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Characterizing the Requirement of the Cmi/Trr COMPASS-Like Complex During Drosophila Development

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CHARACTERIZING THE REQUIREMENT OF THE CMI/TRR COMPASS-LIKE COMPLEX DURING DROSOPHILA DEVELOPMENT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The MLR family of COMPASS-like complexes are histone methyltransferase complexes that are associated with the activation of gene enhancers. In *D. melanogaster*, Cara mitad (Cmi, also known as Lpt) and Trithorax related (Trr) are central subunits of a complex orthologous to mammalian Lysine methyltransferase 2 C and D (KMT2C and KMT2D, also known as MLL3 and MLL2/4) that catalyze H3K4 monomethylation. Previous studies have demonstrated that mutations in these genes are associated with cancer and developmental disorders, but the mechanisms by which these alterations contribute to disease states are unknown. The Cmi-containing COMPASS-like complex and orthologous vertebrate complexes have been identified as necessary co-regulators of multiple critical developmental signaling pathways, and knockout experiments have demonstrated that these complexes are necessary for development and viability. My research shows that Cmi has a distinct expression pattern in developing tissues, and the knockdown abrogates normal developmental patterns in both the embryo and ovary. Ubiquitous shRNA mediated knockdown of Cmi in the embryo results in early embryonic lethality, and shRNA expressed in certain ovary tissues results in reduced egg-laying efficiency and incomplete ovary development. By elucidating the embryogenesis stage at which the COMPASS-like complex performs its function as well as its requirement in the female germline, future experiments aimed at revealing gene targets can be narrowed to these key developmental periods.

CHAPTER ONE

LITERATURE REVIEW

MLR Complexes

COMPASS (Complex of proteins associated with Set1) -like complexes are epigenetic regulators that are associated with the activation of gene enhancers, and they are highly conserved from yeast to mammals (Shilatifard, 2012). In *Drosophila melanogaster*, Cara mitad (Cmi, also known as Lpt) and Trithorax related (Trr) are gene products produced from separate loci that come together to form the enzymatic core of a complex that closely resembles the mammalian orthologs Lysine Methyltransferase 2 C and D (KMT2C and KMT2C) also known as Mixed Lineage Leukemia 3 (MLL3) and Mixed Lineage Leukemia 2/4 (MLL2/4) (Chauhan et al., 2012). This family of complexes (hereafter referred to as MLR) recognize and bind histone tails within transcription enhancer regions and catalyze the addition of a single methyl group onto the fourth lysine of histone 3 (H3K4). Cmi contains the plant homeodomain (PHD) fingers involved in histone interactions and Trr contains the Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain which carries out the methyltransferase catalytic activity. The separation of critical functions in histone regulation allows for the study of the requirement of the domains independently, which can provide valuable insights into possible different functions of Cmi and Trr. Other elements of the complex have been implicated in recruitment, stability, and activity of the complex including a histone lysine demethylase Utx (Kdm6A) which catalyzes the removal of the transcription-repressing H3K27 trimethyl modification (Reviewed in Ford and Dingwall, 2015). MLR complexes have also been shown to recruit other proteins such as histone

acetyltransferases capable of adding the activating H3K27 acetyl modification (Wang et al. 2017) and promote RNA polymerase II loading (Lee et al., 2013; Wang et al., 2016) (Figure 1).

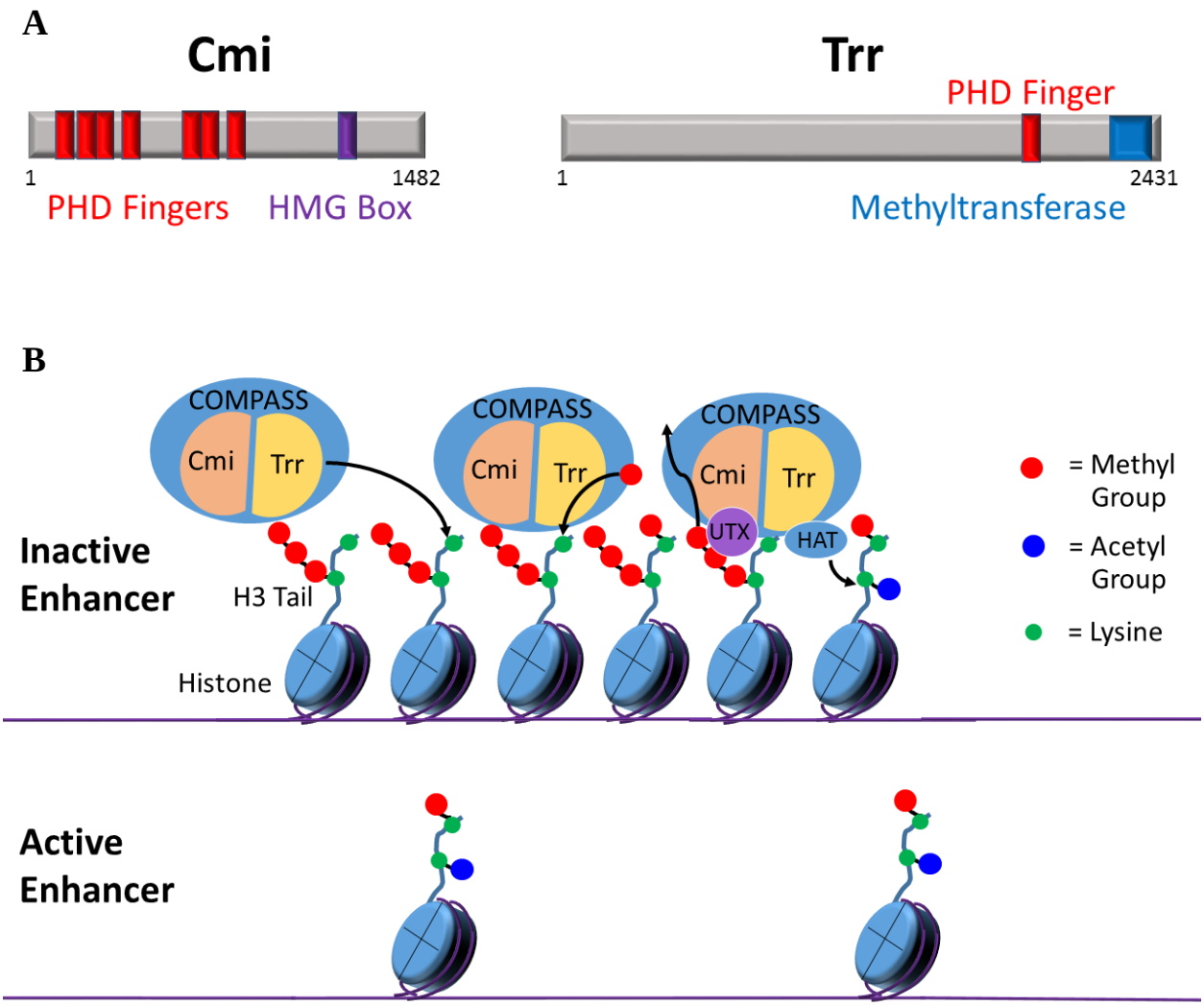


Figure 1. MLR Complexes Activate Enhancers. (A) A schematic of relevant protein domains in Cmi and Trr which are transcribed from different loci but come together to form the enzymatic core of the MLR complex in Drosophila. Cmi contains the histone reading and binding domains (red) and the high mobility group (HMG) nucleic acid binding domain (purple) while Trr contains the methyltransferase catalytic activity (blue) (adapted from Chauhan et al. 2012). **(B)** The MLR complex consisting of the Cmi/Trr enzymatic core as well as COMPASS-Like protein complex components is able to recognize and bind to the histone tails of inactive enhancers, catalyze the addition of the activating H3K4 monomethyl mark, and remove the inhibitory H3K27 trimethyl mark. The complex is also able to recruit histone acetyl transferases (HAT) that can add an activating H3K27 acetyl mark (adapted from Heinz et al., 2015)

MLR Complex Functional Domains

The cores of the MLR complexes contain several protein domains that can account for their known activities. The PHD clusters are made up of groups of zinc fingers that are capable of recognizing and binding to modified histone tails in nucleosomes. The conserved PHD domains contained within COMPASS complex components KMT2A-D have been shown to be involved in domain contacts to facilitate interactions between distant portions of the proteins (e.g., KMT2A), contribute to heterotypic protein interactions with the cyclophilin proline isomerase CYP33 (KMT2A, 2C), contain E3 ubiquitin ligase activity (KMT2A, 2B), as well as recognize several histone modifications including H3K4me3, H3K4me0, H3K14ac, H3R2me2, and H3R2me0 (Ali et al., 2014). Proteins that contain multiple clusters of PHD domains have been shown to have a more complex recognition pattern with varying binding affinities (Sanchez and Zhou, 2011). Recent evidence suggests that the PHD domains are involved in the recruitment of other protein complexes, namely an H2A deubiquitinase BAP1 (Wang et al., 2018). The SET domain is responsible for the catalysis of methyl groups onto H3K4 (Qian and Zhou, 2006). However, catalytically dead mutant KMT2C and KMT2D were still able to facilitate RNA polymerase II activity at enhancer regions indicating that MLR complexes may have separate mechanisms of enhancer activation (Dorighi et al., 2017). MLR complexes also contain FY-rich domain N-terminal (FYRN) and FY-rich domain C-terminal (FYRC) motifs of unknown function that are found in some proteins involved in chromatin stability (García-Alai et al., 2010). Proteins containing a high mobility group (HMG) box similar to the one found in MLR complexes are able to bind to DNA or RNA and have been implicated in a wide range of cellular processes including DNA bending in a sequence specific or non-specific way, recruitment of other proteins to DNA (transcription factors, DNA repair proteins, and silencing

complexes), and piwi-interacting RNA (piRNA) processing (Malarkey and Churchill, 2012; Štros et al., 2007; Genzor and Bortvin, 2015).

Requirement of MLR Complex During Development

Although the histone marks that are indicative of chromatin organization and transcriptional status have been extensively studied, much remains to be determined about the protein complexes that place or remove histone modifications. While KMT2C/D likely act as co-regulators at a large number of transcriptional targets, evidence suggests they are critical for development and influence cell fate transition. Because KMT2C/D are the enzymatic cores of histone modifying complexes, their reduction in a cell leads to a depletion of H3K4 monomethylation—a mark of active enhancers—at enhancer sites (Hu et al., 2013). However, recent evidence suggests that H3K4me1 is not required for MLR complexes to promote transcriptional activation indicating they have functions independent of their catalytic activity (Dorighi et al., 2017; Rickels et al., 2017). MLR complexes are also developmentally essential which makes studying the effects of embryonic depletion difficult (Chauhan et al., 2012, Lee et al., 2013). Embryonic development is a complex and highly-regulated process that involves activation and repression of many enhancers in a cell-type specific and temporal manner, and elucidating the functions and regulatory targets of MLR complexes during development is a daunting task.

