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Characterizing the Dynamic Localization of CMI in Early Drosophila Development

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CHARACTERIZING THE DYNAMIC LOCALIZATION OF CMI IN EARLY DROSOPHILA DEVELOPMENT

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The COMPASS-like family of lysine methyltransferases, MLR/MLX complexes, are epigenetic regulators that are essential for normal development through the methylation of the fourth lysine residue on histone 3 (H3K4), a universal epigenetic mark associated with active transcription. This family of complexes is highly conserved from yeast to mammals and the genes encoding the human MLR complexes have been associated with various developmental diseases and cancers (Dingwall and Fagan, 2019). In *D. melanogaster*, the enzymatic methyltransferase core of this complex is composed of two proteins: Cara Mitad (Cmi, also known as Lpt) and Trithorax-related (Trr). Although these proteins have been shown to be crucial for proper development, little is known about their function in regulating transcription in early animal development. My research shows that Cmi localizes to chromatin coordinately with the activation of zygotic transcription. This contrasts with the female germline where Cmi can be found on the oocyte pronucleus at even the earliest stages of oogenesis and is propagated through later stages of oocyte development. Moreover, we have shown that loss of Cmi disrupts epigenetic marking of chromosomes in the developing oocyte as shown by a reduction of HK27ac, H3K27me3, and H3K4me1 marks on the oocyte pronucleus upon Cmi depletion. Analysis of *Cmi* knockdown embryos containing an *eve-LacZ* reporter transgene revealed that loss of Cmi leads to disrupted transcriptional activation in early embryogenesis. Together, this work implicates the requirement of MLR complex for proper transcriptional activation in early development.

CHAPTER ONE

LITERATURE REVIEW

MLR Complexes

The COMPASS (complex of proteins associated with Set1) family of proteins are histone-lysine methyltransferases (KMTs) that were first discovered in yeast and are highly conserved from yeast to mammals (Cenik and Shilatifard 2021). A subset of COMPASS-like complexes, MLR complexes, catalyze the monomethylation of lysine 4 residues on histone 3 (H3K4me1), an epigenetic mark associated with active enhancers (Cenik and Shilatifard 2021). The genes encoding the catalytic domains of the human MLR complexes, MLL3 and MLL2/4 (also known as KMT2C and KMT2D, respectively), were found to be some of the most frequently mutated genes in a wide variety of cancers and developmental disorders. (Fagan and Dingwall 2019)

The requirement of MLR complexes for enhancer activation during animal development has been investigated in recent years. A study showed that the monomethyltransferase function of Mll4 is required for establishment of adipogenic and myogenic enhancers in mice. Conditional Mll4 knockout mice displayed musculoskeletal abnormalities and died immediately after birth, suggesting that Mll4 mediated enhancer activation is critical for proper murine development. (Lee et al. 2013). Another study done on mouse embryonic stem cells (mESCs) found that Mll4 is required for mESC differentiation by mediating enhancer activation through recruitment of the histone acetyltransferase, p300. However, the same study found that Mll4 is not required to

maintain stem cell identity. This suggests that while Mll4 may be required for initial enhancer activation, it may be dispensable for continued enhancer activity following ESC differentiation (Wang et al. 2016). However, much remains to be discovered regarding the function of MLR complexes in the temporal coordination of transcriptional events in early embryonic development. This may be crucial to uncovering possible mechanisms by which mutations of these genes can drive developmental disorders and cancer phenotypes.

In most eukaryotes, the MLR methyltransferase subunit is a single protein. However, in Drosophila, and related insects, this protein has been split into two independent proteins. The Cmi protein contains the conserved PHD domains implicated in histone recognition and binding. A study found that cancer-associated mutations in the PHD encoding region of the mammalian Mll3 gene were found to disrupt interactions with the tumor suppressor, BAP1 complex. This was correlated with decreased patient survival across multiple malignancies including, lung, skin, and breast cancers (Wang et al. 2018). Cmi also contains the High Mobility Group (HMG). The HMG domain was found to bind UTX, a subunit of the MLR complex, which catalyzes the removal of the repressive H3K27me3 mark (Rickels et al. 2020). The Trr protein contains the SET methyltransferase enzymatic domain responsible for the monomethylation of H3K4. While *trr* null animals are embryonic lethal, lethality can be rescued by the expression of catalytically impaired *trr* transgene constructs and result in normal animal development with no significant changes in gene expression. This result was reinforced in mESCs, where mutations disrupting the catalytic activity of Mll3 and Mll4 did not significantly affect enhancer function. This suggests that there may be methyltransferase-independent functions of MLR complexes that are essential for development (Rickels et al. 2016). This splitting of critical MLL3/4 domains into

two proteins provides us with the unique opportunity to study the functions each protein's domains independently.

Our lab identified the *Cmi* gene and showed it was essential through the development of genetic tools to dissect Cmi roles in transcription (Chauhan et al. 2012). *Cmi* null animals survive into the early third larval instar stage, but die with no obvious defects. Cmi protein and mRNA's are deposited maternally in the oocyte and are present in high amounts with widespread distribution in early embryos. Furthermore, in later stages of drosophila development, Cmi is critically required in hormone dependent gene activation (Chauhan et al. 2012 and Zraly et al. 2020). Additionally, while Cmi is not required for the association of Trr with chromosomal gene enhancers, Cmi is essential for the methyltransferase activity of Trr (Zraly et al 2020). Although zygotic loss of Cmi and Trr through RNA knockdown have been associated with embryonic lethality (unpublished, Nickels, Zraly, Dingwall), Cmi's role in the temporal coordination of enhancer activation in early embryonic development has never been thoroughly characterized.

Enhancers and Histone Modifications in Development

Enhancers are cis-regulatory elements that control precise patterns of gene expression and are typically found in non-coding regions of DNA. The size of these elements can range from several hundred base pairs (bp) to a few kilobases (kb) and contain binding sites for sequence specific transcription factors that can then recruit other factors, such as histone modifying proteins, to control enhancer function and gene expression (Sze and Shilatifard 2016). A main feature of enhancers is that they can act over large distances on a chromosome, potentially bypassing several neighboring genes to act on their target promoters.

The development of ChIP-Seq analysis has allowed for the genome-wide identification of post-translational histone modifications that are characteristic of active/repressed enhancers. Monomethylation of the fourth lysine residue on histone 3 (H3K4me1) was one of the first histone modifications to be associated with both active and poised enhancers. Additionally, the presence of H3K27 acetylation (H3K27ac) and H3K27 trimethylation (H3K27me3), respectively, have also been used to distinguish active enhancers from inactive or poised enhancers. (Sze and Shilatifard 2016). The H3K27me3 mark is associated with transcriptionally inactive chromatin. In drosophila, this mark is maternally deposited and propagated in the early embryo by the maternally supplied E(z) protein. In contrast, H3K27ac is established de novo later in embryogenesis by histone acetyltransferases (HATs) (Zenk et al. 2017).

The Maternal to Zygotic Transition

A commonly shared characteristic in the development of metazoans is the delayed activation of the zygotic genome in early embryogenesis (Harrison et al. 2011). This is due to the deposition of maternal mRNAs and proteins that drive early embryonic development (Lee et al. 2014). As development progresses, transcriptional control passes down to the zygote by a process called the maternal-to-zygotic transition (MZT), where maternally deposited products are degraded coordinately with zygotic genome activation (ZGA) (Schulz and Harrison 2019).

Zygotic genome activation typically occurs in two transcriptional waves. The minor wave corresponds with early gene expression. The major wave corresponds to widespread genome activation as transcriptional control is taken over by the zygote (Shulz and Harrison 2019).

Our lab has shown that Cmi is present in abundance in the female germline (Chauhan et al. 2012), where it appears to be required for oocyte development (Nickels, Zraly; Dingwall

unpublished). In drosophila females, the ovaries take up a large portion of the abdomen (Figure 1A) and are composed of 15-20 strings of oblong units called ovarioles (Figure 1B). At the anterior most tip of the ovariole is the germarium which houses the germline stem cells that divide and initiate oogenesis (Figure 1C) (Robinson and Cooley, 1997). Initial divisions produce a 16-cell cyst containing 15 nurse cells that pump mRNAs and proteins into the developing oocyte. Subsequent divisions undergo incomplete cytokinesis resulting in the development of a mature oocyte (Figure 1D).

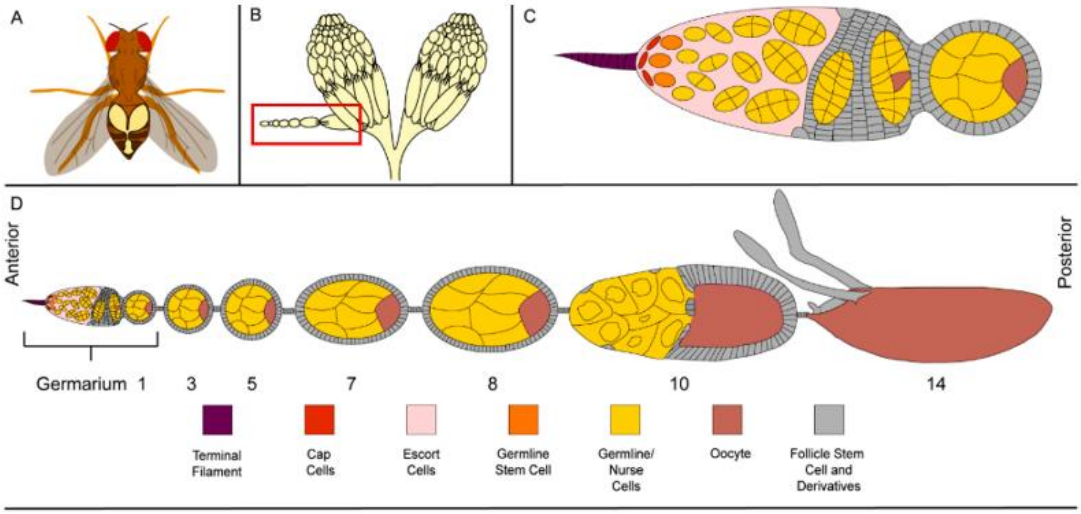


Figure 1: **Drosophila Ovary Diagram. A.** Position of ovaries in female drosophila. **B.** Schematic of the drosophila ovaries with the ovariole boxed in red. **C.** Germarium structure depicting germline stem cells, (refer to D for color scheme). **D.** Schematic of the ovariole at progressing stages of development, starting with the germarium and ending at the mature oocyte. (Adapted from: Lebo and McCall 2021)

Our lab has reported that the maternal deposition of Cmi in the oocyte leads to uniform zygotic expression in the early embryo (Chauhan et al. 2012). When the male and female pronuclei fuse following fertilization, a series of synchronous nuclear divisions take place in the developing embryo. The “cell” replicative cycles rapidly transition between S-phase and mitosis through nuclear division cycle (NC) 8 where the minor wave of zygotic transcription begins, and

maternal products begin to degrade. After NC8, pole cells form at the posterior and nuclei begin migrating to the periphery to form the syncytial blastoderm. Thereafter, nuclear divisions become longer and asynchronous. After NC13, cellularization begins, coinciding with the major wave of zygotic transcription and widespread enhancer activation (Figure 2). (Gilbert SF, 2000).

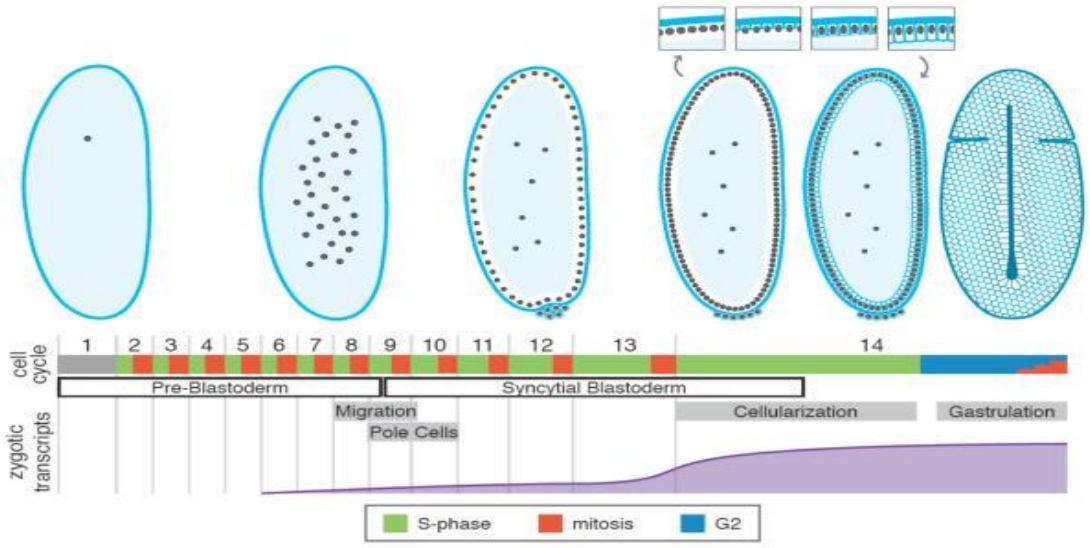


Figure 2: **Schematic of Drosophila Development through Nuclear Cycle (NC) 14.** (Farrell and O'Farrell 2015)

Pioneer Transcription Factors and Early Enhancers

Before enhancers are activated in early drosophila development, the chromatin must be loosened to a less compacted state. This is achieved, in part, through the binding of "pioneer transcription factors" which function to convert condensed chromatin to a more active state. These factors allow for further transcription factor binding and recruitment of chromatin modifying proteins (Small and Arnosti 2020). The pioneer transcription factor Zelda was the first pioneer factor identified in drosophila and found to be a key activator of the early zygotic genome. Zelda was also shown to bind to motifs present on many developmental enhancers (Harrison et al. 2011). GAGA factor (GAF) was recently identified as a pioneer transcription factor in drosophila and

was shown to be required for the accessibility of hundreds of sites in the genome that are activated following NC12/13. This contrasts with Zelda which was shown to be enriched in regions activated earlier at NC10, indicating a possible handoff between Zelda and GAF pioneer-like activity during the MZT (Gaskill et al. 2021). Another pioneer factor, CLAMP, was similarly found to bind to regions of the zygotic genome that are transcribed later than those bound by Zelda. However, the same study found that CLAMP and Zelda act together in the zygotic genome to promote chromatin accessibility and that they regulate each other's binding. (Duan et al. 2021). Together, this indicates a possible need for multiple pioneer factors in the establishment of transcriptionally active chromatin during zygotic development.

Some of the most well characterized enhancers in drosophila are those that control the expression of pair-rule genes involved in segmentation in insects that are expressed in a distinct seven-stripe pattern (Small and Arnosti 2020). The pair-rule gene even-skipped (*eve*) is controlled by five enhancer elements that bind transcription factors which can further recruit histone modifying proteins to drive enhancer activation and eve expression. (Borok et al. 2010). Moreover, eve enhancers are activated early in embryonic development starting at NC12 where broad eve expression occurs. Gradually, eve expression focuses into the characteristic seven-stripe pattern through NC14 following ZGA. Thereafter, eve enhancers are not reactivated later in development (Bothma et al. 2014). This makes eve a suitable reporter to use to evaluate the temporal coordination of enhancer mediated gene transcription during ZGA.

Here, we characterize the dynamic localization of Cmi in early drosophila development and investigate its requirement in zygotic transcriptional activation.

CHAPTER TWO

METHODS

Generation of *eve-lacZ, Cmi IR* Recombinants

Virgin females from two *eve-lacZ* transgenic fly lines, NTG and PTG (Fujioka et al. 2013), were collected and crossed to males containing a *Cmi-IR* transgene fused to a UAS Gal4 region (Chauhan et al. 2012). Virgin females from the progeny were collected and crossed to double-balancer males. Males from the resulting progeny were collected and individual males were crossed to double-balancer females. Once larvae were visible, the male fly was removed and screened for presence of the *eve-lacZ* transgene via PCR as described below. Progeny from the *eve-lacZ* transgene expressing males were allowed to develop, and presence of the *Cmi-IR* transgene was confirmed by crossing virgin females from the progeny to males containing a C765-Gal4 driver expressing UAS-Dcr-2 in the background. The resulting progeny was evaluated for wing phenotypes. UAS-Dcr-2 was added to the background of the resulting *eve-lacZ, Cmi-IR* stock.

Confirming the Presence of *eve-lacZ* Transgene in Recombinants by PCR

PCR analysis was performed using the following *eve-lacZ* transgene primers:

Forward: 5’ – GCTGTGCCGAAATGGTCCATCAAA – 3’,

Reverse: 5’ - TACTGACGAAACGCCTGCCAGTAT – 3’. (Fujioka et al. 2013).

Males of the proper genotype were selected, and DNA was prepared using a modification of the single fly DNA preparation protocol (Gloor and Engels, 1992). Presence of the *eve-lacZ* transgene was detected by PCR using DreamTaq Green PCR (2x) (Thermo Scientific) at cycling conditions of 55°C for 1 minute and 72°C for 30 seconds for a total of 30 cycles. PCR products were run on a 2% agarose gel and analyzed for a band of a (insert) fragment size.

