence encoding a specific RNA or protein, but also of critical transcriptional regulatory elements, including promoters, enhancers, and silencers, that help determine when, where, and to what level genes are expressed.

Production of specific cell types via differential gene expression is by no means unique to the developing embryo. Throughout its lifetime, an organism must replace specific populations of cells, balancing cell death with cell proliferation, to maintain homeostasis. Stem cells of various types play a critical role in maintaining homeostasis. In the adult organism, tissue-specific stem cells have the ability to give rise to the cell types present in the tissue in which they reside; these cells are responsible for replacing cells lost due to

damage or death. Examples include the hematopoietic stem cells that give rise to the cells found in the blood, including red blood cells and lymphocytes, and germline stem cells (GSCs) that are critical for the continued production of sperm or eggs.

Hartman et al. (2015) focus on a population of somatic, or nongermline, stem cells in the fruit fly ovary known as follicle stem cells (FSCs). FSCs produce follicle cells that will surround the germ cells throughout most of oogenesis (reviewed in Spradling 1993). As these cells perform a critical role in supporting germ cell development, it is important to understand how these cells function in the gonad. However, a significant limitation to these studies has been a lack of ways to effectively distinguish the FSCs from other somatic cell populations within the gonad and to manipulate gene expression within specific cell types. Hartman et al. (2015) set out to alleviate this difficulty by identifying genetic elements that regulate gene expression in different cell populations in the ovary, specifically the FSCs. They then can label and manipulate the FSCs and probe the role of specific genes in FSCs.

### **The System: *Drosophila* Ovary**

The fruit fly *Drosophila melanogaster* has proven to be an excellent model organism for scientific research given its 10-day generation time,

conservation of genes (nearly 75% of human disease-associated genes are conserved in flies), and abundance of tools available for genetic manipulation (reviewed in Roote and Prokop 2013). Hartman et al. (2015) utilized these tools to examine gene expression in the adult ovary. *Drosophila* have two ovaries, each composed of 15–20 ovarioles (Fig. 9; reviewed in Spradling 1993). Each ovariole consists of a single germarium and a number of maturing egg chambers that are connected by stalk cells, appearing like beads on a string (reviewed in Spradling 1993). The germarium functions as the source for both germ cells, some of which will give rise to eggs, and somatic gonadal cells, which support the development of the germ cells (reviewed in Spradling 1993). To continue to reproduce, female flies must continue to produce eggs through a process known as oogenesis. Critical to this process are two populations of stem cells: the GSCs and the FSCs. These cell populations each exist in a specialized microenvironment called “the niche” that supplies essential factors specific for their maintenance (reviewed in Morrison and Spradling 2008).

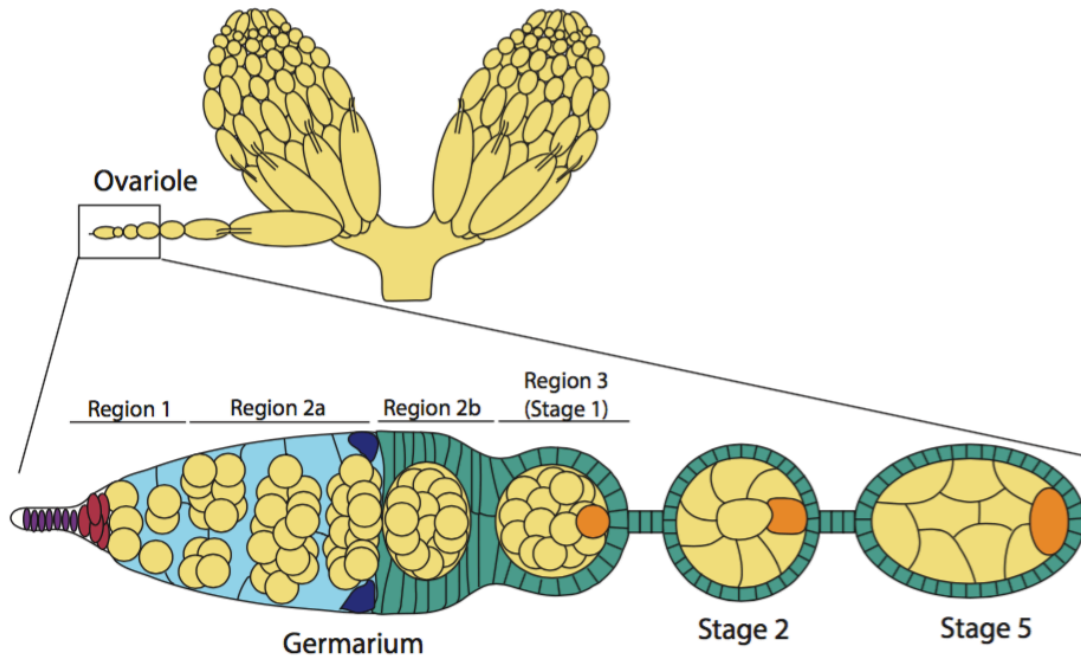
The GSC niche is located in the anterior-most region of the germarium, where five to seven terminal filament cells and three to four cap cells produce factors that regulate the proliferation and maintenance of two to three GSCs (reviewed in Spradling 1993). Upon GSC division, one cell remains in the

niche, thereby self-renewing the GSC population, while the other cell exits the niche and begins differentiation to a gonialblast. This cell undergoes four rounds of synchronized cell divisions in region 1 of the germarium, generating 2-, 4-, 8-, and 16-cell germline cysts that remain interconnected by a structure known as the fusome (Fig. 9; reviewed in Spradling 1993). During this time, a population of somatic cells known as the inner germarial sheath (IGS) cells, or escort cells, wraps around the germline cysts (Fig. 9; King 1970; Schulz et al. 2002; Decotto and Spradling 2005; Morris and Spradling 2011). These cells pass the germline cysts toward the posterior of the germarium, where germline cysts will exchange their interactions with IGS cells for encapsulation by follicle cells as they transition from region 2A to 2B (Decotto and Spradling 2005; Kirilly et al. 2011; Morris and Spradling 2011). The cyst is surrounded by a single layer of follicle cells and will bud off to form an egg chamber. Of the 16 germ cells in the egg chamber, one of these cells, the oocyte, will continue through meiosis to become the egg, while the other 15 cells function as nurse cells to provide RNAs, proteins, and organelles for the oocyte (Spradling et al. 1997).