Studies performed in several organisms demonstrate the involvement of these complexes in developmental processes. For example, Kleefstra and Kabuki syndromes are human developmental disorders characterized by maxillofacial and brain development defects that result from heterozygous inactivating germline mutations in KMT2C and KMT2D respectively (Kleefstra et al., 2012; Hannibal et al., 2011). A similar phenotype was observed in zebrafish

where knockdown of Kmt2D leads to defects in craniofacial structure and brain development (Van Laarhoven et al., 2015). A possible mechanism that could explain these phenotypes is the inability of neuronal precursor cells to fully differentiate when KMT2D is knocked down (Dhar et al., 2012). However, in these cases wild type protein is still made so the phenotypic effects of the absence of MLR complexes during development may be more severe.

Although the role of the MLR complex in the germline has not been extensively studied, researching these proteins in stem cells has led to important insights into the possible mechanisms by which they act. In *C. elegans* germline stem cells, components of the MLR complex wdr-5.1 and rbbp-5 are essential for proper cell development while other components of the complex that are required for embryogenesis were not essential in germline cells (Li and Kelly, 2011). This discovery provides evidence that the MLR complex might have different functions during embryonic development and adult stem cell maintenance. It has also been shown that Kmt2C is likely required for zygotic gene activation in the mouse paternal pronucleus (Aoshima et al., 2015) providing more evidence that this complex likely plays a role in the germline. Currently, the role of MLR complexes in oocyte formation remains unknown.

In planarians, knockdown of the MLR complex leads to defects in neoblast (adult stem cell) differentiation into several cell types; most notably neuronal and epithelial tissue (Mihaylova et al., 2017). In human epithelial cell precursors, KMT2D knockdown leads to a disorganized epithelium and an inability to properly activate lineage-specific enhancers which mirrors the results seen in planarians (Lin-Shao et al., 2018). Kmt2D is also required for mouse fibroblast reprogramming into a pluripotent state using the Yamanaka (Oct3/4, Sox2, Klf4, c-Myc) transcription factors (Wang et al., 2016). These studies demonstrate that the MLR

complexes play a key role in activating enhancers during cell lineage determination and stem cell activity and maintenance.

Drosophila Oogenesis and Early Embryogenesis

Each female *Drosophila* has two ovaries containing approximately 15-20 ovarioles that can each function separately to give rise to a mature egg. The germarium is a structure located at the anterior tip of each ovariole that houses the germline and somatic stem cells that give rise to the nurse cells, the oocyte, and the somatic follicle cells that surround the egg chamber. Oogenesis begins when a germline stem cell asymmetrically divides to self-renew and produce a daughter cell capable of mitosis with incomplete cytokinesis. These daughter cells give rise to a 16-cell cyst, one of which will become the oocyte while the others will become nurse cells. The oocyte undergoes meiosis while the nurse cells become polyploid and pump mRNA and protein into the developing oocyte through ring canals. The egg chambers bud off from the germarium and progress towards the posterior end of the ovary as they develop over 14 stages into a mature egg (Bastock and St Johnston et. al 2008).

The mature oocyte is fertilized as it exits the female oviduct and the two pronuclei fuse into a single zygote nucleus in the embryo. This nucleus then divides eight times to rapidly produce 256 nuclei in about 64 minutes that all share a common cytoplasm. Then, the nuclei move to the periphery as they continue to divide six more times at a slower rate. In this stage, prior to cellularization, the embryo is referred to as a syncytial blastoderm. At this moment, immediately prior to the 14^{th} nuclear division, zygotic transcription begins in a process known as the maternal to zygotic transition. Once all of the nuclei have migrated (about 2 hours after fertilization), the plasma membrane begins to surround each one to create a spherical group of individual cells creating the cellular blastoderm. Each future cell division is now asynchronous

as the endoderm, mesoderm, and ectoderm begin to form and the embryo progresses through well-defined developmental stages (Gilbert and Singer 2000). During this critical time of zygotic gene activation, enhancers are established de novo as changes in the epigenetic landscape lead to an increased number of nucleosome-free regions associated with sites that are bound by maternally deposited transcription factors (Li et al. 2014). This suggests that maternal factors are essential for early enhancer activation and epigenetic modifying complexes may interact with transcription factors during de novo activation.

Our lab has previously generated a null allele of the Drosophila *cmi* gene and demonstrated that *cmi* mutant homozygotes do not survive past the second instar larval stage (Chauhan et al., 2012). However, it is known that maternal *cmi* mRNA and protein are provided to the developing oocyte and the transition to zygotic transcription does not occur until the embryonic blastoderm stage (about 2 hours after fertilization) so it is possible that the maternal contribution of Cmi to the developing embryo may allow for the establishment of early developmental enhancer activation. In this study, we aimed to remove the maternal Cmi component to determine if the MLR complex is contributing to early embryonic enhancer activation and determine the stage at which development is arrested. However, we determined that Cmi is required for the process of oogenesis because *cmi* null germline clones are unable to produce embryos. This led to an attempt to characterize the requirement of the MLR complex in the adult ovary through shRNAi-mediated knockdown in specific cell types. To examine the effects of *cmi* or *trr* depletion in the embryo without the ability to generate a null embryo, we once again used shRNAi controlled by a ubiquitous promoter that is activated early during embryogenesis. Our results show that the MLR complex is required in germline cells in the ovary and prior to gastrulation in developing embryos.

CHAPTER TWO

METHODS

Generation of Germline Clones

The chromosome 2 construct containing the *cmi*^{1} allele at 60A9 previously generated in our lab (Chauhan et al., 2012) was recombined to contain an FRT site at 42B (Chauhan unpublished data). This genetic construct was added to a stock containing a heat-shock inducible FLP recombinase on chromosome 1 (Bloomington Stock Center #1929). Virgin females of this genotype were mated with males containing the *Ovo*^{D1} dominant female sterile allele and an FRT site at 42B on chromosome 2 (Bloomington Stock Center #4434) and offspring were aged at 25°C. On day 5 and day 6 AEL, larvae were subjected to a 37°C heat shock for one hour to induce germline recombination or kept at 25°C as a no heat-shock control. The animals were allowed to develop normally at 25°C, and adult females aged 3-5 days after pupal eclosion were dissected. Ovaries were fixed with 4% formaldehyde in 0.1M PIPES, 2mM $MgSO_4$, and 1mM EGTA (PEM) for 15-20 minutes, washed with phosphate buffered saline containing 0.1% tween-20 (PBT) twice for 2 minutes and then twice for 30 minutes, and mounted in ThermoFisher ProLong™ Gold Antifade Mountant with DAPI (catalog #P36931) and imaged using a fluorescent microscope. A similar procedure was performed using a chromosome 2 construct containing the wild type *cmi* allele at 60A9 and an FRT site at 42B (Chauhan, unpublished data) and a chromosome 2 construct containing a mutant named *cmi*$^{A16\text{-}9}$ obtained from a collaborator which contains an early termination codon in the *cmi* gene within the HMG domain prior to the

HMG box (Daniel St Johnston, University of Cambridge; Claudia Zraly, Loyola University Chciago personal communication).

Immunostaining of Ovary Tissue

Three GAL4 drivers reported to be expressed in the adult ovary (Figure 2B) were used to drive the expression of shRNA inverted repeats targeting *cmi* (Chauhan et al., 2012), *trr* (Chauhan et al., 2012), *vas* (Bloomington Stock Center #38924), or *orb* (Bloomington Stock Center #43143). Virgin females containing UAS-IR transgenes were mated to GAL4 males and offspring were aged at 25°C. Females aged 3-5 days after pupal eclosion were dissected and ovaries were fixed with 4% formaldehyde in PEM for 15-20 minutes. Fixed ovaries were washed with PBT twice for 2 minutes and then twice for 30 minutes and incubated overnight with anti-Cmi guinea pig primary antibody (Chauhan et al., 2012) in 50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% BSA, and 0.1% Triton X-100 (TNBT) at a 1:1000 dilution. Non-specific staining was blocked with 2% normal goat serum (Life Technologies Ref. PCN5000) in PBT, washed in TNBT twice for one hour, and incubated for two hours with Alexa Fluor® 488 goat anti-guinea pig secondary antibody (Life Technologies Ref. A11073). Ovaries were washed in PBT three times for 15 minutes, mounted in ThermoFisher ProLong™ Gold Antifade Mountant with DAPI (catalog #P36931), and imaged using a fluorescent microscope.

Egg Laying and Hatching Assay

Male flies containing the GAL4 drivers described above were mated with virgin females containing either *cmi* or *trr* inverted repeat transgenes (Chauhan et al., 2012). Ten virgin F1 females were collected and mated with wild type males in an embryo collection container with a molasses-agar cap. Flies were given three days to incubate and then embryos were collected

over a 24 hour period. The number of embryos on each cap was recorded and the cap was aged another 24 hours before counting the number of hatched larvae. The process was repeated three times, averaged across the three days, and divided by the number of females in the bottle to obtain the average number of eggs one female can lay in one day. Data shown is an average of two separate experiments.

Immunostaining of Embryos

A GAL4 transgene controlled by the *alphaTub84B* promoter (Bloomington Stock Center #5138) re-balanced over a TM3 chromosome containing GFP-tagged actin (Bloomington Stock Center #) was used to drive ubiquitous expression of shRNA inverted repeats targeting either *cmi* or *trr* (Chauhan et al., 2012). F1 embryos from this cross were collected from 0-2 hours AEL and aged at 25°C. Embryos were dechorionated in 50% bleach, fixed with 1:1 heptane to 4% formaldehyde in PEM for 20-25 minutes, shaken in 1:1 heptane to methanol to remove vitelline, and rinsed and stored in methanol at -20°C. Fixed and frozen embryos were gradually rehydrated with PBT and incubated overnight with one or more of the following primary antibodies in PBS containing 0.1% BSA and 0.1% Triton X-100 (PBSBT): guinea pig anti-Cmi 1:1000 dilution (Chauhan et al., 2012), rabbit anti-GFP 1:1000 dilution (GenScript Cat. A01704), mouse anti-Eve 1:20 dilution (DSHB registry ID AB_528230), mouse anti-En 1:20 dilution (DSHB registry ID AB_528224), mouse anti-Wg 1:20 dilution (DSHB registry ID AB_528512), mouse anti-Antp 1:20 dilution (DSHB registry ID AB_528083), mouse anti-Ubx 1:20 dilution (DSHB registry ID AB_10805300). Embryos were blocked with 2% normal goat serum (Life Technologies Ref. PCN5000) in PBT, washed in TNBT twice for one hour, and incubated for two hours with one or more of the following secondary antibodies: Alexa Fluor® 568 goat anti-guinea pig (Life Technologies Ref. A11075), Alexa Fluor® 488 goat anti-guinea

pig (Life Technologies Ref. A11073), Alexa Fluor® 568 goat anti-mouse IgG (Life Technologies Ref. A11004), Alexa Fluor® 488 goat anti-rabbit (Life Technologies Ref. A11034). Embryos were washed in PBT three times for 15 minutes, mounted in ThermoFisher ProLong™ Gold Antifade Mountant with DAPI (catalog #P36931) and imaged using a fluorescent microscope. The Eve antibody developed by Zinn, K. at the California Institute of Technology; the En antibody developed by Goodman, C. at the University of California, Berkeley; the Wg antibody developed by Cohen, S.M. at the European Molecular Biology Laboratory; the Antp antibody developed by Brower, D. at the University of Arizona; and the Ubx antibody developed by White, R. at the University of Cambridge were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Cuticle Analysis

Embryos were collected from the *cmi* knockdown cross described above. The collection cap was removed after 8 hours and aged at 25°C for 12 more hours before embryos were sorted by GFP signal. Embryos were dechorionated in 50% bleach, shaken in 1:1 heptane to methanol to remove the vitelline membrane, rinsed in methanol, transferred to a microscope slide, and the methanol was removed. A mixture of 9:1 lactic acid to methanol was added to the slide, a cover slip was placed over the embryos, and the slide was incubated overnight at 60°C before observing under a microscope.

qRT-PCR

Procedure was performed as described by Chauhan et al., 2012 using the following *cmi* primers: Forward— CTGATCCTCGAGAGCTTTACG, Reverse— ACACATGATCAGCTTGGACAG. Briefly, embryos of the proper genotypes were selected

and RNA was prepared. RNA was reverse-transcribed and cmi transcripts were qualitatively measured by PCR using the comparative Ct method.