Immunostaining of Embryos

For characterization of native Cmi localization: Oregon R embryos were collected 0-3 hours after egg laying (AEL) and aged at 25°C. For Cmi knock-down embryos: A GAL4 transgene controlled by the alphaTub84B promoter (Bloomington Stock Center #5138, see Table 2) re-balanced over a TM3 chromosome containing GFP-tagged actin was used to drive ubiquitous expression of shRNA inverted repeats targeting either *Cmi* (Chauhan et al., 2012). F1 embryos from this cross were collected from 0-3 hours AEL and aged at 25°C. For Zelda and H3K27ac staining: Embryos were dechorionated in 50% bleach, fixed with 1:1 heptane to 4% formaldehyde in PBT (PBS containing 0.1% Triton X-100) for 15-20 minutes. Embryos were shaken in 1:1 heptane to methanol to remove vitelline membraen, and rinsed and stored in methanol at -20°C. For H3K4me1 and H3K27me3 staining: Embryos were dechorionated in 50% bleach and fixed with 1:1 ice-cold methanol and heptane and washed three times with ice-cold methanol and stored at 4°C overnight. Fixed and frozen embryos were gradually rehydrated with PBT, blocked in PBS containing 0.1% BSA and 0.1% Triton X-100 (PBSBT), and incubated with primary antibody overnight at 4°C (Cmi with Zelda, H3K27ac, or LSM10, respectively) or for 2 hours at room temperature (Cmi with H3K27me3 and H3K4me1, respectively). Details for all primary and secondary antibodies used are listed in Table 1. Embryos were blocked with 2% normal goat serum (Life Technologies Ref. PCN5000) in

PBSBT, washed in PBSBT twice for one hour, and incubated for two hours with appropriate secondary antibody. Embryos were washed in PBT three times for 15 minutes, mounted in ThermoFisher ProLongTM Gold Antifade Mountant with DAPI (catalog #P36931) and imaged using a Zeiss LSM 880 confocal microscope with Airyscan.

Table 1. List of Antibodies Used for Embryo and Ovary Staining.

Antibodies	Dilution	Source	Identifier
Guinea pig anti-Cmi	1:500	Chauhan et al. 2012	N/A
Rabbit anti-H3K27ac	1:100	Active Motif	Cat# 39133
Rabbit anti-H3K27me3	1:100	Active Motif	Cat# 39155
Rabbit anti-H3K4me1[1]	1:100	Active Motif	Cat# 61634
Rabbit anti-H3K4me1[2]	1:100	Active Motif	Cat# 39297
Rabbit anti-Zelda	1:500	Harrison et al. 2010	N/A
Mouse anti-β-galactosidase	1:500	Promega	Cat# Z3781
Rabbit anti-LSM10	1:1000	Liu et al. 2006	N/A
Goat anti-guinea pig, Alexa Fluor® 488	1:1000	Invitrogen	Ref# A11073
Goat anti-rabbit, Alexa Fluor® 568	1:1000	Invitrogen	Ref# A11011
Goat anti-mouse, Alexa Fluor® 568	1:1000	Invitrogen	Ref# A11004

[1]Used for WT and *Cmi* Knockdown Ovary Staining
[2](DISCONTINUED) Used for WT Embryo Staining

Immunostaining of Ovary Tissue

OregonR virgin females were used to assess native Cmi localization in the ovaries. For germline knockdown of Cmi in ovaries, three GAL4 drivers reported to be expressed in the adult ovary were used to drive the expression of shRNA inverted repeats targeting *Cmi* (Chauhan et al., 2012). Males containing *UAS-CmiIR transgene* were mated to GAL4 virgin females (see Table 2.) and offspring were aged at 25°C. Virgin females aged 3-5 days after pupal eclosion were dissected in 1X PBS and ovaries were incubated with 0.375M KCl at 37°C for 30 minutes. Ovaries were washed twice with PBT for 2 minutes each, then fixed with 4% formaldehyde in

PBT for 17 minutes. Fixed ovaries were washed with PBT twice for 2 minutes and then three times for 20 minutes and incubated overnight with primary antibody overnight at 4°C in PBSBT. Details for all primary and secondary antibodies used are listed in Table 1. Non-specific staining was blocked with 2% normal goat serum (Life Technologies Ref. PCN5000) in PBSBT, washed in PBSBT twice for one hour, and incubated for two hours with appropriate secondary antibody. Ovaries were washed in PBT three times for 15 minutes, mounted in ThermoFisher ProLongTM Gold Antifade Mountant with DAPI (catalog #P36931), and imaged using a Zeiss LSM 880 confocal microscope with Airyscan.

Table 2. List of Gal4 Drivers Used in Knockdown. Gal4 drivers were obtained from Bloomington Stock Center and used for embryo and ovary knockdown experiments

Genotype	Stock #	Description
y[1] w[*]; P{w[+mC]=tubP-GAL4}LL7/TM3, Sb[1] Ser[1]	Cat# 5138	Ubiquitous expression of Gal4
w[*]; P{w[+mC]=matalpha4-GAL-VP16}V37	Cat# 7063	Gal4-VP16 fusion protein expressed under the control of the alphaTub67C promoter. Expressed maternally and loaded into eggs.
w[*]; P{w[+mW.hs]=GawB}GR1	Cat# 36287	Expresses Gal4 in all follicle cells including follicle stem cells
y[1] w[1]; betaTub60D[Pin-1]/CyO, P{w[+mW.hs]=GawB}cb16	Cat# 6722	Complex expression pattern including all follicle cells

Image Processing

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.

Fluorescent Quantification of Histone Marks

Corrected nuclear and pronuclear intensity of histone marks was calculated using Image J Fiji project version. Dapi channel was used to manually threshold nuclear regions. Thresholded images were analyzed using “Analyze Particles” function setting size range to >8 pixels. Resulting nuclear ROIs were saved. Remaining non-nuclear region was selected and saved as the background ROI. Corrected nuclear signal for histone marks was calculated by subtracting the mean background signal intensity from the mean nuclear signal intensity. This was done for all nuclei analyzed and averaged for wild-type and *Cmi* knockdown experiments, respectively.

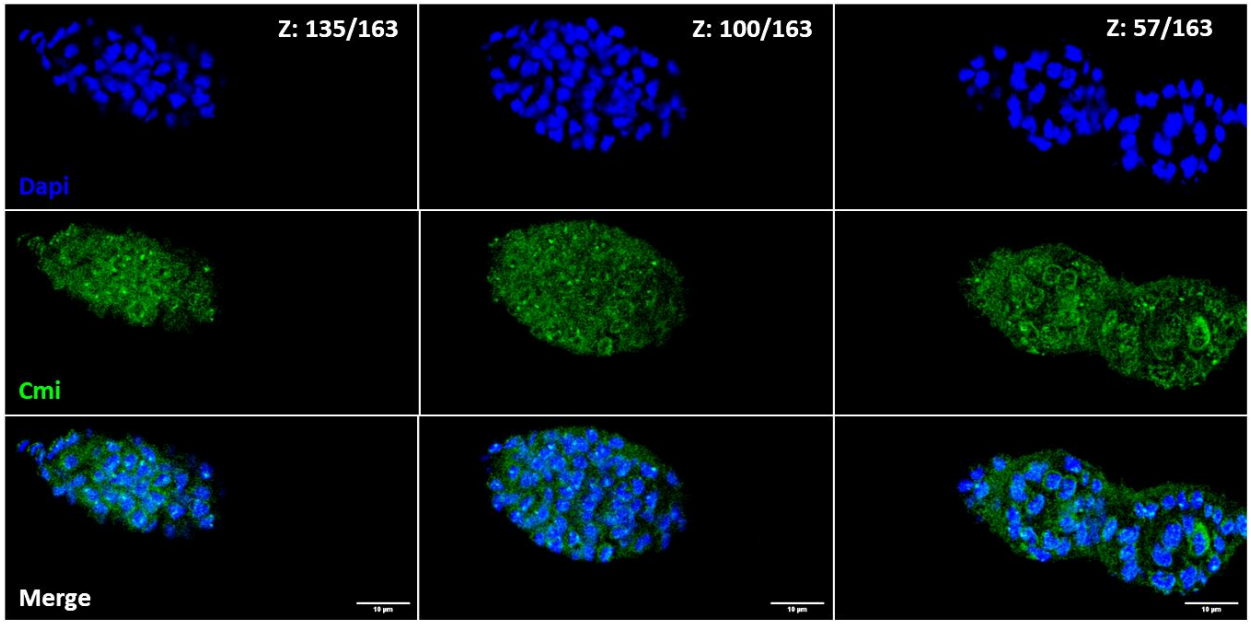
CHAPTER THREE

RESULTS

Cmi may Colocalize with the Oocyte Pronucleus Throughout Oogenesis

Our lab has previously reported that Cmi is maternally deposited into the developing oocyte throughout oogenesis. Unpublished preliminary data from our lab further showed that Cmi localization in the female germline is primarily cytosolic and is present abundantly in the developing oocyte (unpublished, Nickels, Zraly, Dingwall). However, Cmi localization to the oocyte pronucleus was not characterized nor its association with the germline and follicle cells of the germarium. Thus, we first aimed to characterize native Cmi localization during oogenesis. Confocal images of wild-type ovary tissue stained with an antibody targeting Cmi showed even distribution of Cmi and possible colocalization with both the germline and follicle cell nuclei of the germarium (Figure 3A). We further confirmed association of Cmi with nurse and follicle cells throughout oogenesis and observed evidence of Cmi colocalization with the oocyte pronucleus from as early as stage 3 through stage 9 (Figures 3B-3E). This suggests a potential role for Cmi in the bookmarking of enhancers prior to enhancer activation in embryogenesis.

A.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Z: 100/163
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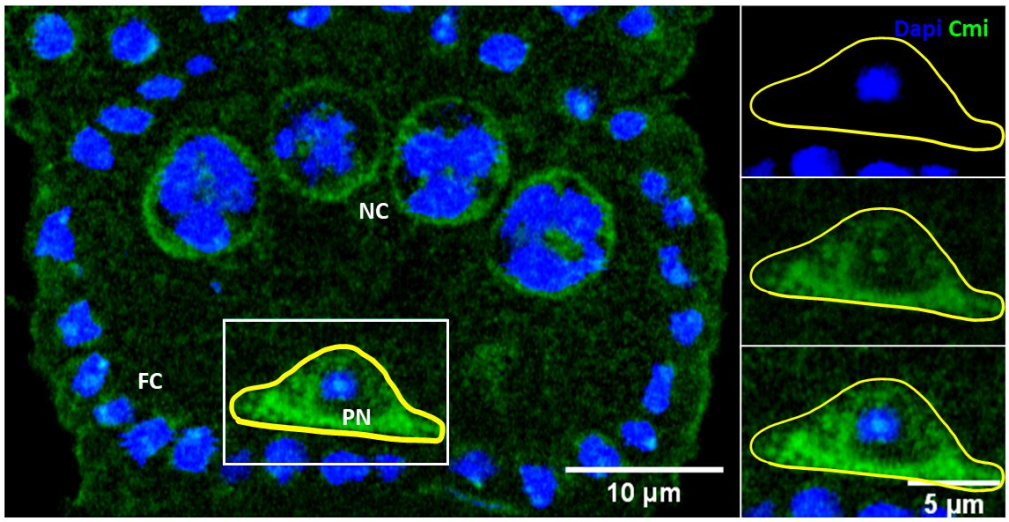
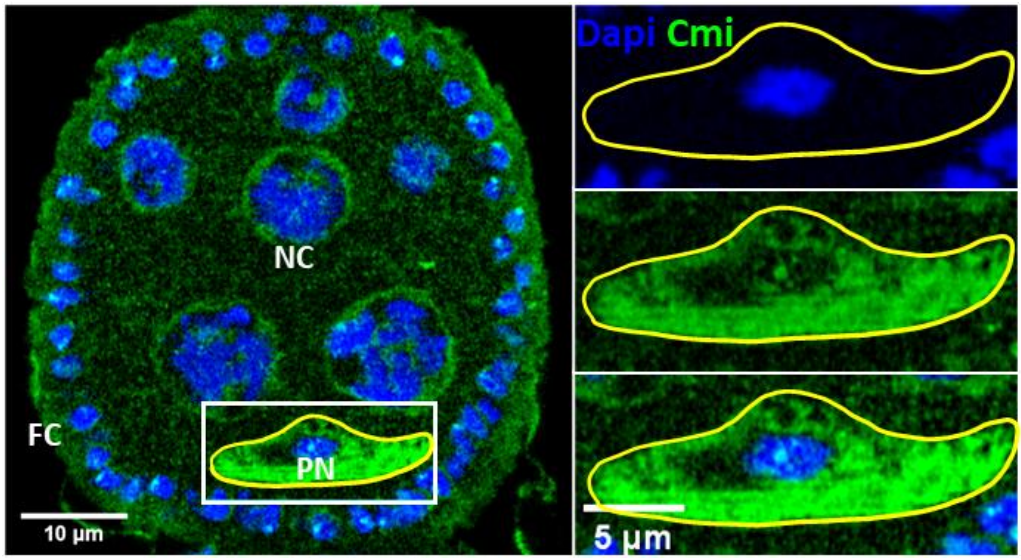
Dapi
Cmi
Merge

B.


NC
FC
PN
10 µm
Dapi Cmi
5 µm

C.



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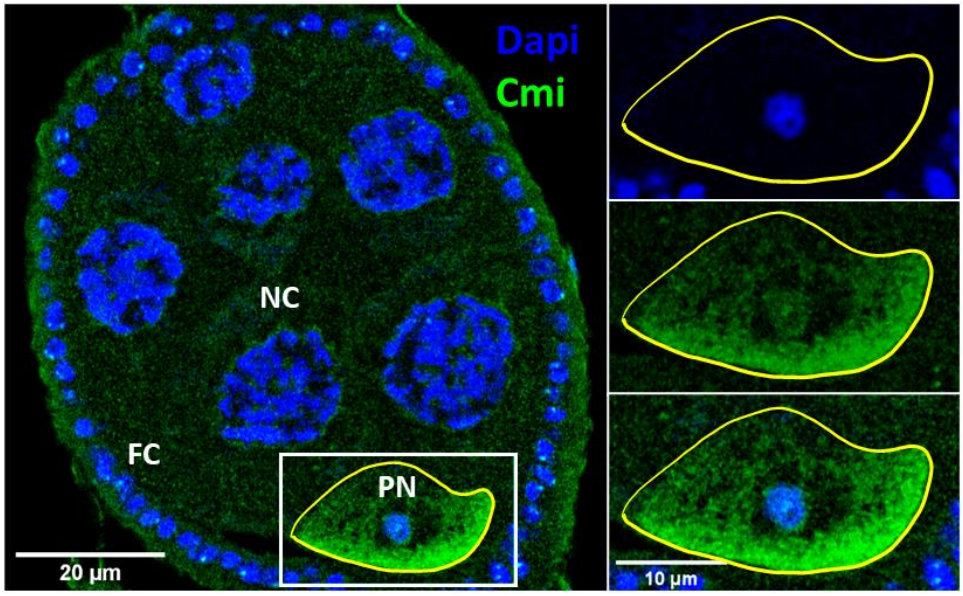


