2011

Roles of Cyclin A and Cyclin B in Drosophila Female Meiosis

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ROLES OF CYCLIN A AND CYCLIN B IN DROSOPHILA FEMALE MEIOSIS

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A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2011

ROLES OF CYCLIN A AND CYCLIN B IN DROSOPHILA FEMALE MEIOSIS

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ABSTRACT

Cyclin Dependent Kinase 1 (CDK1) is a key mitotic regulator that associates with Cyclin proteins to form active Cyclin-CDK1 complexes. The mitotic roles of Cyclin-CDK1 complexes are well understood, but the regulation and function of the meiotic Cyclin-CDK1 complexes remain largely unsolved. This research project explored the roles of Cyclin A-CDK1 and Cyclin B-CDK1 in Drosophila female meiosis. This study found that constitutive activation of Cyclin A-CDK1 causes defects such as chromosome missegregation (meiosis I and II) and abnormal spindle assembly (meiosis II), implying that Cyclin A must be degraded before anaphase I and II. This study also found that Cyclin B-CDK1 is required for maintenance of the metaphase I arrest, proper chromosome segregation (meiosis I and II), proper spindle assembly (meiosis II), repression of DNA replication, and completion of meiosis. The findings of this study also suggest that Cyclin B -CDK1 may promote Cyclin A degradation during meiosis.

DEDICATION

To all those who helped me finish this degree.

ACKNOWLEDGEMENTS

I would like to thank Dr. Swan for giving me the opportunity to work in his lab; his patience and tolerance were greatly appreciated. I would also like to thank my lab mates for their help and support, especially Osama Batiha and Biju Vasavan for teaching me the techniques to carry out this project. I would also like to extend a special thanks to Nila Das for all the useful discussions. I would like to thank Mohammad Haj Dezfulian from the Crosby Lab for help with qRT-PCRs, cloning, and countless other things; I would not have gotten far without his help. A special thanks to Espanta Jalili from the Porter Lab for help with qRT-PCRs and protein quantification. I would also like to thank my family for helping me get through this degree – especially my sister Savita Dhaliwal for driving me back and forth from the lab whenever I asked her to (early in the morning and late at night). A very important thanks to Giani Thakur Singh Ji, without whose advice I would never have finished this degree. And lastly, and perhaps most importantly, I would like to thank Vaheguroo Ji for keeping me alive and sane (I hope that I can make up for the thousands and thousands of flies and embryos that I have killed during the past two years).

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LIST OF ABBREVIATIONS

APC/C	Anaphase Promoting Complex/Cyclosome
BGCN	Benign Gonial Cell Neoplasm
BP	Base Pair
cDNA	Complementary DNA
C_T	Threshold Cycle
DGRC	Drosophila Genomics Resource Centre
DNA	Deoxyribonucleic Acid
DSIR	Designer of Small Interfering RNA
FISH	Fluorescent In-situ Hybridization
GSC	Germline Stem Cell
Hsp70	Heat-shock Protein 70
MTOC	Microtubule Organizing Centre
NEBD	Nuclear Envelope Breakdown
pBS	pBlueScript
PCR	Polymerase Chain Reaction

PGC	Primordial Germ Cell
qRT PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNAi	Ribonucleic acid interference
RPM	Rotations Per Minute
RQ	Relative Quantification
ShRNA	Short Hairpin RNA
T_M	Melting Temperature
UAS	Upstream activating sequence
wt	Wildtype
yw	*yellow white* (wildtype *Drosophila*)

1. INTRODUCTION

1.1 *Drosophila melanogaster* as a model organism

Drosophila melanogaster, the fruit fly, has been used as a model organism for over 100 years. This small insect has a relatively simple diet, short generation time, and produces a large amount of progeny; these qualities have proven advantageous in propagating large numbers of fruit flies under controlled conditions. Furthermore, 120 megabases (Mb) of the 180 Mb Drosophila genome have been fully sequenced, allowing for manipulations at the DNA-level (Adams *et al.*, 2000). The Drosophila genome, which is spread over four chromosomes, is very easy to manipulate compared to other multi-cellular organisms, making this insect readily available for approaches involving transgenesis and reverse genetics (Venken & Bellen, 2005). Approximately 13,600 genes are encoded by the Drosophila genome and many of these share homologues with humans; therefore, certain findings in Drosophila can also be extended to humans (Adams *et al.*, 2000). In this study, Drosophila was used to study mitotic Cyclin function in meiosis. Both humans and Drosophila encode Cyclin homologues that function during meiosis. Furthermore, the Drosophila ovary provides a simple, multi-cellular environment to study the basic regulation of meiosis. Therefore, the findings in Drosophila can help understand meiotic regulation in humans; they may also shed light into the processes that cause infertility or miscarriages, opening the possibility for therapeutic development.

1.2 Mitosis

The eukaryotic cell cycle involves four phases: Gap 1 (G1), Synthesis (S), Gap 2

(G2), and Mitosis (M). During the first three phases of the cell cycle, collectively referred to as interphase, the cell replicates its DNA and prepares to divide. Entry into mitosis and mitosis itself are regulated by heterodimeric protein complexes composed of Cyclin proteins and Cyclin-Dependent Kinase I (CDK1); CDK1 is a mitosis-specific CDK. Together these Cyclin-CDK1 complexes phosphorylate substrates that regulate specific mitotic events, such as chromosome condensation, nuclear envelope breakdown, and spindle assembly (Nigg, 2001). These processes ensure that the replicated DNA is distributed equally among the two daughter cells. Interestingly, exit from mitosis only requires two events in early Drosophila embryos: Cohesin cleavage to allow sister chromatid separation and inactivation of CDK1, implying that Cyclin-CDK1 complexes are the main regulators of mitosis (Oliveria *et al.*, 2010).

1.2.1 Mitotic Cyclin Structure

The *Drosophila melanogaster* genome encodes three mitotic Cyclins: Cyclin A, Cyclin B, and Cyclin B3. Homologues of Cyclin B are found in budding yeast, fission yeast, plants, invertebrates and vertebrates; some organisms contain multiple *Cyclin B* genes. *Cyclin A*, on the other hand, has homologues in most organisms, but not in budding yeast; certain organisms, such as mammals, have two *Cyclin A* genes (Jacobs *et al.*, 1998). *Cyclin B3* homologues are present in many organisms, such as mammals, chicken, and leech, but have not been identified in plants and yeast (Jacobs *et al.*, 1998; Nguyen et al., 2002). Evolutionary conservation of these three types of Cyclins across different phyla implies that each Cyclin must have a specialized role (discussed in detail below).

Historically, Cyclins were first discovered in sea urchin eggs and clam oocytes; it

was noted that upon fertilization or egg activation, certain proteins underwent oscillations in their abundance (Evans *et al.*, 1983). Due to their cyclic synthesis and degradation, these proteins were termed Cyclins. Structurally, all Cyclins share a region of approximately 200 amino acids that contains two Cyclin Boxes (Nugent *et al.*, 1991) (Figure 1). The N-terminal Cyclin Box shows conservation among Cyclins, whereas the C-terminal Cyclin Box is more divergent (Petri *et al.*, 2007). Each Cyclin Box folds into five α-helices , which together form the Cyclin Fold; this Cyclin Fold interacts with CDK1 (Jeffrey *et al.*, 1995). Cyclin binding is a pre-requisite for CDK1 activation (discussed below). Together these Cyclin-CDK1 complexes phosphorylate specific proteins involved in mitotic regulation. Cyclins are also thought to target CDK1 to specific regions within the cell (Miller and Cross, 2001). There is also evidence that the poorly conserved C-terminal region of Cyclins is involved in substrate recognition. A recent study found that the C-terminal region of mammalian Cyclin A interacts with the replication licensing factor Mcm7. This interaction is required for Mcm7 phosphorylation by Cyclin A-CDK2, which, in turn, promotes S-phase entry (Chibazakura *et al.*, 2011). Therefore, it is highly likely that Cyclins provide substrate specificity during mitosis as well.

Inactivation of CDK1 requires Cyclin degradation. Mitotic Cyclins are targeted for degradation by the Anaphase Promoting Complex/Cyclosome (APC/C), which is a multi-subunit ubiquitin ligase that targets specific proteins to the 26S proteasome. Activation of APC/C requires association with either Cdc20 (Fizzy in Drosophila) or Cdh1 (Fizzy-related in Drosophila), both of which are WD40 repeat proteins (Nigg, 2001). $APC/C^{Cdc20/Fzy}$ is activated during the metaphase-anaphase transition, when all

chromosomes are properly aligned at the metaphase plate. APC/$C^{Cdc20/Fzy}$ promotes Securin and Cyclin proteolysis; degradation of Securin releases Separase, which cleaves the Cohesin complexes holding the two sister chromatids together. Degradation of Cyclins is required for mitotic exit. The other APC/C complex, APC/$C^{Cdh1/Fzr}$, is activated in late mitosis and G1; this complex ubiquitinates Cyclins, also promoting mitotic exit (Nigg, 2001).

APC/$C^{Cdc20/Fzy}$- and APC/$C^{Cdh1/Fzr}$-mediated ubiquitination requires the presence of a Destruction-box (D-box), which is a 9 amino acid-long region with the consensus sequence RXXLXXXN. APC/$C^{Cdh1/Fzr}$ can also recognize and ubiquitinate protein substrates containing a KEN box (Pfleger and Kirchner, 2000). All three Drosophila mitotic Cyclins possess at least one D-box (**Figure 1**). Cyclins A and B also possess at least one KEN box (**Figure 1**). However, in the case of Cyclin A, each of the destruction motifs (D-box or KEN box), on their own, are not sufficient to promote degradation. It seems that the N-terminal D-box and KEN box are both required for proper degradation of Cyclin A; therefore, stabilization of Drosophila Cyclin A requires at least 53 amino acids to be deleted from the N-terminus (Jacobs *et al.*, 2001; Kaspar *et al.*, 2001; Parry and O'Farrell, 2001). Cyclins B and B3, on the other hand, can be stabilized by mutating the three conserved amino acids (R, L, N) in the D-box (Jacobs *et al.*, 1998; Raff *et al.*, 2002).

1.2.2 CDK1 Structure and Function

CDK1 is a serine/threonine kinase that is structurally composed of two lobes. The N-terminal lobe contains an alpha-helix called the PSTAIRE helix, whereas the C-terminal lobe contains the activation loop or T-loop. The active site, which is found in a

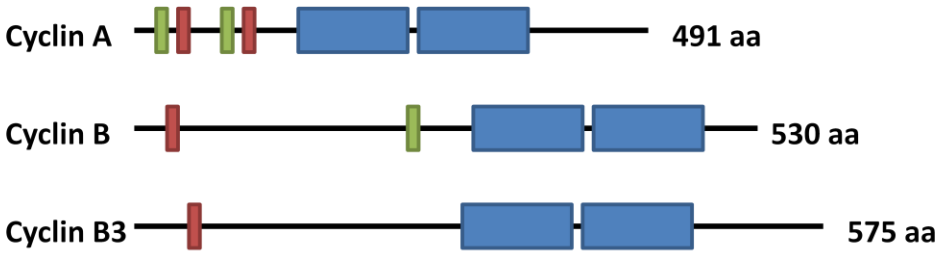

Figure 1 – Mitotic Cyclin protein structure. Blue boxes represent Cyclin Boxes; red boxes represent Destruction Boxes; and green boxes represent KEN boxes. The length of each protein is listed on the right side of the figure.

cleft between the two lobes, is blocked by the T-loop (De Bondt *et al.*, 1993). Activation of CDK1 requires three main events. The first event involves binding of a Cyclin protein; the Cyclin binds to the PSTAIRE helix and the N-terminal portion of the T loop. This causes a conformational change that exposes the active site. The conformational change also positions a glutamic acid within the active site; this residue is involved in coordinating ATP (Jeffrey *et al.*, 1995). The second event involves dephosphorylation of residues Thr14 and Tyr15, which are located within the active site (De Bondt *et al.*, 1993). These residues are phosphorylated by the kinases Wee1 and Myt1 and dephosphorylated by Cdc25 (Nigg, 2001). Dephosphorylation of these residues is essential because phosphorylated residues interfere with ATP-binding within the catalytic cleft and may also interfere with substrate binding. The third event of CDK1 activation requires an activating phosphorylation on Thr160 (found on the T-loop) by CDK-activating kinase (CAK); this phosphorylated residue is thought to stabilize the substrate-enzyme interaction (de Vivo *et al.*, 2006). Unlike Cyclins, which are periodically degraded, CDK1 levels remain constant throughout the cell cycle. Therefore, CDK1 activity oscillates with Cyclin protein levels. Once active, the Cyclin-CDK1 complexes phosphorylate serine or threonine residues on target proteins. The consensus Cyclin-CDK1 phosphorylation sequence is [S/T]PX[K/R] (Holmes and Solomon, 1996).

1.3 Cyclin-CDK1 regulates mitotic entry

Entry into mitosis is regulated by the G2/M checkpoint, which monitors DNA structure. Incomplete DNA replication or DNA damage activates Checkpoint-kinase 1

(Chk1) and Checkpoint -kinase 2 (Chk2), both of which are kinases that phosphorylate and inactivate Cdc25 (Nigg, 2001). When phosphorylated, Cdc25 cannot dephosphorylate Thr14 and Tyr15 within the CDK1 activation site; therefore, entry into mitosis is blocked. Once the DNA has been properly replicated without damage, the rate of CDK1 dephosphorylation via Cdc25 exceeds the rate of phosphorylation by Wee1 and Myt1, and the cell enters mitosis (Nigg, 2001).

1.4 Mitotic Roles of Cyclin-CDK1 complexes

The roles of the mitotic Cyclin-CDK1 complexes have been studied in a variety of organisms, including yeast, fruit flies, frogs, and humans. In some cases, the substrates of Cyclin-CDK1 complexes have also been identified. Cyclin-CDK1 complexes phosphorylate proteins involved in chromosome condensation, nuclear envelope breakdown, spindle assembly, and chromosome segregation. Dephosphorylation of these substrates upon CDK1 inactivation allows mitotic exit by allowing chromosome decondensation, reassembly of the nucleus, and spindle maturation (Nigg, 2001). In human cells, Cyclin B-CDK1 phosphorylates the kinesin-related motor protein Eg5; phosphorylation of Eg5 directs it to the centrosomes, where it regulates centrosome separation during the early phases of mitosis (Blangy *et al.*, 1995). Rat Cyclin B-CDK1 phosphorylates GM130, which is a Golgi transmembrane receptor; this phosphorylation triggers Golgi fragmentation (Lowe *et al.*, 1998). In multiple organisms, Cyclin B-Cdk1 promotes nuclear envelope breakdown (NEBD) by hyperphosphorylating nuclear lamins (Nigg, 2001). Xenopus Cyclin B-CDK1 regulates the DNA supercoiling activity of Condensins, thereby promoting chromosome condensation (Kimura *et al.*, 1998). In

mammalian cells, Cyclin B-CDK1 inactivates the microtubule-destabilizing protein stathmin to promote spindle assembly (Andersen *et al.*, 1997). Xenopus and human CDK1 aid in chromosome separation by phosphorylating Cohesin complexes during the metaphase-anaphase transition (Nigg, 2001). Cyclin-CDK1 complexes also regulate the activity of APC/C through phosphorylation; APC/$C^{Cdc20/Fzy}$ activity is positively regulated by phosphorylation, whereas APC/$C^{Cdh1/Fzr}$ activity is regulated negatively (Kramer *et al.*, 2000). Although Cyclin-CDK1 complexes have been studied extensively in other organisms, this literature review focuses on the roles of these complexes in *Drosophila melanogaster*.

Most studies that address the roles of fly mitotic Cyclin-CDK1 complexes have been carried out in Drosophila embryos, which are easy to manipulate and can be obtained in large numbers. Drosophila early embryogenesis involves a series of 13 syncytial nuclear mitoses in which DNA replication (S-phase) oscillates with nuclear divisions (mitosis). Since there is no detectable zygotic transcription, these syncytial mitoses are controlled by maternally deposited transcripts and proteins. As the nuclei divide in the syncytium, they undergo axial expansion in which they spread out along the anterior/posterior axis of the embryo (Stiffler *et al.*, 1999). Close to the tenth syncytial mitosis, the nuclei migrate from the interior of the embryo to the cortex, forming a syncytial blastoderm. After the 13^{th} mitosis, a G2 phase is introduced and zygotic transcription begins. Zygotic transcription drives blastoderm cellularization, during which the nuclei are sorted into individual cells; this switch to zygotic control is referred to as the mid-blastula transition. At this stage, majority of the maternal stores

have been depleted, thus subsequent development is controlled by zygotic genes. After mitosis 16, the epidermal cells of the embryo exit the cell cycle, but the cells of the nervous system continue to divide via G2-S-M cycles (Lee and Orr-Weaver, 2003).

1.4.1 Cyclin A in Drosophila mitosis

Drosophila Cyclin A is approximately 59 kD in size (Lehner and O'Farrell, 1990). *Cyclin A* mutants die as embryos. In these mutants, the syncytial mitoses, cellularlization, and mitosis 14 are normal due to the presence of maternal Cyclin A. Some epidermal cells also progress through the 15^{th} mitotic division due to small amounts of maternal Cyclin A; however, all epidermal cells fail to enter mitosis 16 and the embryo eventually dies. This phenotype is caused by the premature activation of $APC/C^{Cdh1/Fzr}$ due to absence of Cyclin A (Lehner and O'Farrell, 1989; Reber *et al.*, 2006). In the Drosophila embryo, Cyclin E and Cyclin A inhibit $APC/C^{Cdh1/Fzr}$ activity during late interphase. However, Cyclin E levels are transcriptionally down-regulated in the embryo after cellularization; at this stage, Cyclin A becomes the main inhibitor of $APC/C^{Cdh1/Fzr}$. However, in *Cyclin A* mutants, $APC/C^{Cdh1/Fzr}$ is inappropriately active during G2 phase; this results in premature degradation of Cyclin B, Cyclin B3, and Cdc25/String, all of which promote entry into mitosis 16. Thus, in the terminal mitoses of Drosophila embryonic epidermal cells, Cyclin A promotes mitotic entry by inhibiting $APC/C^{Cdh1/Fzr}$ (Reber *et al.*, 2006). However, Cyclin A does not serve a necessary function in the entire Drosophila embryo since cells of the nervous system can still divide in *Cyclin A* mutants; this may be due to the presence of other $APC/C^{Cdh1/Fzr}$ inhibitors that promote mitotic entry. This implies that Cyclin A may not be required for mitosis, although it is required

for interphase in embryonic cells undergoing the 16th cell cycle (Lehner and O'Farrell, 1989).

Maternally deposited Cyclin A mRNA and protein are evenly distributed throughout the Drosophila embryo. The levels of Cyclin A protein remain fairly constant during the syncytial mitoses, but localized degradation is still believed to occur during each mitosis (Edgar et al., 1994). During prophase of the nuclear division cycles, Cyclin A localizes to the nucleus and associates with the chromatin. This localization pattern implies that Cyclin A may regulate processes such as chromosome condensation and NEBD (Maldonado-Codina and Glover, 1992; Jacobs *et al.*, 1998; Stiffler et al., 1999). After cellularization, the behaviour of Cyclin A is slightly different.

In cellularized Drosophila embryos, in which Cyclin A is made from zygotically expressed transcripts, Cyclin A starts to accumulate during G2 phase (Whitfield *et al.*, 1990). During prophase, Cyclin A localizes to the nucleus, where it may regulate NEBD and chromosome condensation (Whitfield et al., 1990; Stiffler et al., 1999). Cyclin A reaches its maximal protein levels during prophase and is targeted for destruction in prometaphase (Whitfield *et al.*, 1990; Sigrist *et al.*, 1995). Cyclin A is the first mitotic Cyclin to be degraded (Sigrist *et al.*, 1995).