Image Processing

Fluorescent microscopy images were processed using the ImageJ Fiji project version 1.51n (Schindelin et al., 2012). Plugins used: subtract background. Images taken with the Zeiss LSM 880 with AiryScan microscope were processed using ZEN software version 2.3 AiryScan processing script. Figures were arranged in Adobe Photoshop version 19.0.

Statistics

Statistical analysis to compare the difference of means was conducted using a standard student's t-test. Statistical analysis to compare the sample population percentage to the control was conducted using a general z-test. The alpha level used in both cases is 0.05.

CHAPTER THREE

RESULTS

Aim 1: Characterize the Requirement of Cmi in the Germline

Cmi is Required for Oogenesis.

In an effort to eliminate the maternal contribution of *cmi* mRNA and protein to the egg, we generated germline clones with two different alleles (Figure 2): a *cmi* null mutation that eliminates protein expression (cmi^{1}) and an early termination codon mutation obtained from D. St Johnston and A. Plygawko, University of Cambridge ($cmi^{A16\text{-}9}$). Homozygosity for both alleles resulted in failure of *cmi* mutant recombinants to produce eggs while the wild type allele restored egg-laying ability indicating the requirement of *cmi* for proper germline development. Ovaries dissected from both *cmi* mutant recombinants revealed defects in the progression of oogenesis (Figure 3A, Table 1). Without a heat shock induction of FLP recombinase, no recombination was able to occur and the dominant female sterile Ovo^{D1} phenotype was observed. Upon heat shock, cmi^{1} clones looked very similar to the Ovo^{D1} phenotype, so the cmi^{1} homozygous ovarioles exhibited an early stage oogenesis block. In rare cases, cmi^{1} ovarioles are able to develop to a later stage, but the oocytes are malformed and unable to be deposited indicating that the heat shock recombination is occurring (Figure 3B). Clones containing the $cmi^{A16\text{-}9}$ allele, which contains an early termination codon prior to the high mobility group (HMG) box within the HMG domain, contain ovarioles that are able to develop to a later stage than the cmi^{1} clones providing evidence that the $cmi^{A16\text{-}9}$ allele may be partially functional. These data indicate that functional *cmi* is necessary for proper germline development.

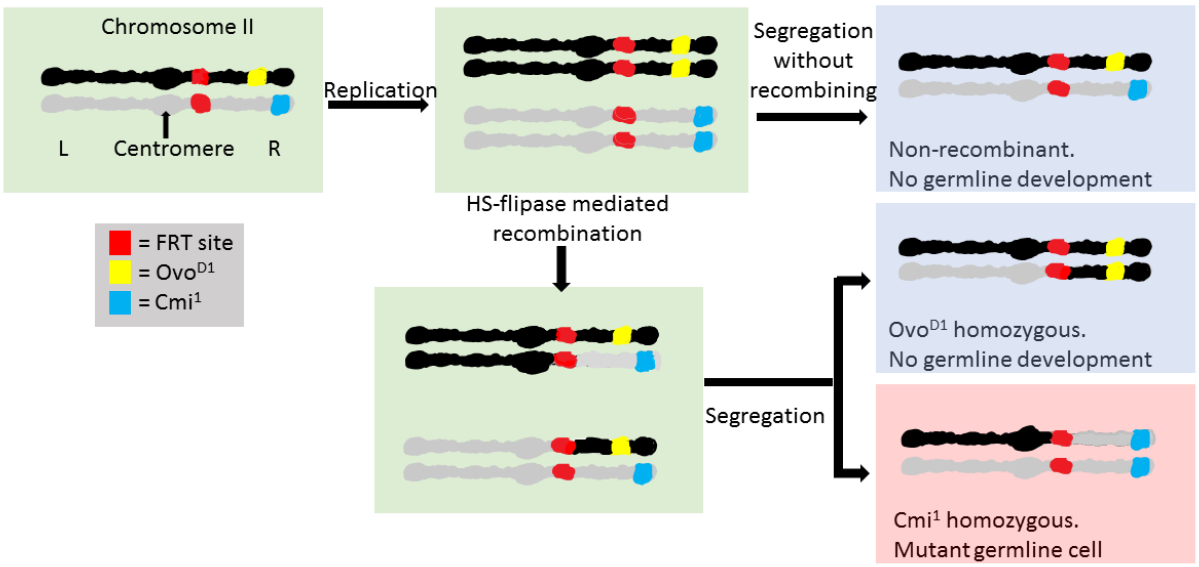


Figure 2. Generation of Germline Clones. Schematic of the dominant female sterile technique used to generate germline clones. FRT42B (red band) indicates an FRT site at position 42B on the right arm of the second chromosome. Ovo^{D1} (yellow band) indicates the *ovo^{D1}* dominant female sterile transgene gene. Cmi^{1} (blue band) indicates the location of the *cmi^{1}* null allele at 60A9 on the right arm of the second chromosome (adapted from Prudêncio and Guilgur, 2015).

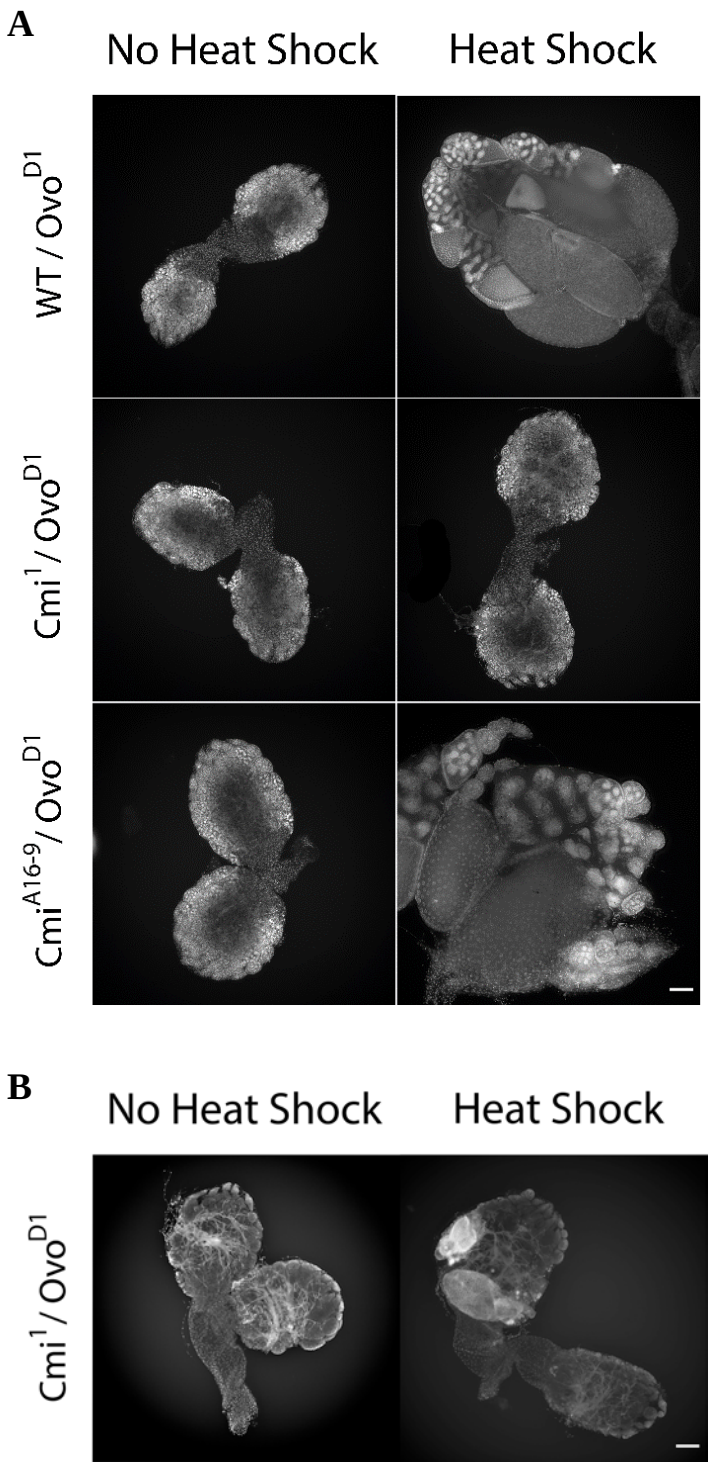


Figure 3. Germline Clone Phenotypes. (A) Germline clone analysis performed as described in Figure 2 using a wild type (WT) allele as a control and two *cmi* null alleles Cmi^{1} and $Cmi^{A16\text{-}9}$. Ovaries were fixed and stained with DAPI to visualize DNA. **(B)** In rare cases, cmi^{1} clones produce later stage oocytes that are not deposited. Ovaries were fixed and stained with DAPI to visualize DNA. Scale bar indicates 100μm.

Genotype	Eggs/Female/Day	
	No Heat Shock	Heat Shock
WT / Ovo^{D1}	0 (n=314)	4.33 (n=230)
Cmi^{1} / Ovo^{D1}	0 (n=198)	0 (n=199)
Cmi^{A16-9} / Ovo^{D1}	0 (n=50)	0 (n=50)

Table 1. Germline Clone Egg Laying Analysis. Germline clone analysis performed as described in Figure 2 using a wild type (WT) allele as a control and two *cmi* null alleles Cmi^{1} and Cmi^{A16-9}. Heat-shock (recombinant) and non heat shock (non-recombinant) animals were mated with WT males and placed in embryo collection containers. Egg laying efficiency is reported as the average number of eggs one female can lay in a 24 hour period. n refers to the number of females assayed.

Cmi Knockdown in Germline Cells Leads to Oogenesis Defects.