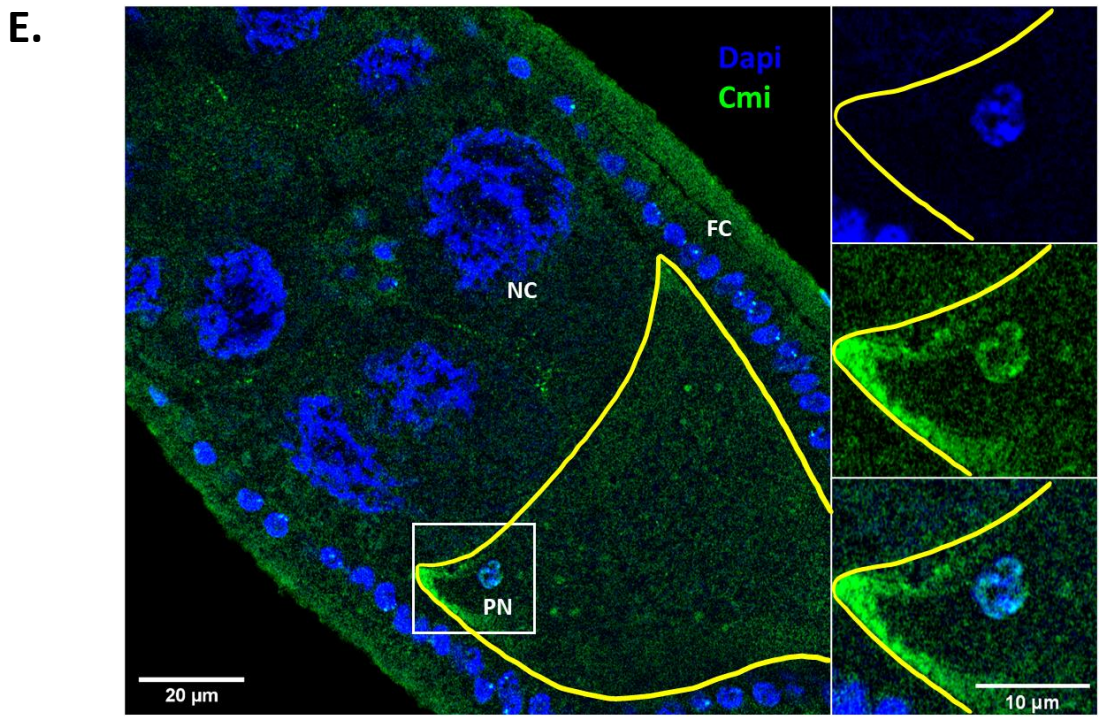


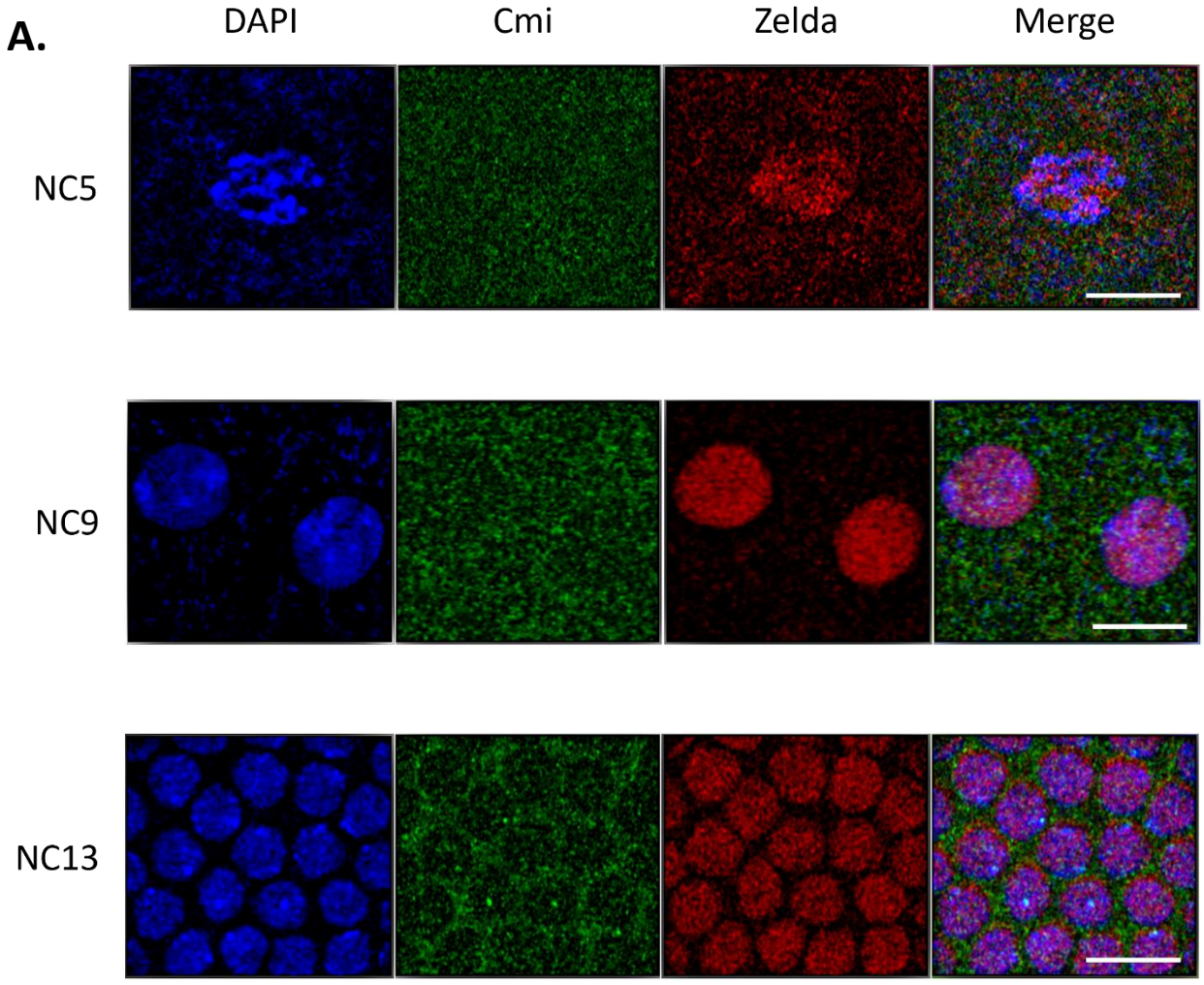
Figure 3. Cmi may Colocalize with the Oocyte Pronucleus throughout Oogenesis. Wild-type ovaries were dissected and stained using polyclonal antibodies to Cmi and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. **(A)** Native Cmi and H3K27ac localization within a single germarium visualized in three separate Z-planes to capture full structure. **(B)** Native Cmi localization within a stage 3 egg chamber **(C)** Stage 5 **(D)** Stage 7 **(E)** Stage 9. Yellow line indicates boundary between the developing oocyte and the rest of the egg chamber. The oocyte pronucleus (PN), follicle cells (FC) and nurse cells (NC).

Cmi Localizes to Chromatin Coordinately with the Major Wave of ZGA

To characterize native Cmi localization during the MZT, wild-type embryos aged 0-3 hours after egg laying (AEL) were immunostained with an anti-Cmi antibody (Chauhan et. al 2012). Based on unpublished ChIPSeq data by our lab suggesting that Cmi binds to positions associated with binding sites of the pioneer transcription factor, Zelda, we further stained with an antibody targeting Zelda protein (Harrison et al. 2010) to determine whether Cmi colocalized with Zelda in the early embryo. Immunostained embryos were analyzed by confocal microscopy

using Z-stack imaging. Confocal images show that embryos collected prior to onset of the major wave of ZGA at NC13, show broad cytosolic distribution of Cmi. This is true for before and during the minor wave of ZGA as shown by embryos staged at NC5 and NC9, respectively. However, embryos staged at the beginning of the major wave of ZGA, at NC13, show punctate areas of Cmi localization on the nuclei (Figure 4A). Nuclear-associated Cmi puncta become more widespread following cellularization at NC14. (Figure 4B). Moreover, Cmi does not seem to directly co-localize with Zelda. Zelda shows association with nuclei at all four stages analyzed. This is consistent with previous ChIP-Seq data published on Zelda, which indicates that it binds to chromatin prior to the minor wave of ZGA (Harrison et al. 2011). This suggests that the majority of Cmi protein in the pre-zygotic embryo is not directly associated with chromatin prior to the major wave of ZGA, when enhancer activation begins. This is consistent with previous unpublished observations made by our lab (Chauhan et al. 2012). However, Cmi appears to begin localizing onto chromatin following the major wave of ZGA, when early enhancers begin to activate. Cmi localization appears gradually, suggesting that Cmi binding onto chromatin may be dependent upon its function in coordinating the activation of early enhancers. We then sought to study the effects of Cmi loss by generating *Cmi* knockdown embryos by using a Gal4/UAS system (Chauhan et al, 2012; Zraly et al, 2020). Male flies containing a *Cmi-inverted repeat (CmiIR)* fused to a UAS Gal4 region were mated to virgin females carrying a tubulin-Gal4 driver to drive widespread knockdown of *Cmi* in the resulting progeny. Embryos from the resulting cross were collected 0-3 hours AEL and immunostained with antibodies to Cmi and Zelda. Cmi knockdown embryos staged at NC14 were used for analysis as the *CmiIR* construct is not expressed until onset of ZGA. Immunostaining results of knockdown embryos show a dramatic decrease in cytosolic Cmi and modest decrease in nuclear

Cmi staining. Zelda levels appear to be largely unaffected by Cmi loss suggesting that Cmi is not required for Zelda binding in the early embryo.



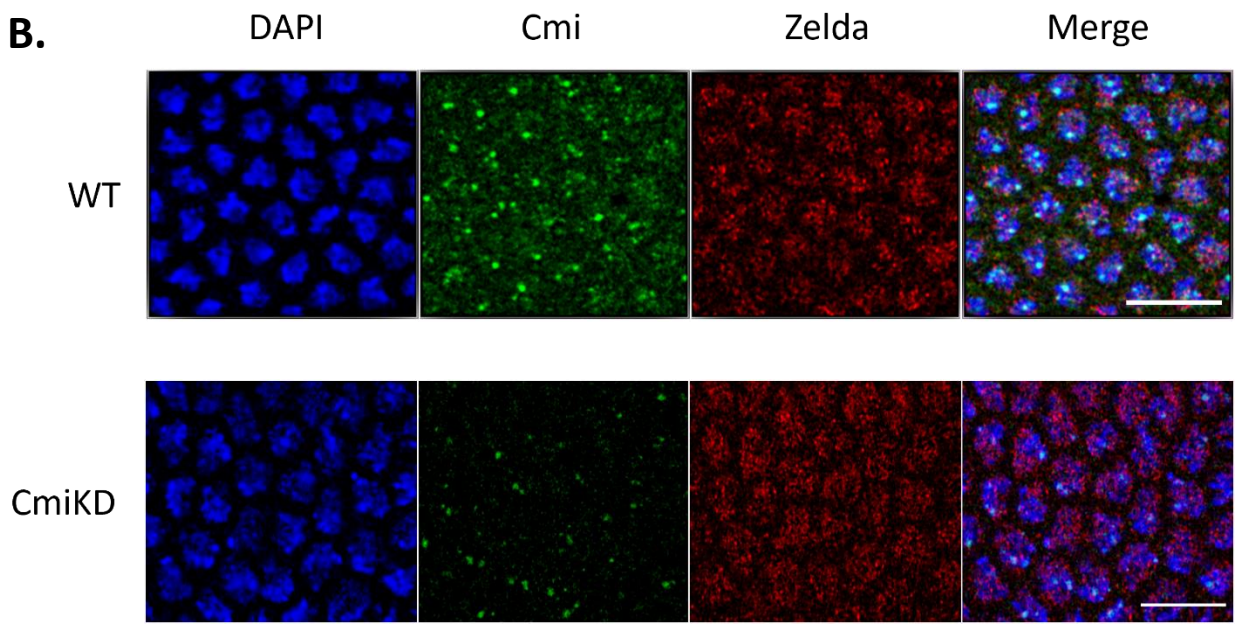


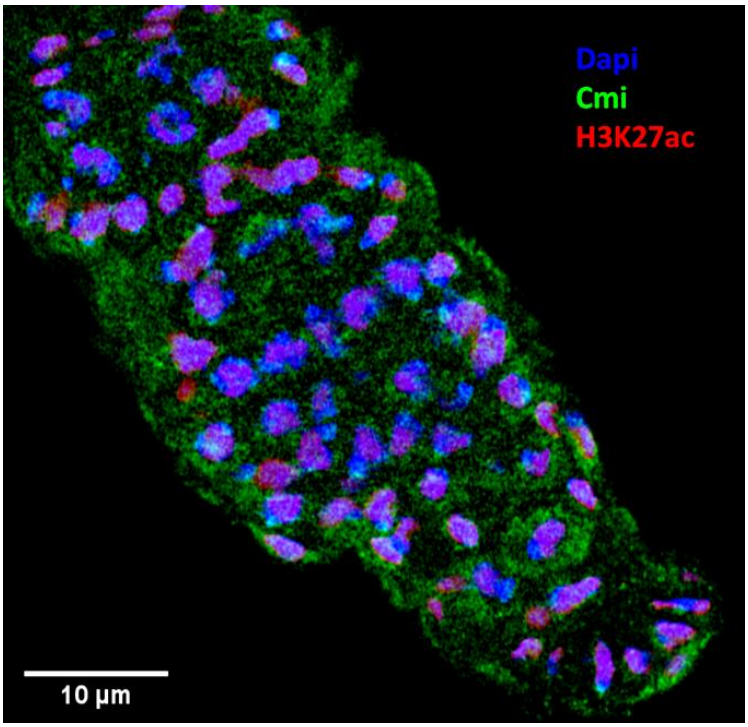
Figure 4. Cmi Localizes onto Chromatin Gradually Starting at the Major Wave of ZGA. Wild-type embryos collected 0-3 hours AEL were stained using polyclonal antibodies to Cmi and H3K27ac and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. **(A)** Native Cmi and Zelda localization prior to major wave of ZGA. Nuclear cycles (NCs) given for each embryo analyzed. Dapi stain is included to visualize nuclei. **(B)** Cmi and Zelda levels in wild-type (WT) versus Cmi knockdown (CmiKD) embryos staged at NC14. Scale bars indicates 10µm.

Cmi may Co-localize with H3K27ac in the Female Germline and Loss of Cmi Leads to Depletion of H3K27ac on the Oocyte Pronucleus

When present with the H3K4me1 mark, H3K27ac is a well-characterized mark of active enhancers. Presence of this mark has been evaluated in female germline and was reported to be present within the germarium and detected through later stages of development (Iovino et al. 2013). Therefore, we aimed to characterize its localization in the female germline with Cmi. Immunostaining results of wild-type ovary tissue confirmed presence of H3K27ac within the germarium and follicle cells (Figure 5A) and nurse cells (not shown), and potential colocalization with Cmi. Results further revealed that H3K27ac is indeed present on the oocyte

pronucleus through Stage 10 and may co-localize with Cmi (Figure 5B). To investigate the effects of Cmi loss on germline H3K27ac, we used shRNAi to knock down *Cmi* in different cell types within the adult ovary. Gal4 drivers 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* in both follicle and somatic cells. The maternal tubulin (Mat-tub) driver is highly expressed in the female germline among follicle and some somatic cells and was used to drive widespread knockdown of *Cmi* in the germline. Two follicle cell specific drivers were also used, GawB-GR1 (GR1) and GawB-Cb16 (Cb16), where GR1 is expressed in all follicle cells and follicle stem cells and Cb16 is expressed more strongly within the follicle cells of the germarium (see Table 2.) Immunstaining results of *Cmi* knockdown ovary tissue show that Cmi loss leads to depletion of H3K27ac on the oocyte pronucleus (Figure 5B). Fluorescent quantification analysis further shows that loss of Cmi leads to an overall reduction in mean pronuclear H3K27ac fluorescence intensity compared to the wild-type signal for all three drivers used (Figure 5C). Results of an unpaired t-test yielded a statistically significant decrease in H3K27ac levels when knocking down *Cmi* using the maternal-tubulin (Mat-Tub) and GawB-Cb16 (Cb16) drivers ($p < 0.01$). While we observed a visible reduction in H3K27ac upon knockdown of *Cmi* with the GawB-GR1 (GR1) driver, fluorescent quantification analysis did not yield a statistically significant difference ($p > 0.05$) as compared to wild-type H3K27ac signal. Together, this suggests that Cmi may be required for maintenance of H3K27ac on the oocyte pronucleus and implicates a possible role for Cmi in bookmarking of enhancer regions.

A.



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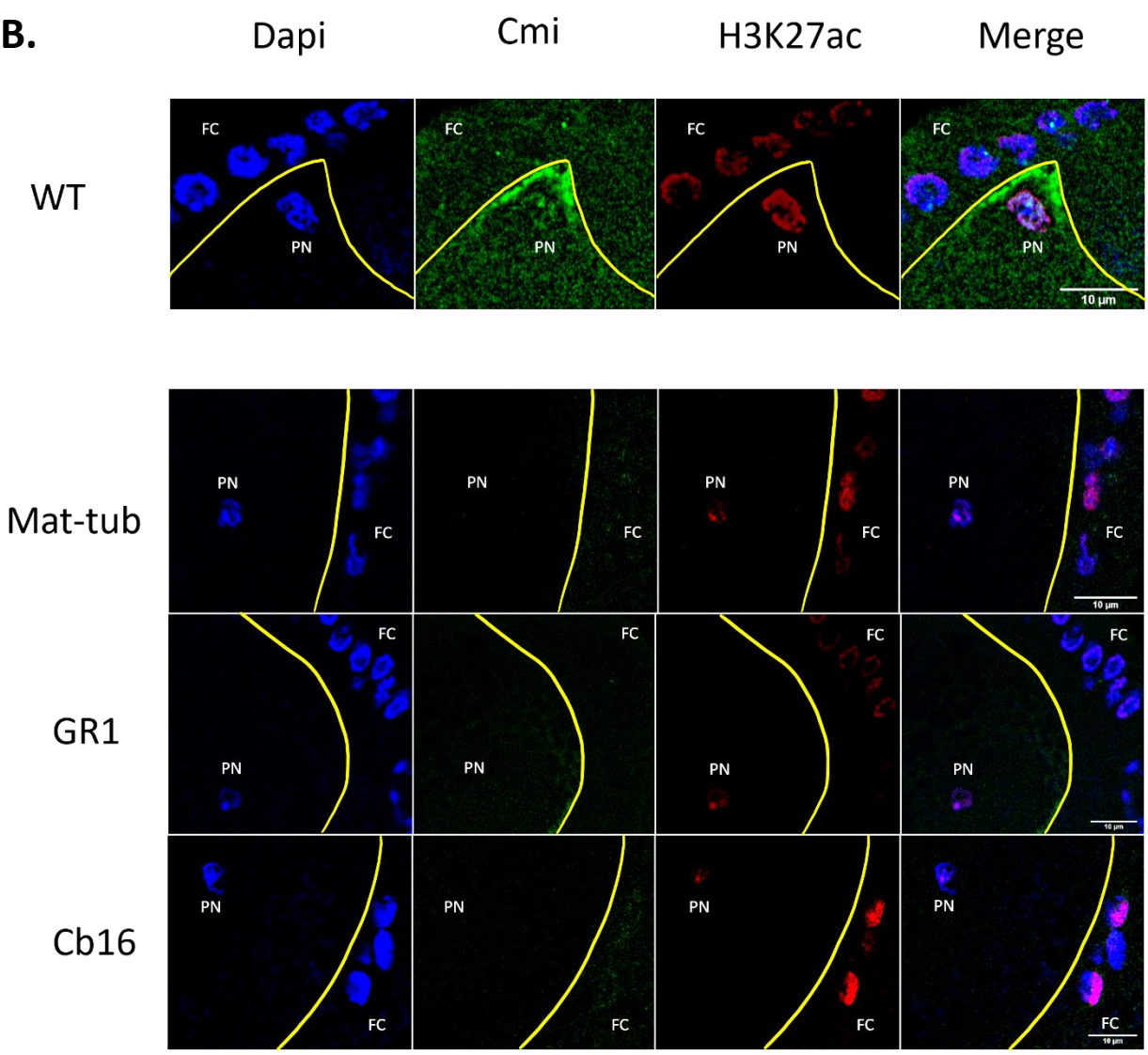
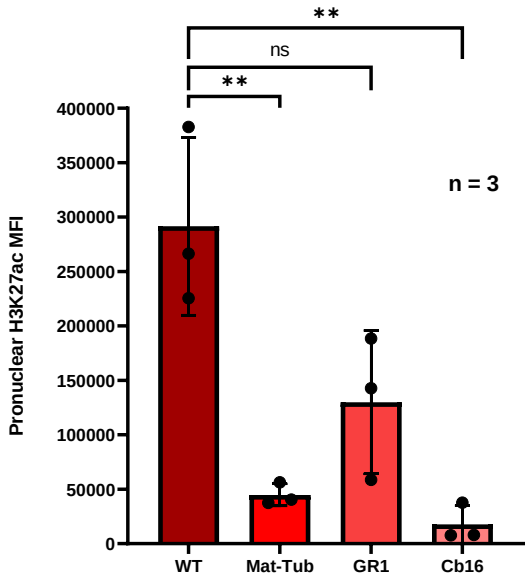