Similar to the continued production of germ cells, continued production of follicle cells depends on a population of FSCs present in the germarium. Two FSCs are located halfway down the germarium at the

junction of regions 2A and 2B (Fig. 9; Margolis and Spradling 1995; Nystul and Spradling 2007). Their proliferation depends on signals received from regions located both anterior and posterior to the FSCs (Sahai-Hernandez et al. 2012; Vied et al. 2012; Sahai-Hernandez and Nystul 2013). Similar to GSCs, FSCs divide asymmetrically, giving rise to one daughter cell that remains in the niche as a FSC, and a second daughter cell that exits the niche and begins to differentiate (Morrison and Spradling 2008). These differentiating daughter cells first give rise to precursor follicle cells, and it is their inward migration that separates germline cysts into the individual egg chambers (Morris and Spradling 2011). Subsequently, precursor follicle cells give rise to polar cells, stalk cells, and the epithelial follicle cells that will encapsulate the 16-cell germline cyst (Fig. 9; Nystul and Spradling 2010). Studies of the characteristics and functions of FSCs have been hampered by a lack of methods for specifically marking these cells. Therefore, Hartman et al. (2015) set out to identify additional tools that can be used to distinguish FSCs from other cell populations within the ovary and to demonstrate how these tools can be used to study the function of specific genes in the FSCs.

Figure 9. The *Drosophila* ovary and oogenesis. The ovary is composed of 15–20 ovarioles. At the anterior end of each ovariole is a structure known as the germarium, which provides the germ cells and somatic gonadal cells that compose the subsequent egg chambers. Terminal filament cells (purple), cap cells (pink), germline stem cells (light pink), gonialblast and germline cysts (yellow), inner germarial sheath cells (light blue), follicle stem cells (dark blue), follicle cells (green), stalk cells (dark green), and oocyte (orange).



## Regulation of Gene Expression

As described above, gene expression is commonly used to distinguish different cell types. A gene that is being expressed is transcribed from DNA to RNA. Transcription requires the presence of transcriptional regulatory elements in the DNA region surrounding and within the gene and a number of proteins, known as transcription factors, that recruit RNA polymerase to

the DNA. Transcriptional regulatory elements are composed of two distinct families: promoters and distal regulatory elements, including enhancers and silencers (Maston et al. 2006). These elements play a critical role in determining when, where, and to what level genes are expressed.

A promoter is a region of DNA located at or just upstream of the transcriptional start site of a gene. The core promoter includes the transcriptional start site and defines the direction of transcription. In addition, it can include the TATA box, Initiator element, Downstream Promoter Element, and the Transcription Factor IIB Recognition Element. These elements bind the general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, and TFIIH) and a multi-subunit complex known as Mediator, which together are responsible for recruiting RNA Polymerase II. These factors initiate a low level of transcription, and therefore another class of transcription factors, known as activators, is required to achieve high levels of gene transcription. Transcriptional activators can bind to transcription factor-binding sites within the proximal promoter, which is located, 1 kb upstream of the core promoter and requires a specific orientation relative to the core promoter for proper function (Fig. 10A; Maston et al. 2006).

Enhancers are distal regulatory elements that contain multiple binding sites for transcriptional activators. However, enhancers differ from promoters

in that they function independently of orientation, can be present both upstream and down-stream of the gene, and are located at a greater distance from the core promoter (up to hundreds of kilobases away), resulting in looping to bring enhancer-bound transcriptional activators in close proximity to promoter elements (Fig. 10A; Pennacchio et al. 2013). Enhancers have the capability of regulating multiple genes; however, enhancer activity is often restricted based on the cell type or age or the specific physiological, pathological, or environmental conditions of the cell (Pennacchio et al. 2013). Finally, silencers share many of the same characteristics of enhancer elements, but are bound by transcriptional repressors rather than transcriptional activators (Maston et al. 2006).



Figure 10. Regulation of gene expression. (A) Transcriptional activation of a target gene is regulated by core promoter elements like the TATA box (orange) that bind to general transcription factors (blue). Binding of activator proteins (green) to transcription factor binding sites (purple) in the proximal promoter and to enhancer elements distally (light green) also regulates gene expression. The Mediator protein complex (red) helps to bridge the gap from enhancer-bound proteins to the general transcription factors. The complex of transcription factors and activators recruits RNA polymerase (RNA Pol; yellow) for transcription. (B) GAL4/UAS System. GAL4 is expressed in a specific cell type based on the enhancer/promoter element located near the GAL4 insertion site in the genome. Flies containing this insertion are mated to another fly line that contains a target gene downstream of the UAS element. The GAL4 protein binds to the UAS element to activate transcription of the target gene. The GAL80 protein functions as an inhibitor of GAL4. If GAL80 is present, transcription of the target gene will be repressed.