As mentioned earlier, Cyclin A has a complex degradation sequence at its N-terminus. This sequence is 53 amino acids long and contains one D-box and one KEN box. Stabilization of Cyclin A requires deletion of these 53 amino acids; once truncated, Cyclin A cannot be targeted for degradation by the APC/C (Jacobs *et al.*, 2001). Expression of non-degradable ΔCyclin A in cellularlized embryos causes a significant

metaphase delay; the length of the delay positively correlates with ΔCyclin A expression levels (Sigrist *et al.*, 1995; Jacobs *et al.*, 2001). However, mitosis is still completed without any detectable defects, indicating that degradation of Cyclin A is not required for mitotic exit. Although ΔCyclin A does not compromise mitosis, it does cause premature entry into S-phase during subsequent cell cycles; thus, degradation of Cyclin A during mitosis is essential for proper regulation of the G1-S transition in the subsequent cell cycle (Jacobs *et al.*, 2001).

The fact that ΔCyclin A causes a significant metaphase delay during mitosis puts forth an interesting possibility concerning the regulation of chromosome separation during anaphase. Chromosome separation was originally thought to be exclusively regulated by the APC/C. As mentioned previously, the APC/C targets Securin for degradation once all the chromosomes are properly attached to the mitotic spindle. The function of Securin is to bind and inhibit the cysteine protease Separase. This protease functions by cleaving the Cohesin complexes and allowing chromosome separation. A recent study in Xenopus egg extracts and mammalian cells has shown that Separase is actually inhibited by two mechanisms: a) inhibitory binding by Securin and b) inhibitory phosphorylation by Cyclin-CDK1. Based on this, activation of Separase requires both degradation of Securin and removal of the inhibitory phosphate group (Stemmann *et al.*, 2001). Thus, it is possible that Cyclin A inhibits chromosome separation in Drosophila embryos by phosphorylating Separase; this may explain the delayed metaphase phenotype observed in the presence of ΔCyclin A (Leismann and Lehner, 2003).

Like other Cyclin-CDK1 complexes, Cyclin A-CDK1 promotes entry into mitosis

(Jacobs *et al.*, 1998). Function-wise, it has also been shown that Cyclin A-CDK1 affects the length of Drosophila syncytial nuclear cycles. Decreasing the dose of Cyclin A increases the length of the nuclear cycles (Stiffler *et al.*, 1999). Studies conducted in Xenopus egg extracts have shown that Cyclin A regulates the microtubule nucleating activity of centrosomes (Buendia *et al.*, 1992). However, this may not be true in Drosophila embryos since Cyclin A fails to co-immunoprecipitate with microtubules (Stiffler *et al.*, 1999). In HeLa cells, Cyclin A2-CDK1 is the main Cyclin-CDK1 complex that phosphorylates nuclear lamins to trigger NEBD (Gong *et al.*, 2007). Since Cyclin A is a nuclear protein across different species, one of its conserved functions may be to trigger NEBD. There is also evidence that Cyclin A is involved in chromosome condensation and this function is shared with Cyclin B3. Both *Cyclin A* and *Cyclin B3* single mutants do not display significant mitotic abnormalities; however, double mutants exhibit extremely delayed chromosome condensation (Lehner and O'Farrell, 1989; Jacobs *et al.*, 1998).

1.4.2 Cyclin B in Drosophila mitosis

Drosophila Cyclin B is a 64 kD protein (Lehner and O'Farrell, 1990). Cyclin B transcripts are enriched at the posterior end of the embryo and around the syncytial nuclei (Lehner and O'Farrell, 1990). The transcripts at the posterior end are incorporated into pole cells, which give rise to future germ cells. The transcripts around the nuclei localize to spindles during the syncytial mitoses (Raff *et al.*, 1990). To some degree, Cyclin B transcript localization correlates with protein localization during the syncytial mitoses. During interphase, Cyclin B protein is cytoplasmic and concentrated on the astral microtubules (Stiffler *et al.*, 1999). During prophase, Cyclin B-CDK1 enters

the nucleus where it promotes NEBD by phosphorylating nuclear pore complexes; Cyclin B-CDK1 may also phosphorylate nuclear lamins, as seen in mammalian cells (Onischenko *et al.*, 2005; Pines and Hunter, 1994). Studies have also shown that Cyclin B may not be involved in chromosome condensation (Jacobs *et al.*, 1998; Onischenko *et al.*, 2005).

Cyclin B protein also localizes to the centrosomes and spindle during the syncytial mitoses, where it may regulate spindle assembly (Maldonado-Codina *et al.*, 1992 ; Raff *et al.*, 2002). Cyclin B is involved in reorganizing the astral microtubules, which radiate from centrosomes during interphase, into mitotic spindles; decreasing the dose of Cyclin B interferes with this process. Furthermore, increasing the dose of Cyclin B results in shorter, more dynamic spindle microtubules; therefore, Cyclin B functions by promoting microtubule instability during mitosis (Stiffler *et al.*, 1999). This function of promoting microtubule dynamics is also conserved in Xenopus egg extracts (Buendia *et al.*, 1992).

During the syncytial cycles, Cyclin B undergoes localized degradation on the mitotic spindle at the metaphase-anaphase transition (Raff *et al.*, 2002). This degradation is mediated by APC/$C^{Cdc20/Fzy}$ since APC/$C^{Cdh1/Fzr}$ is not active in the early embryo. APC/$C^{Cdc20/Fzy}$ degrades Cyclin B in a wave on the mitotic spindle, starting from the centrosomes and ending at the spindle equator (Raff *et al.*, 2002).

In cellularized embryos, Cyclin B accumulates during G2 and drives entry into mitosis; majority of the Cyclin B localizes to the cytoplasm, but punctuate signals are also seen in nuclei (Lehner and O'Farrell, 1990). During prophase, Cyclin B enters the nucleus where it contributes to NEBD; and during metaphase, it localizes to the

metaphase spindle and centrosomes, where it regulates spindle assembly (Lehner and O'Farrell, 1990; Raff *et al.*, 2002). *Cyclin B*$^{-}$ embryos, although viable, have slightly disorganized mitotic spindles; furthermore, spindle assembly is slowed, which results in slower mitoses (Knoblich and Lehner, 1993). Cyclin B degradation occurs during the metaphase-anaphase transition and is mediated by both APC/C$^{Cdc20/Fzy}$ and APC/C $^{Cdh1/Fzr}$. First, APC/C$^{Cdc20/Fzy}$ facilitates the degradation of spindle-associated Cyclin B; this occurs in a wave on the mitotic spindle as seen in syncytial embryos. Second, the cytoplasmic Cyclin B is degraded via APC/C$^{Cdh1/Fzr}$ (Raff *et al.*, 2002).

Cyclin B can be stabilized by mutating the D-box and KEN box. Mutating the D-box alone protects it from APC/C$^{Cdc20/Fzy}$, but not from APC/C$^{Cdh1/Fzr}$ (Raff *et al.*, 2002). Stabilized Cyclin B does not interfere with chromosome separation during anaphase, but it does prevent chromosome segregation to opposite poles; thus, cells expressing non-degradable Cyclin B arrest in early anaphase (Sigrist et al.,1995; Parry and O'Farrell, 2001). Based on this, one can infer that Cyclin B degradation is required for completion of anaphase and mitotic exit (Parry and O'Farrell, 2001).

1.4.3 Cyclin B3 in Drosophila mitosis

In the early syncytial embryo, Cyclin B3 transcripts are distributed uniformly throughout the cytoplasm, but become concentrated near the nuclei during cellularization. The localization of Cyclin B3 protein during the syncytial divisions has not yet been studied, but since Cyclin B3 contains an N-terminal nuclear targeting sequence, it is most likely to be nuclear. In cellularized embryos, Cyclin B3 is a nuclear protein (Jacobs *et al.*, 1998). Like Cyclin B, Cyclin B3 is not absolutely required for mitosis; *Cyclin*

B3^{-} embryos are viable and undergo mitosis without any visible defects (Jacobs *et al.*, 1998). Mutating *Cyclin B3* along with other *Cyclin* genes reveals that Cyclin B3 shares many functions with the other Cyclins. Like Cyclins A and B, Cyclin B3 is involved in mitotic entry; however, analysis of double mutants shows that Cyclins A and B3 are more critical for mitotic entry than Cyclin B. Cyclin B3 is also involved in spindle assembly and chromosomes condensation. Spindles of *Cyclin B*$^{-}$ and *Cyclin B3*$^{-}$ double mutants assemble slowly and are often kinked; this is a more severe phenotype than seen in *Cyclin B* mutants (Jacobs *et al.*, 1998).

Cyclin B3 degradation occurs in late anaphase and is mediated by an N-terminal D-box. Expressing a D-box mutant form of Cyclin B3 causes a block in late anaphase, but chromosome separation and cytokinetic furrow formation still occur. Nonetheless, Cyclin B3 degradation is critical for proper mitotic exit because stabilized Cyclin B3 interferes with spindle disassembly, chromosome decondensation, and nuclear reformation (Sigrist et al., 1995; Parry and O'Farrell, 2001).

1.5 Drosophila Oogenesis and Meiosis

Drosophila melanogaster development begins in the ovaries of the female fruit fly. *Drosophila* females possess two ovaries, each composed of approximately 20 ovarioles. The tip of each ovariole houses the germarium, which contains two or three germline stem cells (GSCs). The GSCs divide asymmetrically to produce a GSC and a cystoblast. The cystoblast undergoes four incomplete synchronous divisions to produce 16 cells or cystocytes that are interconnected via ring canals. A cytoplasmic structure known as the fusome passes through the ring canals and physically connects all the

cystocytes (Lilly *et al.*, 2000).

The 16 cystocytes subsequently enter a premeiotic S phase and up to four cystocytes progress further to form synaptonemal complexes characteristic of prophase I. Ultimately, only the future oocyte retains the synaptonemal complexes. The other 15 cystocytes enter the endocycle, in which DNA replication oscillates with a gap phase. These cells eventually become polyploid nurse cells; they provide nourishment for the oocyte; at stage 11 of oocyte development, the nurse cells transfer the contents of their cytoplasm into the oocyte and undergo apoptosis (Lee and Orr-Weaver, 2003).

Oocyte development has been divided into 14 stages. Stages one through six serve to establish the oocyte, which is the only cystocyte that enters and remains in prophase I. During stages six through 12, the oocyte remains arrested in prophase I; this arrest allows accumulation of the maternal transcripts and proteins that drive the meiotic divisions and early embryogenesis. During stages 12 and 13 of oocyte development, the prophase I arrest is released and oocyte maturation occurs. These stages are also marked by nuclear envelope breakdown, meiotic spindle assembly, and entry into prometaphase I. During stage 14 of oogenesis, the oocytes arrest in metaphase I. This allows coordination between the completion of meiosis and fertilization; however, in *Drosophila*, fertilization is not a pre-requisite for the completion of meiosis because the metaphase I arrest is released upon ovulation or egg activation even if the egg is not fertilized (Lee and Orr-Weaver, 2003). Of the four haploid meiotic products, one combines with the male pronucleus to form the zygotic nucleus; the other three products combine together to form a rosette-like structure

called the polar body (Page and Orr-Weaver, 1997). The fertilized embryo then starts developing (described in **1.4**).

1.6 Mitotic Cyclins in Oogenesis (Meiosis)

Although the mitotic roles of Cyclins A, B, and B3 are gradually coming to light, the meiotic roles of these Cyclins are largely unknown. As mentioned previously, *Cyclin B* mutants are viable and undergo mitosis with minimal defects. However, *Cyclin B*$^{-}$ females and males are sterile, indicating that Cyclin B has a distinct function in the germline. *Cyclin B*$^{-}$ females are reported to have rudimentary ovaries, which produce very few, inviable eggs (Wang and Lin, 2005). Similarly, *Cyclin B3* mutants are viable and show no mitotic defects; however *Cyclin B3*$^{-}$ females are sterile and lay eggs that fail to hatch. Close examination of these eggs reveals that the oocytes fail to exit meiosis after egg activation; thus fertilization is incapable of producing viable embryos (Jacobs *et al.*, 1998). Based on these different phenotypes, one can conclude that Cyclins B and B3 play distinct roles in female oogenesis. The role of Cyclin A in oogenesis is more poorly understood because it is difficult to directly test the function of Cyclin A since *Cyclin A*$^{-}$ embryos die soon after the fifteenth mitotic division (Lehner and O'Farrell, 1989).

1.6.1 Cdc25 homologues in Drosophila meiosis

As mentioned previously, Cdc25 is a phosphatase that removes inhibitory phosphate groups from the CDK1 subunit of Cyclin-CDK1 complexes. The Drosophila genome encodes two Cdc25 homologues: String and Twine. String functions primarily during mitosis, but is also found in ovaries. Interestingly, ovaries lacking String proceed through meiosis normally, implying that String is not required for meiosis (Edgar and

Datar, 1996). Twine on the other hand is a meiosis-specific Cdc25 homologue that is expressed in the male and female germline. *Twine* mutants are sterile indicating that Twine is essential for meiosis (Alphey et al., 1992; Edgar and Datar, 1996). In *Twine* mutants, nuclear envelope break down (NEBD) is significantly delayed (Von Stetina *et al.*, 2008). Mutants also form abnormal, kinked spindles and fail to maintain a metaphase I arrest. Stage 14 oocytes are often seen with a random assortment of DNA, some of which is associated with spindles. Embryonic development is also compromised. Although some embryos contain phenotypically normal mitotic spindles, they also contained a large number of thin spindles associated with small amounts of DNA. Furthermore, a proportion of the embryos are seen with a maximum of five large nuclei. In conclusion, Twine is required for proper timing of NEBD, proper spindle formation and chromosome segregation during meiosis I, and completion of meiosis (White-Cooper *et al.*, 1992). If Twine activates Cyclin-CDK1 complexes during meiosis, one would expect to similar phenotypes in *Cyclin* mutants.

1.6.2 The Role and Regulation of Cyclin A during Drosophila Oogenesis and Early Embryogenesis

Cyclin A is expressed in the primordial germ cells (PGCs) and germline stem cells (GSCs). PGCs are found near the somatic cap cells within the developing larval gonads. The PGCs directly adjacent to the cap cells give rise to GSCs during the larval/pupal transition (Wang and Lin, 2005). Cyclin A degradation is essential for GSC establishment and maintenance. A recent study overexpressed a stabilized form of Cyclin A in the female germline. The stabilized Cyclin A produced severe defects in GSC maintenance:

the adult ovaries, which were rudimentary, either lacked or contained very few GSCs. The stable Cyclin A most likely blocked mitotic progression by causing a delay in metaphase; thus, a prolonged M phase forced the GSCs to differentiate. Furthermore, there is evidence that Cyclin A turnover in GSCs is mediated by the joint action of the ubiquitin conjugating enzyme Effete (Eff) and the ubiquitin ligase APC/C^{Fzy} (Chen *et al.*, 2009).

Cyclin A-specific antibody staining of the germarium reveals that Cyclin A temporally associates with the fusomes of the GSCs and the dividing cysts. In G1 and early S phase, there is very little Cyclin A accumulation within the cytoplasm of the GSCs and cysts. Cyclin A begins to associate with the fusome in late S or early G2, and high levels of fusome-associated Cyclin A are observed before entry into mitosis. In prophase, Cyclin A remains associated with the fusome, but lower levels of Cyclin A are also visible throughout the cytoplasm of the cyst. The cytoplasmic levels of Cyclin A continue to increase until metaphase. Cyclin A is degraded during the metaphase-to-anaphase transition; this is most likely mediated by Eff and APC/C^{Fzy} (Lilly *et al.*, 2000; Chen *et al.*, 2009). Overexpressing Cyclin A or mutating *Eff* results in an extra round of division yielding 32 cystocytes. Thus, it seems that the fusome-associated Cyclin A may synchronize mitotic entry of the interconnected cystocytes, perhaps by equalizing Cdk1 activation during G2 (Lilly *et al.*, 2000). In addition to Cyclin A proteolysis, the actions of Twin, a deadenylase, prevent the inappropriate translation of Cyclin A mRNA within the cystocytes (Morris *et al*., 2004).

Cyclin A transcripts begin to accumulate as soon as meiosis begins. The

translation of *Cyclin A* mRNA is regulated in a temporal manner by multiple proteins. Four forms of Cyclin A protein are present during oogenesis; each form has a different phosphorylation state. During the prophase I arrest, Cyclin A levels are kept low through translational inhibition and protein degradation. Both these processes are essential for the inhibition of CDK1 activity and maintenance of prophase I arrest (Vardy *et al.*, 2009). Translational repression of *Cyclin A* mRNA is mediated by Bruno, which binds to a Bruno Response Element (BRE) in the 3' UTR of the *Cyclin A* mRNA. Bruno protein levels are upregulated during the prophase I arrest when Cyclin A protein levels are low. The other level of regulation occurs at the protein level, where APC/C promotes degradation of Cyclin A protein. When the prophase I arrest is released, Bruno levels are downregulated; this allows Cyclin A to accumulate during oocyte maturation (Sugimura and Lilly, 2006).

The *Cyclin A* mRNA is translationally activated via polyadenylation during late prophase I; the proteins mediating this polyadenylation are unknown. Polyadenylation promotes Cyclin A translation and accumulation during late prophase I and prometaphase I. Translation of *Cyclin A* mRNA ceases at the end of prometaphase I and Cyclin A protein is degraded via APC/C^{Cortex}, where Cortex (Cort) is a female-germline-specific Cdc20 homologue (Vardy *et al.*, 2009; Swan and Schupbach, 2007). There is very little Cyclin A protein present during the metaphase I arrest in stage 14 oocytes, but *Cyclin A* mRNA is poly-adenylated during this stage (Fenger *et al.*, 2000; Vardy *et al.*, 2009).

The *Cyclin A* mRNA poly-adenylation during metaphase I is mediated by a

serine/threonine kinase called PAN GU (PNG). Active PNG is found in a complex with two other proteins called Plutonium (PLU) and Giant Nuclei (GNU). The PNG kinase serves an important function after the completion of meiosis by keeping the haploid meiotic products in a quiescent state. In null mutants of *PNG*, *PLU*, or *GNU*, meiosis proceeds normally, but the four meiotic products undergo repeated rounds of DNA replication, forming polyploid nuclei (Fenger et al., 2000). In *PNG* mutants, the length of the *Cyclin A* mRNA polyA tail is dramatically reduced, but the mechanism by which PNG facilitates *Cyclin A* mRNA polyadenylation is unknown. Polyadenylation results in renewed translation of *Cyclin A* after the metaphase I arrest is released (Vardy *et al.*, 2009). This allows Cyclin A to accumulate during meiosis II. In prometaphase II, Cyclin A is ubiquitinated by APC/C^{Cort} and degraded by the 26S proteasome (Swan *et al.*, 2005). During embryogenesis, PNG kinase promotes Cyclin A translation by displacing (directly or indirectly) the translational repressor PUMILIO (PUM) from the 3'UTR of *Cyclin A* mRNA (Vardy *et al*., 2009). The resulting Cyclin A protein is required for the embryonic syncytial divisions.

Based on its expression profile, it seems that Cyclin A is required for late prophase I, the prophase I-to-prometaphase I transition, prometaphase I (during which nuclear envelope breakdown and spindle assembly occurs), and for the early phases of meiosis II (during which the meiosis I spindle undergoes reorganization and the chromosomes start aligning at the metaphase II plates). It is also possible that Cyclin A may serve an important function during the metaphase I arrest even though it is present at low levels at this stage. Narrowing down these possible functions requires

examination of meiosis in the absence of Cyclin A.

1.6.3 The Role and Regulation of Cyclin B during Drosophila Oogenesis

Cyclin B is expressed in the primordial germ cells and the germline stem cells. In the mitotic divisions of the PGCs, Cyclin B accumulates during G2 to promote the G2/M transition. It is degraded in early anaphase to allow exit from mitosis. *Cyclin B*$^{-}$ PGCs arrest in G2; as a result, adults contain very few GSCs and these are gradually lost during oogenesis. Thus, Cyclin B is essential for the formation and maintenance of selfrenewing GSCs. Because of this, Cyclin B is also required for the formation of functional ovaries; *Cyclin B* mutants contain rudimentary ovaries that produce very few eggs (Wang and Lin, 2005).