Dapi
Cmi
H3K27ac
Merge
WT
Mat-tub
GR1
Cb16
FC
PN
10 µm

C.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**
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350000
300000
250000
200000
150000
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50000
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Pronuclear H3K27ac MFI
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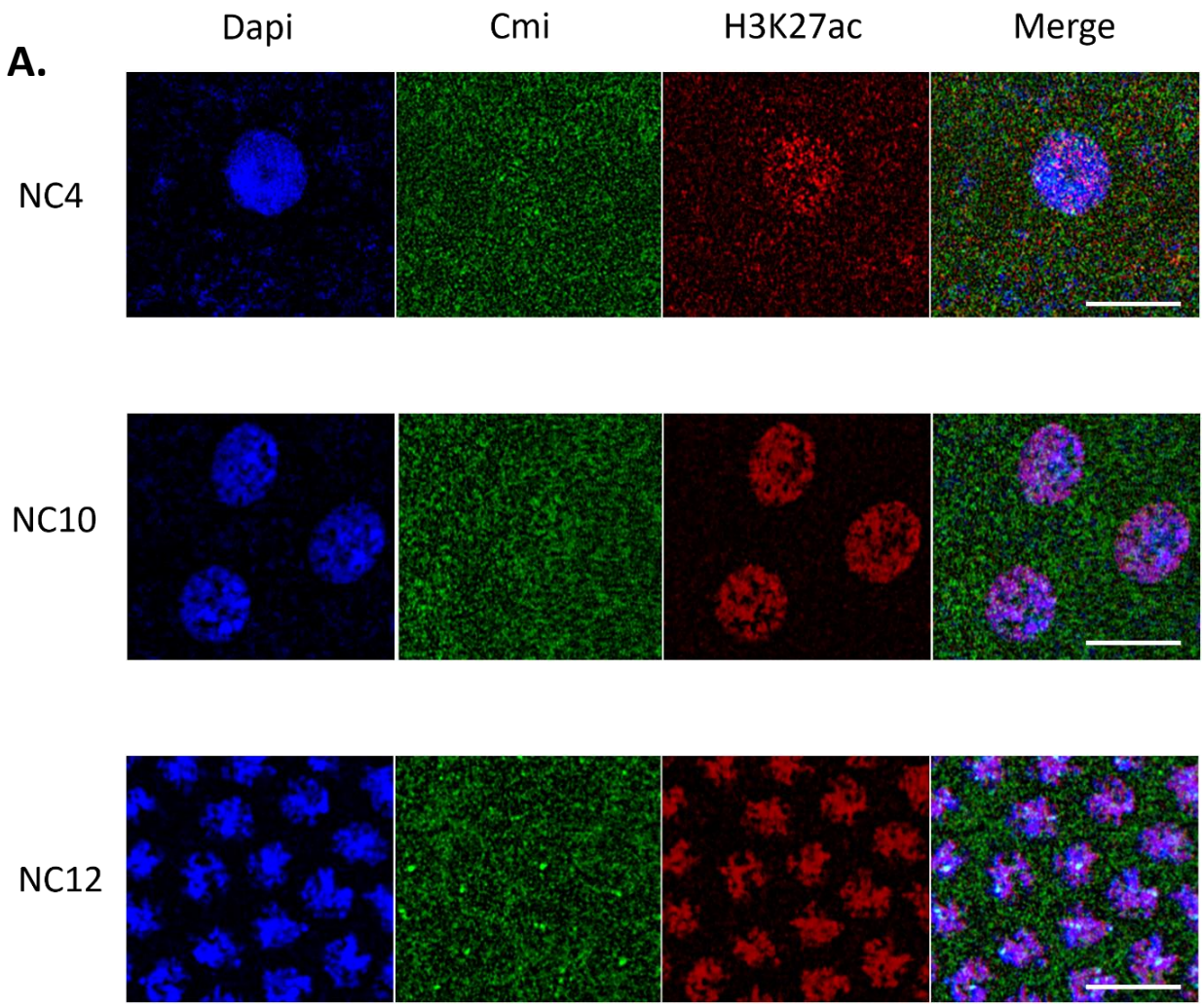
WT
Mat-Tub
GR1
Cb16

Figure 5. Cmi may Colocalize with H3K27ac during Oogenesis and Loss of Cmi Leads to Depletion of Pronuclear H3K27ac. (A) Native Cmi and H3K27ac localization within the germarium. Wild-type ovaries were dissected and stained using polyclonal antibodies to Cmi and H3K27ac and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. **(B)** H3K27ac levels in wild-type (WT) versus shRNAi mediated *Cmi* knockdown egg chambers using three Gal4 drivers: Maternal-tubulin (Mat-Tub), GawB-GR1 (GR1), and GawB-Cb16 (Cb16). Egg chambers at stage 9 (wild-type) and stage 10 (knockdowns) were used for analysis. Yellow line indicates boundary between the developing oocyte and the rest of the egg chamber. The oocyte pronucleus (PN) and follicle cells (FC) are indicated. Scale bars indicate 10µm. **(C)** Fluorescent quantification of pronuclear H3K27ac signal between wild-type and *Cmi*-knockdown egg chambers based on analysis of pronuclei from three biological replicates for each treatment. Result is displayed as mean fluorescence intensity (MFI) with individual data points and standard error bars shown. An unpaired t-test was used to determine statistical significance between WT and *Cmi*KD treatments, results displayed on graph.

Cmi Co-localizes with H3K27ac at Onset of ZGA and Loss of Cmi Leads to a Reduction of H3K27ac in the Early Embryo

Our lab has previously shown that the drosophila MLR complex is involved the recruitment of histone acetyltransferases (HATs) which catalyze the addition of H3K27ac (Zraly et al 2020). When present with H3K4me1, H3K27ac is known to be a mark of active enhancers. Thus, we sought to investigate potential co-localization of Cmi with H3K27ac in the early embryo. Confocal images of wild-type embryos stained with antibodies targeting Cmi and H3K27ac show that Cmi co-localizes with H3K27ac beginning at the major wave of ZGA (Figure 6B) while H3K27ac shows nuclear association at all stages analyzed. Interestingly, while H3K27ac has been previously reported to begin appearing on chromatin at ~NC8 through ChIPSeq analysis (Li et al. 2014) and immunofluorescence (Zenk et al. 2017), we see evidence of H3K27ac appearing on chromatin as early as NC4 (Figure 6A), suggesting that our previously observed maternal deposition of the H3K27ac mark on the oocyte pronucleus may be maintained

through early embryogenesis. Upon knockdown of *Cmi*, we see a modest decrease in levels of H3K27ac (Figure 6B), and fluorescent quantification analysis further shows a statistically significant reduction ($p < 0.0001$) in mean fluorescence intensity as compared to wild-type (Figure 6C). This suggests that Cmi may be required for maintenance of the H3K27ac mark at onset of zygotic transcription and further supports that loss of Cmi may lead to disrupted enhancer activation in early embryogenesis.



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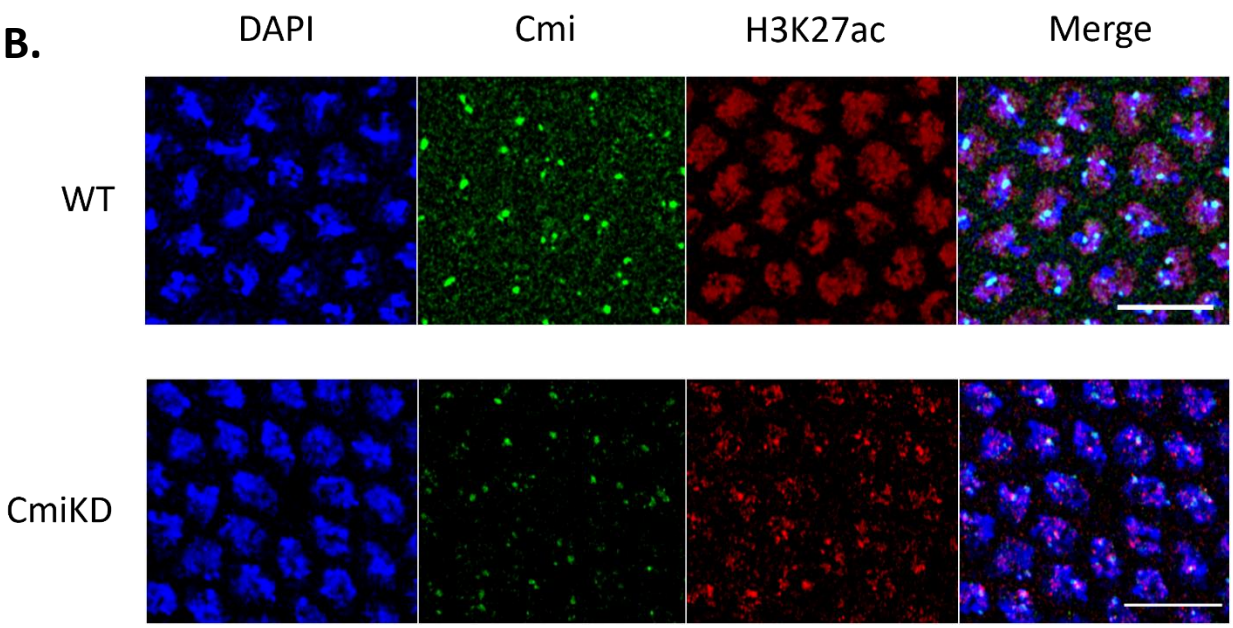
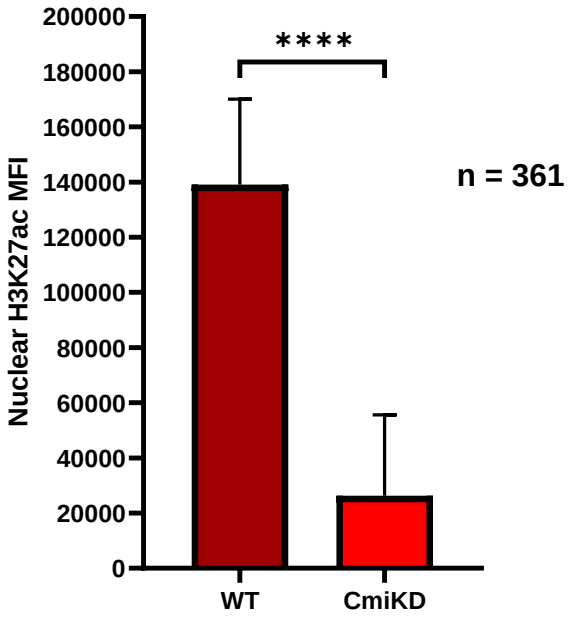

DAPI
Cmi
H3K27ac
Merge
WT
CmiKD

C.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Nuclear H3K27ac MFI
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180000
160000
140000
120000
100000
80000
60000
40000
20000
0
WT
CmiKD

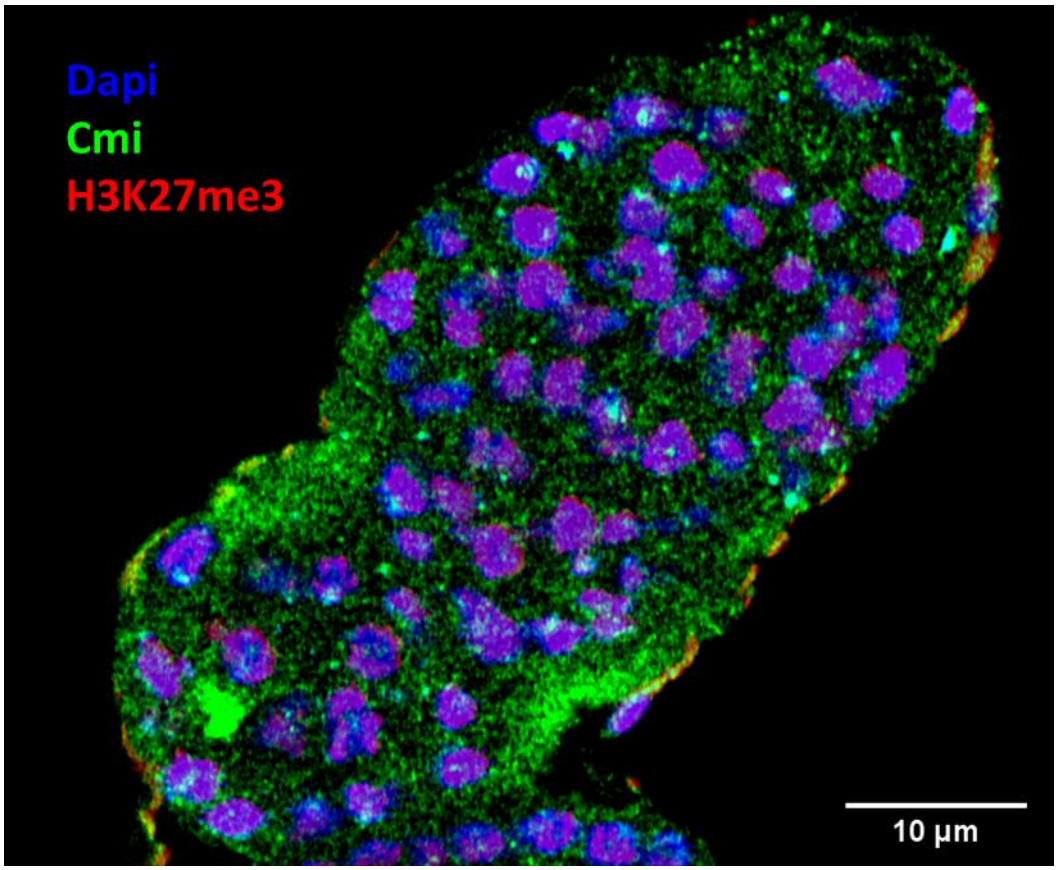
Figure 6. Cmi Colocalizes with H3K27ac at the Major Wave of ZGA and Loss of Cmi Leads to a Reduction of H3K27ac in the Early Embryo. (A) Native Cmi and H3K27ac localization prior to major wave of ZGA. Wild-type mbryos collected 0-3 hours AEL were stained using polyclonal antibodies to Cmi and H3K27ac and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. Nuclear cycles (NCs) given for each embryo analyzed. Dapi stain is included to visualize nuclei. **(B)** H3K27ac levels in wild-type (WT) versus Cmi knockdown (CmiKD) embryos staged at NC14. Scale bars indicate 10μm **(C)** Fluorescent quantification of nuclear H3K27ac signal between wild-type and *Cmi*-knockdown embryos based on analysis of 361 nuclei across three biological replicates for each treatment. Results are displayed as mean fluorescence intensity (MFI) with individual data points and standard error bars shown. An unpaired t-test was used to determine statistical significance between WT and CmiKD treatments, results displayed on graph.