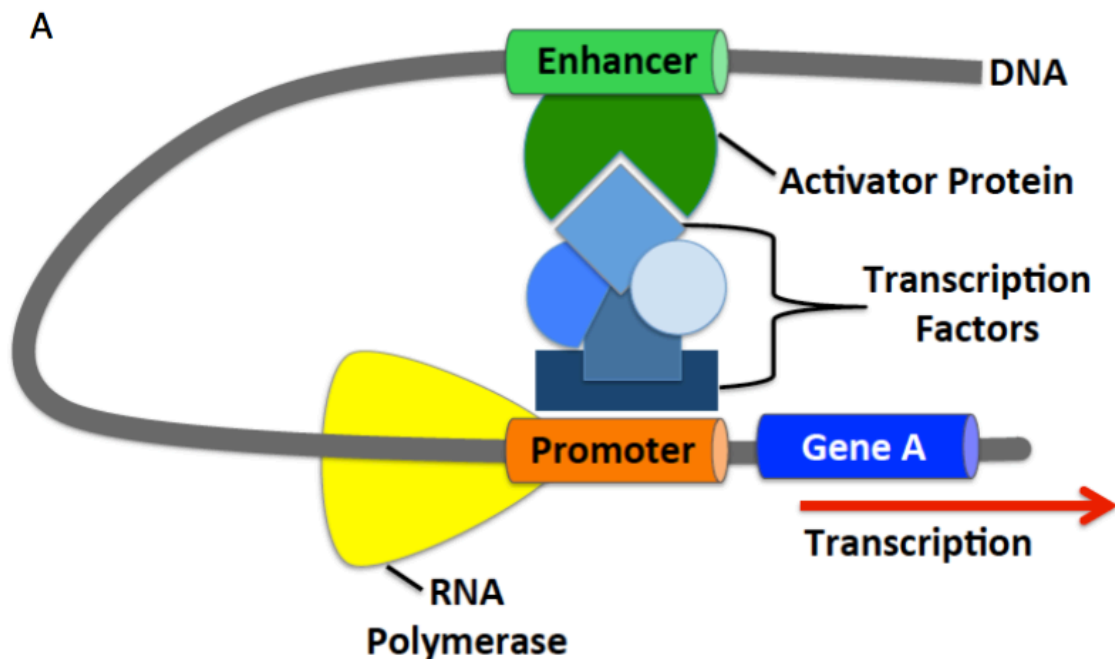
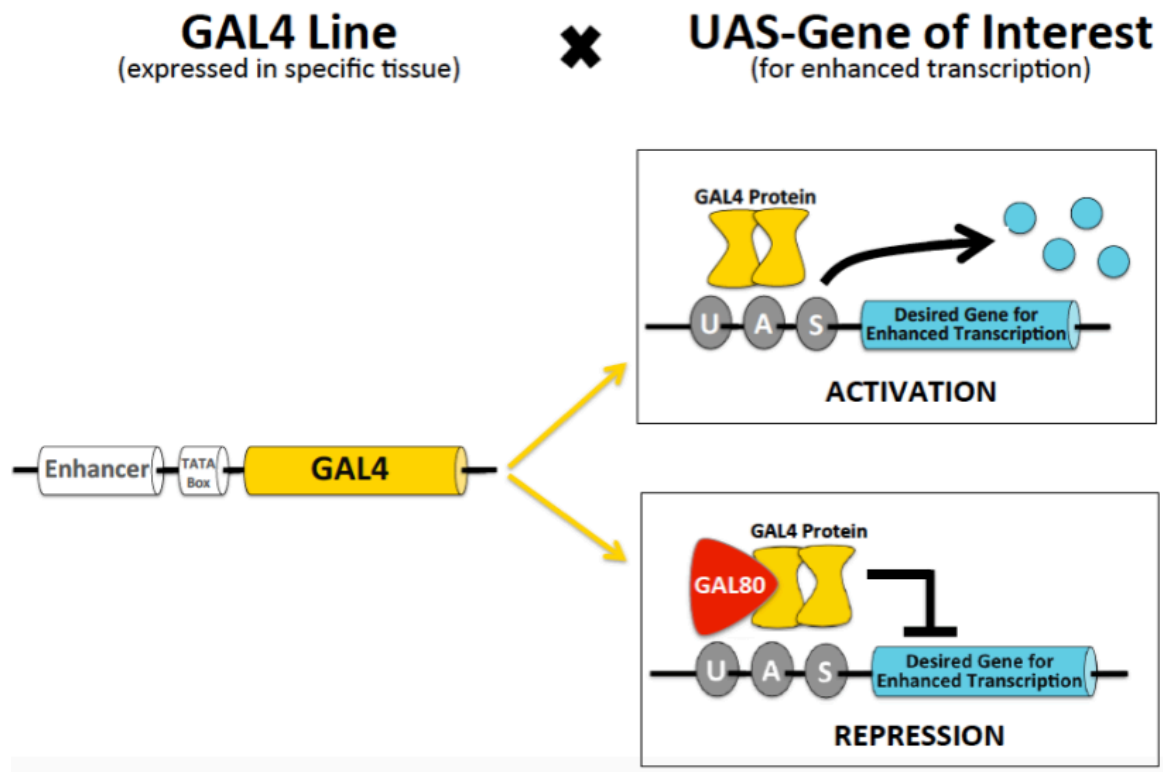


Figure 10. Regulation of gene expression. Continued.

B



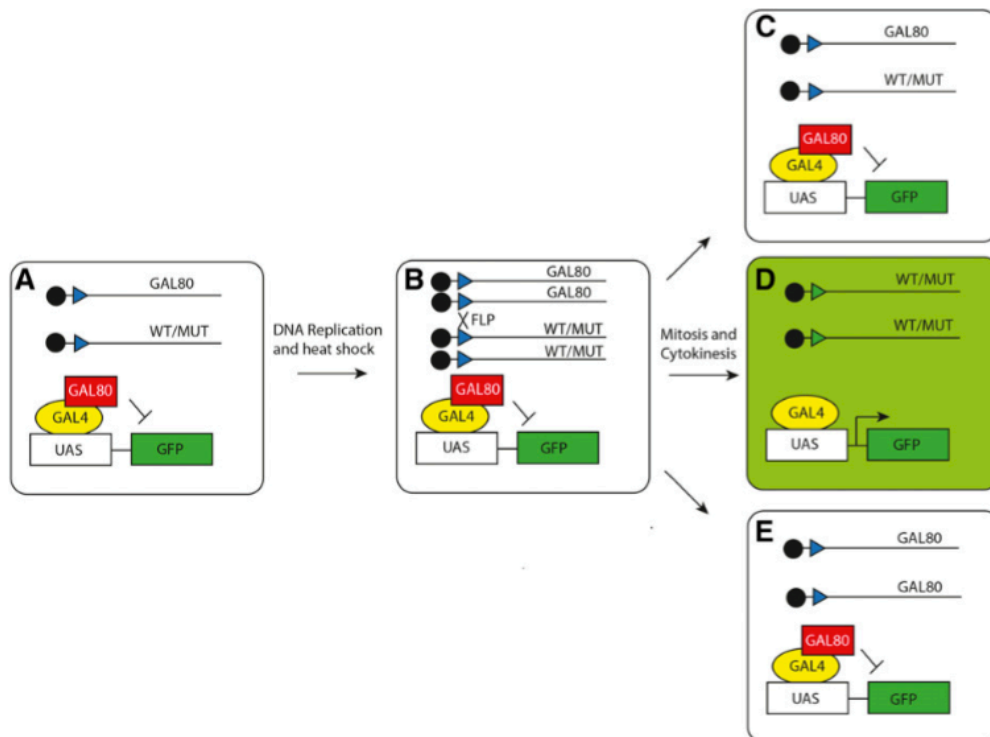
### Methods for Labeling Cells

Hartman et al. (2015) utilized promoter and enhancer elements to manipulate expression of genes to mark specific cell types. In particular, they used a set of tools, collectively known as the GAL4/UAS system from baker's yeast, *Saccharomyces cerevisiae*, to identify distinct cell types in the *Drosophila* ovary. GAL4 is a transcriptional regulator that functions by binding to a specific enhancer element, known as the Upstream Activating Sequence (UAS) element, to promote transcription of downstream genes (Fig. 10B;

reviewed in Duffy 2002). Previous studies generated *Drosophila* strains in which the GAL4 gene was inserted at sites throughout the genome (reviewed in Duffy 2002). As a result, GAL4 is expressed in specific cell types, reflecting control by nearby transcriptional regulatory elements, including promoters and enhancers. The lines are referred to as GAL4 drivers, as different regulatory elements promote or “drive” expression of the GAL4 gene. In addition, the UAS element has also been inserted upstream of genes of interest, reporter genes, and sequences encoding RNA hairpins, and these sequences have been integrated into the *Drosophila* genome. When the GAL4 gene is expressed, it binds and activates expression of the gene downstream of the UAS element (Brand and Perrimon 1993). By mating flies containing the GAL4 gene under the control of different enhancer/promoter elements with flies carrying a UAS element with a desired downstream gene, it is possible to express genes in a variety of different patterns (Fig. 10B; reviewed in Duffy 2002). The GAL4/UAS system has played an important role in research using many model organisms. Hartman et al. (2015) utilized an extensive collection of fly lines from multiple sources with insertions of GAL4 throughout the genome and a UAS element upstream of the Green Fluorescent Protein (GFP) gene to label specific populations of cells within the germlarium and visualized them using immunofluorescence microscopy, as

described below.