Immunostaining wild type ovary tissue revealed Cmi localization in the developing oocyte (Figure 4). Cmi protein is observed in germline stem cells at the anterior tip of the germarium, and in both the cytoplasm and nuclei of nurse cells. At later stages of oogenesis, Cmi is still seen in the nurse cells but begins to localize in the cytoplasm of the oocyte in large quantities. Throughout oogenesis, Cmi levels seem to be relatively constant throughout the nuclei and cytoplasm of the somatic follicle cells. To characterize further the function of the MLR complex in oocyte development, we used shRNAi to knock down *cmi* and *trr* in different cell types within the adult ovary. We included a *trr* knockdown based on evidence that catalytically dead MLR complexes can still activate enhancers during development and we wanted to assess a possible differential function of *cmi* and *trr* during oogenesis (Dorighi et al., 2017). GAL4 expressed under the control of tissue-specific enhancers reported to be expressed in the ovary (Hudson and Cooley, 2014) were used to drive the knockdown of *cmi*, *trr*, *vas*, and *orb* in both germline and somatically derived cells (Figure 5, Table 2). The GAL4 expression pattern of three drivers was verified with a UAS-GFP transgene; the maternal tubulin (*mat-tub*)

driver is highly expressed in the germline with some somatic expression, the first follicle cell driver (*GawB-GR1*) is only expressed in the somatic follicle cells, and the second follicle cell driver (*GawB-Cb16*) is also expressed exclusively in the somatic follicle with a stronger expression within the germarium (Figure 6A). Two other knockdown constructs against *vasa* (*vas*) and *orb* were included as controls because they are required in germline cells but not somatic cells during oogenesis (Kai et al., 2005). Knocking down *cmi* or *trr* in the germline derived cells using the *mat-tub* driver resulted in both structural and functional phenotypes in the

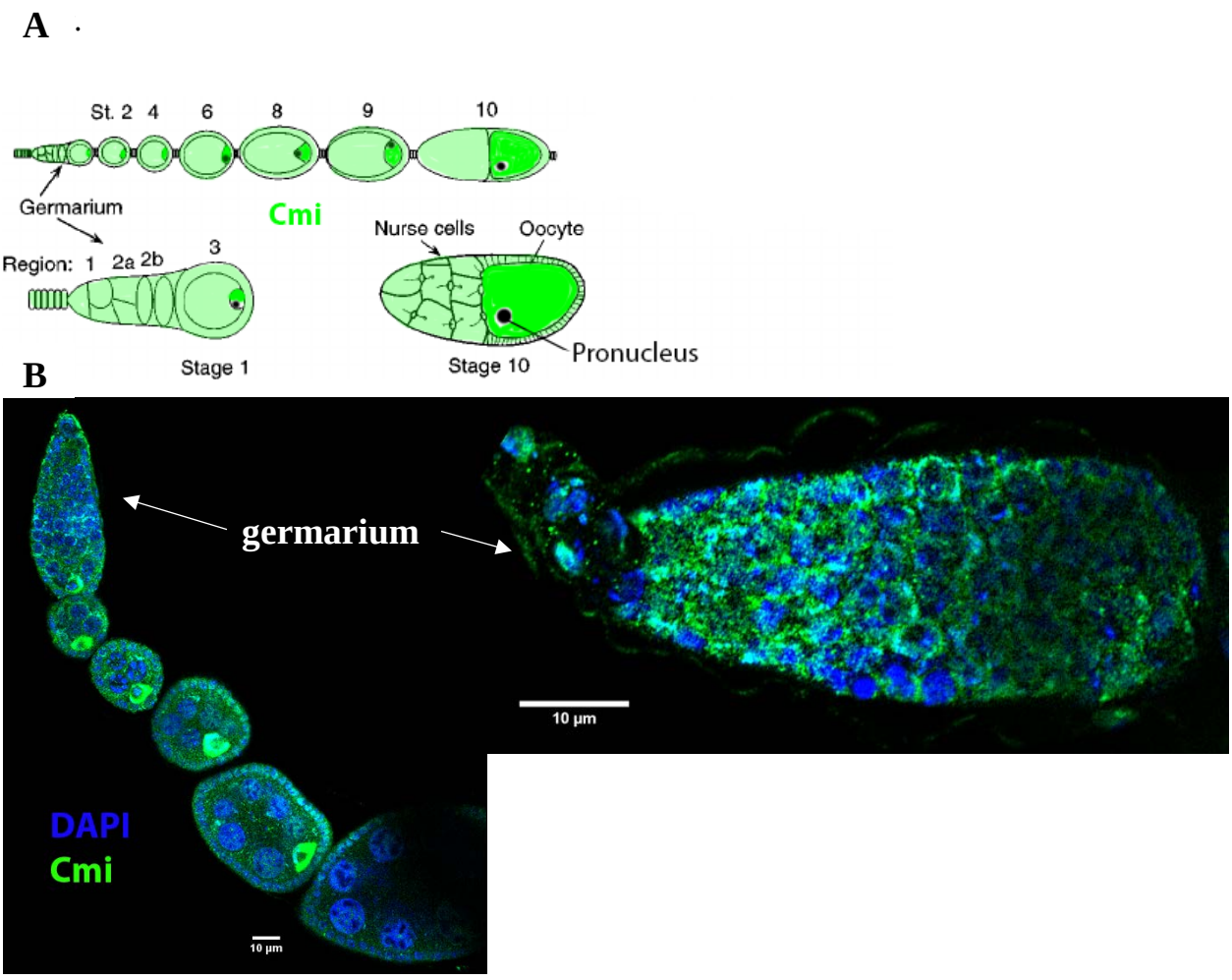


Figure 4. Cmi Localizes in the Developing Oocyte. **(A)** Schematic of an ovariole indicating developmental stages. Cmi localization pattern is shown in green. (adapted from Robinson et al., 1997). **(B)** Wild type Drosophila ovariole showing native expression pattern of Cmi. Blue indicates DNA, and green indicates Cmi protein. Ovaries were fixed and incubated with anti-Cmi primary polyclonal antibody, detected using a fluorescent secondary antibody, and mounted in a solution containing DAPI. Scale bar indicates 10µm.

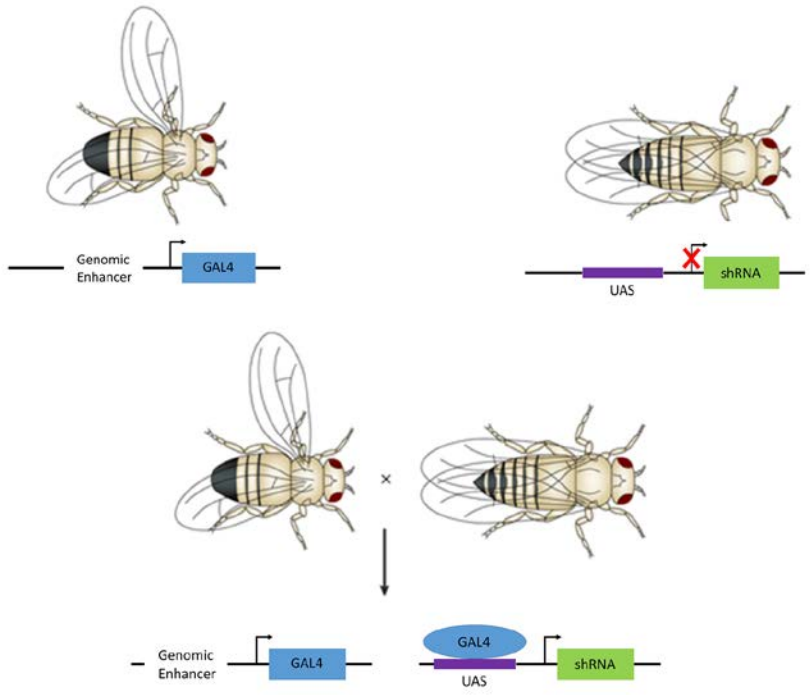
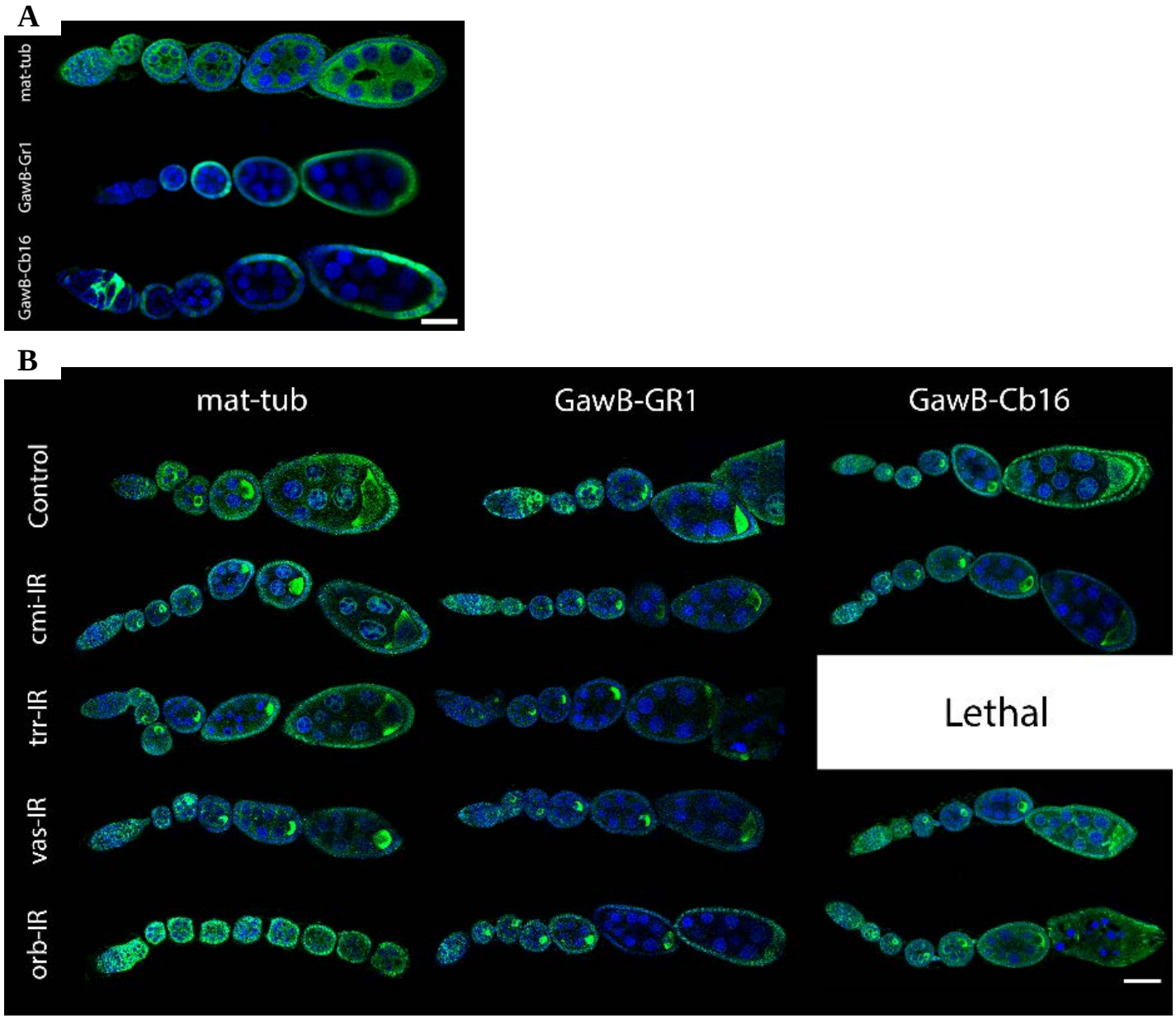


Figure 5. Using the Gal4 System to Express shRNA Inverted Repeats. GAL4 is a yeast transcription factor that binds to an upstream activating sequence (UAS) and induces transcriptional activation of the target gene. The expression of GAL4 is regulated by a genomic enhancer so it can be cell-type specific. Fly on the left is male and on the right is female (adapted from St Johnston, 2002).