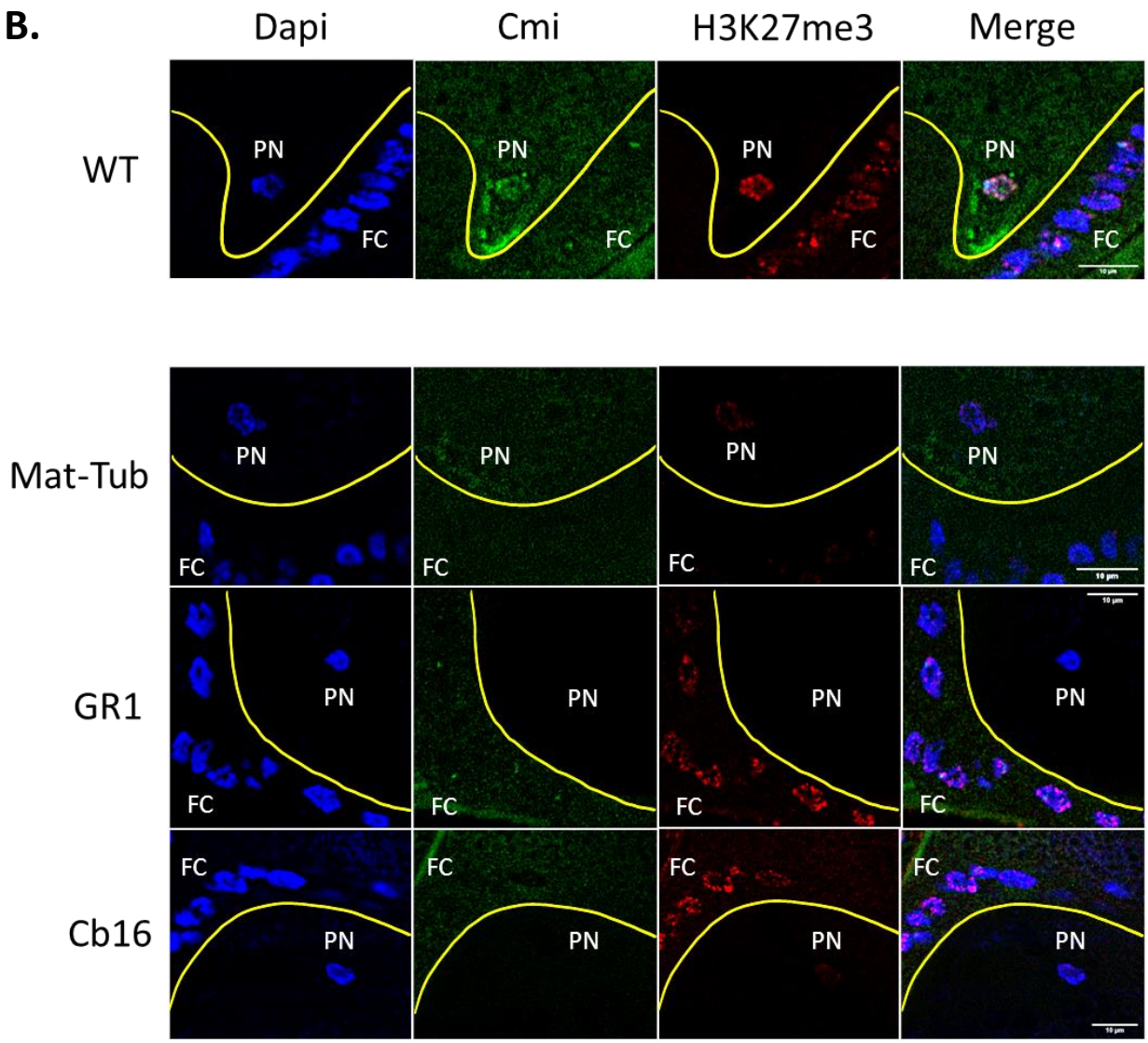
Cmi may Colocalize with H3K27me3 in the Female Germline and Loss of Cmi Leads to Depletion of H3K27me3 on the Oocyte Pronucleus

The H3K27me3 mark is a well-characterized mark of transcriptional repression and closed chromatin. When present with H3K4me1 it is characteristic of poised enhancers. H3K27me3 has been previously shown to be maternally deposited on the oocyte pronucleus during oogenesis and is maintained on the chromatin through early embryogenesis (Zenk et al. 2017). Moreover, H3K27me3 has been reported to be expressed within the germline and follicle cells of the germarium (Iovino et al. 2013). Thus, we sought to investigate possible colocalization of Cmi with H3K27me3 in the female germline and the effect of Cmi loss on germline H3K27me3 levels. Confocal images of wild-type ovary tissue stained with antibodies targeting Cmi and H3K27me3 confirm H3K27me3 localization within the follicle and germline cells of the germarium and may colocalize with Cmi (Figure 7A). We further confirmed that H3K27me3 is maintained on the oocyte pronucleus through stage 10 of oogenesis and may colocalize with Cmi (Figure 7B). While H3K27me3 has been shown to begin dissociating from the nurse cells following stage 4, we observed maintenance of H3K27me3 on the nurse cells through stage 10 (not shown) and confirmed its association with the follicle cells through later

stages of oogenesis. Confocal images of similarly stained shRNAi-mediated *Cmi* knockdown ovary tissue showed that Cmi loss leads to depletion of H3K27me3 on the oocyte pronucleus for all three drivers used (Figure 7B) and some retention of H3K27me3 on nurse cells (not shown) and follicle cells. Fluorescent quantification analysis further revealed that Cmi loss leads to a statistically significant reduction in pronuclear H3K27me3 signal compared to wild-type for all three drivers used ($p < 0.001$ for Mat-Tub and Cb16, $p < 0.01$ for GR1) (Figure 7C). This suggests that Cmi may be required for maintenance of H3K27me3 on the oocyte pronucleus, indicating a possible role for Cmi in the bookmarking of enhancer regions.

A.

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H3K27me3
Merge
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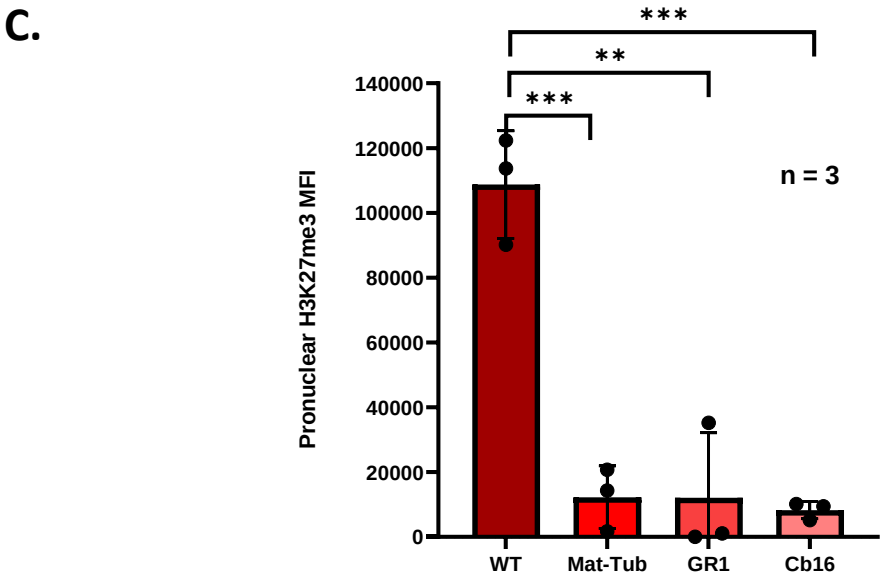
Mat-Tub
GR1
Cb16
PN
FC


C.
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***
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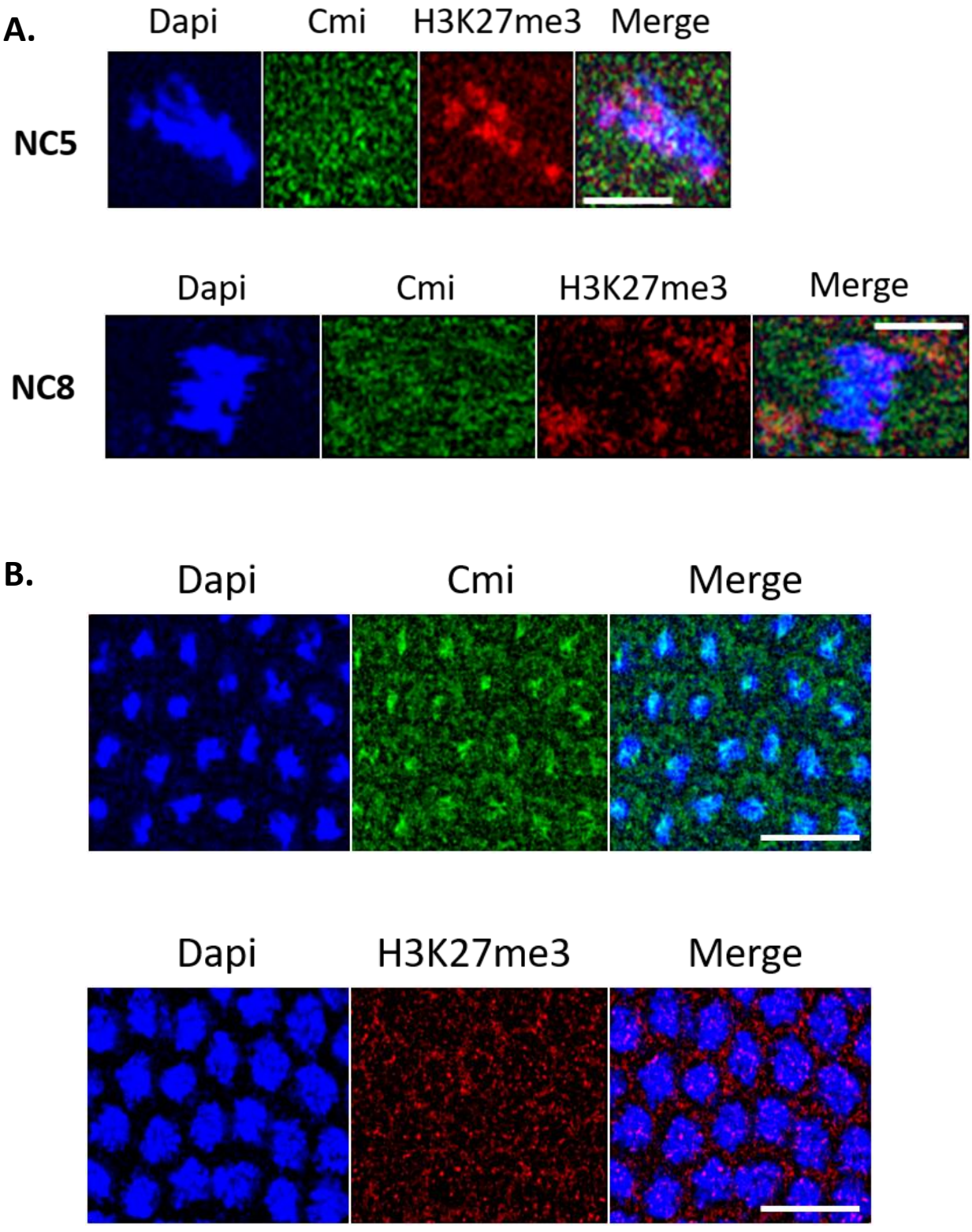
120000
100000
80000
60000
40000
20000
0
Pronuclear H3K27me3 MFI
n = 3
WT
Mat-Tub
GR1
Cb16

Figure 7. Cmi may Colocalize with H3K27me3 during Oogenesis and Loss of Cmi Leads to Depletion of Pronuclear H3K27me3. (A) Native Cmi and H3K27ac localization within the germarium. Wild-type ovaries were dissected and stained using polyclonal antibodies to Cmi and H3K27me3 and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. **(B)** H3K27ac levels in wild-type (WT) versus shRNAi mediated *Cmi* knockdown egg chambers using three Gal4 drivers: Maternal-tubulin (Mat-Tub), GawB-GR1 (GR1), and GawB-Cb16 (Cb16). Egg chambers at stage 10 were used for analysis. Yellow line indicates boundary between the developing oocyte and the rest of the egg chamber. The oocyte pronucleus (PN) and follicle cells (FC) are indicated **(C)** Fluorescent quantification of nuclear H3K27me3 signal between wild-type and *Cmi*-knockdown embryos based on analysis of pronuclei from biological replicates for each treatment. Results are displayed as mean fluorescence intensity (MFI) with individual data points and standard error bars shown. An unpaired t-test was used to determine statistical significance between WT and CmiKD treatments, results displayed on graph.

Cmi does not Colocalize with H3K27me3 in the Early Embryo and Loss of Cmi leads to a Decrease in H3K27me3 Levels in Early Embryogenesis

Presence of the H3K27me3 mark in embryogenesis and oogenesis has been previously characterized and was shown to be maternally deposited on the oocyte pronucleus and propagated through early embryogenesis (Zenk et al. 2017). Because the MLR complex has previously been implicated in the removal of this mark, we sought to characterize colocalization of Cmi with H3K27me3 and assess the effect of Cmi loss on H3K27me3 levels in early embryogenesis. Confocal images of wild-type embryos stained with antibodies to Cmi and H3K27me3 confirmed presence of H3K27me3 in the earliest stages of embryogenesis (Figure 8A) through ZGA at NC14 (Figure 8B). This contrast with localization pattern of Cmi which does not appear on the chromatin until onset ZGA. We further did not observe colocalization between Cmi and H3K27me3 in the early embryo as shown by optimal visualization of Cmi and H3K27me3 in separate Z-planes. Interestingly, confocal images of similarly stained *Cmi*-knockdown embryos show a visible decrease in nuclear H3K27me3 staining (Figure 8C)

suggesting that loss of Cmi may affect factors contributing to the maintenance of H3K27me3 mark at onset of zygotic transcription.



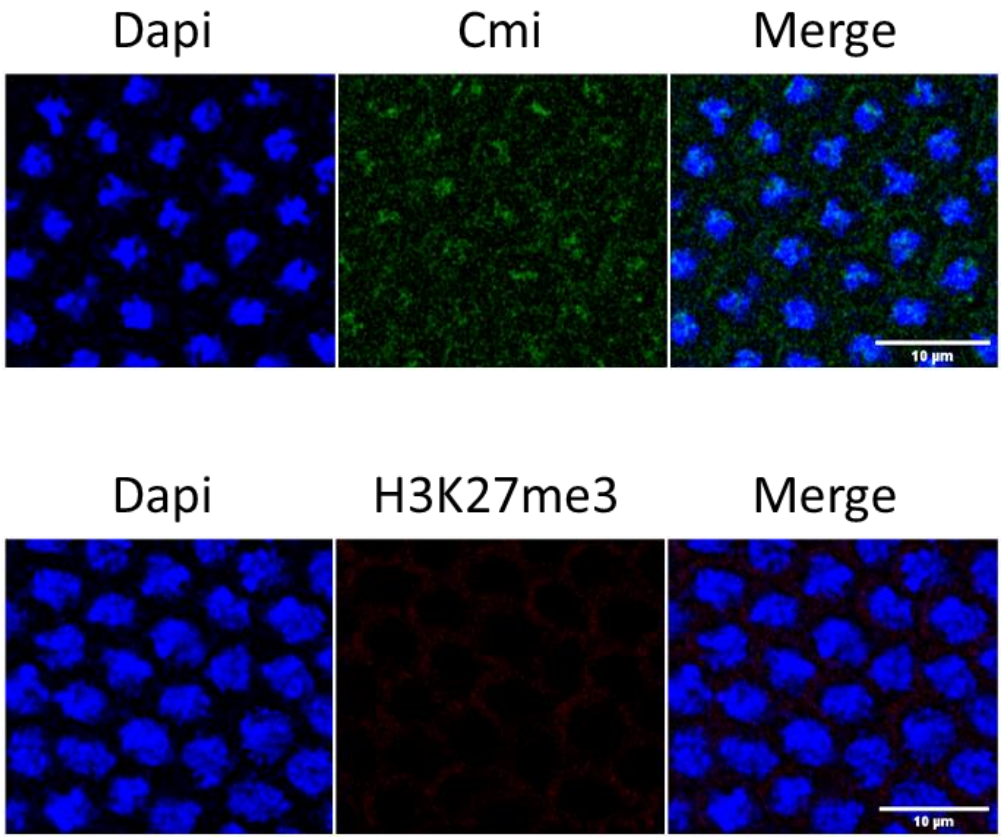


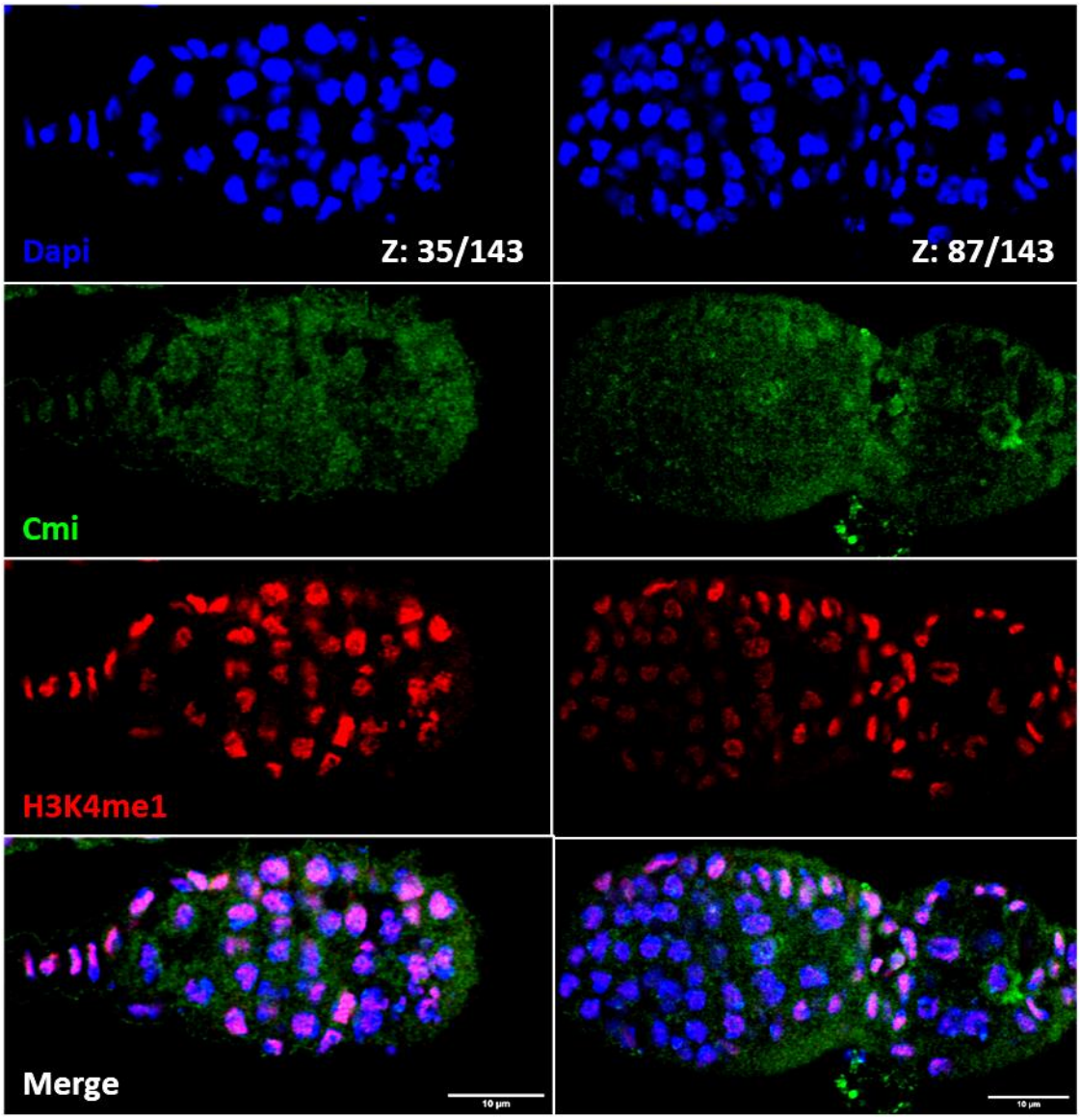
Figure 8. Cmi does not Colocalize to with H3K27me3 in the Early Embryo and Loss of Cmi Leads to a Decrease in H3K27me3 Levels. (A) Native Cmi and H3K4me1 localization prior to major wave of ZGA. Wild-type embryos collected 0-3 hours AEL were stained using polyclonal antibodies to Cmi and H3K27me3 and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. Nuclear cycles (NCs) given for each embryo analyzed. Dapi stain is included to visualize nuclei. **(B)** Cmi and H3K27me3 levels in wild-type embryos staged at NC14. Respective Cmi and H3K4me3 localization visualized in independent Z-planes within a single embryo. **(C)** Cmi and H3K27me3 levels in Cmi knockdown embryos staged at NC14. Respective Cmi and H3K27me3 localization visualized in independent Z-planes within a single embryo. Scale bars indicates 10µm.