Figure 11. MARCM technique. (A) The cell carries a GAL4 insertion and a UAS-GFP insertion within the genome on a chromosome different from those pictured (not shown). The two copies of the chromosomes shown both contain a FRT site (blue arrowhead) near the centromere (black circle) on one chromosome arm. One of the FRT chromosomes also carries the GAL80 gene, while the other chromosome lacks the GAL80 gene and either is wild type or carries a genetic mutation. Even though GAL4 is produced in this cell, GFP transcription is inhibited by GAL80. (B) Following heat shock at 37 C, FLP recombinase is expressed and can induce recombination between the FRT sites. The continued presence of GAL80 results in repression of GFP transcription. The cell undergoes mitosis and cytokinesis. Depending on the ways in which chromosomes segregate, one can generate a cell of the genotype(s) (illustrated in C–E). (C) The resulting cell inherits one chromosome lacking the GAL80 gene, and one chromosome carrying the GAL80 gene, resulting in the repression of GFP transcription. (D) The resulting cell inherits two chromosomes lacking the GAL80 gene, allowing GAL4 to activate GFP transcription. (E) The resulting cell inherits two chromosomes carrying the GAL80 gene, resulting in repression of GFP transcription.



While the GAL4/UAS system restricts gene expression to a subset of cells, it is often desirable to limit expression to just a couple of cells at a time. Imagine that you are studying the shape of cells in a given tissue and that you have labeled the membranes of those cells. A problem arises when you need to distinguish one specific cell from its neighbor; you need a way to label just a few cells within the tissue rather than all of them. One method that has proven particularly useful for labeling a few cells in a tissue is known as Mosaic Analysis with a Repressible Cell Marker (MARCM) (Fig. 11; Lee and Luo 1999). This method allows for tighter control of the GAL4/UAS system with the introduction of an inhibitor of GAL4, known as GAL80. GAL80 binds to GAL4, preventing it from activating transcription of a gene, like GFP, downstream of the UAS element (Fig. 10B; reviewed in Duffy 2002). Two additional elements derived from yeast were also incorporated to generate mosaically labeled tissue: an enzyme known as FLP recombinase and FLP recombinase target (FRT) sites (Golic and Lindquist 1989). FLP expression was under the control of a promoter from the heat-shock protein Hsp70. Therefore, this enzyme was produced only when flies were incubated at a temperature of 37, known as heat shocking (Golic and Lindquist 1989). The production of FLP and the presence of FRT sites in the same position on both copies of homologous chromosomes allow for crossing-over events to occur

at the FRT sites during mitosis (Golic and Lindquist 1989). This is unique, as it allows for the induction of mitotic recombination, a process normally limited to meiosis. In the MARCM system, the gene encoding the GAL80 repressor protein must be present on one of the chromosomes containing the FRT site (Lee and Luo 1999). Thus, following DNA replication, FLP promotes a recombination event at the FRT sites. Chromosome segregation during mitosis can result in cells with three different genotypes (Fig. 11C–E). The first cell has the same genotype as the starting cell, thus expression of the GFP marker is repressed due to the presence of GAL80 (Fig. 11C; Lee and Luo 1999). The second cell inherits two copies of the chromosome containing the FRT site but lacking the GAL80 gene, thereby allowing GAL4 to activate transcription of the GFP reporter gene (Fig. 11D; Lee and Luo 1999). The third cell inherits two copies of the chromosome containing the FRT site and the GAL80 gene, thereby repressing transcription of the GFP marker (Fig. 11E; Lee and Luo 1999). In the MARCM system, the GAL4 gene and the UAS elements are integrated at varying locations in the genome, but cannot be on the same chromosome arm as the FRT site or the GAL80 gene. This method can also be used to analyze cells mutant for a gene of interest by incorporating a mutant allele for the gene of interest on the non-GAL80 FRT chromosome (Fig. 11).

Although MARCM is useful for marking single cells, it also labels all daughter cells that arise from that single cell, thereby labeling a population of adjacent cells. As one of the authors' goals was to examine the shape, or morphology, of cells, it was disadvantageous to have adjacent cells labeled as they become difficult to distinguish. Therefore, Hartman et al. (2015) further refined the MARCM technique to control when labeled daughter cells are generated. Following the induction of clones by heat shock, they allowed the labeled daughter cells to differentiate into follicle cells and exit the germarium. Then they cultured these flies using grape juice plates, which are a poor source of proteins and lipids, to arrest cell division in the specific cells of interest. These cells are said to be quiescent. Addition of nutrient-rich yeast paste to the plates resulted in a transition back to proliferation. Using this technique, it was possible to label a limited number of cells and to inhibit the generation of similarly labeled daughter cells, allowing for analysis of cell morphology by immunofluorescence microscopy.