Stock Number	Genotype	Description
5138	y[1] w[*]; P{w[+mC]=tubP-GAL4}LL7/TM3, Sb[1] Ser[1]	ubiquitous expression of GAL4
7063	w[*]; P{w[+mC]=matalpha4-GAL-VP16}V37	GAL4-VP16 fusion protein expressed under the control of the alphaTub67C promoter. Expressed maternally and loaded into eggs.
36287	w[*]; P{w[+mW.hs]=GawB}GR1	Expresses GAL4 in all follicle cells including follicle stem cells
6722	y[1] w[1]; Pin[1]/CyO, P{w[+mW.hs]=GawB}cb16	Complex expression pattern including all follicle cells

Table 2. List of Gal4 Drivers Used in Knockdown. Gal4 drivers obtained from the Bloomington Drosophila Stock Center were utilized in knockdown experiments.

We observed fewer late stage oocytes per ovary (Figure 6B), a reduction of Cmi protein, an increase in the number of early stage chambers (Figure 6C), and a decrease in both egg laying and hatching rate (Figure 7A). Knocking down *cmi* in somatic cells using either of the two drivers within the ovary did not result in a phenotype indicating that *cmi* is not required in the somatic follicle cells. However, *trr* knockdown in follicle cells produced a strong phenotype and abrogated egg-laying ability indicating a possible cmi independent function (Figure 6, Figure 7B-C).


A
mat-tub
GawB-Gr1
GawB-Cb16
B
mat-tub
GawB-GR1
GawB-Cb16
Control
cmi-IR
trr-IR
vas-IR
orb-IR
Lethal

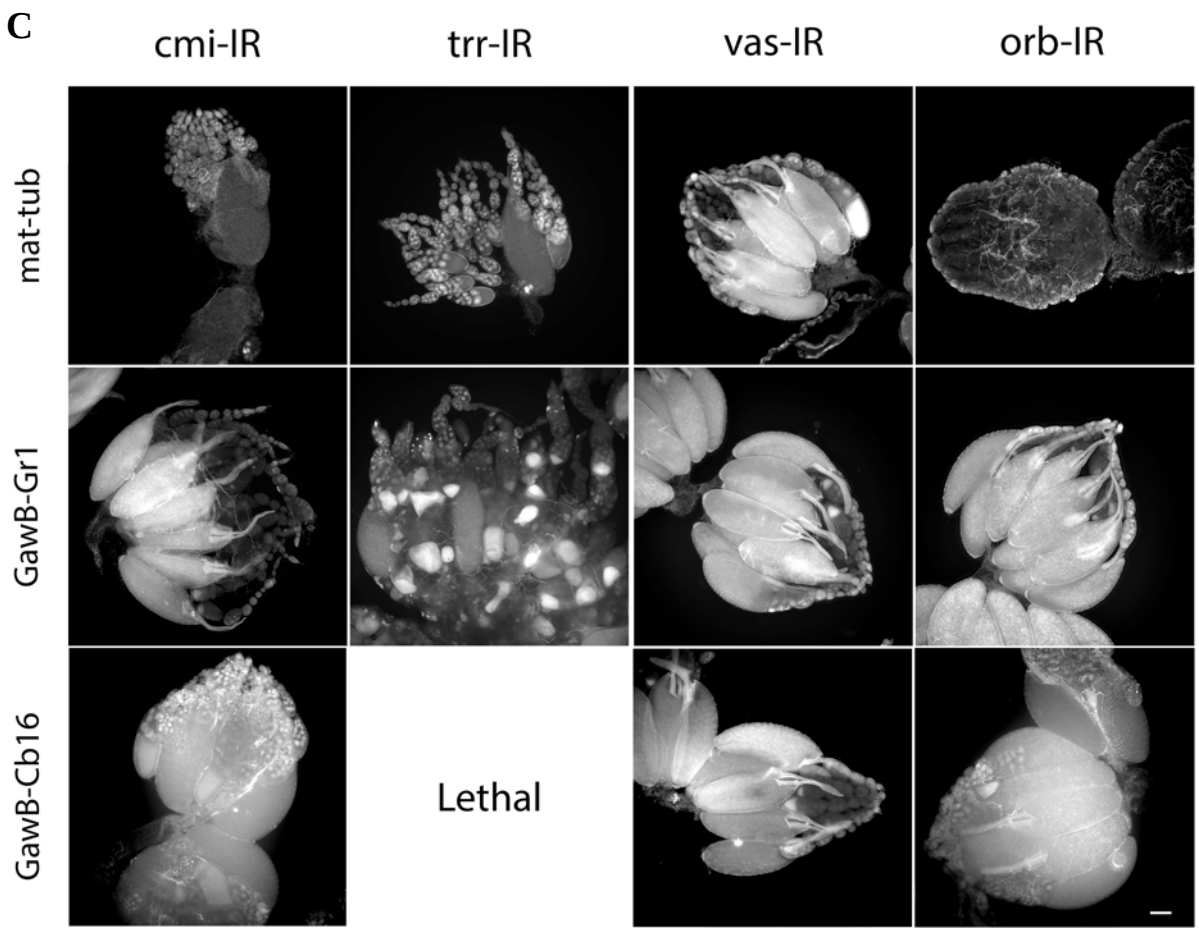


Figure 6. Knockdown of Cmi and Trr in Germline and Somatic Cells Within the Adult Ovary. (A) Immunostaining of Gal4 driving expression of GFP in the ovary for three drivers reported to be expressed in the adult ovary: *mat-tub*, *GawB-GR1*, and *GawB-Cb16* (Hudson and Cooley 2014). Tissues were fixed and stained with anti-GFP antibody (green), and DAPI (blue). Scale bar indicates 50 µm. **(B)** Gal4 lines driving the expression of shRNAi inverted repeat (IR) targeting *vasa, orb, cmi* or *trr* in the ovary. Tissues were fixed and stained with DAPI. Scale bar indicates 100 µm. **(C)** Representative images of ovarioles in knockdown animals fixed and stained with DAPI (blue) and anti-Cmi antibody (green). Scale bar indicates 100µm. Knockdown of *trr* using the *GawB-Cb16* Gal4 line was female lethal. Scale bar indicates 50 µm.

Aim 2: Characterize the Requirement of Cmi in Embryogenesis

Cmi Knockdown in the Early Embryo Leads to Arrested Development.

According to the modENCODE database, there are relatively high amounts of *cmi* mRNA in the adult female and early embryo compared to other developmental stages (data not shown) which indicates there may be a large amount of maternal *cmi* mRNA in the embryo. Since *cmi* null germline clones did not produce embryos, we theorized that using a ubiquitous GAL4 driver in the early embryo might remove enough of the maternal mRNA to cause embryonic lethality and allow us to visualize phenotypes associated with loss of the MLR complex during embryonic development. We discovered that driving the knockdown of *cmi* under the control of the tubulin promoter (*tub-GAL4*) led to a decreased hatch rate (Figure 8A). However, the tub-GAL4 construct is a homozygous lethal insertion and needs to be kept heterozygous over a balancer chromosome so the hatch rate is artificially high due to 50% of the progeny containing the balancer which restores wild type gene expression (Figure 8B). Utilizing a balancer containing a GFP allele, we sorted the GFP-positive larvae (those without GAL4) from the GFP-negative larvae (those with GAL4 driving knockdown) and performed a lethal phase experiment (Figure 8C-E). Knockdown animals that complete embryogenesis exhibit delayed development and die prior to reaching adulthood. While there is death at every stage, a small proportion of *cmi* knockdown animals are able to pupate while *trr* knockdown animals are unable to. The mRNA reduction in the embryo was verified by qRT-PCR showing less than 0.5-fold expression in knockdown embryos aged 8-14 hours compared to the similarly aged GFP-positive sibling control (Figure 8F).

In order to learn the stage at which the portion of *cmi* and *trr* knockdown embryos that die stop developing, we designed a series of experiments to utilize key developmental milestones to determine if embryonic development is progressing. First, we characterized the expression of Cmi in wild type animals using well-studied patterning genes to stage them (Figure 9). Gap genes such as *even-skipped* (*eve*), *engrailed* (*eng*), *wingless* (*wg*), *antennapedia* (*antp*), and *ultrabithorax* (*ubx*) display a recognizable pattern at specific stages during embryogenesis and disruptions in this pattern are indicative of developmental abnormalities. Cmi protein is present in all cells in the blastoderm stage, and after gastrulation is highly expressed in the ventral nerve cord and epithelial cells. Another developmental milestone is cuticle production, and wild type animals produce cuticle during stage 16 of development from 13-16 hours after egg laying (AEL) (Ostrowski et al., 2002). *Cmi* knockdown embryos that failed to hatch did not produce a cuticle indicating lethality prior to the point of cuticle production (Figure 10A). The germ band in the Drosophila embryo is another easily visible structure that forms after 3 hours of embryogenesis (Tyler, 2000). Embryos aged 4-6 hours AEL were fixed and immunostained to visualize DNA, verify that Cmi protein content was lower, and ensure they were GFP negative. The results show that the knockdown embryos were fertilized but failed to exhibit proper cellular organization based on DAPI signal, Cmi protein was depleted, and the germ band did not form based on DIC imaging while the GFP-positive sibling control exhibited wild type morphology (Figure 10B). Next, we decided to check for proper localization of Eve which is a well-studied early patterning protein in the embryo that is produced shortly after the maternal to zygotic transition and is activated in seven distinct parasegments to affect gene expression in those regions (Manoukian and Krause, 1992). Embryos aged 2-4 hours AEL were fixed and immunostained to detect Eve protein in the nuclei during cellularization of the blastoderm.

Knockdown embryos verified by GFP antibody (data not shown) failed to activate eve in a segmented pattern when compared to the GFP positive sibling control (Figure 10C). Additionally, DAPI staining revealed that the knockdown embryos have fewer nuclei in the blastoderm, and the nuclei are larger with fragments of DNA surrounding the condensed chromatin. These results indicate that the MLR complex is required early on during embryogenesis and knockdown of *cmi* or *trr* leads to arrested development, early patterning defects, and genomic instability. The portion of knockdown animals that are able to hatch have reduced body size compared to similarly aged wild type animals (data not shown) and are unable to reach adulthood (Figure 8D-E) indicating possible variation in knockdown efficiency at different developmental stages.