The *Cyclin B* mRNA is transcribed and accumulates in the cytoplasm during meiosis; however, it is masked at various stages, such as during early prophase I, to prevent inappropriate translation. In stage 14 oocytes, *Cyclin B* mRNA is unmasked via ORB-mediated polyadenylation (Vardy and Orr-Weaver, 2007). ORB (Oo18 RNA binding) is a Cytoplasmic Polyadenylation Element Binding protein (CPEB protein) that binds to cytoplasmic polyadenylation elements at 3' ends of specific mRNAs; once bound, ORB recruits proteins involved in mRNA polyadenylation (Castagnetti and Ephrussi, 2003). Polyadenylation of *Cyclin B* mRNA corresponds to increased levels of Cyclin B protein during metaphase I. After the metaphase I arrest is released, Cyclin B is targeted for destruction; however, the overall levels of Cyclin B protein do not change due to further translational activation of *Cyclin B* mRNA via the PNG kinase (Vardy and Orr-Weaver, 2007).

Protein-wise, Cyclin B has an interesting localization pattern during meiosis. It accumulates at the meiotic spindle midzone during metaphase I and metaphase II (Swan and Schupbach, 2007). However, before degradation during anaphase II, Cyclin B is found uniformly distributed along the meiotic spindle. Based on its localization pattern, one may speculate that Cyclin B is involved in spindle assembly, chromosome alignment, and/or chromosome segregation during meiosis. Two APC/C complexes are required for the local destruction of Cyclin B during meiosis II: APC/C^{Cort} and APC/$C^{Cdc20/Fzy}$. APC/C^{Cort} mediates Cyclin B destruction during metaphase II when Cyclin B is concentrated at the spindle midzone; and APC/C^{Fzy} mediates Cyclin B destruction during anaphase II when Cyclin B is more uniformly distributed along the meiotic spindle (Swan and Schupbach, 2007). Expression of non-degradable Cyclin B in the germline results in a variable phenotype. Some oocytes arrest in anaphase I; others arrest in metaphase II or anaphase II. Very few eggs complete meiosis, but, those that do, subsequently arrest in the first mitosis. Thus, these phenotypes indicate that Cyclin B destruction is essential for the proper completion of meiosis (Swan and Schupbach, 2007).

In the early *Drosophila* embryo, *Cyclin B* transcripts become enriched at the posterior end of the egg and around the syncytial nuclei. The posterior-end *Cyclin B* transcripts are translationally repressed by PUM, which is distributed throughout the egg's cytoplasm. PUM forms a complex with NANOS, which is concentrated at the posterior end of the embryo. Together, this PUM-NANOS complex binds to a Nanos Response Element (NRE) in the 3' UTR of the *Cyclin B* mRNA and represses translation. As previously mentioned, unlike the posterior *Cyclin B* transcripts, the transcripts near

the nuclei are translated, but are also thought to undergo PUM-mediated translational repression. Translational activation of these particular transcripts is controlled by PNG, which antagonizes the activity of the translational repressor PUM (Vardy and Orr-Weaver, 2007).

1.7 Objectives of Research Study

The purpose of this research study was to examine the roles of Cyclin A and Cyclin B in Drosophila female meiosis. Although the regulation of these proteins and their transcripts has been studied during oogenesis, their meiotic roles have not yet been examined. Nonetheless, the mitotic roles of these Cyclins may provide clues to their roles in meiosis. For example, Cyclin B-CDK1 is involved in spindle assembly during mitosis in many different species. Thus, one may expect that Cyclin B-CDK1 also serves a similar function during meiosis. The results of this study may also aid in understanding human meiosis and possible causes of infertility, birth defects, and miscarriages.

This study had six objectives:

a) Examine the role of Cyclin A in meiosis by observing Cyclin A localization.

b) Examine the role of Cyclin A in meiosis by examining *Cyclin A* mutant ovaries.

c) Examine the role of Cyclin A in meiosis by expressing a stabilized form of Cyclin A during meiosis.

d) Examine the role of Cyclin B in meiosis by observing Cyclin B localization.

e) Examine the role of Cyclin B in meiosis by examining *Cyclin B*$^{-}$ ovaries.

f) Examine the role of Cyclin B in meiosis by expressing a stabilized form of Cyclin B during meiosis.

2.0 MATERIALS AND METHODS

2.1 Fly stocks

The *Cyclin B* mutants were generated by crossing flies harbouring the deficiency *Df(2R)59AB* with flies containing the $CycB^{2}$ allele. The *HsCycB-50* transgene (from Dr. C. Lehner) was used to rescue the early germline stem cell requirement for Cyclin B. All experiments with these $Cyclin\ B^{-}$; *HsCycB* flies were conducted at 29°C unless otherwise stated. Sterile males were obtained by crossing wildtype (*yw)* females with X-Y;*ry* males. The X-Y;*ry* males have attached X-Y chromosomes. The progeny that inherit this attached chromosome become females. The male progeny do not inherit the X-Y chromosome; therefore they have the genotype XO. These males are sterile because they are missing the Y chromosome.

For gain of function studies involving non-degradable Cyclin B, the *UASp-CyclinB-TPM:GFP* transgene (from Dr. J. Raff) was expressed using the *nanos-Gal4:VP16* (*nos-Gal4:VP16*) driver at 29°C either in a wildtype or *Cyclin B* background.

To create *Cyclin A* mutants, the following alleles were used: $CycA^{H17b}$ and $CycA^{C8LR1}$, also referred to as $CycA^{L7R1}$ (Bloomington Drosophila Stock Center). In an attempt to rescue these mutants, *HsCycA* transgenes (from Dr. C. Lehner) were introduced into the *Cyclin A* mutant background. Other methods of *Cyclin A* mutant rescue employed the following transgenes: *actin-Gal4* (from Bloomington Drosophila Stock Center) in combination with *UASt-Cyclin A* (from C. Lehner), and *daughterless-Gal4 (da-Gal4)* (Bloomington Drosophila stock center) in combination with *UASp-Flag:Cyclin A* line *31b* or *UASp-Venus:Cyclin A* line *21c* (generation of these transgenic

lines is described below). Since the *da-Gal4* gene was on the third chromosome, a recombinant was made by combining the $CycA^{H17b}$ allele with this *da-Gal4* driver. The driver *prd-Gal4* was used to test transgenic lines containing *UASp-Venus:ΔCyclin A* (described below).

2.1.1 Generation of *UASp-Venus:Cyclin A* and *UASp-Venus:ΔCyclin A* transgenic lines

To examine localization of Cyclin A, a Venus-tagged *Cyclin A* transgene was created. *Cyclin A* cDNA (LD44443) was used as a PCR template. A one-step PCR was performed to attach attB recombination sites to each end of the *Cyclin A* gene. For the first step, the *Cyclin A* gene was amplified using Finnzymes Phusion high-fidelity DNA polymerase and the following primers: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCAGTTTC-3' (forward *Cyclin A*-specific primer containing full attB1 sequence) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATTAAAACTTATAAAA-3' (reverse *Cyclin A*-specific primer containing full attB2 sequence). The PCR reaction conditions were as follows: initial denaturation at 98°C for 30s, 25 cycles of denaturation at 98°C for 10s, annealing at 58°C for 30s, and elongation at 72°C for 50s, and final elongation at 72°C for 5 minutes. The resulting PCR product was diluted 1:5 in water and cloned into pDONR221 (from Dr. W.L. Crosby) using Invitrogen Gateway BP Clonase enzyme. The resulting pENTRY vector was recombined with pPVW (obtained from Drosophila Genomics Resource Center (DGRC)) using Invitrogen Gateway LR Clonase enzyme to generate a *UASp-Venus:Cyclin A* transgene. Multiple transgenic fly lines were generated via random P-element insertions. The *UASp-Venus:Cyclin A* line *21c* (transgene on

second chromosome) was used for majority of the experiments. For some experiments a similar line, *15c* (transgene on third chromosome), was also used. Both lines show equal amounts of transgene expression in ovaries when expressed using the *mat67-Gal4* driver. In addition to the *UASp-Venus:Cyclin A* transgene, Flag-tagged and Protein A-tagged *Cyclin A* transgenes were also generated by cloning the *Cyclin A* cDNA sequence into vectors pPFW (DGRC) and pUASp-NPrA (from Dr. V. Archambault). To examine the localization of Cyclin A, *UASp-Venus:Cyclin A 21c* or *15c* were expressed under the *nos-Gal4:VP16* driver.

The non-degradable *Cyclin A* transgene was generated by deleting 53 amino acids from the N-terminus and cloning into the pPVW via the Gateway method (Jacobs *et al.*, 2001). The following gene-specific primers with full attB sequences were used for cloning: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGCAACAACAAT-3' (forward primer) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATTAAAACTTATAAAA-3' (reverse primer).

2.1.2 Generation of *UAS-Venus:CyclinB* transgenic lines

For Cyclin B localization studies, a Venus-tagged *Cyclin B* transgene was generated. Genomic DNA was extracted from wildtype (*yw*) female flies. A two step PCR was performed to attach attB recombination sites to each end of the *Cyclin B* gene. For the first step, the *Cyclin B* gene was amplified from the genomic DNA using Finnzymes Phusion high-fidelity DNA polymerase and the following primers: 5'-AAAGCAGGCTTAATGGTGGGCACAACACTGAAAATG-3' (forward *Cyclin B*-specific primer containing partial attB1 sequence) and 5'-

GAAAGCTGGGTACTATTTCCTCTGGCTCTGGCCCAC-3' (reverse *Cyclin B*-specific primer containing partial attB2 sequence). The PCR reaction conditions were as follows: initial denaturation at 98°C for 30s, 12 cycles of denaturation at 98°C for 10s, annealing at 60°C for 30s, and elongation at 72°C for 75s, and final elongation at 72°C for 5 minutes. 1.5uL of the PCR product was used in a second PCR reaction using the same DNA polymerase and the following primers: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTA-3' (forward primer containing partial attB1 sequence) and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA-3'. The PCR cycling conditions were the same, except for the number of cycles were increased to 24. The resulting PCR product was diluted 1:5 in water and cloned into pDONR221 using Invitrogen Gateway BP Clonase enzyme. The resulting pENTRY vector was recombined with pPVW (obtained from DGRC) using Invitrogen Gateway LR Clonase enzyme to generate a *UASp-Venus:CyclinB* transgene. Multiple transgenic fly lines were generated via random P-element insertions. The line 31a was used for localization experiments. In addition to the *UASp-Venus:CyclinB* transgene, Flag-tagged and Protein A-tagged *Cyclin B* transgenes were also generated by cloning the *Cyclin B* sequence into vectors pPFW (DGRC) and pUASp-NPrA.

2.1.3 Generation of *bgcn-Gal4:VP16* transgenic lines

For construction of the *bgcn (benign gonial cell neoplasm)-Gal4* driver, the 3' UTR region of *bgcn* was amplified from genomic DNA from *nos-Gal4:VP16*-transgene-containing flies using the following primers: 5'-TCTCAAGCTTTAAAGCCCGCTTCCTCGCGATATTGC-3' (forward primer with *Hind*III

overhang) and 5’-TCTCGGTACCTGAATTCAATTTACCACTTAGGC-3’ (reverse primer with *Kpn*I overhang). The PCR product was digested and cloned into *Hind*III and *Kpn*I sites in pBlueScript II SK +/- (pBS) to generate pBS-3’bgcn. The following primers were used to amplify Gal4:VP16 (sequence kindly provided by Dr. Y. Kamachi): 5’-TCTCGGATCCAAGCTACTGTCTTCTATCGAACAAGC-3’ (forward primer with *Bam*HI overhang) and 5’-TCTCAAGCTTCTACCCACCGTACTCGTCAATTCC-3’ (reverse primer with *Hind*III overhang). This Gal4:VP16 PCR product was digested and cloned into *Bam*HI and *Hind*III sites in pBS-3’bgcn to generate pBS-Gal4:VP16-3’bgcn. The 5’ promoter region of *bgcn* was amplified using the following primers: 5’-TCTCGCGGCCGCCTTTAAGGCGGTGCCTGTCACC-3’ (forward primer with *Not*I overhang) and 5’-TCTCGGATCCCATTTCTGCTAGCTTCAAACAGATTGAC-3’ (reverse primer with *Bam*HI overhang). The resulting PCR was digested and cloned into pBS-Gal4:VP16-3’bgcn to generate pBS-5’*bgcn-Gal4:VP16*-3’bgcn. The entire transgene was excised from pBS using *Not*I and *Kpn*I and cloned into vector pBI-G (from Dr. B. McCabe). Sequencing was used to confirm proper cloning. The purified vector was then sent to Genetic Services Inc (Cambridge, MA) for site-directed insertion into sites attp2 (chromosome 3L) and attp40 (chromosome 2L).

2.1.4 Generation of *hsp70-Gal4-SCS* transgenic lines

For construction of the *hsp70-Gal4-SCS* driver, the Gal4 sequence was amplified from vector F89 (kindly provided by Dr. A. Brand) using the following primers: 5’-TCTCGAATTCATGAAGCTACTGTCTTCTATCGAA-3’ (forward primer with *Eco*RI overhang) and 5’-TCTCGAATTCTTACTCTTTTTTTGGGTTTGGTGG-3’ (reverse primer with *Eco*RI

overhang). The resulting PCR product was digested and cloned into the *Eco*RI site in pELBA-Hsp70-SCS (from Dr. P. Schedl), which contains a *Hsp70* promoter and *SCS* and *SCS'* insulator sequences. Next, the *hsp70-Gal4-SCS* fragment was excised from pELBA-Hsp70-SCS using *Not*I and *Kpn*I and cloned into these respective sites in the vector pattB. The transgene sequence was confirmed by sequencing. The vector was then sent to Genetic Services Inc (Cambridge, MA) for site-directed insertion into sites attp2 (chromosome 3L) and attp40 (chromosome 2L).

2.1.5 Generation of RNAi transgenic lines

Short-hairpin RNA (shRNA) constructs were generated using the protocol provided on the Transgenic RNAi Project (TRiP) website (http://www.flyrnai.org/supplement/2ndGenProtocol.pdf). The shRNA sequences were designed using the Designer of Small Interfering RNA (DSIR) algorithm on http://biodev.extra.cea.fr/DSIR/DSIR.html. The shRNAs against *Cyclin A* and *Cyclin B* were designed to target all transcripts produced by these respective genes. The primers used for shRNA cloning are listed in **Table I**. The numbers 1508, 1289, and 196 indicate the position each shRNA targets on a particular mRNA. The 3'UTR shRNAs are designed to target sequences within the 3'UTR region of the mRNAs.

2.1.6 Testing the *UASp-Venus:ΔCyclin A* transgene

Different insertion lines of *UASp-Venus:ΔCyclin A* were generated via P-element-mediated transgenesis, which is a method that randomly inserts transgenes into the fly genome (**Table 4** – see Results). The resulting transgenic fly lines were tested by crossing them with flies containing the drivers *nos-Gal4:VP16, mat67-Gal4* and *prd-Gal4*. It has

Target Gene	Name of shRNA construct	Top/ Bottom Primer	Sequence of Primers
Cyclin A	Cyclin A (1508)	Top	ctagcagtCTCCCTAGTCGACAAAGAGAAtagttata ttcaagcataTTCTCTTTGTCGACTAGGGAGgcg
	Cyclin A (1508)	Bottom	aattcgcCTCCCTAGTCGACAAAGAGAAtatgcttga atataactaTTCTCTTTGTCGACTAGGGAGactg
	Cyclin A (3'UTR)	Top	ctagcagtCCCGTTGCAAGTGCCGGACAAtagttata ttcaagcataTTGTCCGGCACTTGCAACGGGgcg
	Cyclin A (3'UTR)	Bottom	aattcgcCCCGTTGCAAGTGCCGGACAAtatgcttga atataactaTTGTCCGGCACTTGCAACGGGactg
Cyclin B	Cyclin B (1289)	Top	ctagcagtCCGACCAAGACCACTGTAGAAtagttata ttcaagcataTTCTACAGTGGTCTTGGTCGGgcg
	Cyclin B (1289)	Bottom	aattcgcCCGACCAAGACCACTGTAGAAtatgcttga atataactaTTCTACAGTGGTCTTGGTCGGactg
	Cyclin B (3'UTR)	Top	ctagcagtCCGGTAAGACTAGGAACCGAAtagttat attcaagcataTTCGGTTCCTAGTCTTACCGGgcg
	Cyclin B (3'UTR)	Bottom	aattcgcCCGGTAAGACTAGGAACCGAAtatgcttg aatataactaTTCGGTTCCTAGTCTTACCGGactg
Cyclin B3	Cyclin B3 (196)	Top	ctagcagtTAGCGAACGACTCAAGTTCTAtagttata ttcaagcataTAGAACTTGAGTCGTTCGCTAgcg
	Cyclin B3 (196)	Bottom	aattcgcTAGCGAACGACTCAAGTTCTAtatgcttga atataactaTAGAACTTGAGTCGTTCGCTAactg
	Cyclin B3 (3'UTR)	Top	ctagcagtCACAATCAATCTAAACTCGAAtagttatat tcaagcataTTCGAGTTTAGATTGATTGTGgcg
	Cyclin B3 (3'UTR)	Bottom	aattcgcCACAATCAATCTAAACTCGAAtatgcttga atataactaTTCGAGTTTAGATTGATTGTGactg

Table 1 – Primer sequences used for shRNA cloning. Capital letters indicate the gene-specific regions of the shRNA constructs.

been reported that expression of non-degradable Cyclin A in the germline leads to loss of germline stem cells (Chen *et al.*, 2009). When all transgenic lines were crossed with *nos-Gal4:VP16*, a gradual loss of GSCs was observed (the flies stopped laying eggs after a few days). The egg-laying capacity and egg viability was examined by crossing flies with the driver *mat67-Gal4*, which does not express in the GSCs. The results of this test are listed in **Table 4** (see Results). Certain lines were found to lay more eggs than others, and the egg viability also varied. The line that laid the most eggs that did not hatch (*2a*) was chosen for further experiments. The biological functionality of the non-degradable *Cyclin A* transgene was tested by crossing with the driver *prd-Gal4*, which expresses in stripes in the late embryo. Previous studies have shown that expression of stable Cyclin A causes a delay in metaphase of mitosis (Sigrist *et al*., 1995). The *prd-Gal4-UASp-Venus-ΔCyclin A* embryos were collected for 4-8 hours on apple juice agar plates. Embryos were dechorionated with 50 % bleach, fixed in 4% formaldehyde (540 uL PBST and 60 uL of 37% formaldehyde), and devitellinized using a 50:50 mix of methanol and heptane. The embryos were then rehydrated and washed several times with PBST and block in 1% BSA in PBST. The embryos were then stained with rabbit anti-Phosphohistone H3 antibody (Santa Cruz) at 1:1000.

2.1.7 Testing the *bgcn-Gal4:VP16* transgene

Insertion lines for the *bgcn-Gal4:VP16* transgene were generated using site-directed recombination into attP sites on chromosomes 2 and 3. Different insertion lines were tested by crossing transgenic flies with flies containing the reporter contructs *UASp-GFP* or *UASp-LacZ*. Ovaries were dissected from *bgcn-Gal4:VP16-UASp-GFP* and

bgcn-Gal4:VP16-UASp-LacZ flies and fixed in 4% formaldehyde (540 uL PBST and 60 uL 37% formaldehyde). The *bgcn-Gal4:VP16-UASp-LacZ* ovaries were stained with mouse β-galactosidase antibody (Promega) at 1:1000. Both types of ovaries were also stained with propidium iodide (PI) at 1:1000 or Quant-i-T OliGreen (Invitrogen) at 1:500 (from a 1:10 working stock) in the presence of RNase A (Invitrogen) at 1:250. Ovaries were visualized via confocal microscopy.