### **Visualization of Gene Expression**

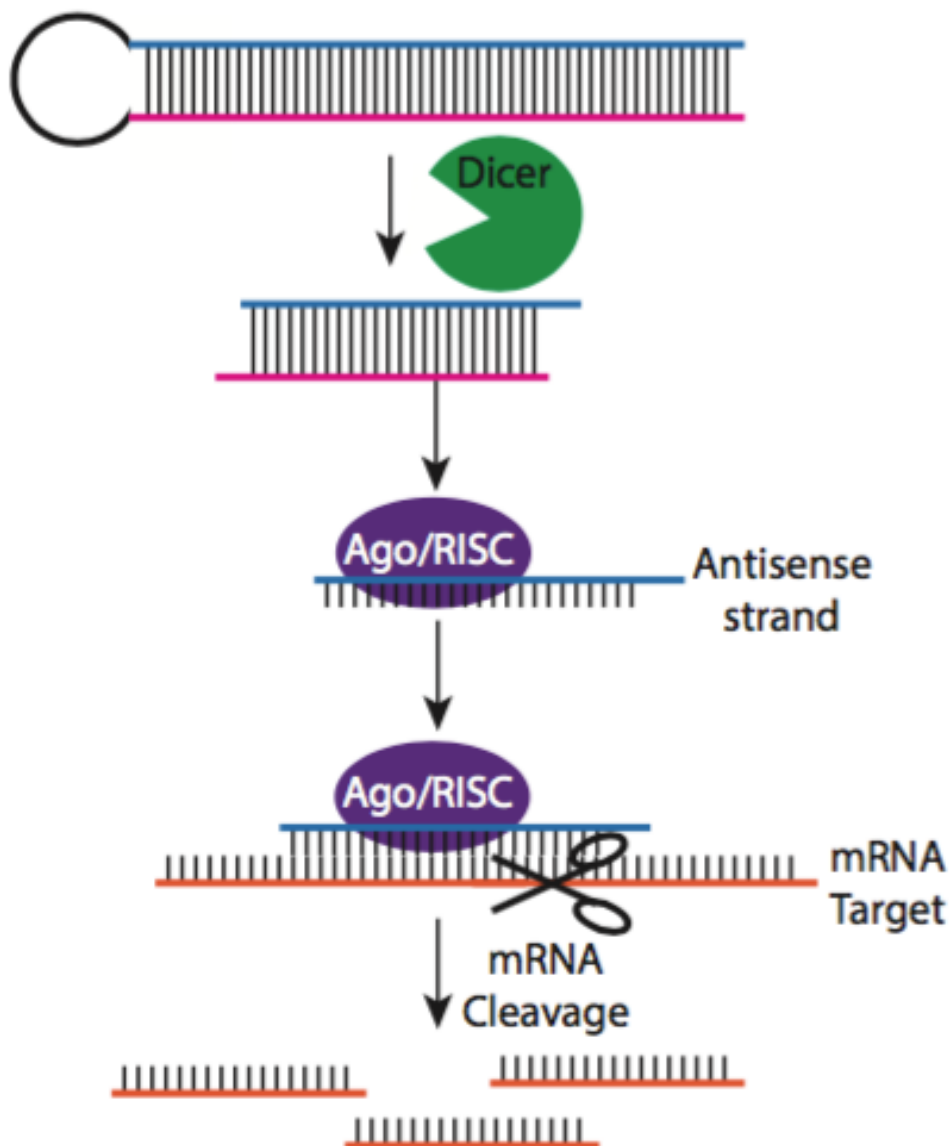
To be able to see the GFP reporter and to identify the cell and tissue types expressing the reporter, immunofluorescence microscopy is used. This technique allows one to identify where a protein of interest is expressed with

the help of additional markers for other cell types and specific cellular structures. Before tissues or cells can be used for immunofluorescence microscopy, they are fixed to preserve and stabilize the tissue structure. Following fixation, the sample is incubated with the desired primary antibodies. A primary antibody recognizes a specific antigen, like GFP, and is generated by injecting a protein, or a portion of that protein, into a host animal, typically rabbit, mouse, guinea pig, rat, or chicken. Hartman et al. (2015) used not only a GFP antibody generated in the chicken, but also a Fasciclin 3 (Fas 3) antibody generated in mouse and a Vasa antibody generated in rabbit to mark the germ cells. After washing out any primary antibody that does not bind to antigen, the sample is incubated with secondary antibodies. Secondary antibodies are typically generated in goat or donkey by injecting the animal with the common region of an antibody from rabbit, mouse, guinea pig, rat, or chicken. Therefore, the secondary antibody recognizes the conserved region of the primary antibody. It is also linked to a detectable marker like a fluorescent molecule, known as a fluorophore, which can be visualized following exposure to light of a specific wavelength and a photosensitive detector in a confocal microscope. When using multiple primary antibodies, it is critical that each of the primary antibodies be generated in a different animal and that each secondary antibody be



conjugated to a different fluorophore, making it possible to distinguish each of the different proteins. Using immunofluorescence microscopy combined with the GAL4/UAS system or MARCM, Hartman et al. (2015) were able to label different cell types or examine cell morphology in the germarium.

Figure 12. Mechanisms for RNAi. RNA forms a hair-pin and is trimmed to a shortened length of 70 nucleotides in some cases. This dsRNA is exported from the nucleus and cleaved by Dicer into the mature 21- to 25-nt dsRNA. This dsRNA is loaded onto the Ago/RNAi RISC, and one of the strands is discarded. Loaded Ago/RISC binds to target mRNA (orange). If complementarity is perfect, the mRNA target is cleaved. If complementarity is imperfect, the target mRNA is often destabilized by removal of the 5'-methylguanosine cap or poly(A) tail-binding proteins, reducing its translation.



## Reducing Gene Expression with RNA Interference

In addition to developing tools for labeling specific cell populations, Hartman et al. (2015) were also interested in using these tools to manipulate gene expression in these cells. While gene expression is regulated at the level of transcription, it can also be regulated post-transcriptionally by controlling the availability of a given messenger RNA (mRNA) for translation. Studies in a variety of organisms have demonstrated the use of RNA interference (RNAi) to control gene expression levels (reviewed in Ipsaro and Joshua-Tor 2015). RNAi is a mechanism by which cells fine-tune the levels of available RNA using microRNAs (miRNAs) and short interfering RNAs (siRNAs). In both of these mechanisms, RNA is produced that has the ability to undergo complementary base pairing, forming a double-stranded RNA (dsRNA) hairpin (Figure 12; Ipsaro and Joshua-Tor 2015). An enzyme called Dicer cuts the dsRNA into a mature 21- to 25-nt dsRNA. This dsRNA is loaded into a complex called the RNA-Induced Silencing Complex (RISC), which contains the Argonaute (Ago) protein, and one of the RNA strands is discarded (Fig. 12; Ipsaro and Joshua-Tor 2015). The remaining single stranded RNA undergoes complementary base pairing with its target mRNA, resulting in post-transcriptional gene silencing. While siRNAs typically undergo perfect base pairing with their targets, miRNAs often undergo perfect binding with a

critical sequence known as the seed sequence and imperfect binding elsewhere (Ipsaro and Joshua-Tor 2015). This results in different mechanisms of regulation. siRNAs usually promote slicing of the target mRNA, while miRNAs lead to translational repression by removing the 5'-methylguanosine cap and/or Poly(A)-binding proteins, two critical factors for recruiting proteins needed for efficient RNA translation (Ipsaro and Joshua-Tor 2015). This mechanism has been harnessed for use in the lab. Injection of short dsRNAs can promote RNAi. In addition, one can design a gene that encodes RNA capable of undergoing hairpin formation and is complementary to the mRNA from a gene of interest. If this sequence is inserted downstream of the UAS element described earlier, one can specifically control when and where RNAi occurs. Thus, there are a variety of tools available for manipulating gene expression in *Drosophila* that can be harnessed to study protein function.