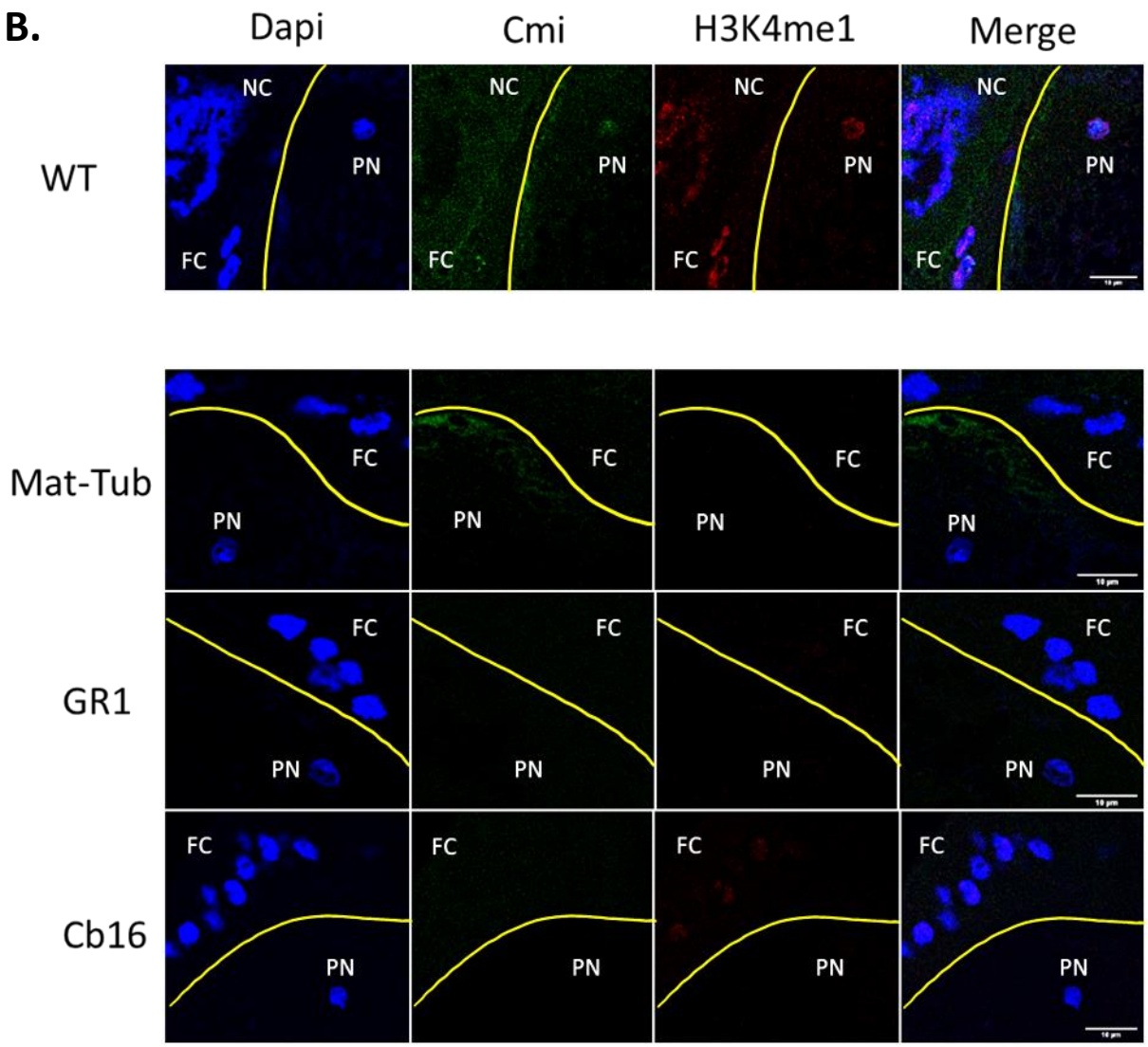
Cmi may Colocalize with H3K4me1 in the Female Germline and Loss of Cmi Leads to Depletion of Germline H3K4me1 Levels

The H3K4me1 mark is a well-characterized mark of active and poised enhancers. This mark has been studied previously in the female germline and was detected by

immunofluorescence in the germarium (Yan et al. 2014) and on the oocyte pronucleus and follicle cells at later stages of oogenesis (Iovino et al. 2013). Because the MLR complex is responsible for deposition of the H3K4me1 mark, we sought to characterize localization of H3K4me1 with Cmi during oogenesis and investigate the effect of Cmi loss on germline H3K4me1. Confocal images of wild-type ovary tissue stained with antibodies targeting Cmi and H3K4me1 confirmed presence of H3K4me1 in the germline and follicle cells of the germarium and may colocalize with Cmi (Figure 9A). We further confirmed presence of H3K4me1 on the follicle cells and oocyte pronucleus at later stages of oogenesis which may co-localize with Cmi (Figure 10B). Upon shRNAi mediated knockdown of *Cmi,* we observed depletion of H3K4me1 on the oocyte pronucleus, follicle cells (Figure 9B) and nurse cells (not shown) for all three drivers used. Fluorescent quantification analysis further showed that Cmi loss leads to a reduction of mean pronuclear H3K4me1 signal compared to wild-type (Figure 9C). However, this difference was not determined to be statistically significant ($p > 0.05$). This suggests that Cmi may be required for deposition and maintenance of the H3K4me1 mark in the developing oocyte and implicates a possible role for Cmi in bookmarking of enhancer regions.

A.

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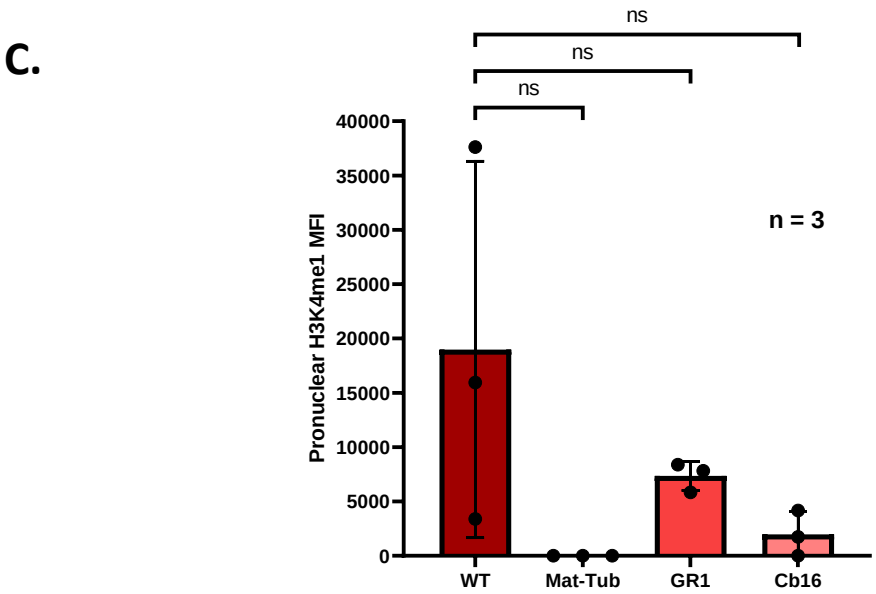
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Pronuclear H3K4me1 MFI
n = 3
WT
Mat-Tub
GR1
Cb16

Figure 9. Cmi may colocalize with H3K4me1 during Oogenesis and Loss of Cmi Leads to Depletion of Germline H3K4me1. (A) Native Cmi and H3K4me1 localization within a single germarium shown in two Z-planes to capture full structure. Wild-type ovaries were dissected and stained using polyclonal antibodies to Cmi and H3K4me1 and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. **(B)** H3K4me1 levels in wild-type (WT) versus shRNAi mediated *Cmi* knockdown egg chambers using three Gal4 drivers: Maternal-tubulin (Mat-Tub), GawB-GR1 (GR1), and GawB-Cb16 (Cb16). Egg chambers at stage 10 were used for analysis. Yellow line indicates boundary between the developing oocyte and the rest of the egg chamber. The oocyte pronucleus (PN) follicle cells (FC), and nurse cells (NC, wild-type only) are indicated. **(C)** Fluorescent quantification of nuclear H3K4me1 signal between wild-type and *Cmi*-knockdown embryos based on analysis of pronuclei from biological replicates for each treatment. Results are displayed as mean fluorescence intensity (MFI) with individual data points and standard error bars shown. An unpaired t-test was used to determine statistical significance between WT and CmiKD treatments, results displayed on graph.

Cmi Localizes to Chromatin Coordinately with the Appearance of H3K4me1

H3K4me1 is a well-characterized mark of active and poised enhancers which is placed by MLR complexes. While Trr is responsible for adding the monomethyl mark to H3K4, our lab shown that Cmi is required for the methyltransferase function of Trr (Zraly et al. 2020). Thus, we investigated co-localization of H3K4me1 with Cmi in during the MZT. Confocal images of embryos stained with antibodies to H3K4me1 and Cmi show that H3K4me1 is absent from the chromatin prior to the major wave of ZGA (Figure 10A) and begins localizing to the nuclei following at onset of zygotic transcription at NC14 (Figure 10B). While Cmi also begins localizing to the nuclei at NC14 we did not see evidence of co-localization between Cmi and H3K4me1. Rather, nuclear localization Cmi and H3K4me1 were visualized in separate Z-planes within the same embryo. This suggests that following deposition of the H3K4me1 mark at enhancers, retention of the MLR complex may be dispensable for subsequent enhancer activation.

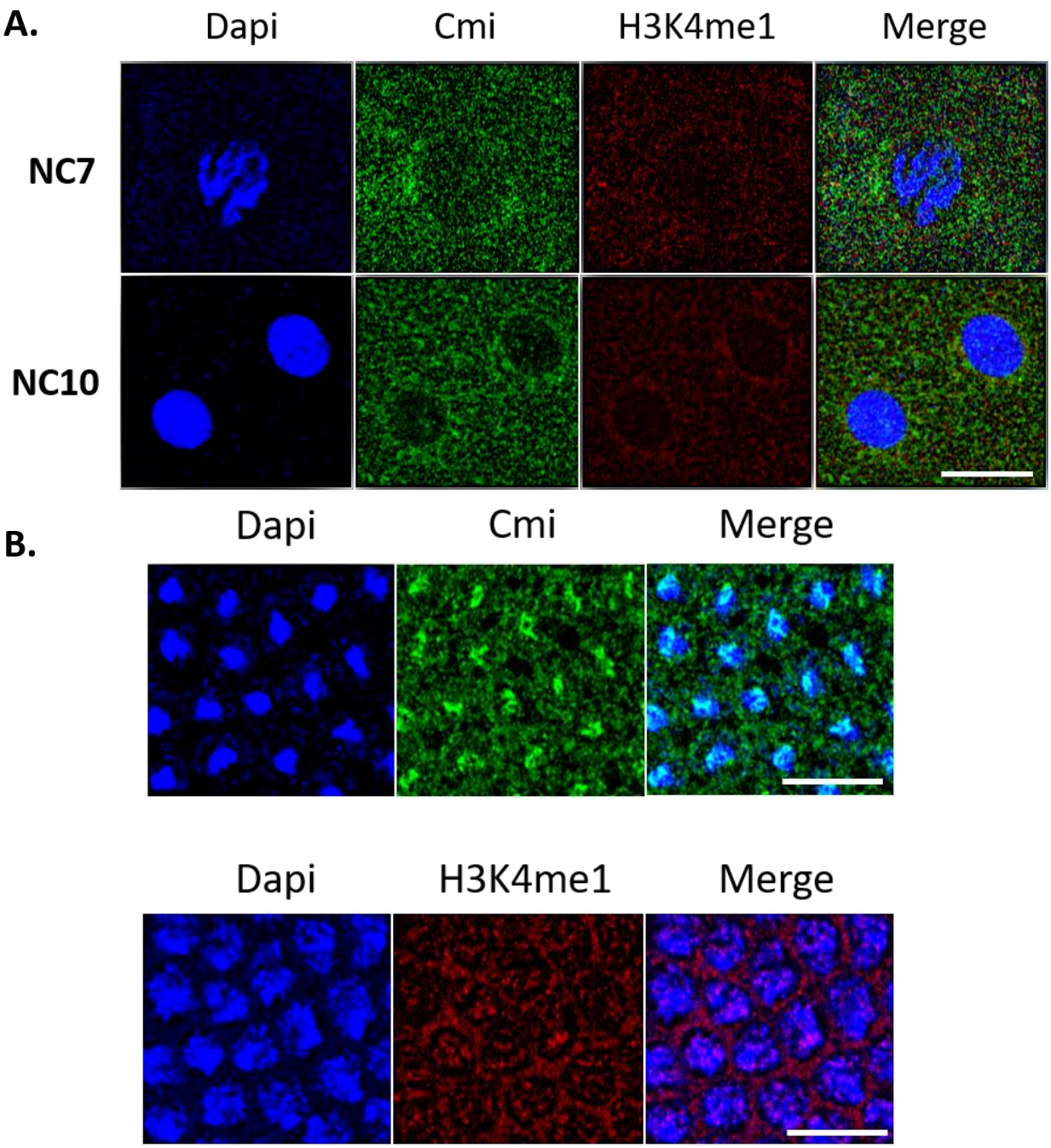
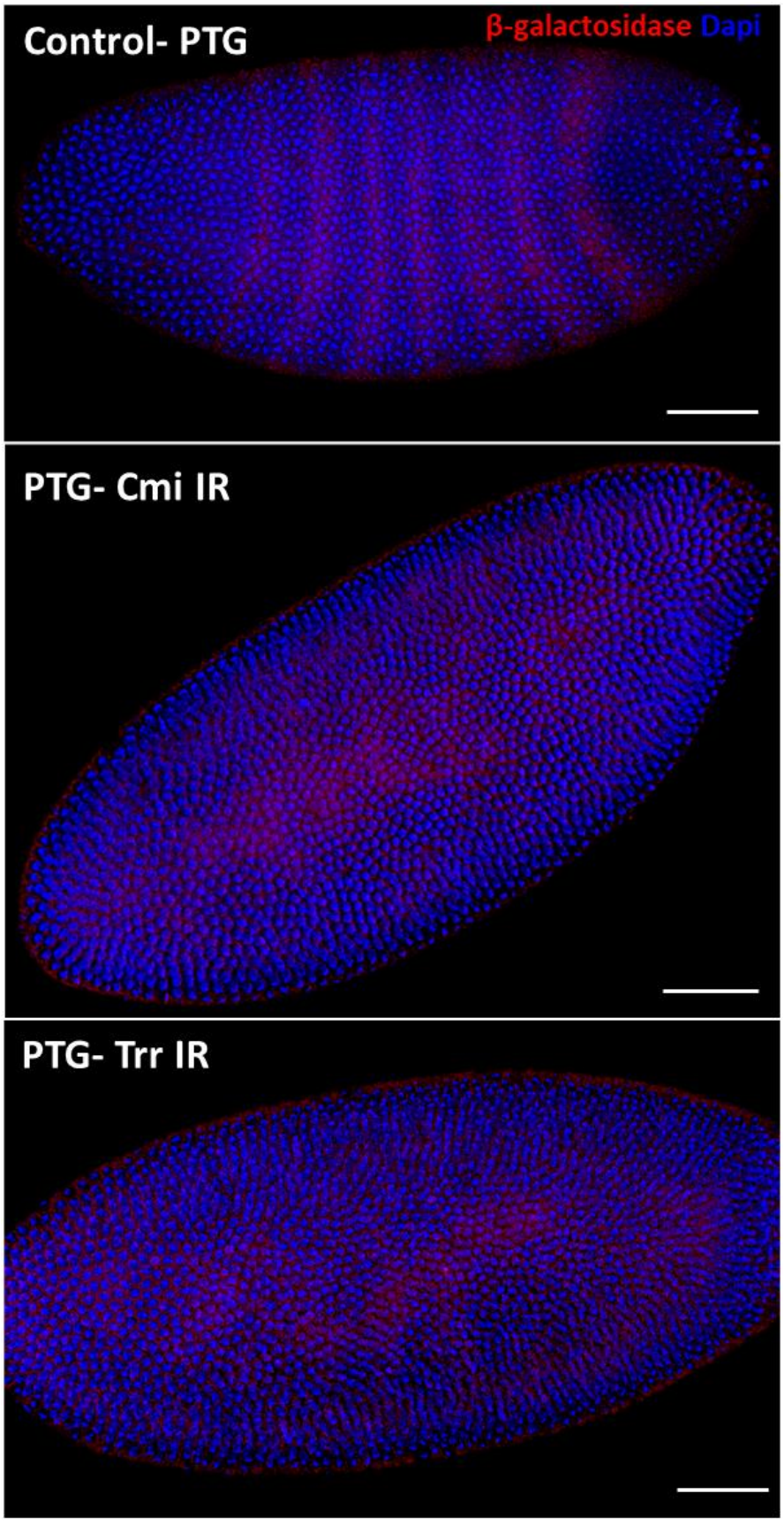


Figure 10. Cmi Localizes to Chromatin Coordinately with the Appearance of H3K4me1. **(A)** Native Cmi and H3K4me1 localization prior to major wave of ZGA. Wild-type embryos collected 0-3 hours AEL were stained using polyclonal antibodies to Cmi and H3K4me1 and analyzed by confocal microscopy using Z-stack imaging under oil at 63x magnification. Nuclear cycles (NCs) given for each embryo analyzed. Dapi stain is included to visualize nuclei. **(B)** Cmi and H3K4me1 levels in wild-type embryos staged at NC14. Respective Cmi and H3K4me1 localization visualized in independent Z-planes within a single embryo. Scale bars indicates 10µm.