2.1.8 Testing the *hsp70-Gal4-SCS* transgene

Insertion lines for this transgene were generated using site-directed recombination into attP sites on chromosomes 2 and 3. Different insertion lines were tested by crossing transgenic flies with flies containing the reporter contructs *UASp-GFP* or *UASp-LacZ*. The progeny of the crosses were either kept at 29°C for two days or were subjected to 30 minute heat-shocks at 37°C twice a day for two days, after which they were put at 29°C to recover. The flies heat-shocked at 37°C were dissected three hours after the last heat-shock. Ovaries were fixed in 4% formaldehyde. Ovaries expressing *LacZ* were stained with mouse anti-β-galactosidase (Promega) at 1:1000. Transgene expression was visualized via confocal microscopy. (The driver *nos-Gal4:VP16* was used as a positive control.)

2.2 Heat-shocks rescue of *Cyclin B* and *Cyclin A* single mutants

On the first day of heat-shocking, *Cyclin B^{-}; HsCycB* and *Cyclin B^{-}* flies were heat-shocked for 20 minutes at 37°C. From the second day onwards, flies were heat-shocked for 1 hour twice a day, 12 hours apart for a total of 12 days. The flies were put at 29°C to recover. Egg laying and egg viability were monitored.

Many different heat-shock approaches were used for the attempted rescue of *Cyclin A* mutant embryos. Flies were put in a 29°C incubator; however, this did not rescue the embryos. Eggs from crosses listed in **Table 3** (see Results) were heat-shocked in vials twice a day for 30 minutes at 37°C and then put at 29°C to recover; this also did not rescue the mutant embryos. It seemed as if putting the eggs at 29°C after the heat-shocks reduced either the hatching rate or the larva viability because very few larvae survived to form adults. Thus, heat-shocked vials were kept at 25°C after heat-shocks. Heat-shocking in vials twice a day for 30 minutes at 37°C and then putting at 25°C did not produce any mutant adult flies. Heat-shocking in bottles twice a day for 30 minutes at 37°C and then putting at 25°C did not rescue the *Cyclin A* mutant embryos.

2.3 Antibody Staining of Ovaries, Oocytes and Embryos

For ovary dissections, flies were raised in fresh vials with yeast for 4-5 days. Dissected ovaries were fixed with 4% formaldehyde (540 uL PBST and 60 uL 40% formaldehyde). The DNA of ovaries was stained with Propidium Iodide at 1:1000 or Quant-i-T OliGreen (Invitrogen) at 1:500 (from a 1:10 working stock) in the presence of RNase A (Invitrogen) at 1:250. The reversion to mitosis in nurse cells expressing Venus:ΔCyclin A was visualized using rat anti-α-Tubulin (Santa Cruz or Millipore) at 1:500.

For stage 14 oocyte collection, flies were dissected for ovaries in collagenase-containing Isolation Buffer (2 uL of collagenase per mL of Isolation Buffer) (Page and Orr-Weaver, 1997). Stage 14 oocytes were enriched by suspending all oocytes in Isolation Buffer in a microcentrifuge tube, allowing the heavy oocytes to sink to the

bottom of the tube, and removing the lighter, suspended oocytes; the enrichment process was performed several times. Stage 14 oocytes were fixed in 100% methanol. Oocyte DNA was stained with Propidium Iodide at 1:1000 or Quant-i-T OliGreen (Invitrogen) at 1:500 (from a 1:10 working stock) in the presence of RNase A (Invitrogen) at 1:250. For visualization of spindle and DNA morphology, the outer chorion and vitelline membrane of stage 14 oocytes were removed in 100% methanol using sharp forceps. The oocytes were then rehydrated, washed several times with PBST, blocked in 1% BSA in PBST, and stained with rat anti-α-Tubulin (Santa Cruz or Millipore) at 1:100 and mouse anti-Histone (Chemicon International or Millipore) at 1:10000.

Embryos and unfertilized eggs were collected on apple juice agar plates. Embryos/eggs were dechorionated with 50 % bleach, devitellinized using a 50:50 mix of methanol and heptane, and fixed in 100% methanol. Embryos/eggs were then washed several times with PBST and blocked for at least 1 hour in 1% BSA in PBST. They were stained rat anti-α-Tubulin (Santa Cruz) at 1:100 and mouse anti-Histone (Chemicon International or Millipore) at 1:10000.

For FISH, stage 14 oocytes, eggs, and embryos were obtained using the methods described above. These samples were fixed in 100% methanol. The samples were then incubated with a FISH probe designed against a 359-bp sequence that repeats on the X-chromosome (Dernberg, 2000). Oocytes were then stained with Quant-i-T OliGreen (Invitrogen) at 1:500 (from a 1:10 working stock) in the presence of RNase A (Invitrogen) at 1:250. Eggs and embryos were stained with rat anti-α-Tubulin (Santa Cruz or Millipore) at 1:100 and mouse anti-Histone (Chemicon International or Millipore) at

1:10000.

2.4 Confocal microscopy

DNA-stained and/or immunostained ovaries, stage 14 oocytes, unfertilized eggs, and embryos were visualized with an Olympus FluoView FV1000 laser scanning confocal microscope. The Olympus PLAPO20 x WLSM-NA1.0 was the primary objective used. Olympus Fluoview software version 1.5 was used to draw scale bars on the acquired images. Scanned images were compiled together using Adobe Photoshop Elements 7.0.

2.5 Western analysis

Extracts of stage 14 oocytes, unfertilized eggs, embryos, and third-instar larvae were prepared in 2x sample buffer. Western blotting was performed using standard techniques. Western blots were probed with the following antibodies: mouse anti-Cyclin A (Developmental Studies Hybridoma Bank) at 1:5, mouse anti-Cyclin B (Developmental Studies Hybridoma Bank) at 1:20, rabbit anti-Cyclin B3 (from C. Lehner) at 1:500, rabbit anti-PSTAIRE (Santa Cruz) at 1:1000, and mouse anti-Actin (Developmental Studies Hybridoma Bank) at 1:500. Densitometry analysis was performed using the Spot Denso tool of FluorChemTM HD2-AlphaEase FC software. Protein levels were normalized based on the loading control (Actin or PSTAIRE) and 'relative' normalized protein levels were calculated by dividing values of test samples with those of *yw*.

2.6 Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Unfertilized eggs were collected on apple juice agar plates and frozen in liquid nitrogen (without dechorionation or devitillenization). Each frozen sample contained approximately 100 eggs. Too many eggs reduce the RNA yield. RNA was extracted using

Qiagen RNeasy Plus Mini Kit. The protocol for RNA extraction was modified from http://furlonglab.embl.de/methods/extras/Expression-profiling.pdf. In a microcentrifuge tube, 600 uL of Buffer RLT were mixed with 6 uL of β-mercaptoethanol. 300 uL of this solution was added to frozen eggs, when were then homogenized using a pestle. The remaining 300 uL of RLT-β-mercaptoethanol were added to the homogenized eggs. This mixture was incubated at room temperature for 5 minutes. The lysate was centrifuged for 3 minutes at maximum speed. The supernatant was put into a new microcentrifuge tube. One volume of 70% ethanol was added and mixed immediately by pipetting. This mixture was applied to an RNeasy Mini Spin Column. The column was centrifuged at 10,000 rpm for 15 seconds and the flow-through was discarded. This was repeated for the remaining mixture. 700 uL of Buffer RWI was added to the column, which was then centrifuged at 10,000 rpm for 15 seconds. The column was put in a new 2 mL collection tube. 500 uL of Buffer RPE were added to the column. The column was centrifuged at 10,000 rpm for 15 seconds. The flow through was discarded. Another 500 uL of Buffer RPE was added to the column, which was centrifuged for 2 minutes (2 min) at 10,000 rpm. The flow-through was discarded and the column was centrifuged for 1 minute at 10,000 rpm. For elution, the column was put in a microcentrifuge tube and 32 uL of RNase-free water was added to the centre. The column was incubated at room for 1 minute and centrifuged at 10,000 rpm for 1 minute. The elution was repeated in a new microcentrifuge tube by adding 30 uL of RNase-free water to the centre of the column. The concentration of the RNA was determined using a NanoDrop 1000 Spectrophotometer. The RNA yield ranged between

20-150 ng/uL. The RNA was stored at -80°C.

The RNA was converted to cDNA using SuperScript III First-Strand Synthesis SuperMix. The following were mixed in a 0.2 mL RNase-free PCR tube: 6 uL of total RNA, 1 uL of Oligo$(dT)_{20}$ nucleotides, and 1 uL of Annealing Buffer. This was incubated in a thermocycler at 65°C for 5 minutes and placed immediately on ice afterwards. The following were added to the PCR tube on ice: 10 uL of 2x First-Strand Reaction Mix and 2 uL of SuperScript III/RNaseOUT Enzyme Mix. The mixture was vortexed, centrifuged, and incubated in a thermocycler for cDNA synthesis under the following conditions: 50°C for 50 minutes then 85°C for 5 minutes. The cDNA was stored at -20°C. The cDNA was used for a conventional PCR to confirm successful reverse-transcription.

The qRT-PCR was performed using the ABI 7500 Real-Time PCR System. The PCR reaction was performed in a 96-well plate using the Qiagen QuantiTect SYBR Green PCR Kit. Each reaction contained the following components: 10 uL of SYBR Green, 1.5 uL of 4 uM forward primer, 1.5 uL of 4 uM reverse primer, 6 uL of RNase-free water, and 1 uL of cDNA. A negative control containing RNase-free water instead of cDNA was also used. The primers were suspended in RNase-free water. The primers used for the qRT-PCR were designed against exon-exon junctions (**Table 2**). This was not the case for *Cyclin B3* primers since the *Cyclin B3* gene does not have any introns. Primers against genomic DNA (*Cyclin A* intron) were also designed to test for the level of genomic DNA contamination. The ribosomal protein gene *RP49*, which is a housekeeping gene, was used as an endogenous control for normalization of data. The Relative Quantification

Primer Name	Primer Sequence	Product Length (bp)	T_M (°C)
Rp49 (RpL32) For a	CGTGAAGAAGCGCACCAAGCAC	73	59.5
Rp49 (RpL32) Rev a	GCGCCATTTGTGCGACAGCTTAG	73	59.65
Rp49 (RpL32) For b	AGCTGTCGCACAAATGGCGCAA	111	60.74
Rp49 (RpL32) Rev b	GGGTGCGCTTGTTCGATCCGT	111	60.05
Cyclin A For	TCAGCGTGGGCACTGAAACGG	148	59.92
Cyclin A Rev	GGGCGATGTTTCTTCTCGCTCTCC	148	59.66
Cyclin B For	GCCGAGGACGAGCACCATACG	103	59.66
Cyclin B Rev	GTGAGGCAGCTGCAATCTCCGA	103	59.23
Cyclin B3 For	TTGCCGCCGTCAAGCCAGTT	133	59.83
Cyclin B3 Rev	CGCGCCGCCTCAAAGTTTCC	133	59.51
Cyclin A gDNA For	GGAAGTCGGACCCTGTCGCAC	141	59.46
Cyclin A gDNA Rev	CGGACGCAGACAGACAAGCGT	141	59.73

Table 2 – Primer sequences used for qRT-PCRs.

(RQ) mode was used for the qRT-PCR. The following conditions were used for the PCR reaction: first step at 50°C for 2 minutes, second step at 95°C for 15 minutes (to activate the HotStarTaq DNA polymerase), and then 50 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). The data was exported as an Excel file and RQ values were used for mRNA expression analysis.

3.0 RESULTS

3.1 Cyclin A localization during oogenesis

A *UASp-Venus:Cyclin A* transgene was used to examine the localization of Cyclin A in the Drosophila ovary (**Figure 2A**). To ensure that the Venus-tag did not interfere with the function of Cyclin A, the transgene was first tested by introducing it into the *Cyclin A* mutant background and assessing its ability to rescue *Cyclin A* mutants. The transgene, which was expressed using the *daughterless-Gal4* (*da-Gal4*) promoter, successfully rescued the null embryos to adulthood; therefore, this indicated that Venus:Cyclin A functions in the same manner as endogenous Cyclin A during mitosis.

In the germarium, Venus:Cyclin A localized to the spectrosome and fusome (**Figure 2B**). The spectrosome is a germline stem cell (GSC)-specific organelle, which gives rise to the fusome. The observed fusome-specific localization has been previously reported (Lilly *et al.*, 2000). Venus:Cyclin A also localized to the oocyte centrosome during prophase I (**Figure 2C,D**). Centrosomal localization of Cyclin A has been previously observed in mammalian and Xenopus cells during mitosis (Pascreau *et al.*, 2009).

The main purpose of this localization experiment was to determine whether Cyclin A enters the nucleus and aids in nuclear envelope breakdown (NEBD) during meiosis. Experiments revealed that Venus:Cyclin A did not localize to the oocyte nucleus during stage 13 of meiosis (**Figure 2E**), which is when NEBD occurs (von Stetina *et al.*, 2008). Furthermore, no specific Venus:Cyclin A localization was observed during stages 11 to 14 even though Cyclin A protein is present during most of these later stages

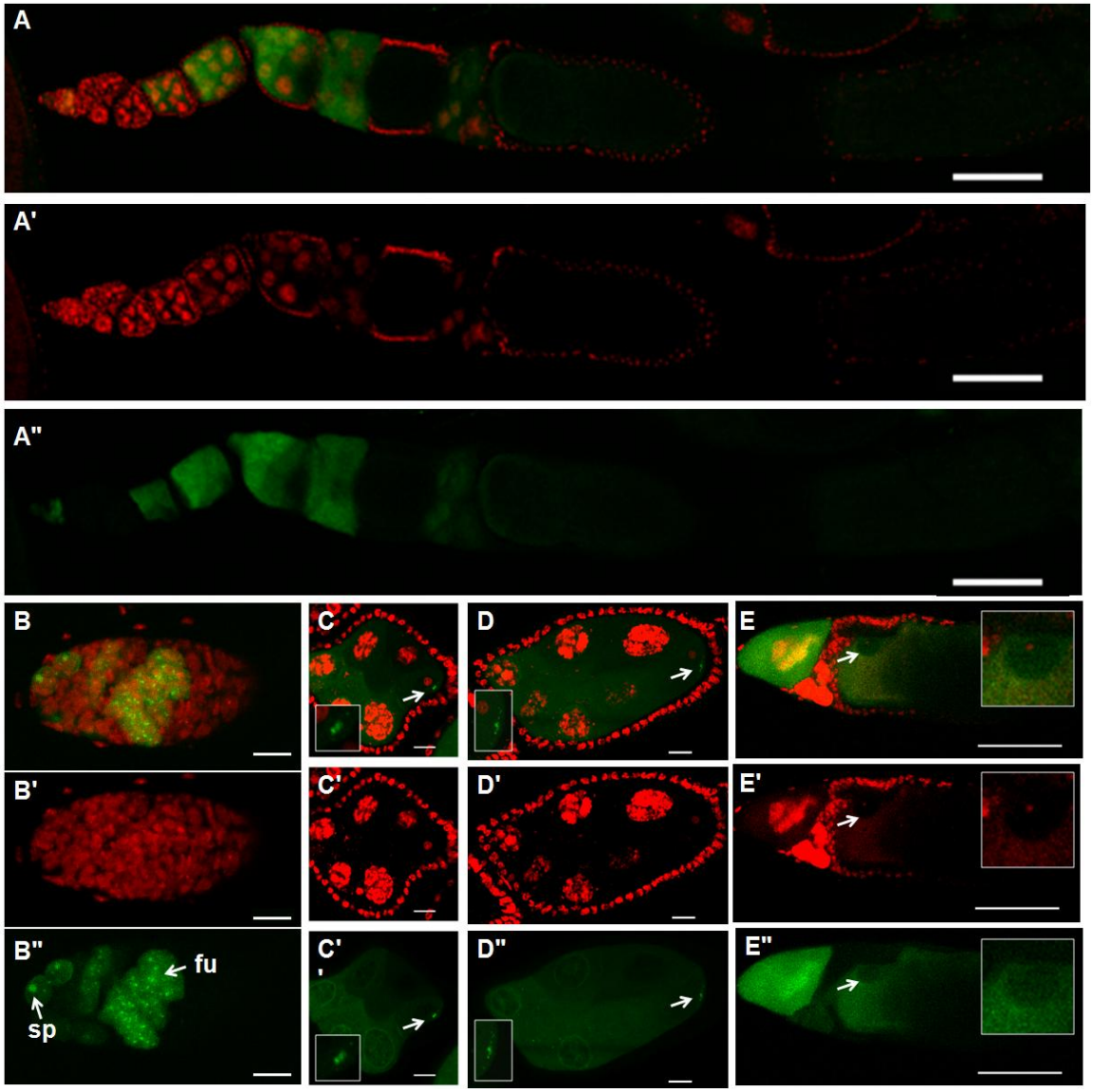
A
A'
A"
B
C
D
E
B'
C'
D'
E'
B"
fu
sp
C'
'
D"
E"

Figure 2 – Characterization of Cyclin A localization during oogenesis. Venus:Cyclin A is green and DNA is red. The *UASp-Venus:Cyclin A* transgene was expressed using the *nos-Gal4:VP16* driver. (A-A'') shows the localization pattern of Venus:Cyclin A within the ovariole. Specific localization is observed within the germarium (B-B'') and during prophase I (C-D'') of meiosis. (B- B'') show that Venus:Cyclin A localizes to spectrosomes (sp) and fusomes (fu) in the germarium. (C – D'') show that, during prophase I, Venus:Cyclin A localizes to the oocyte centrosomes (arrows). Insets show magnified views of centrosomes. (E-E'') shows that Venus:Cyclin A does not localize to the oocyte nucleus (arrow) before NEBD. Insets show magnified views of oocyte nuclei. For all panels, the germarium is oriented to the left. Scale bars are 100 um for (A-A''', E-E'') and 10 um for (B-D'').

(Vardy *et al.*, 2009). Several attempts were made to examine the post-metaphase I localization of Venus:Cyclin A using 0-1 hour embryo collections; however, no specific Cyclin A signal was observed in the collected embryos.

3.2 *Cyclin A*$^{-}$ embryos cannot be rescued using *Cyclin A* transgenes

As mentioned previously, *Cyclin A* mutant embryos fail to enter mitosis 16 of embryogenesis; as a result, they die very early in development (Lehner and O'Farrell, 1989). Therefore, to examine meiosis in *Cyclin A* mutants, the *Cyclin A*$^{-}$ embryos must be rescued to adults. In this study, several attempts were made to rescue the *Cyclin A*$^{-}$ embryos to adulthood. **Table 3** outlines the different crosses that were used in the rescue attempt. First, a heat-shock inducible *Cyclin A* transgene (*HsCycA*) was introduced into the *Cyclin A* mutant background. The resulting *HsCycA*; *Cyclin A*$^{-}$ embryos were heat-shocked in a 37°C water-bath, twice a day for 30 minutes, and then placed at 25°C to recover; originally the embryos were placed at 29°C, but this was found to reduce larva viability. This heat-shock method failed to rescue the *Cyclin A*$^{-}$ embryos. Two, three, and, finally, four *HsCycA* transgene copies were also introduced in the *Cyclin A* mutant background; the resulting embryos were heat-shocked using the same method. Again, this failed to rescue the mutant embryos (even to larval stage). Occasionally, non-*Tubby* (apparently rescued *Cyclin A* mutants) larvae were observed in heat-shocked bottles, but Western Blot analysis revealed that they were not *Cyclin A* mutants, but had lost the *Tubby* allele on their TM6 balancer. Thus, this rescue technique was unsuccessful. It appeared as if the embryo itself was not rescued during mitosis 16 because all methods failed to produce *Cyclin A* mutant larvae.