### **Generating Transgenic Flies**

Many of the genetic elements described above are derived from other organisms, like yeast, or are created in the lab, like gene-specific RNAi. To utilize these reagents, it is critical to create stable fly lines containing these genetic elements. The use of transposable elements/transposons carrying

these genetic elements and the transposase enzyme allows for the incorporation of modified genetic elements into the fly genome (Rubin and Spradling 1982; Bachmann 2008). Once integrated into the genome, they are treated as endogenous genes. To generate flies carrying the GAL4 element near different enhancers and promoters, researchers first inserted the GAL4 gene into the most commonly used transposable element in flies, the *P* element. While transposons normally encode an enzyme called transposase, which helps them hop around the genome, the *P* element was modified to no longer harbor transposase activity (reviewed in Bachmann 2008). Therefore, once the *P* element is inserted in the genome it maintains a relatively stable position. To generate a transgenic fly, the DNA containing the modified *P* element and a temporary source of transposase are injected into the fly embryo at the posterior end (reviewed in Bachmann 2008). This is done at a time before the embryo has formed distinct cells via the process of cellularization. The posterior end of the embryo is where the pole cells will form, which will later give rise to sperm or eggs. Thus, the embryo that is injected will carry the DNA only in a subset of cells, but, importantly, this includes the cells that will be used to generate gametes for reproduction. In this way, the injected fly can pass on the newly inserted DNA to its offspring, resulting in a fly that will have the modified DNA in every cell of its body.

## Unpacking the Experiments

One of the challenges in studying stem cells is distinguishing stem cells from other populations of cells in the tissue. Hartman et al. (2015) were interested in exploring the morphology of FSCs and how FSCs are maintained in the germarium. To do so, they developed new tools to genetically manipulate and identify individual FSCs within the germarium, starting with the GAL4/UAS system. First, they screened flies with different GAL4 insertions for lines expressing the GFP reporter in subpopulations of somatic cells within the germarium. Once fly lines were identified that expressed the reporter in the FSCs, Hartman et al. (2015) used the MARCM system to label a subset of FSCs and to analyze their morphology. They analyzed the function of one integrin subunit, encoded by the myospheroid (*mys*) gene, in the FSCs using their newly developed techniques. Finally, RNAi was used as a genetic tool to reduce gene expression of *mys* in FSCs to determine if *mys* is required for FSCs to transition from quiescence to proliferation. Thus, Hartman et al. (2015) developed and utilized a variety of genetic tools followed by immunofluorescence microscopy to improve the accuracy of somatic cell identification in the ovary and to define the roles of genes required for FSC function.

## Utilizing the GAL4/UAS System for Cell Identification

Hartman et al. (2015) took advantage of an extensive collection of fly lines from multiple sources with insertions of GAL4 throughout the genome. By combining these GAL4 insertions and a UAS-GFP reporter, Hartman et al. (2015) were able to identify GAL4 insertions that were expressed in specific populations of cells within the germarium. They focused on GAL4 insertion lines near genes previously found to be expressed or to function in somatic cell populations in the ovary. Using this candidate approach, they identified lines expressing GAL4 in terminal filament and cap cells, stalk and polar cells, follicle cells, and IGS cells using immunofluorescence microscopy (Hartman et al. 2015). In many cases, GAL4 expression was observed in multiple somatic cell types and at multiple stages of oogenesis (see figures 1–4 and tables 1 and 2 in Hartman et al. 2015). These studies not only identified new ways of marking subsets of somatic cells within the germarium, but also resulted in the identification of GAL4 insertions that can be used to activate expression of other genes of interest at varying expression levels downstream of a UAS, including genes whose ubiquitous expression is lethal.

The next step was to find a GAL4 fly lineage that would distinguish IGS cells from FSCs at the region 2A/2B border of the embryo to analyze the genetic mechanisms controlling their behavior. Previously used fly lines

expressed GAL4 not only in IGS cells, but also in FSCs and their daughter cells. Hartman et al. (2015) identified 15 GAL4 lines capable of expression in the IGS cells. While many of these insertions were expressed in other somatic cell populations as well, two of the GAL4 insertions, one in the forked ends (fend) gene and the other in the engrailed (en) gene, are expressed primarily in IGS cells, with sporadic cap cell and FSC expression (see figure 3 in Hartman et al. 2015). These new GAL4 lines are useful for altering gene expression within a more limited range of somatic cells.

### **From Quiescent to Proliferating FSCs**

Hartman and colleagues used GAL4 expression in FSCs to study their characteristics. Previously, the distinction of FSCs from their prefollicle daughters necessitated the use of features like location, morphology, and gene expression levels (reviewed in Sahai-Hernandez et al. 2012). In an earlier study, O'Reilly et al. (2008) could not definitively say that the defects observed upon integrin mutation affected the FSCs or their prefollicle daughters using these characteristics, demonstrating the need for additional ways of distinguishing cells. As Hartman et al. (2010) had previously observed expression of 109-30-GAL4 in FSCs and all their daughters through stage 3 of egg chamber development, this GAL4 line was a good candidate for



labeling FSCs. Combining the 109-30-GAL4 with the MARCM system allowed them to label a smaller population of FSCs (see figure 5 in Hartman et al. 2015). While IGS cells were also labeled when recombination was induced during larval stages, recombination induced during adult stages labeled few IGS cells due to their decreased proliferation in the adult (see figure 5 in Hartman et al. 2015). The use of 109-30-GAL4 within the MARCM system allowed Hartman et al. (2015) to analyze the morphological characteristics of FSCs, resulting in the identification of a microtubule-based cytoplasmic extension that extends across the germarium (see figure 5, K–M, in Hartman et al. 2015).

While the MARCM approach with 109-30-GAL4 successfully labeled FSCs and their immediate daughters, it remained difficult to assess dynamics in a single FSC when its neighboring prefollicle daughter cells were also labeled. Using nutrient deprivation, Hartman et al. (2015) generated a smaller number of labeled cells, allowing them to assess the morphological changes of FSCs in the germarium during their transition from quiescent to proliferating cells (see figure 6 in Hartman et al. 2015). An examination of the region 2A/2B border of the germarium revealed that FSCs from nutrient-deprived flies remained close to the surface of the germarium with short, thick projections. Six hours after the return to a nutrient-rich diet, FSC

projections were more elongated (see figure 7 in Hartman et al. 2015). They continued to perform immunofluorescence microscopy at different time points following a return to a nutrient-rich diet to examine FSC location during their transition from quiescence to proliferation and found that FSCs and their daughter cells remain localized at the surface of the germarium during this transition (see figure 7 in Hartman et al. 2015).

One continued challenge was the labeling of both FSCs and their daughters, as well as some IGS cells, using the 109-30- GAL4 line. However, Hartman et al. (2015) observed that many of the GAL4 drivers expressed in the IGS cells were also expressed in the FSCs, but not in the differentiating follicle cells (see figure 3 in Hartman et al. 2015). As IGS cells rarely undergo cell division in the adult, Hartman et al. (2015) proposed that this class of GAL4 insertions might be useful for labeling FSCs within the MARCM system. Following the generation of GFP-positive cells by MARCM, the authors found that they were able to successfully label FSCs (see figure 8 in Hartman et al. 2015). In the case of 109-30-GAL4, the authors had observed labeling of some IGS cells at the region 2A/2B border, while IGS cells labeled by fend-GAL4 were located more anteriorly, decreasing the possibility of mistakenly identifying an IGS cell as a FSC. Similar to observations with 109-30-GAL4, FSCs were observed to send out projections across the germarium

when labeled using the fend-GAL4 line following the feeding-induced transition from quiescence to proliferation (see figure 8, G–J, in Hartman et al. 2015).