Loss of Cmi Leads to Disrupted Transcriptional Activation in the Early Embryo

Unpublished ChIP-Seq data from our lab shows evidence of Cmi binding to *even-skipped (eve)* enhancers in 0-8 hour embryos, but not at later stages of development. This makes *eve* a suitable reporter to assess the requirement of Cmi in zygotic transcriptional activation in early embryos. Preliminary data from our lab showed that knock down of Cmi in embryogenesis resulted in disrupted eve expression as shown by confocal images of knockdown embryos stained with anti-Eve antibody (unpublished, T. Nickels, C. Zraly, A. Dingwall). However, there may be other factors that contribute to proper *eve* expression which could be affected by knockdown of Cmi in the early embryo. Therefore, we sought to specifically target analysis of Cmi function on transcriptional activation using an *eve-LacZ* reporter. To determine the requirement of Cmi in embryonic transcriptional activation we generated recombinant fly lines containing a *Cmi* or *trr inverted-repeat (IR) transgene,* fused to a UAS-Gal4 region, with an *eve-LacZ* reporter gene (Fujioka et al. 2013). Recombinant males were then crossed to virgin females containing a tubulin-Gal4 driver (see Table 2.) and embryos were collected from this cross at 0-3 hours AEL. Embryos were then fixed and stained with antibodies targeting Cmi and β-galactosidase to assess the effects of Cmi loss on *eve* transcription. Confocal images of stained embryos show that loss of Cmi and Trr lead to abnormal *eve* expression that does not resolve into its characteristic seven-stripe pattern (Figure 7A). Cmi and Trr loss was evaluated based on visible depletion of Cmi stain in embryos analyzed (Figure 7B). Together, this suggests that loss of Cmi and Trr lead to disrupted transcriptional activation in early embryogenesis.

A.



B.

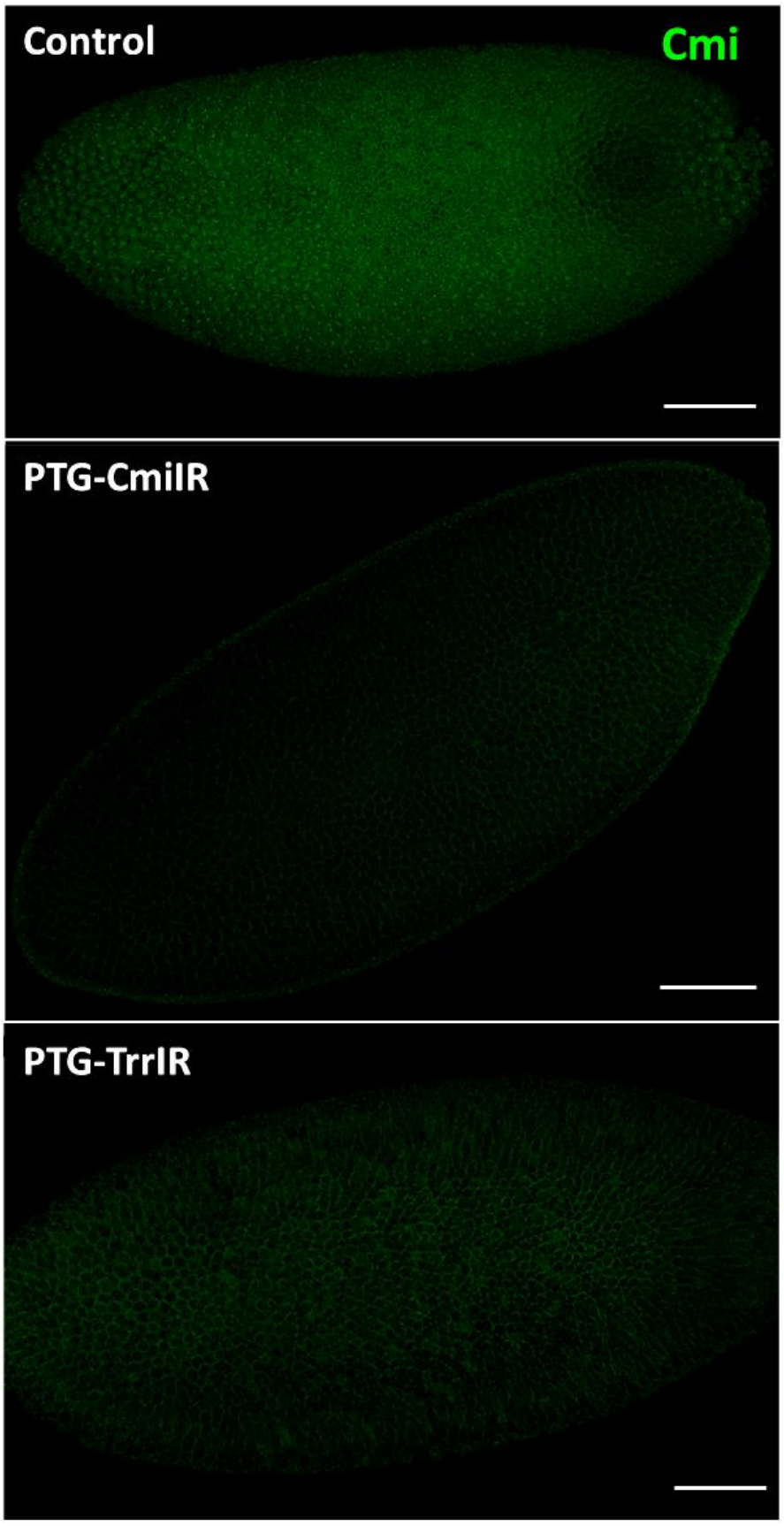


Figure 11. Loss of Cmi Leads to Disrupted *eve* Transcription. Embryos collected 0-3 hours AEL were stained using polyclonal antibodies to Cmi and β-galactosidase and analyzed by confocal microscopy using Z-stack imaging at 20x. **(A)** Parental (PTG) control displayed a "wild-type" *eve* expression pattern while *Cmi* and *trr* knockdown embryos were unable to express *eve* in a segmented pattern. **(B)** Cmi stain used to assess visible depletion of Cmi indicative of knockdown. Embryos are lined up anterior (left) to posterior (right). Scale bars indicates 50μm.

CHAPTER FOUR

DISCUSSION

The genes encoding the mammalian MLR complex methyltransferase subunits are among the most frequently mutated in a wide variety of cancers and developmental disorders. Despite their critical importance as drivers of oncogenesis, much remains to be discovered regarding MLR complex functions in the temporal coordination of early developmental transcriptional events, which may be crucial to uncovering possible mechanisms by which mutations of these genes drive cancer phenotypes.

Our studies have indicated a potential role of the drosophila MLR complex in the preliminary book marking of enhancer regions by deposition and maintenance of histone marks characteristic of active and poised enhancers through ovary development and re-establishment of those marks into embryogenesis. Immunostaining of wild-type ovary tissue confirmed previous unpublished reports that Cmi is abundantly present in the cytoplasm of the developing oocyte and seen in both the cytoplasm and the nucleus of the nurse cells. It may be that maternally deposited Cmi is required in abundance to facilitate efficient transport to the nucleus in response to signaling events in early embryogenesis. This is supported by our results showing that Cmi begins to localize to chromatin coordinately with activation of the zygotic genome. Contrary to previous unpublished reports, we observed evidence of Cmi association with the oocyte pronucleus through later stages of oogenesis. While the native localization pattern of Cmi in the developing ovary may show association with the oocyte pronucleus, that association is not

maintained in the earliest stages of embryogenesis. Rather, Cmi association with chromatin is not re-established until onset of ZGA. This may be due to nuclear levels of Cmi falling below detection limits of immunofluorescence and confocal microscopy prior to onset of ZGA. This could also be the result of major epigenetic reprogramming that occurs following fertilization of the mature oocyte (Iovino 2014), leading to dissociation of the MLR complex from the chromatin.

While unpublished ChIPSeq data by our lab had suggested that Cmi binds to positions associated with binding sites of the pioneer transcription factor, Zelda, we did not observe Cmi association with Zelda until onset of zygotic transcription. Pioneer transcription factors function to convert closed chromatin to a more open state allowing for recruitment of transcriptional machinery. However, additional activating transcription factors may be required to facilitate the recruitment of transcriptional machinery to enhancer and promoter regions (Small and Arnosti 2020). It may be that additional transcription factors, along with Zelda, are required to recruit the MLR complex to enhancer sites in early embryogenesis. Additionally, loss of Cmi did not appear to effect Zelda levels in the early embryo suggesting that Cmi may not be required for Zelda binding in early embryogenesis thus reinforcing Zelda's role in the establishment of areas of active chromatin prior to recruitment of the MLR Complex and onset of ZGA. However, it should be noted that, following onset of zygotic transcription, additional pioneer factors such as GAF (Gaskill et al. 2021) and CLAMP (Duan et al. 2021) are binding to chromatin and promoting transcription of genes expressed later in embryogenesis, suggesting the possibility of Zelda dissociation from the chromatin beginning after onset of zygotic transcription as additional pioneer transcription factors become more active in later stages of embryogenesis. The MLR complex may serve to fine tune gene activation following establishment of open areas of

chromatin by Zelda at onset of ZGA, possibly by re-activating enhancers used at multiple points in development. This is supported by previous work published by our lab showing that the MLR complex may regulate hormone-dependent gene activation/re-activation by priming hormone responsive enhancers via its methyltransferase function (Zraly et al. 2020). Based on our results, we propose a model for MLR complex-facilitated transcriptional activation where initial binding of Zelda, prior to the minor wave of ZGA, facilitates binding of additional transcription factors which, together, recruit the MLR complex to facilitate transcriptional activation at the major wave of ZGA (Figure 12A-C).

H3K27ac is a well-characterized mark associated with active enhancers when present with H3K4me1. Contrary to previously published ChIP-seq data reporting that H3K27ac is established de novo later in embryogenesis (Zenk et al. 2017), we observed presence of H2K27ac as early as NC4 in wild-type embryos which became more prevalent through later stages of embryogenesis. This increase in H3K27ac later into embryogenesis could be facilitated, in part, through recruitment of histone acetyltransferases (HATs) by Zelda, as Zelda has previously been speculated to directly recruit HATs due to the enrichment of H3K27ac at Zelda binding sites (Moshe and Kaplan, 2017). Because we also confirmed presence of H3K27ac on the oocyte pronucleus into later stages of oogenesis, we propose that H3K27ac is a maternally deposited mark. We further observed that upon knockdown of *Cmi* there was a reduction in H3K27ac on the oocyte pronucleus and in the early embryo at onset of ZGA. Our lab has similarly observed a decrease in H3K27ac by immunofluorescence in larval salivary glands following knockdown of *Cmi* and implicated Cmi in the recruitment of histone acetyltransferases (HATs) that catalyze the addition of the H3K27ac mark (Zraly et al. 2020). It may be that the MLR complex contributes to the bookmarking of enhancer regions in oogenesis and subsequent

enhancer activation in embryogenesis through the recruitment of HATs which catalyze the deposition of H3K27ac. Upon knockdown of *Cmi* in embryogenesis we observed a reduction in H3K27ac at which could be due to a decrease in HAT recruitment and lead to disrupted enhancer activation. To note, we observed visible retention of H3K27ac on the nuclei in *Cmi* knockdown embryos, particularly in regions that retained Cmi, further supporting a model in which the MLR complex recruits HATs at onset of zygotic transcription through Cmi (Figure 12C).

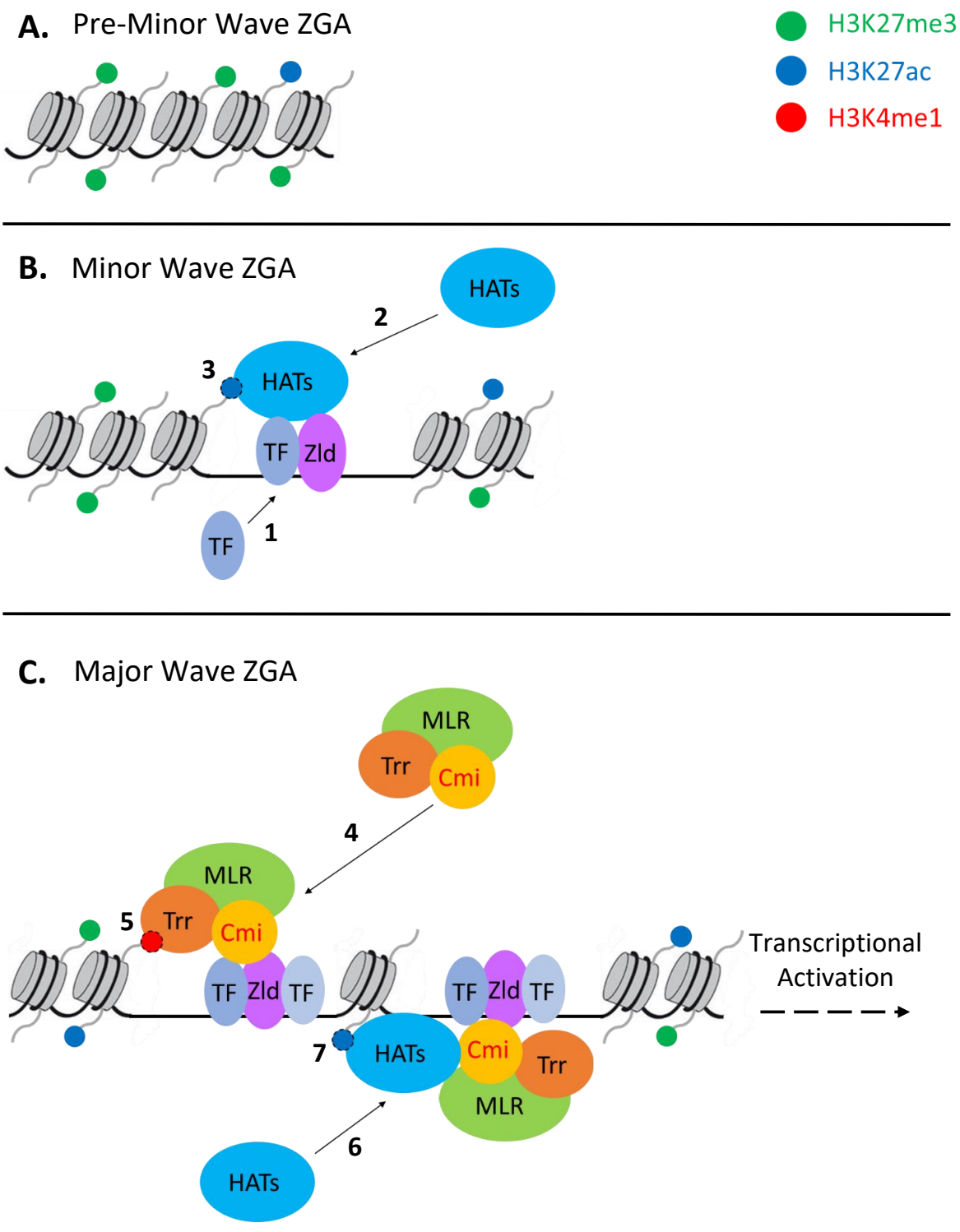

A. Pre-Minor Wave ZGA
H3K27me3
H3K27ac
H3K4me1
B. Minor Wave ZGA
HATs
2
3
HATs
TF
Zld
TF
1
C. Major Wave ZGA
MLR
Trr
Cmi
4
MLR
5
Trr
Cmi
TF
Zld
TF
TF
Zld
TF
Transcriptional
Activation
7
HATs
Cmi
Trr
MLR
6
HATs