Genotypes of Flies Crossed	Genotype(s) of Progeny of Interest	Outcome
HsCycA/CyO*; CycA^{L7R1}*/TM6 x *HsCycA*/CyO*;CycA^{H17b}*/TM6	*HsCycA*/CyO; *CycA^{L7R1}/CycA^{H17b}* or *HsCycA/HsCycA*; *CycA^{L7R1}/CycA^{H17b}*	No rescue at 29°C or 37°C
HsCycA/CyO*; CycA^{L7R1}*/TM6 x CyO*/S; HsCycA, CycA^{H17b}*/TM6	*HsCycA*/CyO; *CycA^{L7R1}/HsCycA, CycA^{H17b}*	No rescue at 29°C or 37°C
HsCycA/CyO*; CycA^{L7R1}*/TM6 x *HsCycA*/CyO*; HsCycA, CycA^{H17b}*/TM6	*HsCycA*/CyO; *CycA^{L7R1}/HsCycA, CycA^{H17b} or HsCycA/HsCycA*; *CycA^{L7R1}/HsCycA,CycA^{H17b}*	No rescue at 29°C or 37°C
HsCycA/HsCycA; CycA^{L7R1}/TM6 x *HsCycA/HsCycA; HsCycA, CycA^{H17b}*/TM6	*HsCycA/HsCycA*; *CycA^{L7R1}/HsCycA, CycA^{H17b}*	No rescue at 29°C or 37°C
HsCycA/HsCycA; HsCycA, CycA^{H17b}/TM6 *(stock)*	*HsCycA/HsCycA*; *HsCycA, CycA^{H17b}/HsCycA-CycA^{H17b}*	No rescue at 29°C or 37°C
*actin-Gal4/*CyO; *CycA^{L7R1}*/TM6 *x UASt-Cyclin A/*CyO*; CycA^{H17b}*/TM6	*actin-Gal4/UASt-Cyclin A; CycA^{L7R1}/CycA^{H17b}*	Flies die as pharate adults
CyO*/S*; *CycA^{H17b},da-Gal4/*TM6 *x UASp-Flag:Cyclin A/*CyO*; CycA^{L7R1}*/TM6	*UASp-Flag:Cyclin A/*CyO *or S; CycA^{L7R1}/CycA^{H17b},da-Gal4*	Flies overexpress Cyclin A in ovary
CyO*/S*; *CycA^{H17b},da-Gal4/*TM6 *x UASp-Venus:Cyclin A/*CyO*; CycA^{L7R1}*/TM6	*UASp-Venus:Cyclin A/*CyO *or S; CycA^{L7R1}/ CycA^{H17b},da-Gal4*	Flies overexpress Cyclin A in ovary

Table 3 – Attempted rescue of *Cyclin A*$^{-}$ embryos. This table lists the genotypes of flies used for rescue of *Cyclin A* mutant embryos and the outcome of each cross.

Another method was also attempted to rescue the *Cyclin A* mutant embryos. This method employed a *UASt-Cyclin A* transgene, which was expressed using the *actin-Gal4* driver in the *Cyclin A* mutant background. The *UASt* promoter consists of upstream activation sequence (UAS) elements followed by the *heat shock protein 70* (*hsp70*) basal promoter (Duffy, 2002). While the *UASt* promoter can drive high levels of transgene expression in somatic cells, it does not function in the Drosophila germline; therefore, any rescued flies would be expected to undergo meiosis in the absence of Cyclin A. This combination rescued the embryos to pupal stage; however, the flies died as pharate adults (enclosed in the pupae). Dissection of the pupae revealed that the flies had developed to adulthood. Western blot analysis of rescued third instar larvae showed that the *UASt-Cyclin A* transgene was being expressed at very high levels (**Figure 3A**). Therefore, constitutive activation of Cyclin A-CDK1 may have been the cause of fatality.

A similar approach involving different transgenes was also attempted. A *UASp-Flag:Cyclin A* transgene was expressed using the *da-Gal4* driver in the *Cyclin A* mutant background. The *UASp* promoter consists of UAS elements followed by a *P transposase* basal promoter (Duffy, 2002). Genes with this promoter are expressed both in the germline and in somatic cells. In this case, the specificity of expression (within the somatic cells) was dependent on the *da-Gal4* driver, which was thought to be only expressed in somatic cells and not in the germline. This transgene combination successfully rescued the mutant embryos to adulthood; however, the adult flies contained tiny ovaries and failed to lay eggs (**Figure 3B**). Western blotting of third

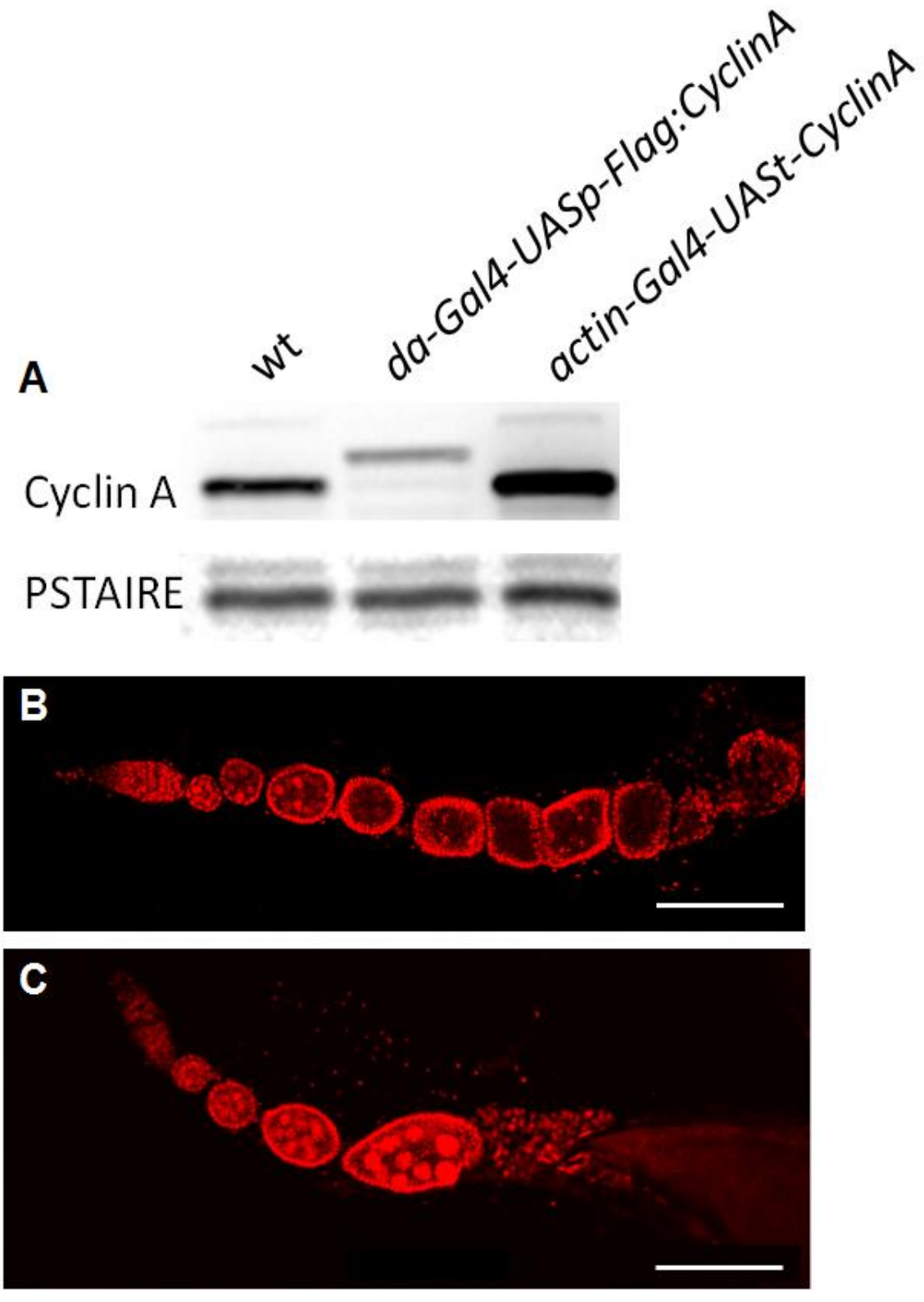
A
wt
da-Gal4-UASp-Flag:CyclinA
actin-Gal4-UASt-CyclinA
Cyclin A
PSTAIRE
B
C

Figure 3 – Different combinations of transgenes do not allow rescue of *Cyclin A*$^{-}$ embryos and examination of meiosis in the absence of Cyclin A. DNA is shown in red. (A) depicts a Western blot on third instar larvae, which was probed for Cyclin A. PSTAIRE (CDK1) was used as the loading control. This Western blot was performed to determine the cause of the phenotypes observed in the crosses listed in **Table 3**. The Western blot shows that *da-Gal4-UASp-Flag*:*Cyclin A* larvae were rescued to adulthood because they expressed close to normal levels of Cyclin A (Flag:Cyclin A), However, *actin-Gal4-UASt-Cyclin A* larvae died as pharate adults due to Cyclin A overexpression. (B) depicts an ovariole overexpressing Flag-Cyclin A. The DNA morphology reveals that this transgene combination interferes with normal oogenesis. It seems that the egg chambers degenerate and fail to produce eggs. (C) depicts a wildtype ovariole. (B, C) are shown with the germarium oriented to the left. The scale bar in (B, C) is 100 um.

instar larvae revealed that Cyclin A was expressed at low levels (**Figure 3A**). To determine if the observed ovarian phenotype was due to low levels of Cyclin A, the *UASp-Flag:Cyclin A* transgene was introduced into wildtype flies and expressed using the *da-Gal4* driver. These flies also developed small ovaries and failed to lay eggs, indicating that the observed phenotype was not due to a lack of Cyclin A. Next, the expression pattern of *da-Gal4* was observed in the ovaries using the *UASp-LacZ* reporter gene. β-galactosidase-specific immunostaining revealed that this driver was expressed in the follicle cells and throughout the ovary. Thus, the observed phenotype was due to overexpression of Cyclin A. This same phenotype was also seen when *UASp-Venus:Cyclin A* was expressed in *Cyclin A*$^{-}$ flies using *da-Gal4*. However, when Venus:Cyclin A was expressed using the germline-specific driver *nos-Gal4:VP16*, the ovaries were phenotypically normal; thus, the defect may be due to Cyclin A overexpression in the follicle cells that surround the egg chambers. Therefore, these *Cyclin A* transgenes are capable of rescuing the *Cyclin A*$^{-}$ embryos, but they cannot be used to examine the function of Cyclin A during meiosis because Gal4 drivers that express only in the somatic cells are currently not available.

A group at Harvard Medical School recently developed plasmids that express short hairpin RNAs (shRNAs) in the Drosophila germline (Ni *et al.*, 2011). Previously, RNA interference (RNAi) techniques were not available for the Drosophila germline. This new development has opened up the possibility of knocking down *Cyclin A* only in the ovary, therefore bypassing the need to rescue the *Cyclin A*$^{-}$ embryo. Two shRNA sequences targeting the *Cyclin A* mRNA were designed using the Designer of Small Interfering RNA

(DSIR) algorithm. One of the shRNAs was designed to target the 3'UTR. The other was designed to target a region within the coding sequence of all four *Cyclin A* transcripts. These shRNAs were cloned into the Valium22 shRNA expression vector. The resulting transgenic flies will be used to examine the consequences of Cyclin A-CDK1 inactivation during meiosis.

3.3 Cyclin A degradation is important for proper completion of meiosis

Non-degradable Cyclin A causes a delay in metaphase during mitosis (Sigrist *et al.*, 1995; Jacobs *et al.*, 2001). This suggests that Cyclin A degradation is required for proper chromosome segregation. To determine the effects of non-degradable Cyclin A on meiosis, a Venus-tagged nondegradable *Cyclin A* transgene was constructed by deleting 53 amino acids from the N-terminus, which is the minimum deletion required to stabilize this protein (Jacobs *et al.*, 2001).

Several Drosophila insertion lines of *UASp-Venus:ΔCyclin A* were generated. The insertion lines were tested by crossing them with the *nos-Gal4:VP16* driver (which is expressed in the germarium and egg chambers), *mat67-Gal4* driver (which is expressed from prophase I and onwards in the egg chambers), and *prd-Gal4* (which is expressed in stripes in the late embryo). The tested insertion lines are listed in **Table 4**.

A recent study showed that when non-degradable Cyclin A is expressed in GSCs, stem cell renewal is impaired (Chen *et al.*, 2009). All transgenic lines exhibited compromised germline stem cell renewal when crossed with *nos-Gal4:VP16*. The flies initially developed ovaries and laid eggs, but they gradually lost their ovaries over time (due to loss of GSCs).

Transgene (Level of Expression)	Insertion Line	Chromosome	Phenotypic Data After Crossing with the Driver *mat67-Gal4*
UASp-Venus:Cyclin A (high)	*21a*	3	Lay many eggs that hatch.
UASp-Venus:Cyclin A (medium)	*15c*	3	Lay many eggs that hatch.
UASp-Venus:Cyclin A (medium)	*21c*	2	Lay many eggs that hatch.
UASp-Venus:ΔCyclin A (high)	*16x*	3	Lay very few eggs that fail to hatch.
UASp-Venus:ΔCyclin A (medium)	*2a*	2	Lay many eggs and very few hatch.
UASp-Venus:ΔCyclin A (low)	*16f*	3	Lay many eggs, most of which hatch.
UASp-Venus:ΔCyclin A (lowest)	*6d*	2	Lay many eggs that hatch.
UASp-Venus:ΔCyclin A (lowest)	*7e*	2	Lay many eggs that hatch.

Table 4 – Characterization of *UASp-Venus:Cyclin A* and *UASp-Venus:ΔCyclin A* transgenic lines. Each type of *Cyclin A* transgene is listed in order from highest to lowest expression as determined by egg-laying and in some cases, also immunostaining and Western blotting.

The transgenic lines were also crossed to the *mat67-Gal4* driver to examine egg laying and egg viability. All *mat67-Gal4-UASp-Venus:ΔCyclin A* progeny (except those derived from 6d and 7e) were delayed in egg-laying (from time of eclosion), compared to wildtype. The two insertion lines 6d and 7e laid a large amount of eggs that hatched. Three insertion lines (*2a*, *16f*, *16x*) laid eggs, the majority of which failed to hatch. The *16x* insertion line laid the least number of eggs; the *16f* line produced an intermediate amount; and the *2a* line produced the greatest number of eggs and almost all of them failed to hatch.

The function of Venus:ΔCyclin A (line *2a*) was tested using the *paired-Gal4* driver. Previous studies have shown that non-degradable Cyclin A delays cells in metaphase of mitosis (Jacobs et al., 2001). To determine whether the *UASp-Venus:ΔCyclin A* transgene behaves in a similar manner, the *2a* insertion line was crossed with the *paired-Gal4* driver, which induces transgene expression in stripes in the late Drosophila embryo. Embryos were stained with Phosphohistone-H3-specific antibody; this antibody labels cells that have entered mitosis. If Venus:ΔCyclin A functions as previously described, the cells within the stripes should be delayed in mitosis; therefore, the stripes should have many more cells that stain positive for Phosphohistone-H3 than the wildtype control stripes. As expected, an enrichment of mitotic cells was not observed in stripes expressing wildtype Cyclin A (**Figure 4A**). However, there was an obvious enrichment of mitotic cells within the stripes expressing nondegradable Cyclin A (**Figure 4B**), implying that Venus:ΔCyclin A indeed does cause a mitotic delay. Therefore, the *UASp-Venus-*

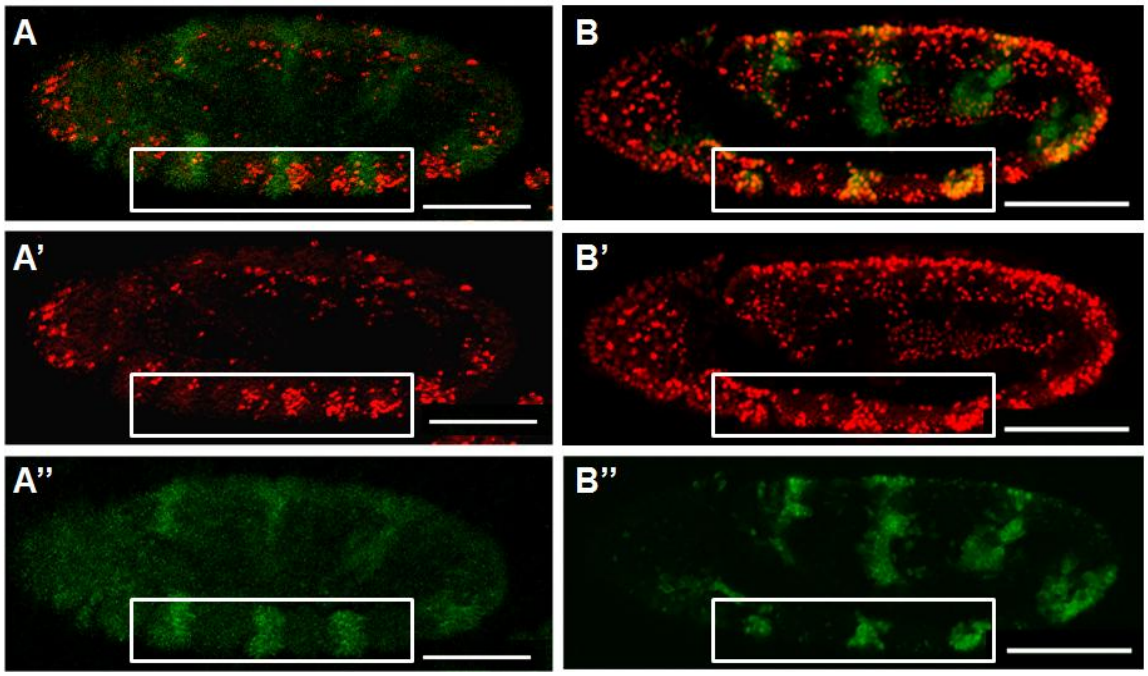

Figure 4 – Venus:ΔCyclin A delays cells in mitosis. Phosphohistone-H3 is shown in red, whereas Venus:Cyclin A (A, A'') and Venus:ΔCyclin A (B, B'') are shown in green. Phosphohistone-H3 antibody specifically stains cells that are in mitosis. The boxes outline the regions of interest. When wildtype Venus:Cyclin A is over-expressed using the *paired-Gal4* driver, there is no significant enrichment of mitotic cells in the stripes expressing this fusion protein (A-A''). However, when Venus:ΔCyclin A is expressed using the *paired-Gal4* driver, there is an obvious enrichment of mitotic cells in the regions expressing the stabilized Cyclin A (B-B'') (yellow signal in B). All embryos are oriented with anterior end to the left. All scale bars are 100 um.

ΔCyclin A (*2a*) transgene behaves as previously described. All subsequent experiments were done using the *2a* insertion line.

When expressed in the ovaries using *mat67-Gal4*, Venus:ΔCyclin A delayed oogenesis. Close examination of ovaries revealed that in stage five and later egg chambers, the DNA of nurse cells was condensed and associated with microtubules (**Figure 5B, D-D''**). This is similar to the *Morula* mutant phenotype. In *Morula* mutants, the APC/C is non-functional; therefore, Cyclins accumulate within the endocycling nurse cells; as a result, the nurse cells exit the endocycle and enter mitosis, as evidenced by DNA condensation and spindle formation (Reed and Orr-Weaver, 1997; Kashevsky *et al.*, 2002). However, in this case, the mitotic entry was temporary since oogenesis continued and stage 14 oocytes were produced. Therefore, it seems that meiosis can proceed normally until metaphase I in the presence of Venus:ΔCyclin A.