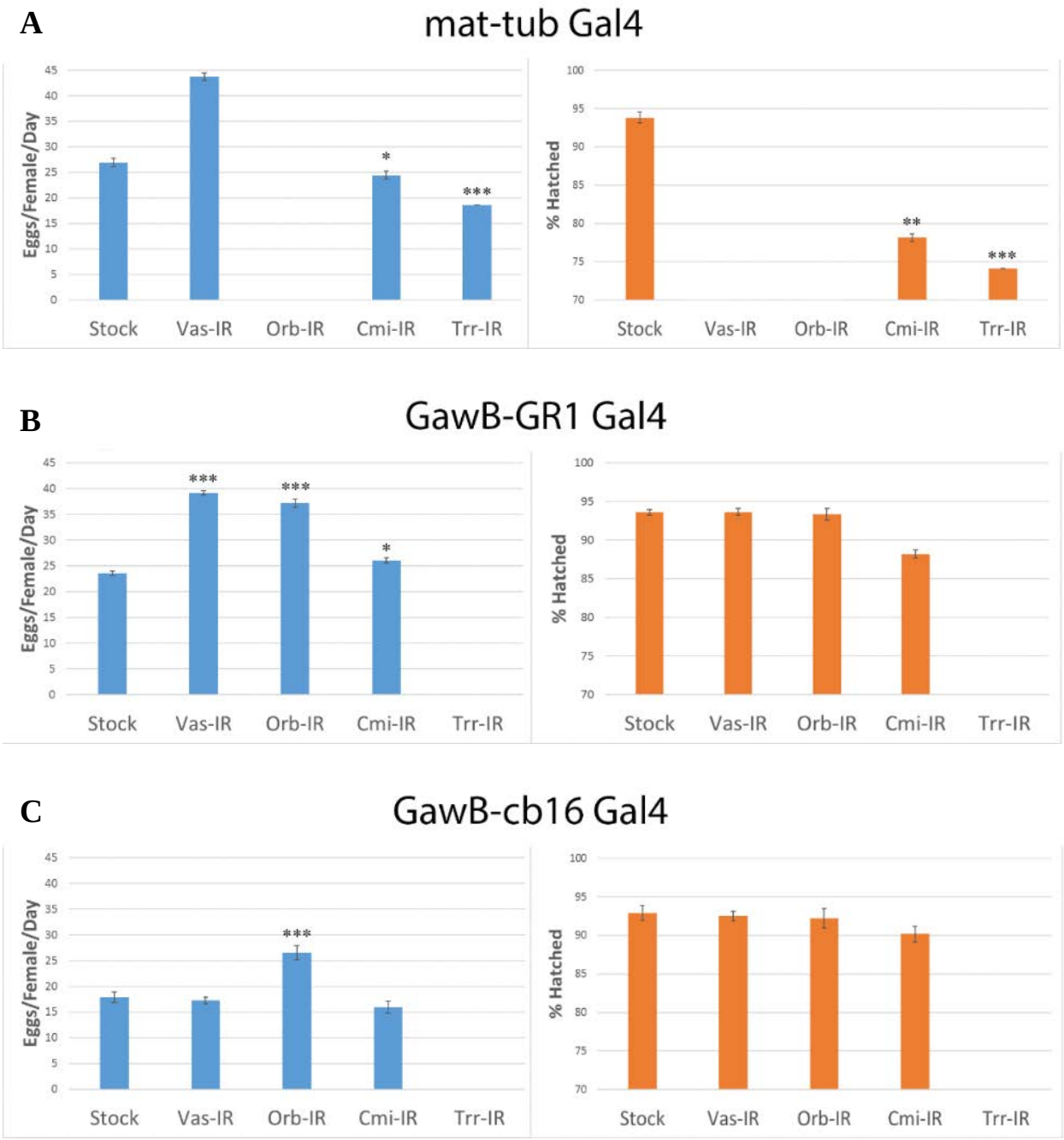


Figure 7. Effects of Cmi or Trr Knockdown on Ovary Function. Egg laying (blue) and hatching (orange) rates for knockdown animals. *mat-tub* **(A)** *GawB-GR1* **(B)** and *GawB-cb16* **(C)** drivers were crossed with IR lines to knock down *vas*, *orb*, *cmi*, and *trr* within the adult ovary. F1 females were mated with WT males. P<0.05*, p<0.01**, p<0.001***

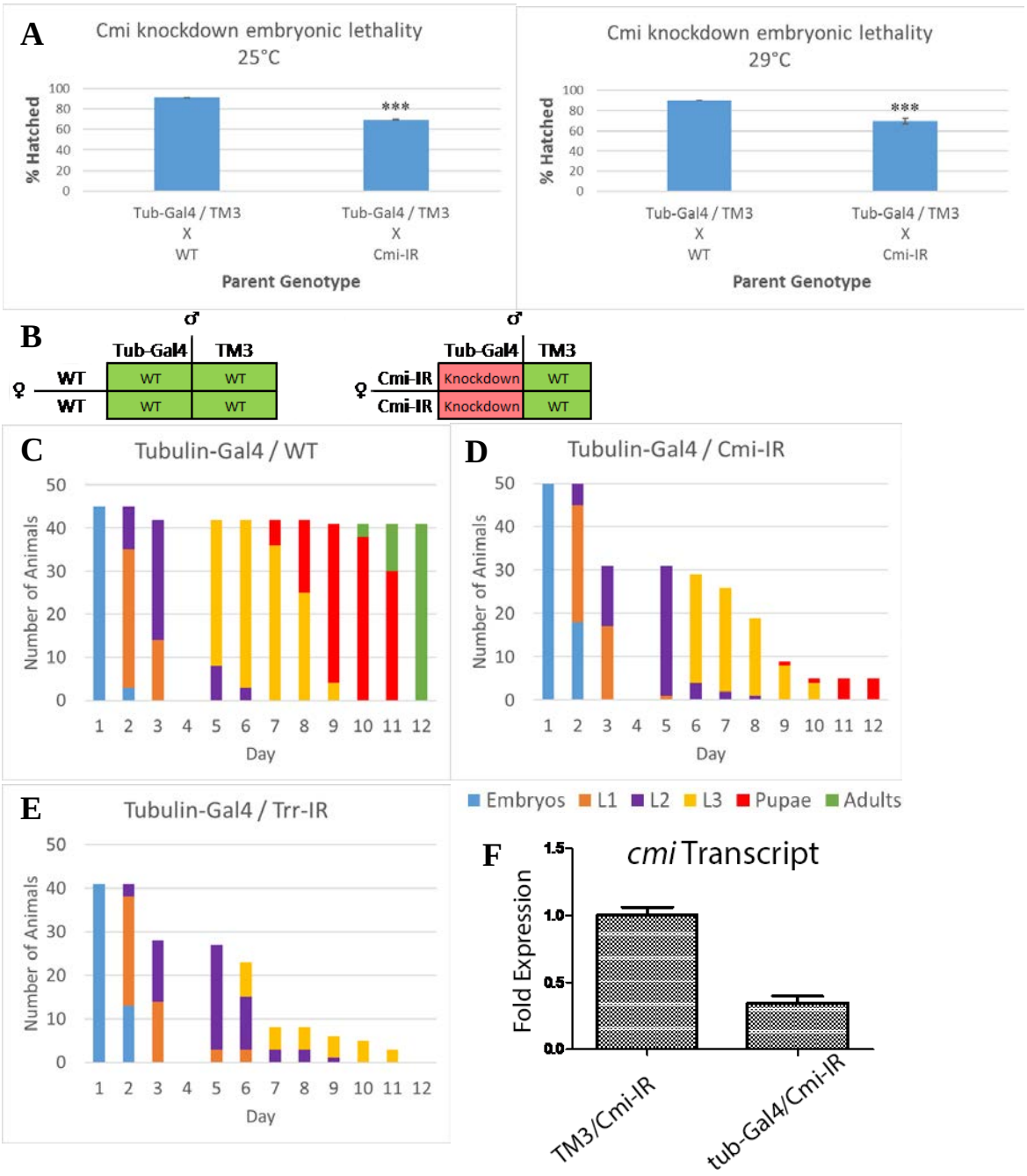


Figure 8. Cmi or Trr Knockdown Effects on Embryo Development. (A) Hatch rate of tubulin-Gal4 driver compared to tubulin-Gal4 driven knockdown of Cmi at 25°C and 29°C. **(B)** Diagram describing the mating scheme in which the expected ratio of WT embryos is 50%. WT embryos were included in the data. Embryos collected from three crosses—tubulin-Gal4 mated with WT **(C)**, tubulin-Gal4 driven knockdown of Cmi **(D)**, and tubulin-Gal4 driven knockdown of Trr **(E)**—were kept at 25°C and the number of animals at each stage of development was recorded each day in order to determine the lethal phase of Cmi and Trr knockdown. **(F)** qRT-PCR data comparing *cmi* transcript levels in GFP-positive TM3 / *cmi-IR* control to GFP-negative Tub-Gal4 / *cmi-IR* knockdown embryos. $p<0.001$***