Figure 12. Proposed Model for MLR Complex Mediated Transcriptional Activation. (A) Prior to the minor wave of zygotic genome activation (ZGA) the chromatin is in a closed/inactive state as characterized by widespread repressive H3K27me3 and minimal H3K27ac **(B)** By the minor wave of ZGA, the pioneer transcription factor, Zelda (Zld), is establishing areas of open chromatin and may recruit additional transcription factors (TFs) **(1)** as well as histone acetyltransferases (HATs) **(2)** to catalyze the further addition of H3K27ac **(3) (C)** By the major wave of ZGA, Zelda widely establishes areas of open chromatin at enhancer/promoter sites and is further recruiting activating transcription factors which, together, recruit the MLR complex through Cmi mediated chromatin binding **(4)** and facilitate the addition of H3K4me1 at enhancer sites by Trr **(5)**. Chromatin-bound MLR complex may also facilitate recruitment of histone acetyltransferases (HATs) **(6)** to further catalyze the addition of H3K27ac at enhancer sites **(7)**. Together, MLR complex-mediated methylation and acetylation at enhancer sites contribute to transcriptional activation in early development. Adapted from (Panzeri et al. 2016) and (Moshe and Kaplan, 2017)

H3K27me3 is a well characterized mark associated with transcriptional repression. Our results show that H3K27me3 is maintained on the oocyte pronucleus in wild-type ovaries and propagated through early embryogenesis and the major wave of ZGA, confirming previously published results characterizing presence of H3K27me3 in drosophila development (Zenk et al. 2017). Interestingly, we observed that upon knockdown of *Cmi* in the female germline, H3K27me3 is significantly reduced on the oocyte pronucleus and in embryogenesis following onset of zygotic transcription. This was unexpected as Cmi has been implicated in the recruitment of UTX, a subunit of the drosophila MLR complex that catalyzes the removal the repressive H3K27me3 mark (Rickels et al. 2020). Moreover, a previous study investigating H3K27me3 levels in early drosophila development found that the H3K27me3 mark was retained on the oocyte pronucleus following knockdown of E(z), the enzyme responsible for deposition of the H3K27me3 mark, in stage 10 egg chambers (Zenk et al. 2017). It may be that that loss of the MLR complex function in the female germline effects factors that contribute to the deposition and/or maintenance of pronuclear and embryonic H3K27me3. The same study found that loss of H3K27me3 prior to onset of zygotic transcription results in embryonic lethality. Notably, we

were unable to assess the effects of *Cmi* knockdown prior to onset of zygotic transcription. Previous attempts by our lab in generation of *Cmi*-null germline clones were unsuccessful, as Cmi was found to be essential for oogenesis and females were unable to produce embryos (unpublished, Nickels, Zraly and Dingwall). However, based on our results, it may be that the MLR complex plays a role in the stabilization of the H3K27me3 mark throughout early development and that loss of H3K27me3 following loss of Cmi could lead to aberrant enhancer activation into embryogenesis due to loss of transcriptional repression.

H3K4me1 is a well-characterized mark of active and poised enhancers places by MLR complexes. Similar to Cmi, we observed that wild-type pronuclear association of H3K4me1 is not maintained into the earliest stages of embryogenesis and is not re-established until after onset of zygotic transcription when Cmi association with chromatin is also re-established. While fluctuations in H3K4me1 levels could also be due to detection limitations, this suggests that while the MLR complex may serve to preliminarily bookmark enhancers during oogenesis through its methyltransferase function, the MLR complex may be required for maintenance of the H3K4me1 mark during oogenesis and its re-establishment at enhancer regions for subsequent enhancer activation following onset of zygotic transcription. This is further supported by our observation that H3K4me1 is depleted in all ovarian cells analyzed following knockdown of *Cmi*.

While the presence of H3K4me1 in the female germline has been observed previously (Iovino et al. 2013) (Yan et al. 2014), its function in the germline is unclear. Moreover, the necessity of H3K4me1 for activation of enhancers in early development remains poorly understood. Interestingly, a previous study showed that catalytic-inactivating mutations in the MLR complex do not substantially affect enhancer activation (Rickels et al. 2017), suggesting

monomethylation of H3K4 facilitated by the MLR complex at enhancer sites may be dispensable for subsequent activation. Additionally, a more recent study alluded to a potential role for the H3K4me1 mark in partial maintenance of chromatin accessibility and establishing developmentally specific gene expression programs necessary for in vitro germline competence while also confirming previous studies pointing to the dispensability of the H3K4me1 mark for enhancer activation (Bleckwehl et al. 2021). While our results indicate a requirement for the MLR complex in the deposition of the H3K4me1 mark, they do not indicate a causal role for the H3K4me1 mark for enhancer activation. To note, the H3K4me1 mark alone is not indicative of enhancer activation, its presence along with H3K27ac has been shown to be characteristic of active enhancers. It may be that that deposition of H3K4me1 by the MLR complex in the germline may serve to maintain some areas of open chromatin allowing for additional factors to bind and facilitate proper germline differentiation. While we did not observe maintenance of H3K4me1 on the nuclei into embryogenesis, it may be that similar to Cmi, the decrease could be the result of major epigenetic reprogramming following fertilization (Iovino 2014). Overall, our results point to a necessity for the MLR complex in the deposition of H3K4me1 throughout early development which may contribute to its role in regulating enhancer function.

Overall, our results (summarized in Table 3.) indicate a requirement for the MLR complex in the maintenance and stability of enhancer-associated histone marks in early drosophila development, suggesting a broader mechanism by which the MLR complex regulates transcription in addition to its methyltransferase function.

Table 3. Presence of Histone Marks by Developmental Stage. Comparison of nuclear histone mark levels between wild-type (WT) and *Cmi* knockdown (*Cmi*KD) treatments in ovaries and embryos.

	Ovaries (Ooctye Pronucleus)		Embryos (MZT and ZGA)			
	WT	*Cmi*KD	WT Pre-Minor Wave	WT Minor Wave	WT Major Wave	*CmiKD* Major Wave
H3K27me3	✓	↓	✓	✓	✓	↓
H3K27ac	✓	↓	✓	✓	✓	↓
H3K4me1	✓	↓	X	X	✓	**NA[1]**

[1]H3K4me1 levels not evaluated in *Cmi*KD embryos

Importantly, *Cmi* knockdown phenotype was characterized by a decrease in the production late-stage egg chambers following ovary-specific knockdown. To note, knockdown animals are still able to produce functional protein at a lower concentration, and knockdown efficiency may vary across different cell types within the ovary. *Cmi* knockdown in early embryogenesis was characterized by depletion of cytosolic Cmi and some depletion of nuclear Cmi. We showed that Cmi begins localizing to chromatin gradually as early as NC12 before widespread transcriptional activation occurs. Because the *CmiIR* construct used to knockdown *Cmi* in the early embryo is not expressed until onset of zygotic transcription at NC14, it may be that any nuclear bound Cmi is stabilized in that state prior to expression of the *CmiIR* construct and is more resistant to depletion resulting in some retention of nuclear localized Cmi that can be detected by immunofluorescence and confocal microscopy.

To directly address the role of the MLR complex in embryonic transcriptional activation we generated recombinant fly lines containing our *CmiIR* and *trrIR* transgene constructs, respectively, with and an *eve-LacZ* reporter transgene (Fujioka et al. 2013) to assess the effect of Cmi loss on *eve* transcription. We used the *eve* gene as a target to assess the effect Cmi/Trr loss on transcriptional activation as expression of the *eve* gene is controlled by five enhancer elements which are some of the first to be activated at onset of zygotic transcription (Borok et al. 2010). We observed abnormal *eve* expression pattern following knockdown of *Cmi* and *trr*, reinforcing previous unpublished data by our lab showing abnormal expression patterns of even-skipped protein following knockdown of *Cmi* and *trr* (unpublished, Nickels, Zraly, and Dingwall). Together, this suggests that loss of Cmi and Trr result in disrupted *eve* transcription that may be due to its role in activation of *eve* enhancers. However, a previous study showed that *trr*-null animals can be rescued by a catalytically deficient *trr-transgene* and that the monomethyltranserase function of the MLR complex at enhancer regions is dispensable for animal viability and proper gene expression (Rickels et al. 2016). It may be that loss of Cmi and Trr disrupt *eve* transcription by a mechanism independent of its function to monomethylate at enhancer sites. While our results do not establish a definitive link between the role of the MLR complex in transcriptional activation with its methyltransferase function, our results implicate the requirement of the MLR complex in the epigenetic marking of chromosomes during oogenesis and proper transcriptional activation in early animal development.

REFERENCE LIST

Berrocal A, Lammers NC, Garcia HG, Eisen MB. Kinetic sculpting of the seven stripes of the Drosophila *even-skipped* gene. *Elife*. 2020;9:e61635. Published 2020 Dec 10. doi:10.7554/eLife.61635

Bleckwehl, T., Crispatzu, G., Schaaf, K., Respuela, P., Bartusel, M., Benson, L., Clark, S. J., Dorighi, K. M., Barral, A., Laugsch, M., van IJcken, W. F., Manzanares, M., Wysocka, J., Reik, W., & Rada-Iglesias, Á. (2021). Enhancer-associated H3K4 methylation safeguards in vitro germline competence. *Nature Communications*, *12*(1). https://doi.org/10.1038/s41467-021-26065-6

Borok MJ, Tran DA, Ho MC, Drewell RA. Dissecting the regulatory switches of development: lessons from enhancer evolution in Drosophila. *Development*. 2010;137(1):5-13. doi:10.1242/dev.036160

Bothma JP, Garcia HG, Esposito E, Schlissel G, Gregor T, Levine M. Dynamic Regulation of eve stripe 2 expression reveals transcriptional bursts in living drosophila embryos. Proceedings of the National Academy of Sciences. 2014;111(29):10598-10603. doi:10.1073/pnas.1410022111

Cenik BK, Shilatifard A. COMPASS and SWI/SNF complexes in development and disease. *Nature Reviews Genetics*. 2021;22(1):38-58. https://www.nature.com/articles/s41576-020-0278-0. Accessed May 19, 2021. doi: 10.1038/s41576-020-0278-0.

Chauhan C, Zraly CB, Parilla M, Diaz MO, Dingwall AK. Histone recognition and nuclear receptor co-activator functions of drosophila cara mitad, a homolog of the N-terminal portion of mammalian MLL2 and MLL3. *Development*. 2012;139(11):1997-2008. https://doi.org/10.1242/dev.076687. Accessed May 19, 2021. doi: 10.1242/dev.076687.

Duan J, Rieder L, Colonnetta MM, et al. CLAMP and Zelda function together to promote *Drosophila* zygotic genome activation. *Elife*. 2021;10:e69937. Published 2021 Aug 3. doi:10.7554/eLife.69937

Fagan RJ, Dingwall AK. COMPASS ascending: Emerging clues regarding the roles of MLL3/KMT2C and MLL2/KMT2D proteins in cancer. *Cancer letters*. 2019;458:56-65. http://dx.doi.org/10.1016/j.canlet.2019.05.024. doi: 10.1016/j.canlet.2019.05.024.

Farrell JA, O'Farrell PH. From egg to gastrula: how the cell cycle is remodeled during the Drosophila mid-blastula transition. *Annu Rev Genet*. 2014;48:269-294. doi:10.1146/annurev-genet-111212-133531

Gaskill MM, Gibson TJ, Larson ED, Harrison MM. GAF is essential for zygotic genome activation and chromatin accessibility in the early *Drosophila* embryo. *Elife*. 2021;10:e66668. Published 2021 Mar 15. doi:10.7554/eLife.66668

Gilbert SF. Developmental Biology. 6th edition. Sunderland (MA): Sinauer Associates; 2000. Early Drosophila Development. Available from: https://www.ncbi.nlm.nih.gov/books/NBK10081/

Harrison MM, Li XY, Kaplan T, Botchan MR, Eisen MB. Zelda binding in the early drosophila melanogaster embryo marks regions subsequently activated at the maternal-to-zygotic transition. *PLoS Genet*. 2011;7(10):e1002266. doi: 10.1371/journal.pgen.1002266 [doi].

Hoppe C, Ashe HL. Live imaging and quantitation of nascent transcription using the MS2/MCP system in the drosophila embryo. *STAR Protoc*. 2021;2(1):100379. doi: 10.1016/j.xpro.2021.100379 [doi].

Hudson, A. M., & Cooley, L. (2014). Methods for studying oogenesis. Methods, 68(1), 207–217. https://doi.org/10.1016/j.ymeth.2014.01.005

Iovino, N., Ciabrelli, F., & Cavalli, G. (2013). PRC2 controls drosophila oocyte cell fate by repressing cell cycle genes. Developmental Cell, 26(4), 431–439. https://doi.org/10.1016/j.devcel.2013.06.021

Iovino, N. (2014). Drosophila epigenome reorganization during oocyte differentiation and early embryogenesis. *Briefings in Functional Genomics*, *13*(3), 246–253. https://doi.org/10.1093/bfgp/elu007

Lebo DPV, McCall K. Murder on the Ovarian Express: A Tale of Non-Autonomous Cell Death in the *Drosophila* Ovary. *Cells*. 2021;10(6):1454. Published 2021 Jun 10. doi:10.3390/cells10061454

Lee JE, Wang C, Xu S, et al. H3K4 mono- and di-methyltransferase MLL4 is required for enhancer activation during cell differentiation. *Elife*. 2013;2:e01503. Published 2013 Dec 24. doi:10.7554/eLife.01503

Lee MT, Bonneau AR, Giraldez AJ. Zygotic genome activation during the maternal-to-zygotic transition. *Annu Rev Cell Dev Biol*. 2014;30:581-613. doi:10.1146/annurev-cellbio-100913-013027

Li, X.-Y., Harrison, M. M., Villalta, J. E., Kaplan, T., & Eisen, M. B. (2014). Establishment of regions of genomic activity during the drosophila maternal to zygotic transition. ELife, 3. https://doi.org/10.7554/elife.03737

Moshe, A., & Kaplan, T. (2017). Genome-wide search for zelda-like chromatin signatures identifies GAF as a pioneer factor in early fly development. *Epigenetics & Chromatin*, *10*(1). https://doi.org/10.1186/s13072-017-0141-5

Panzeri, I., Rossetti, G., & Pagani, M. (2016). Basic principles of noncoding RNAS in epigenetics. *Medical Epigenetics*, 47–63. https://doi.org/10.1016/b978-0-12-803239-8.00004-1

Rickels R, Wang L, Iwanaszko M, et al. A small UTX stabilization domain of Trr is conserved within mammalian MLL3-4/COMPASS and is sufficient to rescue loss of viability in null animals. *Genes Dev*. 2020;34(21-22):1493-1502. doi:10.1101/gad.339762.120

Robinson DN, Cooley L. Genetic analysis of the actin cytoskeleton in the drosophila ovary. *Annu Rev Cell Dev Biol*. 1997;13:147-170. doi: 10.1146/annurev.cellbio.13.1.147 [doi].

Schulz KN, Harrison MM. Mechanisms regulating zygotic genome activation. *Nature Reviews Genetics*. 2019;20(4):221-234. https://www.nature.com/articles/s41576-018-0087-x. Accessed May 19, 2021. doi: 10.1038/s41576-018-0087-x.

Small S, Arnosti DN. Transcriptional enhancers in drosophila. *Genetics*. 2020;216(1):1-26. doi: 10.1534/genetics.120.301370 [doi].

Sze CC, Shilatifard A. MLL3/MLL4/COMPASS family on epigenetic regulation of enhancer function and cancer. *Cold Spring Harb Perspect Med*. 2016;6(11):10.1101/cshperspect.a026427. doi: a026427 [pii].

Wang C, Lee JE, Lai B, et al. Enhancer priming by H3K4 methyltransferase MLL4 controls cell fate transition. *Proc Natl Acad Sci U S A*. 2016;113(42):11871-11876. doi:10.1073/pnas.1606857113

Wang L, Zhao Z, Ozark PA, et al. Resetting the epigenetic balance of Polycomb and COMPASS function at enhancers for cancer therapy. *Nat Med*. 2018;24(6):758-769. doi:10.1038/s41591-018-0034-6

Yan, D., Neumüller, R. A., Buckner, M., Ayers, K., Li, H., Hu, Y., Yang-Zhou, D., Pan, L., Wang, X., Kelley, C., Vinayagam, A., Binari, R., Randklev, S., Perkins, L. A., Xie, T., Cooley, L., & Perrimon, N. (2014). A regulatory network of drosophila germline stem cell self-renewal. *Developmental Cell*, *28*(4), 459–473. https://doi.org/10.1016/j.devcel.2014.01.020

Zenk F, Loeser E, Schiavo R, Kilpert F, Bogdanović O, Iovino N. Germ line-inherited H3K27me3 restricts enhancer function during maternal-to-zygotic transition. *Science*. 2017;357(6347):212-216. doi:10.1126/science.aam5339

Zraly CB, Zakkar A, Perez JH, et al. The Drosophila MLR COMPASS complex is essential for programming cis-regulatory information and maintaining epigenetic memory during development. *Nucleic Acids Res*. 2020;48(7):3476-3495. doi:10.1093/nar/gkaa082

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