Next, the post-metaphase I affects of Venus:ΔCyclin A were examined by collecting embryos. Wildtype and Venus:ΔCyclin A embryos were collected for 0-2 hours. In wildtype embryos, completion of meiosis is indicated by the presence of a polar body, which is derived from the haploid meiotic products (**Figure 6A**). All wildtype embryos (n=45) had completed meiosis and were undergoing embryonic development (**Table 5**). Venus:ΔCyclin A embryos, however, displayed a range of phenotypes (**Table 5**). Approximately 70% (n=60) of embryos were not undergoing embryonic development (**Figure 6 B-G''**). Regardless, approximately 23% contained normal-looking polar bodies, indicating that they had completed meiosis successfully (**Figure 6B-B''**). Some embryos also contained two polar bodies (**Figure 6C-C''**). 58% of embryos had not completed

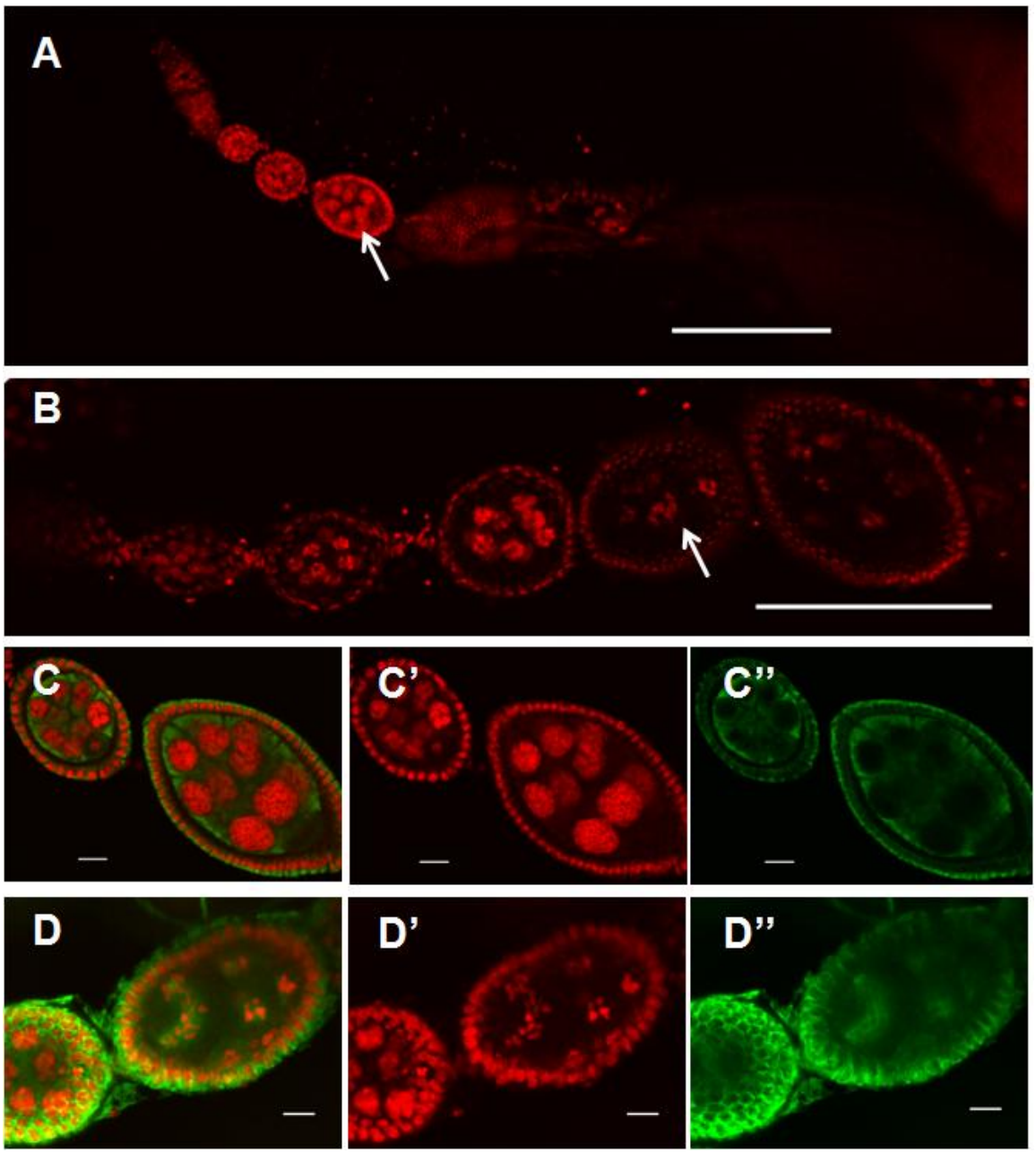
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Figure 5 – Stabilized Cyclin A causes endocycling nurse cells to enter mitosis. DNA is shown in red and microtubules are shown in green. (A) shows a wildtype ovariole in which nurse cell morphology is normal (arrow). Overexpression of ΔCyclin A (*mat67-Gal4-UASp-Venus:ΔCyclin A*) in the ovaries causes condensation of nurse cell DNA (arrow in B, D, D') and formation of mitotic spindles during stage 5 of oogenesis (D, D''). As a result, the nurse cells exit the endocycle and arrest in mitosis. (C-C'') depicts wildtype egg chambers which are spindle-free and contain polyploid, endocycling nurse cells. In all panels, the ovarioles are oriented with the germarium to the left. Scale bars in (A, B) are 100 um and scale bars in (C'-D'') are 10 um.

	Meiosis Incomplete	Meiosis Complete	Difficult to Assess Meiosis Completion	Synchronous Syncytial Mitoses	Asynchronous Syncytial Mitoses	Post-cellular Blastoderm	N
wildtype (25°C) (0-2 hours)	0%	100%	0%	82%	0%	18%	45
***mat67-Gal4-Venus:ΔCyclin A* (0-2 hours)**	58%	30%	12%	13%	17%	0%	60

Table 5 – Summary of developmental stages observed in Venus:ΔCyclin A embryos. All embryos were collected for 0-2 hours from flies kept at 25°C. . 'Meiosis Incomplete' refers to the presence of meiotic spindles. 'Meiosis Complete' refers to the presence of a polar body. For some developing embryos, it was difficult to find a polar body; these embryos are scored under the category "Difficult to Assess Meiosis Completion". Fly genotype is indicated in the left column. N represents the total number of embryos counted.

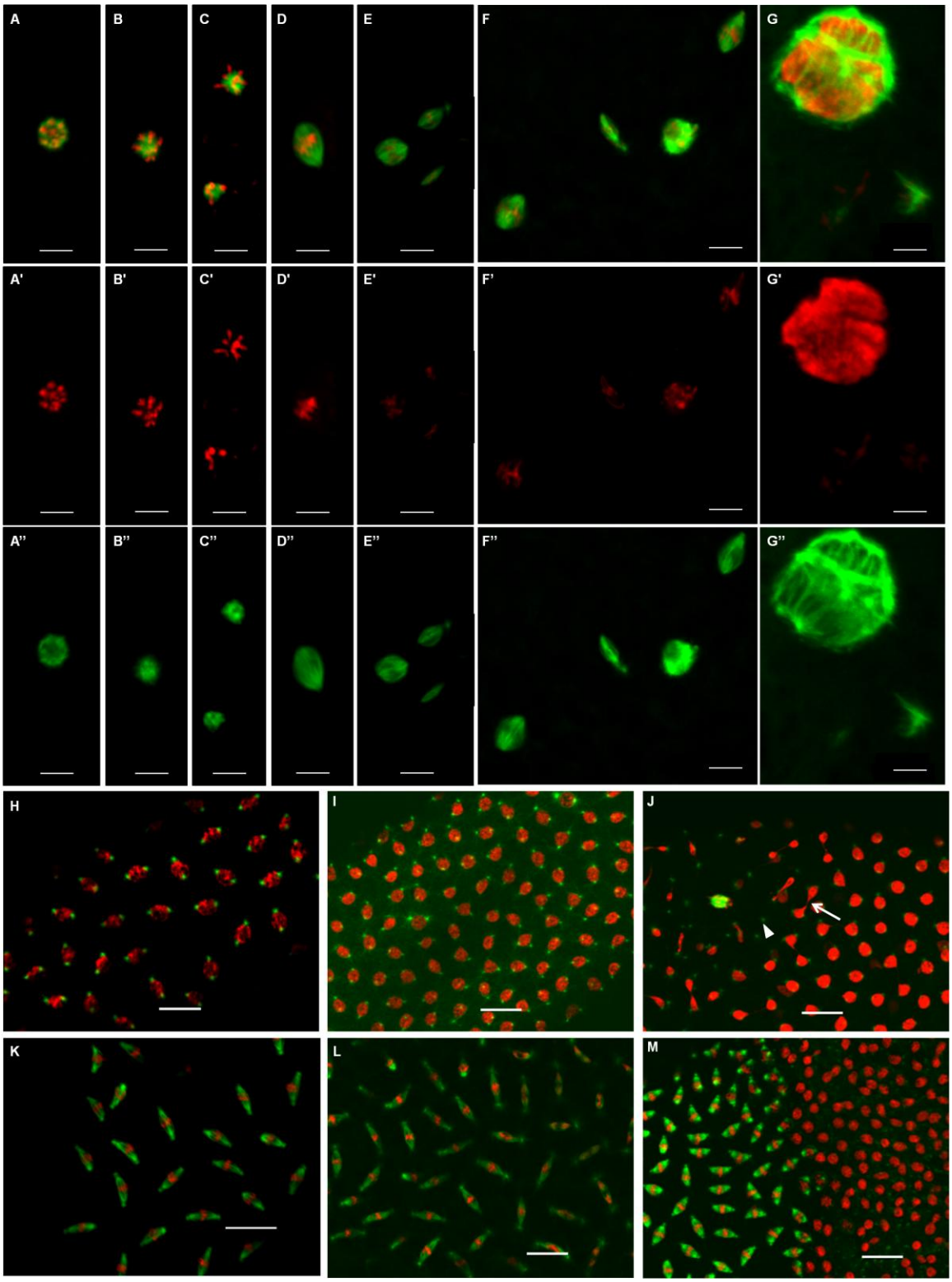
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Figure 6 – Cyclin A needs to be degraded for proper completion of meiosis and proper embryonic development. DNA is red and microtubules are green. All embryos are fertilized and derived from 0-2 hour collections from flies (wildtype or *mat67-Gal4-UASp-Venus:ΔCyclin A*) kept at 25°C. (A-A'') shows a polar-body in wildtype embryos. The presence of a polar body indicates proper completion of meiosis. (B-G'') depict the various phenotypes observed in the presence of Venus:ΔCyclin A. (B-B'') shows a normal polar body. (C'C'') shows two polar bodies. (D-D'') depicts a single meiotic spindle structure. (E'-E'') and (F-F'') show three and four meiotic spindle structures respectively. (G'-G'') depicts a large mass of DNA associated with microtubules. (H) shows a wildtype embryo during interphase, in which nuclei are associated with two centrosomes each. (I) shows a phenotypically normal Venus:ΔCyclin A embryo in interphase. (J) shows an abnormal interphase embryo that contains meiotic products, nuclei connected via chromatin bridges (arrow), and multiple free centrosomes (arrowhead). (K) shows a wildtype embryo in mitosis. (L) shows a phenotypically normal Venus:ΔCyclin A embryo in mitosis. (M) shows a Venus:ΔCyclin A embryo that is both in mitosis and in interphase. Scale bars for (A-G'') are 10 um and for (H-M) are 20 um.

meiosis (**Figure 6D-G''**). Among these, some contained single spindle structures associated with DNA (**Figure 6D-D''**), while others contained two to four spindle structures that were different in shape and associated with different amounts of DNA (**Figure 6E-F'')**. Large masses of DNA associated with spindles were also observed (**Figure 6G-G''**).

Wildtype embryos undergoing syncytial mitoses contain evenly spaced nuclei that divide synchronously. During interphase, the embryo contains nuclei, which appear as DNA masses, associated with two centrosomes each (**Figure 6H**). During mitosis, the embryo contains astral spindles associated with chromosomes (**Figure 6K**). Among the 30% of Venus:ΔCyclin A embryos that underwent embryonic development, 44% exhibited normal mitoses (**Figure 6I,L**). However, 56% of embryos displayed abnormal, asynchronous mitoses (**Figure 6J,M**). Most appeared to contain remnants of meiotic spindles, suggesting that meiosis had not completed and the paternal DNA was involved in the mitotic divisions. The phenotypes of the asynchronously dividing embryos also varied. In the less severe phenotypes, nuclei connected via chromatin bridges were observed (arrow in **Figure 6J**); centrosomes devoid of nuclei were also observed (arrowhead in **Figure 6J**). The more severe phenotypes involved sharp gradients of asynchrony within the embryo, in which different regions of the embryo were found in different stages of mitosis (**Figure 6M**). In the embryo shown in **Figure 6M**, half the DNA is in a metaphase state whereas the other half is in interphase. Furthermore, the interphase nuclei are unevenly distributed and do not appear to be associated with centrosomes. Overall, these phenotypes suggest that Cyclin A degradation is essential

for proper meiosis completion and for proper embryonic development.

Timed collections (0-20 minutes, 20-40 minutes, and 40-60 minutes) were performed to determine how meiosis proceeded in the presence of Venus:ΔCyclin A (**Figure 7** and **Table 6**). These timed collections were meant to give a better understanding of the sequence of events that took place from meiosis to mitosis in embryos expressing the stabilized Cyclin A. In the early 0-20 minute collections, 3% of wildtype eggs (n=44) were in meiosis II (**Figure 7A-A''** and **Table 6**). The example shown in **Figure 7A** is in metaphase II of meiosis. Although there is some bleed-through in the DNA channel, the DNA is aligned at the metaphase plates and the spindles are fairly symmetrical and organized. During this same time interval, 52% of wildtype eggs had completed telophase II and formed four haploid meiotic products; 45% had completed meiosis and formed polar bodies (**Table 6**). The percentage of meiotic figures were higher in Venus:ΔCyclin A embryos. Approximately 2% of embryos were in meiosis I (**Figure 7B-B''** and **Table 6**). The embryo shown in **Figure 7B** appears to be undergoing an aberrant anaphase I, in which DNA is distributed along the meiotic spindle. 24% of Venus:ΔCyclin A embryos were in meiosis II. 92% of these meiosis II embryos demonstrated abnormalities in DNA arrangement and/or spindle morphology. In almost all the meiosis II embryos observed, each meiosis II spindle was associated with different amounts of DNA. In many of these embryos, the DNA was found at the spindle equators, similar to what is seen during metaphase II (**Figure 7C-C''**). However, in majority of the embryos, the DNA was found at the spindle equators and it was also found at the outer poles of the spindles and/or at the central spindle pole body (**Figure**

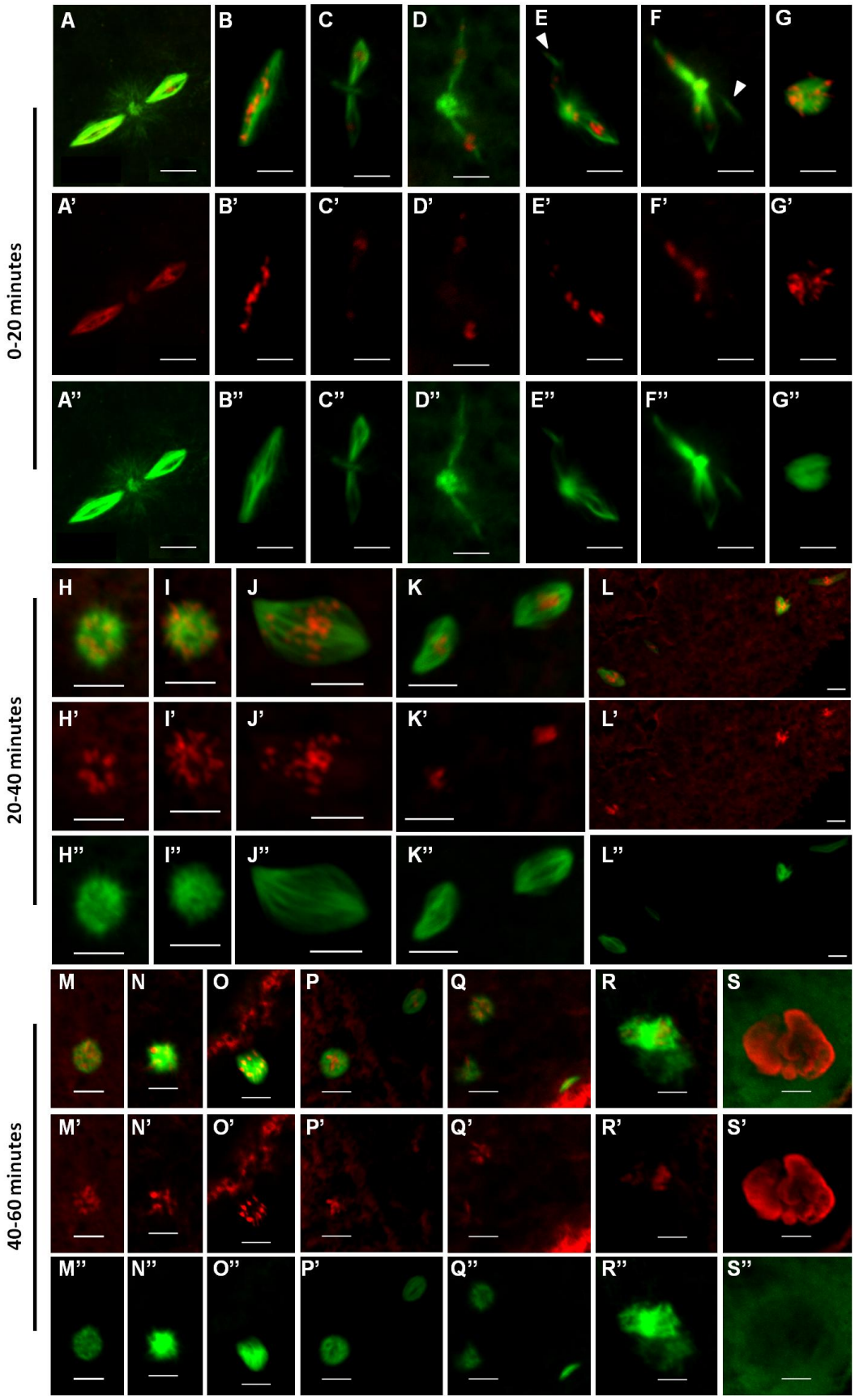
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Figure 7 – Cyclin A needs to be degraded for proper completion of meiosis. DNA is red and microtubules are green. The collection times are indicated on the left side of the corresponding figures. Fertilized eggs were collected from flies (wildtype or *mat67-Gal4-UASp-Venus:ΔCyclin A*) kept at 25°C. (A-A'') show a wildtype embryo in metaphase II. There is some bleed-though in the DNA channel. In the presence of Venus:ΔCyclin A, meiosis I and II were defective. (B-B'') show an aberrant anaphase I spindle with DNA distributed along its length. (C-F'') show meiosis II spindles. The spindle morphology of the meiosis II spindles is abnormal. (C-C'') has a dispersed spindle pole body. (D-F'') have prominent spindle pole bodies associated with DNA. (E-F'') also have spindle fibers dissociating from the main spindle (arrowheads). The two meiosis II spindles in (C-F'') are each associated with different amounts of DNA. In (C-C''), the DNA is arranged at the spindle equators; thus, this embryo is in metaphase II. In (D-F''), however, DNA is found at spindle equators and also at outer spindle poles and/or spindle pole bodies. Some of the DNA signal at the spindle pole body in (F-F'') may be bleed-through from the Tubulin channel. (G-G'') show a polar-body like structure. In later 20-40 minute collections, wildtype embryos contained a polar body (H-H''). Some Venus:ΔCyclin A embryos also contained polar bodies (I-I''). Single spindle- (J-J''), two spindle- (K-K''), three spindle-, and four spindle- (L-L'') structures were also observed in the presence of Venus:ΔCyclin A. (K-K'') resembles a metaphase II spindle. The 40-60 minute collections showed similar phenotypes to the 20-40 minute collections. Wildtype embryos contained polar bodies (M-M''). Embryos containing Venus:ΔCyclin A contained normal polar bodies (N-N''), single spindle structures (O-O''), two spindle structures (P-P''), three spindle structures

(Q-Q’’), and four spindle structures. Some embryos also contained small amounts of DNA attached to large amounts of microtubules (R-R’’) or large amounts of DNA devoid of microtubules (S-S’’). All scale bars are 10 um.