### **Role of Integrins in FSCs**

Hartman et al. (2015) set out to further examine the function of a protein called integrin in the FSCs using their new tools. Integrins are cell-surface receptors composed of two subunits, an  $\alpha$ -subunit and a  $\beta$ -subunit, that serve a variety of functions, including linking the extracellular matrix outside the cell to the actin cytoskeleton inside the cell. Previously, it was shown that integrins are necessary for FSC localization, morphology, and proliferation, as integrin mutant FSCs were displaced and had altered cell shape (O'Reilly et al. 2008). Mislocalization of FSCs carrying an integrin mutation made it difficult to distinguish them from daughter cells. Hartman et al. (2015) set out to more closely examine the function of integrin in the FSCs, using a mutant in the *mys* gene. *mys* encodes the  $\beta$ PS-integrin subunit, one of two  $\beta$ -subunits in flies. MARCM was used to generate GFP-positive FSCs that were also mutant for *mys*. Similar to previously published results, *mys* mutant FSCs had altered cell shape, reduced proliferation, and mislocalization in the germarium (see figure 9 in Hartman et al. 2015). In addition, cellular projections in *mys* mutant cells exhibited a more random

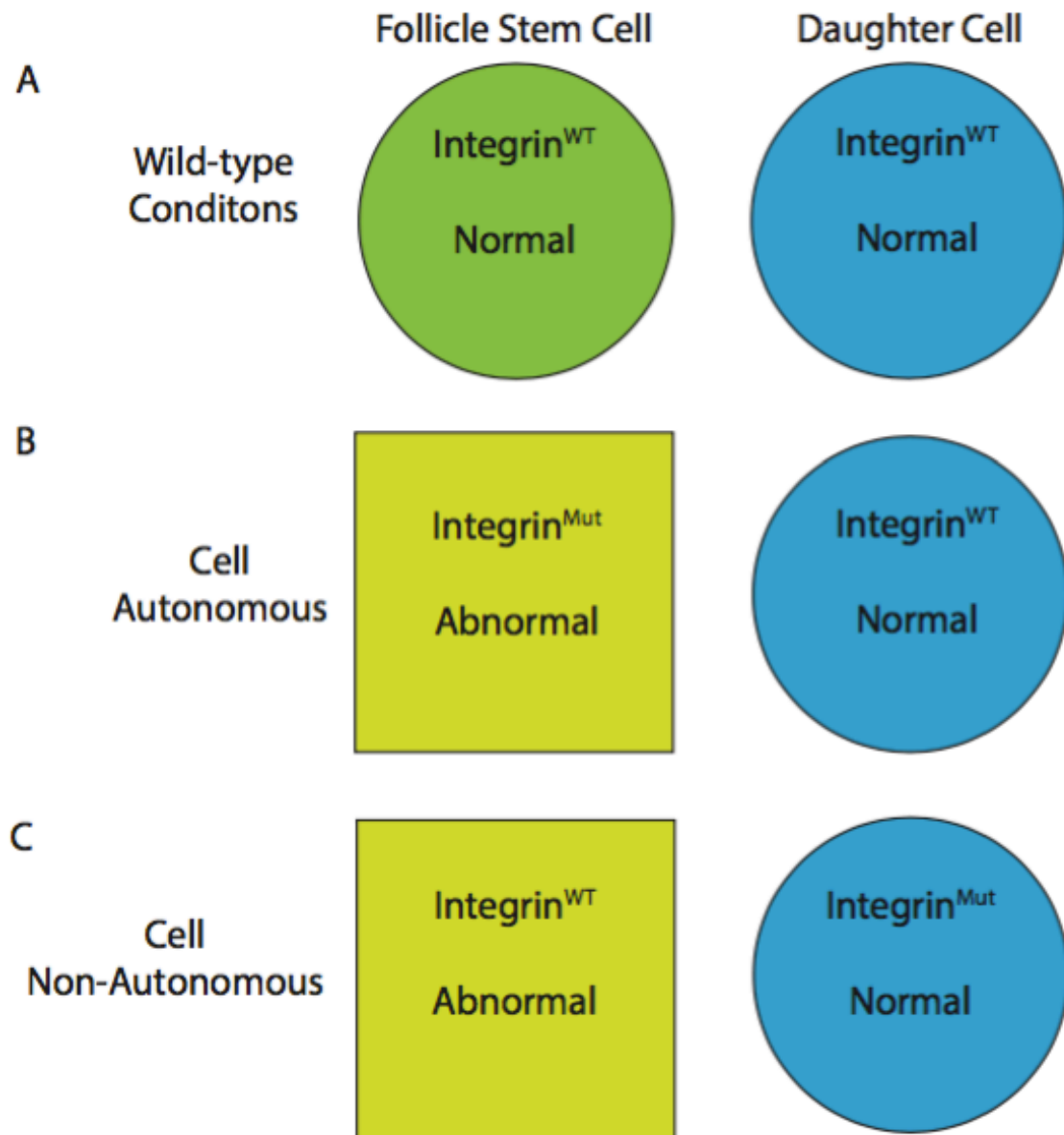
orientation as compared to controls (see figure 9 in Hartman et al. 2015).

These results suggest that the integrin mutant FSCs are less likely to be maintained in the niche and have entered a quiescent state (Hartman et al. 2015).

While mutation of integrin clearly affects FSC function, it is formally possible that this effect could be an indirect effect from loss of integrin in the differentiating daughter follicle cells (Hartman et al. 2015). Thus, the question arises, “Is integrin regulation of FSCs cell autonomous or nonautonomous?” A protein required in the same cell in which it is produced is said to function cell-autonomously (Fig. 13B), while a protein required for the proper function of another cell is said to function cell-nonautonomously (Fig. 13C). Previous studies demonstrated that daughter cells influence FSCs, supporting the possibility that integrins could function cell-nonautonomously to regulate FSC function (Vied et al. 2012). To examine if integrin functions within FSCs or within the daughter cells to influence the FSCs, Hartman et al. (2015) induced marked FSC clones and prevented further production of follicle daughter cells by nutrient restriction. Following a return to a nutrient-rich diet and FSC division, the daughters of integrin mutant FSCs showed dramatic differences in morphology and positioning within the germarium relative to wild-type controls. The displacement of daughter cells and

changes in FSC morphology were also observed when integrin levels were reduced in FSCs using RNAi (see figure 10 in Hartman et al. 2015). Taken together, these results support previous observations that integrins regulate FSC function cell-autonomously to promote FSC proliferation and maintenance in the niche (Fig. 13B; O'Reilly et al. 2008).