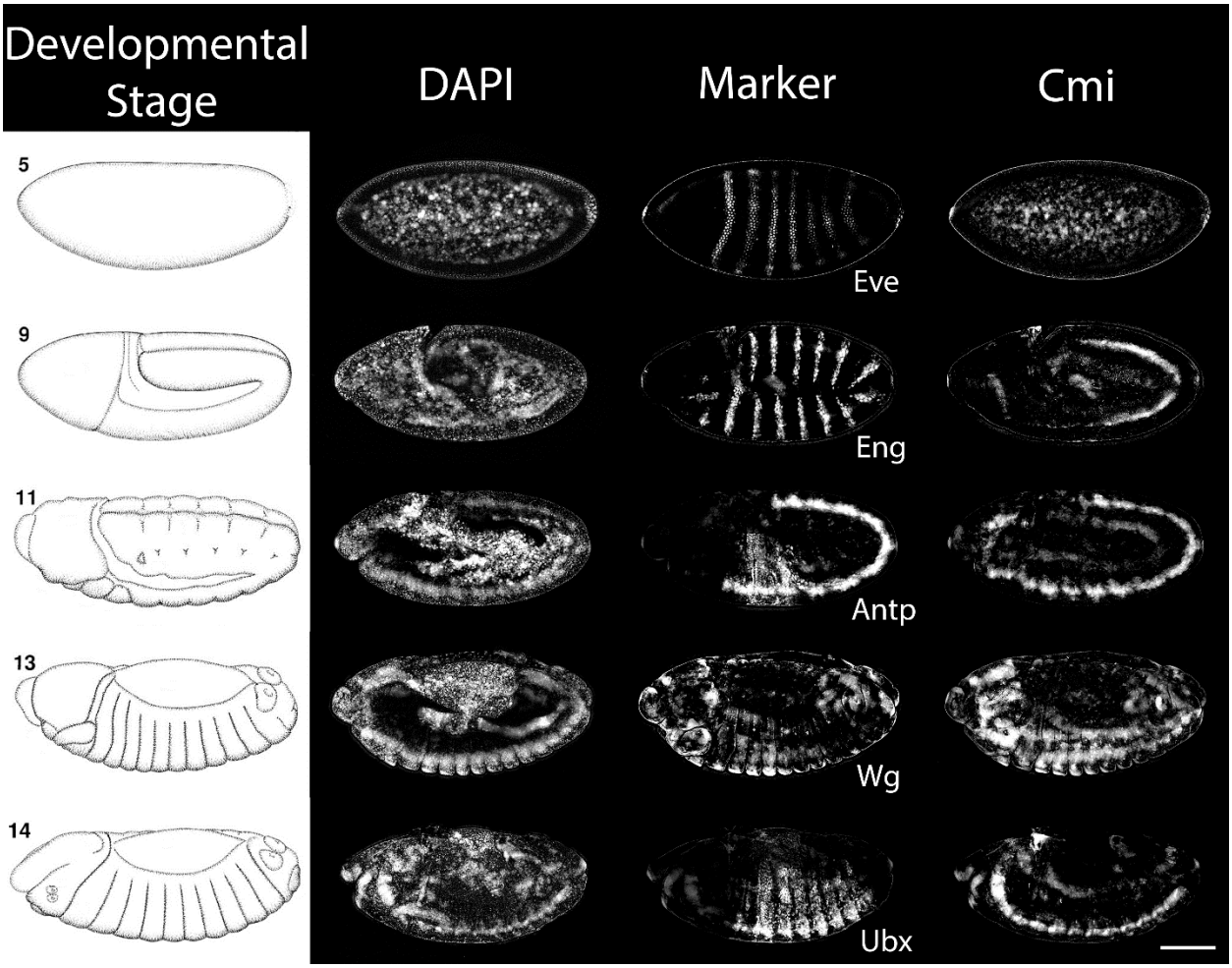
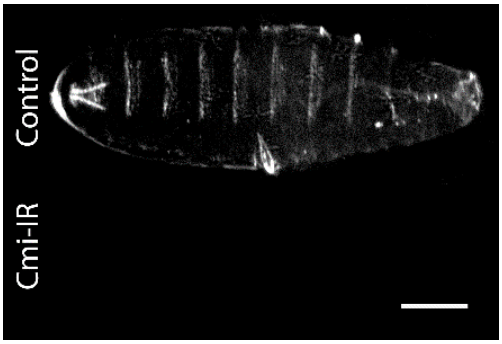
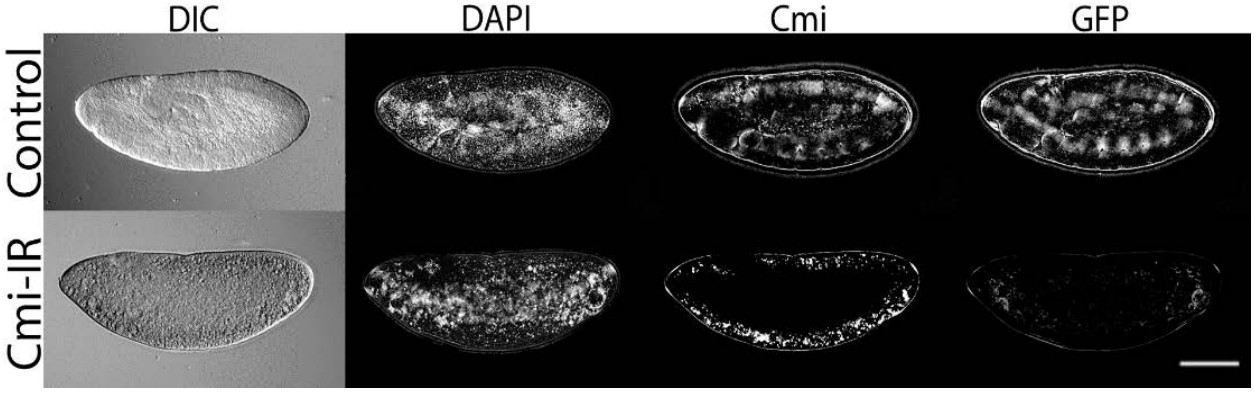


Figure 9. Cmi Expression During Embryo Development. Wild type Drosophila embryos showing native expression pattern of Cmi and developmental markers; even skipped (Eve), engrailed (Eng), antennapedia (Antp), wingless (Wg), and ultrabithorax (Ubx). Marker antibodies are monoclonal and Cmi antibodies are polyclonal. DAPI nuclear stain included for staging. Embryos oriented with anterior to the left, dorsal on top (lateral view). Schematic of developmental stage indicated on the left (adapted from Hartenstein, 1993). Scale bar indicates 100 µm.

A



B



C

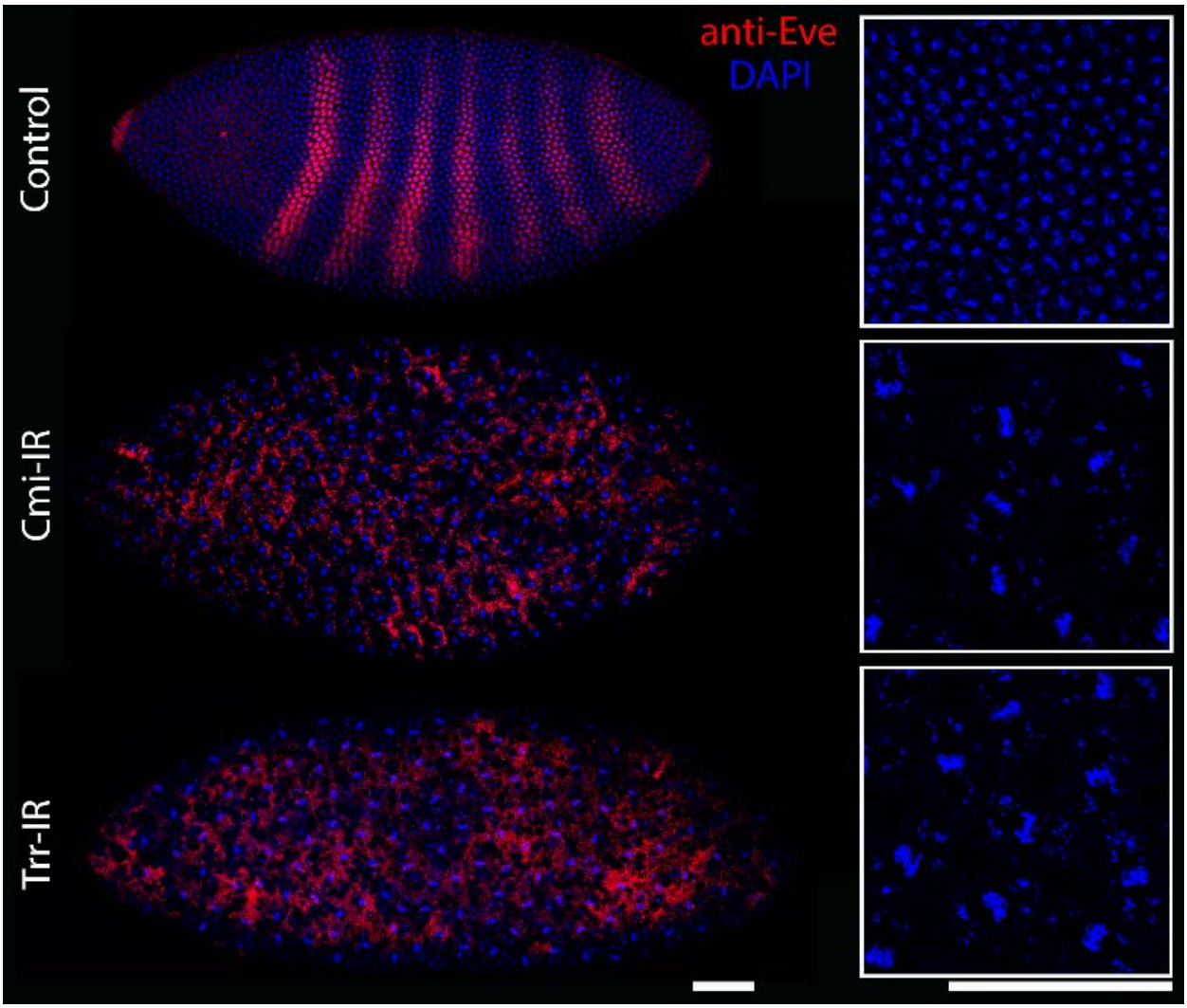


Figure 10. Cmi or Trr Knockdown in the Early Embryo. (A) Cuticle analysis of TM3 / Cmi-IR sibling control compared to tub-GAL4 / Cmi-IR knockdown embryo indicating knockdown embryos do not produce a cuticle. Scale bar indicates 100 µm. **(B)** Analysis of unhatched knockdown embryos from Figure 10 (verified by lack of GFP balancer) revealed that embryos aged 4-6 hours after egg laying (AEL) failed to form key structures, such as the germ band, when compared to the GFP positive sibling control. Scale bar indicates 100 µm. **(C)** Embryos aged 2-4 hours AEL were fixed and stained with anti-Eve antibody (red) and DAPI (blue). GFP balancer positive sibling control (GFP not shown) displayed a WT Eve expression pattern while Cmi knockdown embryo was unable to express Eve in a segmented pattern. Scale bar indicates 50 µm.

Paternal Contribution of Cmi Null Allele Worsens Phenotype of Cmi Depleted Embryos.

Certain alleles that lead to maternal-effect lethality can be rescued by paternal contribution of a wild type allele (Tomkiel et al. 1991). To assess whether or not zygotic rescue is occurring, we tested the hatch rate of embryos produced from *cmi* or *trr* knockdown ovaries that were fertilized with the *cmi*^{1} allele. Virgin F1 females containing *mat-tub* GAL4 driven knockdown of *cmi* or *trr* were mated to males carrying the cmi^{1} allele over a balancer chromosome so the expected ratio of embryos containing the cmi null chromosome is 1:1. Introducing the *cmi*^{1} allele significantly lowered the hatch rate of embryos produced from both *cmi* and *trr* knockdown ovaries compared to introducing the wild type allele (Figure 11) indicating zygotic rescue is partially restoring embryonic viability when the MLR complex is depleted during oogenesis.