	Metaphase I	Anaphase I	Metaphase II	Anaphase II	Telophase II Complete	In Meiosis But Not Decipherable	Polar Body	N
wildtype (0-20 min)	0%	0%	3%	0%	52%	0%	45%	44
wildtype (20-40 min)	0%	0%	0%	0%	1%	0%	99%	95
wildtype (40-60 min)	0%	0%	0%	0%	1%	0%	99%	26
***mat67-Gal4-Venus:ΔCyclin A* (0-20 min)**	2%	3%	16%	8%	3%	43%	25%	100
***mat67-Gal4-Venus:ΔCyclin A* (20-40 min)**	0%	0%	3%	0%	0%	88%	9%	34
***mat67-Gal4-Venus:ΔCyclin A* (40-60 min)**	0%	0%	0%	0%	0%	50%	50%	26

Table 6 – Summary of developmental stages observed in timed collections of Venus:ΔCyclin A embryos. All embryos were fertilized and collected from flies kept at 25°C. Fly genotype is indicated in the left column. N represents the total number of embryos counted.

7D-F''). These phenotypes indicated that chromosome missegregation was taking place in the presence of stabilized Cyclin A. In some cases, the spindle morphology was also abnormal. Many spindles had very prominent spindle pole bodies, which contained a large number of microtubules (**Figure 7D-F''**), while others were either missing spindle pole bodies or had ones that appeared somewhat dispersed. The spindles with abnormal DNA distributions did not appear straight and organized. In some embryos, small, thin spindle strands had dissociated from the main meiotic spindle (arrowheads in **Figure7E,F**). Regardless of these meiosis II phenotypes, 25% of embryos completed meiosis to form normal-looking polar bodies (**Table 6**). Embryos that did not complete meiosis properly often contained one to four spindles, which varied in size, shape and DNA content; some spindles were more like meiotic spindles whereas others were more rounded and polar body-like (**Figure 7G-G''**). Thus, because there are many more Venus:ΔCyclin A embryos in meiosis II than wildtype embryos, it seems that the progression of meiosis is slightly delayed in the presence of stabilized Cyclin A. A control wildtype Venus:Cyclin A transgene was also tested in a similar manner and did not significantly interfere with meiosis completion.

In the 20-40 minute collections, almost all wildtype embryos had completed meiosis and formed polar bodies (**Figure 7H-H''** and **Table 6**). The phenotypes were more varied in Venus:ΔCyclin A embryos. Many embryos had formed polar bodies (**Figure 7I-I''**), indicating that meiosis was complete. Some embryos were in recognizable stages of meiosis (**Figure 7J-K''**). The embryo shown in **Figure 7J** appears to be in metaphase I of meiosis, whereas the embryo in **Figure 7K** is in metaphase II. Other

embryos were in indiscernible stages of meiosis and contained up to four spindle structures, which varied in shape and DNA content (**Figure 7L-L''**).

A similar pattern was observed in the 40-60 minute collections. All wildtype embryos had completed meiosis, as indicated by the presence of polar bodies (**Figure 7M-M''** and **Table 6**). Most Venus:ΔCyclin A embryos had also completed meiosis (**Figure 7N-N''** and **Table 6**), while the remainder were in indiscernible stages of meiosis (**Figure 7O-S''** and **Table 6**). Embryos with more than four meiotic spindle structures were also observed in these later collections. A few embryos contained large amounts of spindles associated with small amounts of DNA (**Figure 7R-R''**) or large amounts of DNA with or without spindles (**Figure 7S-S''**); the DNA mass shown in **Figure 7S** is not associated with spindles. The amount of DNA in some of these embryos suggested that DNA replication was taking place.

Studies suggest that Separase may be inhibited by two mechanisms: through inhibitory binding by Securin or inhibitory phosphorylation by Cyclin-CDK1 complexes (Stemmann *et al.*, 2001). Among the different Cyclin-CDK1 complexes, Cyclin A-CDK1 is the best candidate for Separase inhibition since stabilized Cyciln A can effectively delay chromosome separation in Drosophila embryos (Leismann and Lehner, 2003; Jacobs *et al.*, 2001). To determine whether Cyclin A-CDK1 might affect chromosome segregation during meiosis, FISH was performed on Venus:ΔCyclin A embryos (collected for 0-1 hours). The FISH probe was specific for a 359 bp repeat region found in the centromeres of X-chromosome. Successful completion of meiosis is indicated by the presence of a polar body associated with four FISH dots in unfertilized eggs or three FISH dots in

embryos. All fertilized wildtype embryos had three FISH dots associated with their polar bodies, indicating that meiosis had completed successfully (**Figure 8A-A''**). However, FISH staining of Venus:ΔCyclin A embryos revealed defects in meiosis completion and meiotic product arrest. The FISH results indicated that meiotic exit was successful in some embryos due to the presence of round spindle structures with three (**Figure 8B-B''**) or four (**Figure 8C-C''**) FISH signals. Spindle structures with two FISH signals were also observed at low frequencies, implying that chromosome segregation was not always successful. Multiple spindles, each associated with one or more FISH dots were also seen (**Figure 8D-G''**). Interestingly, some embryos contained spindle structures with a large number of FISH dots (**Figure 8E-F''**), indicating that the meiotic products underwent DNA replication after the completion of meiosis. Therefore, the FISH results suggest that that meiosis can be completed successfully in the presence of Venus:ΔCyclin A; however, this is not the case in all embryos, implying that Cyclin A-CDK1 also has the capacity to interfere with proper chromosome segregation. Overall, it appears that Cyclin A degradation is required for proper and timely meiotic exit, the prevention of polyploidy, and normal embryonic development.

3.4 Cyclin A-CDK1 does not regulate APC/C activity

There is evidence that Cyclin-CDK1 complexes regulate APC/C activity via phosphorylation. Studies have shown that APC/$C^{Cdc20/Fzy}$ is positively regulated by phosphorylation whereas APC/$C^{Cdh1/Fzr}$ is negatively regulated (Kramer *et al.*, 2000). Studies have also shown that Cyclin A-CDK1 specifically inhibits APC/$C^{Cdh1/Fzr}$ activity in Drosophila embryos (during mitosis) to allow accumulation of Cyclins B and B3 (Reber *et*

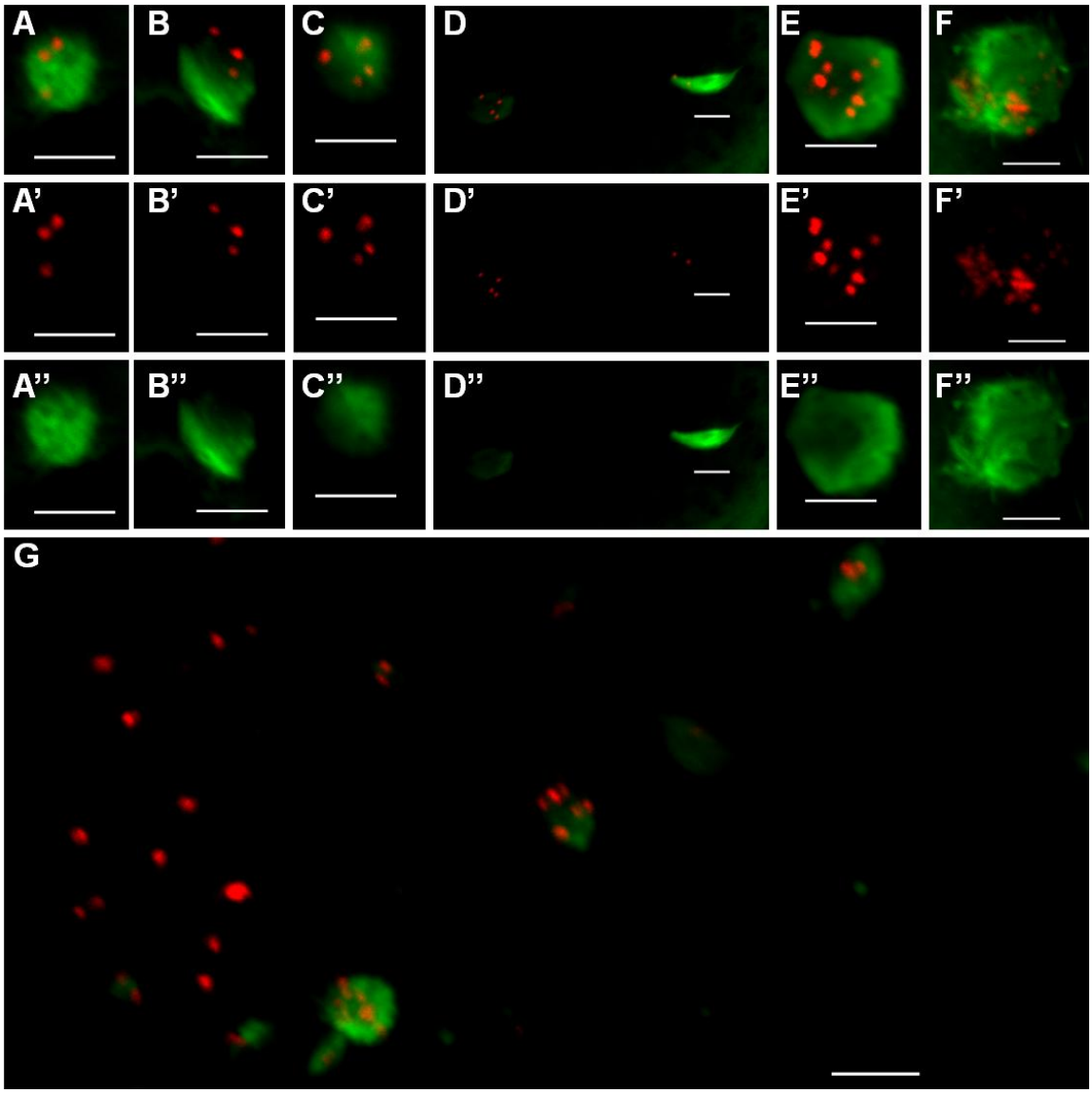
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Figure 8 – Non-degradable Cyclin A promotes DNA replication of meiotic products.

FISH probe specific for the centromeric region of the X-chromosome is shown in red and microtubules are shown in green. All eggs were 0-1 hours old, fertilized, and collected from flies (wildtype or *mat67-Gal4-UASp-Venus:ΔCyclin A*) kept at 25°C. (A-A'') show a wildtype polar body with three FISH dots, as seen in fertilized eggs. (B-F'') depict Venus:ΔCyclin A embryos arrested with meiotic products. (B-B'') resembles a wildtype polar body with three FISH dots. (C-C'') resembles a normal polar body that would be seen in unfertilized eggs (with four FISH dots). (D-D'') shows two spindle structures associated with two and four FISH dots. The meiotic products in (E-G) are associated with more than four FISH dots due to DNA replication. Many spindle-free FISH dots are also present in (G). All scale bars are 10 um.

al., 2006). However, during meiosis, APC/$C^{Cdh1/Fzr}$ is inactive; thus, proteins are targeted for degradation by APC/$C^{Cdc20/Fzy}$ and APC/C^{Cort} (Swan and Schupbach, 2007).

To determine whether Cyclin A regulates APC/C activity during meiosis, the levels of select APC/C substrates (Cyclin A, Cyclin B, Cyclin B3) were examined in the presence of Venus:ΔCyclin A. Interestingly, ΔCyclin A did not affect the levels of any of these Cyclins (**Figure 9A-C**). Therefore, it seems that Cyclin A-CDK1 does not regulate APC/C activity during meiosis.

Next the meiotic role of Cyclin B was examined using similar experiments.

3.5 Cyclin B localization during oogenesis

To determine the localization of Cyclin B during meiosis, a *UASp-Venus:CyclinB* transgene was constructed and expressed in the germline using the *nos-Gal4:VP16* driver. Venus-Cyclin B was expressed in the germarium, where it is known to regulate the mitotic divisions that give rise to the oocyte (**Figure 10A- B''**) (Wang & Lin, 2005). Venus-Cyclin B localized around the oocyte DNA in metaphase I-arrested stage 14 oocytes (**Figure 10C-C''**). It has been previously reported that Cyclin B localizes to the spindle midzone during metaphase I; therefore, this localization is in agreement with previous studies (Swan & Schupbach, 2007). A D-box mutant form of Cyclin B (CyclinB-TPM:GFP) also exhibited a similar localization pattern (**Figure 10D-D''**).

As with Venus:Cyclin A, the primary purpose of this localization experiment was to determine whether Cyclin B contributes to nuclear envelope breakdown during meiosis. If Cyclin B directly contributes to NEBD, it would be expected to enter the nucleus slightly before mid-stage 13, which is when NEBD occurs. However, Venus-

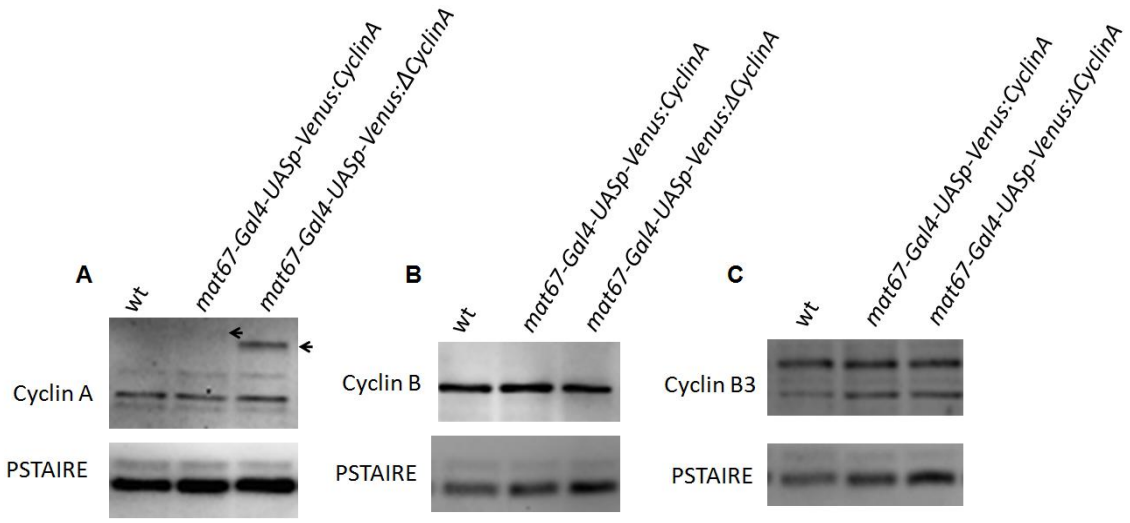

Figure 9 – Cyclin A-CDK1 does not regulate the activity of APC/C during meiosis.

Western blotting was performed on unfertilized eggs collected for 0-2 hours from flies (wildtype, *mat67-Gal4-UASp-Venus:Cyclin A* or *mat67-Gal4-UASp-Venus:ΔCyclin A*) kept at 25°C. Western blots were probed for Cyclin A (A), B (B), or B3 (C), all of which are APC/C substrates. PSTAIRE (CDK1) was used as a loading control. Arrows in (A) point to Venus:Cyclin A or Venus:ΔCyclin A. Multiple bands seen in (A, C) represent different phosphorylation states of Cyclin A (A) and Cyclin B3 (C). Western blotting showed that the levels of all endogenous Cyclins were unaffected in eggs expressing Venus:Cyclin A or Venus:ΔCyclin A, implying that Cyclin A-CDK1 does not regulate APC/C activity.

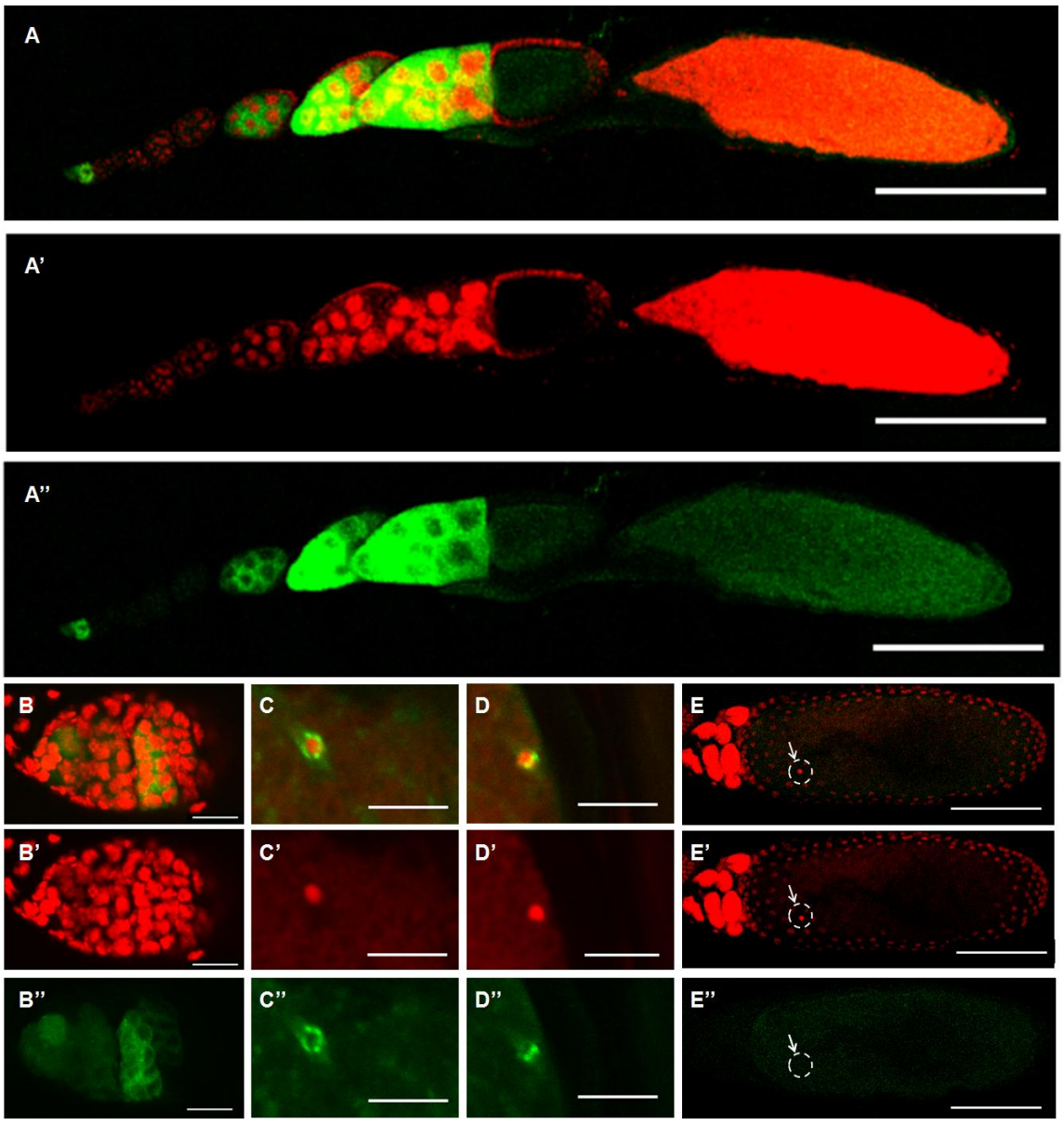
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Figure 10 – Characterization of Cyclin B localization during oogenesis. DNA is shown in red whereas Venus-Cyclin B (A-C'', E-E'') and Cyclin B-GFP-TPM (D-D'') are shown in green. (A-A'') shows the localization pattern of Venus-Cyclin B within the ovariole. (B-B'') shows that Venus-Cyclin B is present in the germarium, specifically in the germline stem cells and in the cystocytes. (C–C'') depicts localization in stage 14 oocytes, where Venus-Cyclin B localizes around the oocyte DNA. (D-D'') shows the localization of a D-box mutant Cyclin B-TPM:GFP, which also localizes around the oocyte DNA. (E-E'') shows that Venus-Cyclin B does not localize to the oocyte nucleus (arrowhead) before NEBD. All ovarioles are oriented with the germarium to the left. Scale bars for (A-A'', E-E'') are 100 um, while scale bars for (B-D'') are 10 um.