Figure 13. Cell-autonomous and cell-nonautonomous regulation by integrin. (A) Normal function of the FSC and daughter cell in the presence of wild-type integrin. (B) Loss-of-function integrin mutation in FSC leads to abnormal FSC function, demonstrating a cell autonomous requirement for integrin. (C) Loss-of-function integrin mutation in the daughter cell leads to abnormal function of the FSC, demonstrating a cell-nonautonomous requirement for integrin.



## **Suggestions for Classroom Use**

Regulation of gene expression is a key topic that relates not only to genetics, but also cell, molecular, and developmental biology. Organ development and function requires the cooperation of multiple cell types that perform diverse roles. The expression of different genes is one characteristic that distinguishes one cell type from another, causing it to assume a specific shape and function. Gene expression is regulated by transcription factors that bind regulatory elements found both proximal (promoters) and distal (enhancers, silencers) to the protein-coding sequence. While many classes focus on general transcriptional factors, students are often left with questions about the role that promoters and enhancers perform in transcriptional regulation. Hartman et al. (2015) nicely demonstrate how transcriptional regulatory elements play a critical role in distinguishing one cell from its neighbor, and how these elements can be used to generate valuable tools to be used in a research setting. It is recommended that this Primer article and Hartman et al. (2015) be read and discussed when covering regulation of gene expression in a genetics or advanced genetics course. Expression of different genes is one feature that often distinguishes a cell from its neighbor, and this is particularly important when it comes to stem cell populations, given the need to isolate stem cells to explore their therapeutic potential. Hartman et al.

(2015) focused on a critical population of somatic stem cells in the *Drosophila* ovary, the FSCs. To allow students to more easily follow the experiments described in Hartman et al. (2015), this Primer article describes the *Drosophila* ovary as an experimental system, discussing the types and functions of cells found in the ovary that Hartman et al. (2015) are aiming to distinguish. The heart of this Primer article focuses on the tools developed by Hartman et al. (2015) that utilize different promoter and enhancer elements to direct gene expression. Thus, these articles are useful for introducing the concept of transcriptional regulatory elements to students in a classroom and a research setting. This Primer explains the experimental tools utilized by Hartman et al. (2015) to explore when and where these elements promote transcription. In addition, this Primer introduces students to commonly used techniques for altering gene expression in specific cell types, including RNAi and the induction of mosaic clones. It is recommended that students read the introduction to Hartman et al. (2015) and the introduction and techniques portion of this Primer article and discuss these portions of the articles in small groups. Each group can take a section of the techniques and present them in a classroom setting. The figures could then be discussed in the following class period. For discussion of the figures from Hartman et al. (2015), it is recommended that each group be assigned one to two figures for the class



period to lead the discussion, wrapping up with how the genetic tools could be used for future research studies. As Hartman et al. (2015) describe how these tools are utilized for understanding the genes that function in FSCs, it is recommended that students describe how these tools could be used to explore the role of other genes in the variety of cell types found in the developing ovary. There are a multitude of articles that explore the roles of specific genes, epigenetic regulation of gene expression, and chromosomal inheritance in stem cell populations that could be incorporated for further discussion in an advanced genetics class (Jemc 2011; Sahai-Hernandez et al. 2012; Tran et al. 2012; Yadlapalli and Yamashita 2013; Luyten et al. 2014; Slaidina and Lehmann 2014).

### **Questions for Review and Discussion**

1. In Hartman et al. (2015), the authors are focused on identifying ways to distinguish FSCs from other somatic cell populations in the ovary. How could a failure to effectively distinguish FSCs from IGS cells or daughter follicle cells impact their results? Why is the ability to distinguish different cell types so important for studying how organs function?
2. Why was it important for Hartman et al. to focus on region 2A/2B of the germarium in their identification of tools?
3. How could one develop a GAL4 line in the lab that is expressed in the

same pattern as a gene of interest?

4. Why was it important to use MARCM to analyze FSC characteristics with the 109-30-GAL4 driver, as opposed to analyzing FSCs in flies containing only the GAL and UAS elements (see figure 5 in Hartman et al. 2015)? Why is the timing of clone induction important?
5. In figure 6 in Hartman et al. (2015) the authors observe that a nutrient-poor diet induces quiescence. Why do you think lipids and proteins are important for the process of cell division to take place?
6. Hartman et al. (2015) use UAS-GFP to label cells throughout their article. In their figure 8, they use UAS-Tau-GFP to examine cell morphology. What insights do they gain by using UAS-Tau-GFP that they would not have gained had they used a UAS-GFP containing a nuclear localization sequence (UAS-GFPnls)?
7. In regard to the cell-autonomous requirement for integrin in FSCs, how would you have expected the results of the RNAi experiment to differ if integrin function were required cell-nonautonomously?
8. Provide students with a gene of interest and have them design a DNA sequence that could be used for RNAi for the gene of interest.
9. In figure 2 in Hartman et al. (2015) the authors identify weak GAL4 lines that promote low levels of expression of reporter genes, as opposed to

high levels of reporter genes. Why might these weak GAL4 lines be useful for UAS-RNAi studies?

10. The *punt* gene is located on the third chromosome and is required for the maintenance of the FSCs in the ovary (Kirilly et al. 2005). However, it is unknown if the FSCs mislocalize or have altered morphology. As a mutation in the *punt* gene is lethal to the fly, it is necessary to generate a small group of mutant cells using the MARCM technique to examine these characteristics. Using Figure 11 as a guide, what genetic elements are needed to generate FSCs that are mutant for *punt* and express a GFP reporter gene? What elements need to be on the third chromosome? Draw out the scheme as in Figure 11.

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## VITA

Diane Silva was born in Des Plaines, Illinois, to Daniel and Monica Silva. She is the oldest of three children. After graduating from Maine West High School in Des Plaines, Illinois, in 2009, she continued her education by attending DePaul University in Chicago, Illinois where she graduated in 2013 with a Bachelor's of Science in Biology. After DePaul University she decided to attend Loyola University Chicago to work on a Master of Science in Biology in order to expand her science knowledge and provide her with research experience

At Loyola University Diane had the opportunity to work in Dr. Jennifer Mierisch's lab, with the lab being a developmental biology lab she was able to learn a lot of new techniques and skills in biochemistry, cell and molecular biology, but most importantly she developed a passion for research that lead her to discover that research was something she wanted in her future career. She graduated from Loyola University with a Master of Science degree in Biology on May 2016 and will be attending The University of Illinois at Chicago College of Medicine for their Graduate Education in

Medical Sciences Program in the fall of 2016. At U.I.C. Diane plans to get further training in the biomedical sciences where she plans to enter a cancer research lab. Ultimately, her career goals are to enter the medical research field for the development of new preventions, therapies, and cures for cancer patients.