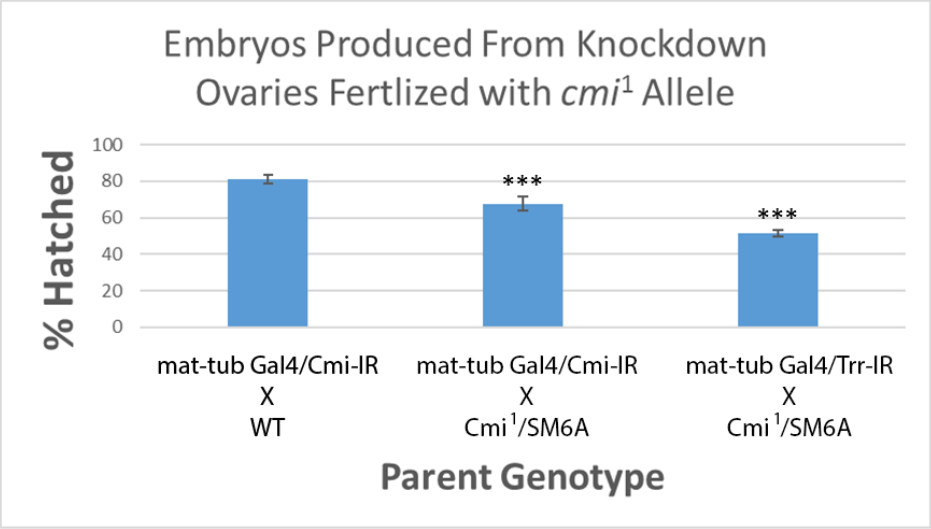


Figure 11. Zygotic Rescue of Cmi. Virgin F1 females from the cross described in Figure 7A with ubiquitous knockdown of *cmi* or *trr* via mat-tub Gal4 were mated with either WT males or *cmi*^{1}/SM6A males (*cmi* null allele over a balancer). The expected ratio of balancer (WT) to knockdown is 50% and embryos were not sorted. Fertilizing the embryos with the null allele lowered the hatch rate of both the *cmi* and *trr* knockdown. $p<0.001$***

CHAPTER FOUR

DISCUSSION

The mechanisms of chromatin remodeling and enhancer activation during development have remained elusive. The complex patterns of gene activation and repression that are critical for cell fate transitions and tissue patterning are regulated by numerous activities such as transcription factor binding, cell signaling pathways, and histone modification, but it is unclear how COMPASS-like complexes interact with and contribute to these mechanisms. With a growing number of disease states associated with mutations in chromatin remodeling and modifying complexes, it is crucial that we expand our understanding of how these complexes function in order to screen for or prevent developmental diseases and cancer. While it is known that the MLR family complexes mediate enhancer specific histone modifications and regulate the transition of inactive or poised enhancers to the active state, little is known about how the complexes recognize specific enhancers in a temporal and cell-type specific way. Because inactivating mutations in the MLR complexes are developmentally lethal and haplo-insufficient for normal development, they likely have broad roles throughout the genome and are required in many cell types.

Our studies have revealed an essential role of the Drosophila MLR complex for ovary development and extended the observation of embryonic lethality in other organisms. Importantly, we were only able to achieve partial lethality with an embryonic knockdown and an incomplete penetrance of phenotype characterized by some but not all of the ovarioles failing to produce late stage oocytes upon ovary-specific knockdown. This indicates that a minimal

threshold level of the MLR complex may be required to exert an effect because the knockdown animals are still able to produce functional protein at a lower concentration, and there may be variation in the knockdown efficiency in different cells within the ovary. Immunostaining of wild type ovary tissue revealed that Cmi is present in the cytoplasm of the oocyte and not associated with chromatin in the nucleus, while Cmi can be seen in both the cytoplasm and the nucleus of the nurse cells. It is possible that the maternal Cmi is required in high amounts so that it can be rapidly transported to the nucleus in response to early signaling events in the developing embryo during cell differentiation.

Since MLR complexes have been shown to be downstream components of hormone-responsive signaling pathways (Chauhan et al., 2013), it is likely they are involved in regulation of developmental progression. Ecdysone is a steroid hormone in flies that regulates developmental transitions during both oogenesis and embryogenesis (Reviewed in Yamanaka et al., 2013), and the Cmi/Trr complex has been shown to directly interact with the activated ecdysone receptor (Sedkov et al., 2003; Chauhan et al. 2012). During oogenesis, ecdysone is required at multiple stages. It is involved in germline stem cell maintenance and differentiation, somatic follicle cell development and cyst formation, and late stage nutrient sensing and border cell migration (Reviewed in Belles and Piulachs 2015). In the absence of functional MLR complexes, these hormonal signaling events during oogenesis may not be activated which would affect egg development and deposition similar to the phenotypic effects of *cmi* or *trr* knockdown. During embryogenesis, ecdysone is required for germ band retraction, head involution, dorsal closure, organ development, and certain transcription factor expression (Chavoshi and Moussian 2010). It is also required during larval and pupal stages for molting and metamorphosis (reviewed in Yamanaka et al., 2013). The depletion of MLR in the embryo may lead to a

decreased ability for the animals to progress through ecdysone-mediated developmental transitions which accounts for the decreased survival over time during the lethal phase analysis.

The A16-9 mutant of Cmi (*cmi*$^{A16-9}$), which lacks the HMG box, was identified in a screen for mutations that affect the localization of Staufen: a protein that is required in the germline to localize mRNA required for oocyte anterior-posterior polarity (St Johnston et al., 1991). This mutant led to the inability for Staufen to localize in the posterior of the developing oocyte (St. Johnston, University of Cambridge personal communication). Since germline clones containing this mutation failed to deposit eggs, it is possible this phenotype is related to the inability to properly polarize the egg through Staufen mRNA localization. Furthermore, other proteins containing an HMG domain such as DssRP (Hsu et al., 1993) and DSP1 (Decoville et al., 2000) localize in the nurse cells of the ovary, and DSP1 localizes in the ventral nerve cord during embryonic development mimicking the expression pattern of Cmi. The MLR complex may influence oogenesis through the process of mRNA localization either by directly interacting with RNA localization machinery or by influencing the expression of proteins that are involved in the process.

Another class of RNA that influences both oogenesis and embryogenesis is Piwi-interacting RNA (piRNA). These small RNAs are involved in the silencing of transposable elements through the recognition and targeting of RNA for cleavage or DNA for methylation, and are heritable through the maternal germline (Shpiz and Kalmykova 2009). In this way, piRNAs are able to prevent catastrophic germline mutations caused by mobilization of transposable elements. Furthermore, piRNAs and interacting proteins are critical for continuous epigenetic repression throughout germline development and localize in the germplasm (Marie et al. 2016; Megosh et al. 2006). The generation of piRNAs involves a protein called Maelstrom

(Mael) which can selectively convert retrotransposon RNA into piRNA in the germline, and Mael contains an HMG box domain (Genzor and Bortvin 2015) similar to the one found in Cmi. Problems with retrotransposon regulation and piRNA activity lead to DNA damage, meiotic errors, embryonic lethality, and defects in chromosome segregation (Genzor and Bortvin 2015; Malki et al. 2014). It is possible that the embryonic lethality and apparent chromosomal abnormalities observed in Cmi or Trr depleted embryos are due to the disruption of the piRNA associated proteins. In support of this hypothesis, RNA-seq data taken from *cmi* depleted larval fat bodies revealed that multiple proteins involved in piRNA generation and processing are dysregulated (D. Ford, Loyola University Chicago personal communication).

Our results show that early embryonic knockdown of Cmi or Trr leads to possible DNA fragmentation and defects in chromatin condensation. A similar phenotype along with apoptotic cell death was observed in various tumor types upon MLL4 knockdown (Ansari et al., 2012). Because histone modification readers are involved in the double-strand DNA damage response, it is likely that this phenotype is the result of the cell's inability to recruit DNA repair proteins to sites of DNA breakage (Reviewed in Gong and Miller, 2017). In addition, knockdown of proteins that are critical for piRNA function leads to DNA fragmentation through multiple centriole formation during mitosis and destabilization of the telomere protection complex in the early syncytial blastoderm (Orsi et al. 2010; Khurana et al. 2010). These studies provide two possible mechanisms for the contribution of the MLR complex to DNA damage; either MLR knockdown affects the cell's ability to repair DNA damage induced by general genomic stress during periods of rapid, synchronous cell divisions, or the knockdown leads to reduced piRNA function causing chromosome fragmentation and defects in maternal transposon silencing in the early embryo.

We also showed that embryonic death associated with *cmi* or *trr* knockdown occurred prior to gastrulation indicating that the MLR complex is required early during embryogenesis. The epigenetic landscape during this early period is dynamic, and the activation of enhancers is an intricately regulated process involving gradients of activating and repressing factors. For example, Eve expression is regulated by several different enhancers that respond to a balance of broad activators and localized repressors to form a pattern of seven distinct segments in the blastoderm (reviewed in Levine 2010). The activation of these enhancers is contingent on the JAK-STAT signaling pathway as well as the transcription factor Zelda (Struffi et al., 2011). Interestingly, ChIP-seq data previously generated in our lab shows strong Cmi binding peaks at the enhancers bound by pioneer transcription factors such as Zelda and Grainyhead. Pioneer factors are able to bind to inactive chromatin and begin to affect gene expression of key regulatory elements such as those required for cellularization and pattern formation (Iwafuchi-Doi and Zaret, 2016; Liang, 2008). Since *cmi* and *trr* knockdown both affect cellularization and disrupt the Eve expression pattern, it is possible that the MLR complex regulates early zygotic enhancer activation by either directly modifying histones at the enhancers of these genes or regulating the activity of pioneer transcription factors.

Another mechanism that may explain the observed phenotypes in both the ovaries and embryos is the involvement of MLR complexes in chromatin structure. Recent evidence suggests that catalytically dead MLR complexes are still able to contribute to the loading of RNA polymerase II at enhancers (Dorighi et al., 2017), and wild type complexes lead to the recruitment of other proteins such as CBP/p300 which is a transcriptional activator (Lai et al., 2017) and the cohesin complex which is involved in chromatin looping (Yan et al., 2017). The difference in phenotype observed between somatic knockdown of *cmi* and *trr* in the ovary may

be due to the formation of a relatively stable MLR complex in the absence of Cmi. This partial complex may still be able to recruit other proteins and participate in chromatin structural changes while *trr* knockdown destabilizes the entire complex and abrogates this function.

While these experiments do not provide much insight in relation to the mechanism by which the MLR complex influences development, they begin to characterize the phenotypes associated with its loss and elucidate the time window in which it is required. Future endeavors into understanding the specificity and activity of MLR complexes during development can be focused on the time between the maternal to zygotic transition and gastrulation. Gene expression profiles during this time in knockdown animals may provide valuable insights into which enhancers the MLR complex is activating and how loss of function can lead to developmental abnormalities. To better understand the functions in the adult ovary, in-situ hybridization to test for delocalization of mRNAs in the context of Cmi or Trr loss will determine if this is the mechanism of action in the oocyte. To determine if MLR complexes are involved in DNA repair within the embryo, genetic interaction studies with damage response proteins and immunostaining for indicators of apoptotic cell death and cell cycle arrest can be performed. With so many possible functions of MLR complexes in the germline and embryonic development, it will take considerable effort to elucidate their role. However, it is important to understand their native functions so we can learn more about the process of development and how mutations might contribute to various disease states.

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VITA

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