Cyclin B did not localize to the oocyte nucleus in any stage of oogenesis, suggesting that it may not be involved in NEBD (**Figure 10E-E''**).

To confirm the finding that Cyclin B may not be involved in NEBD, the timing of NEBD was examined in *Cyclin B*$^{-}$*; HsCycB* flies (discussed below). Anti-lamin antibodies were used to visualize the oocyte nuclei, but were unsuccessful at staining the nuclei of later-stage oocytes. Thus, nuclei were visualized by examining the yolk autofluorescence around the oocyte DNA; since yolk is excluded from the oocyte nucleus, the absence of autofluorescence indicates that the nucleus is still intact and NEBD has not occurred. In wildtype meiosis, NEBD occurred during mid-stage 13 of oogenesis (**Figure 11C** and **Table 7**). In mutant oocytes, NEBD also occurred during mid-stage 13 (**Figure 11D and Table 7**); therefore, the timing of nuclear envelope breakdown was not affected in the absence of Cyclin B-CDK1 activity. This suggests that Cyclin B is either not involved in nuclear envelope breakdown or it is redundant with other proteins that contribute to this process.

3.6 Cyclin B is required for proper completion of meiosis

It is difficult to examine meiosis in the absence of Cyclin B because *Cyclin B* mutants are sterile. The ovaries of these mutants contain very few germline stem cells, which fail to self-renew (Wang *et al*., 2005). To overcome this problem, a heat-shock inducible *Cyclin B* (*HsCycB*) transgene was introduced into *Cyclin B*$^{-}$ flies, which were made by combining the *CycB*^{2} allele with the deficiency *Df(2R)59AB*. The purpose of this *HsCycB* transgene was to provide Cyclin B during the early germline stem cell divisions to promote ovary development and meiosis. The *Cyclin B*$^{-}$*; HsCycB* flies and their *Cyclin*

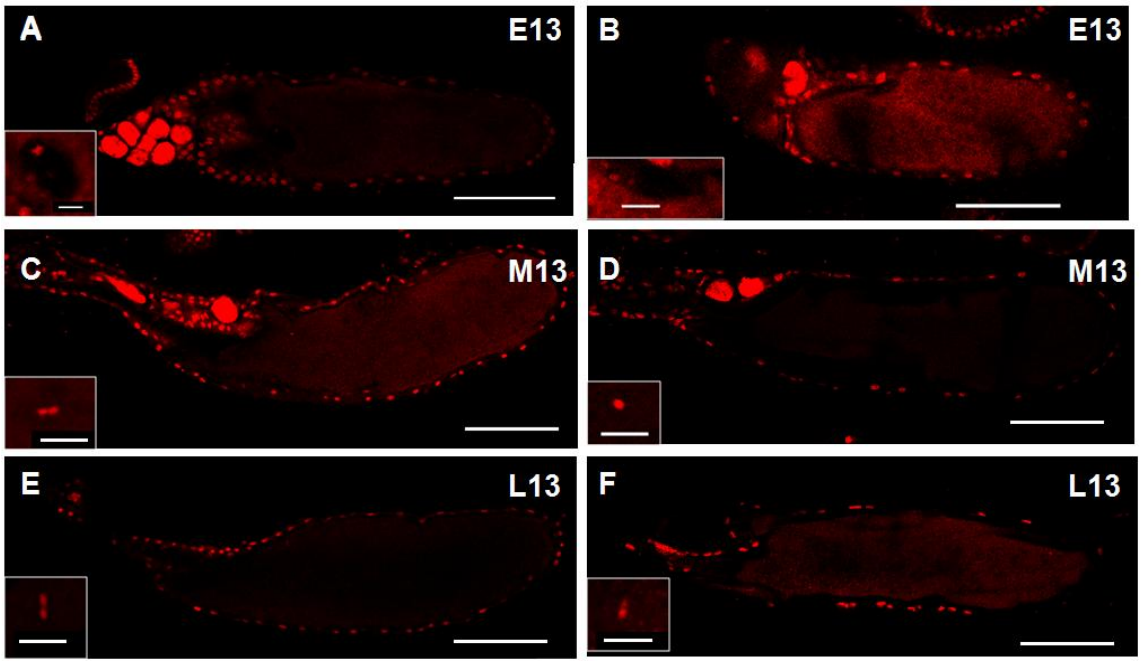

Figure 11 – Cyclin B is not required for NEBD during meiosis. DNA is shown in red. E13 refers to early stage 13 oocytes; M13 refers to mid-stage 13 oocytes; and L13 refers to late state 13 oocytes. NEBD occurs during mid-stage 13 (C). Oocytes were obtained from wildtype or *Cyclin B*$^{-}$; *HsCycB* flies kept at 29°C. Insets show close-ups of oocyte DNA. Nuclei appear as holes in the staining pattern. (A,C,E) depict wildtype stage 13 oocytes. There is a nucleus present during early stage 13 (A), but none is present in mid-stage 13 (C) and late stage 13 (E) oocytes from wildtype flies. (B, D, F) show *Cyclin B*$^{-}$; *HsCycB* stage 13 oocytes. There is a nucleus present in early stage 13 oocytes (B), but none during mid-stage 13 (D) and late stage 13 (F). Therefore, Cyclin B is not required for NEBD. Scale bars of insets are 10 um, while all other scale bars are 100 um.

	Early Stage 13		Mid-Stage 13		Late Stage 13	
Genotype	**Nucleus**	**No Nucleus**	**Nucleus**	**No Nucleus**	**Nucleus**	**No Nucleus**
Wildtype	4/4	0/4	0/10	10/10	0/18	18/18
Cyclin B^{-}; *HsCycB*	4/4	0/4	0/9	9/9	0/18	18/18

Table 7 – Cyclin B is not required for NEBD during meiosis. The table lists frequencies of early-, mid-, and late-stage 13 oocytes with or without nuclei. All stage 13 oocytes were obtained from wildtype or *Cyclin B*$^{-}$; *HsCycB* flies kept at 29°C. The NEBD timing was not affected in *Cyclin B*$^{-}$; *HsCycB* mutants.

B^{-} siblings were subjected to various temperatures to examine ovary development and egg laying (**Table 8**). The *Cyclin B*$^{-}$ flies failed to develop ovaries, and, therefore, failed to lay eggs at all temperatures tested. However, the *Cyclin B*$^{-}$*; HsCycB* flies laid eggs at 25°C and at 29°C. The amount of eggs laid at 25°C was significantly reduced (approximately 30% of eggs laid at 29°C). Furthermore, the number of eggs laid at 29°C was also low compared to wildtype (approximately 10-30% of wildtype). The eggs failed to hatch at all temperatures tested. As a control, wildtype flies expressing the *HsCycB* transgene were also tested; and they were found to lay viable eggs at all temperatures, indicating that the lethality was not due to Cyclin B overexpression.

The *Cyclin B*$^{-}$*; HsCycB* flies were also subjected to heat-shocks at 37°C to determine whether a higher temperature could fully rescue the *Cyclin B* mutant. However, these heat-shocks were insufficient to rescue the inviable embryo phenotype. This may be due to the poor expression of the *Hsp70* promoter in the germline. Since it was more convenient to raise the flies at 29°C (versus the periodic 37°C heat-shocks), the experiments hereafter were performed at 29°C.

To determine the cause of embryonic lethality, fertilized eggs (0-4 hours) were collected from *Cyclin B*$^{-}$*; HsCycB* flies and examined via immunostaining. While all wildtype embryos contained polar bodies and were undergoing synchronous, syncytial mitoses (**Figure 12A**), all mutant embryos were abnormal and did not contain normal polar bodies (**Figure 12B-C**). These mutant embryos did not contain any mitotic spindles. Instead, they contained multiple, abnormal DNA-associated spindles and many centrosomes (**Figure 12B-C**). Centrosomes are known to originate from the paternal

Temperature (°C)	Egg Laying	
	***Cyclin B*$^{-}$**	***Cyclin B*$^{-}$*; HsCycB***
18 °C	Do not lay eggs	Do not lay eggs
22 °C	Do not lay eggs	Do not lay eggs
25 °C	Do not lay eggs	Lay eggs (~30% of 29°C)
29 °C	Do not lay eggs	Lay eggs
37 °C	Do not lay eggs	Lay eggs

Table 8 – Characterization of *Cyclin B*$^{-}$*; HsCycB* mutant flies. *Cyclin B*$^{-}$ and *Cyclin B*$^{-}$*; HsCycB* flies were placed at various temperatures after eclosing and egg-laying was examined. The *Cyclin B*$^{-}$*; HsCycB* flies laid the most eggs at 29°C and after periodic heat-shocks of 37°C than at other temperature tested. The *Cyclin B*$^{-}$ flies did not lay eggs at any temperature examined.

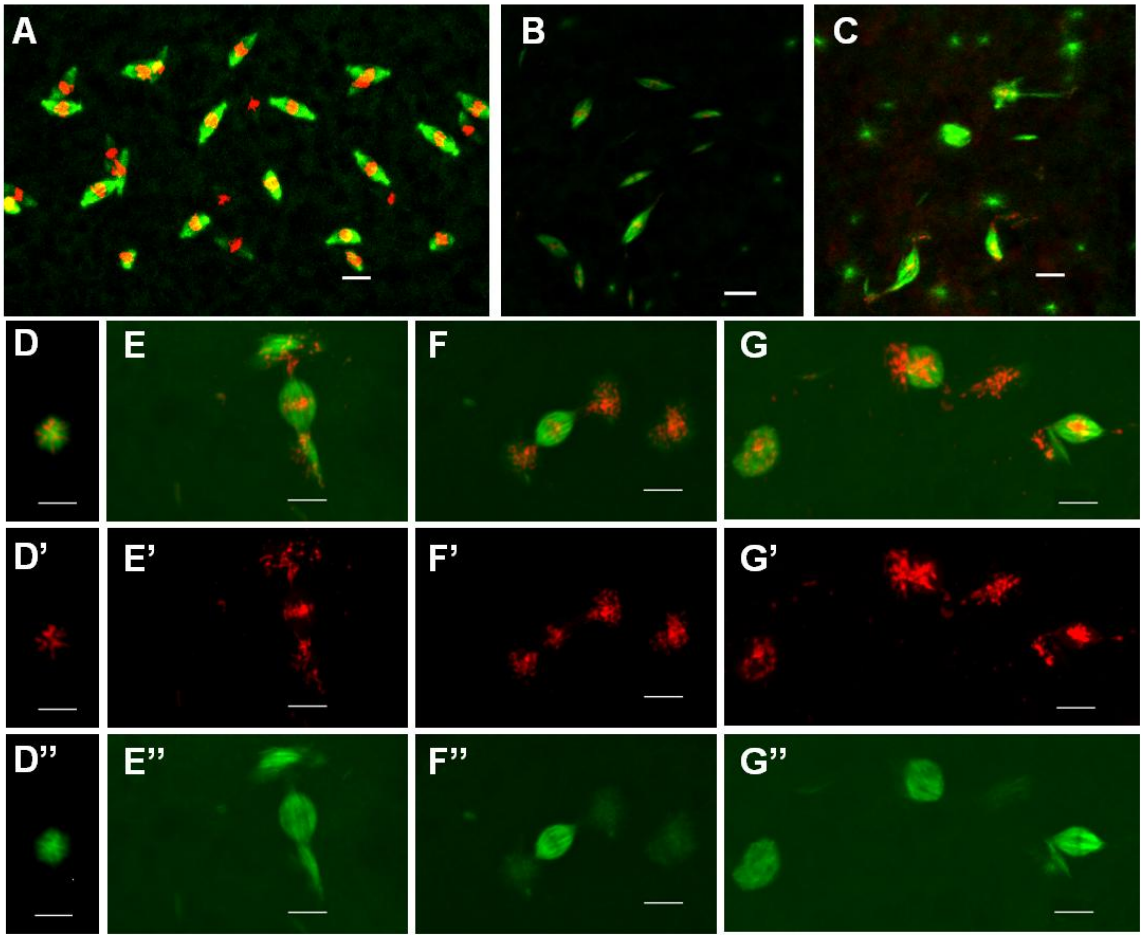
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Figure 12 – Cyclin B is required for proper completion of meiosis and for initiation of embryonic development. DNA is shown in red and microtubules are shown in green. (A-C) show fertilized eggs obtained from 0-4 hour collections from flies kept at 29°C. (A) shows a wildtype embryo in which all nuclei are in metaphase. (B, C) show *Cyclin B^{-}; HsCycB* embryos. These embryos contain abnormal spindles and multiple free centrosomes. (D-G'') depict unfertilized eggs obtained from 0-2 hour collections from wildtype or *Cyclin B^{-}; HsCycB* flies kept at 29°C. (D-D'') show a polar body in a wildtype egg. (E-G'') show *Cyclin B^{-}; HsCycB* eggs. These eggs do not contain discernible polar bodies; instead, they contain multiple spindles associated with large amounts of DNA. Scale bars for all figures are 10 um.

pronucleus, however, it was unclear whether the observed spindles were maternally or paternally derived.

To eliminate phenotypic-contribution from the male pronucleus, unfertilized eggs were collected from mutant flies. All wildtype eggs contained polar bodies (Figure **12 D-D''** and **Table 9**). None of the mutant eggs had polar bodies, indicating that meiosis had not completed successfully (**Figure 12E-G''** and **Table 9**). The mutant eggs also did not contain any centrosomes; instead, they contained many, abnormal DNA-associated spindles (**Figure 12E-G''**), indicating that the spindle structures observed previously in the fertilized eggs were maternally derived. To better understand the events leading to this complex phenotype, meiosis was examined more closely.

First, stage 14 oocytes were examined to determine whether a proper metaphase I arrest could be achieved in the absence of Cyclin B. Western blotting analysis revealed that in the mutant stage 14 oocytes , Cyclin B protein levels were at 4% of wildtype levels (**Figure 13A**). To determine whether metaphase I was defective, stage 14 oocytes were stained with OliGreen and DNA morphology was examined via confocal microscopy (**Figure 13C-F**). All wildtype oocytes were normal (n=149): DNA was found as either one dot or two equal dots (**Figure 13C**). However, 12% of mutant oocytes were abnormal (n=182): these oocytes contained two, three, or four unequal DNA dots (**Figure 13D-F**).

To observe this DNA defect more closely, oocytes were hand-dissected (to remove the chorion and vitelline membrane) and immunostained for DNA and microtubules (**Figure 13G-J**). 7 of 10 of examined mutant oocytes exhibited a normal

	Meiosis Incomplete	Meiosis Complete	N
wildtype (29°C) (0-2 hours)		100%	103
Cyclin B^{-}***; HsCycB*** **(0-2 hours)**	100%		74

Table 9 – Summary of developmental stages observed in *Cyclin B*$^{-}$*; HsCycB* unfertilized eggs. All unfertilized eggs were collected for 0-2 hours from flies kept at 29°C. 'Meiosis Incomplete' refers to the presence of meiotic spindles. 'Meiosis Complete' refers to the presence of a polar body. Genotypes of flies are indicated in the left column. N represents the total number of eggs counted.

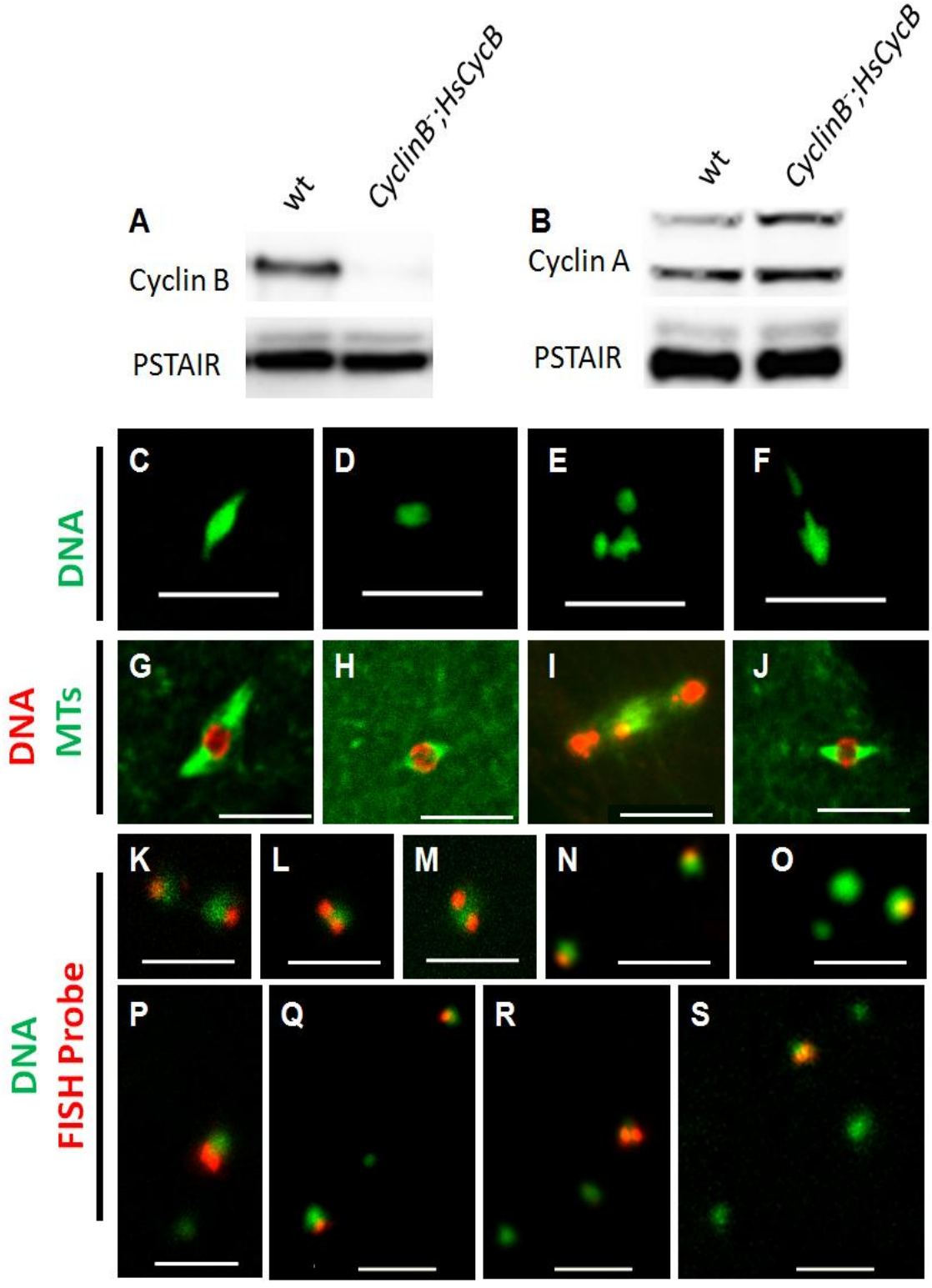
wt
CyclinB⁻;HsCycB
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Figure 13 – Cyclin B is required for maintenance of metaphase I arrest in stage 14 oocytes and for proper chromosome segregation during anaphase I. The Western blots in (A, B) were done on stage 14 oocytes collected from wildtype or *Cyclin B^{-}; HsCycB* flies kept at 29°C. The blots were probed for Cyclin B (A) and Cyclin A (B). PSTAIRE (CDK1) was used as a loading control. (A) shows that the levels of Cyclin B are drastically reduced in *Cyclin B^{-}; HsCycB* oocytes, whereas (B) shows that Cyclin A levels are not significantly altered. Densitometry analysis shows that Cyclin B levels are reduced to 2-4% of wildtype (see Methods and Materials). (C-D) depicts DNA staining of oocytes using OliGreen. (C) depicts a wildtype oocyte, whereas (D-F) depict *Cyclin B^{-}; HsCycB* oocytes. The DNA staining pattern in (D) is similar to that of wildtype; however, the staining patterns of (E, F) are clearly abnormal. (G-J) show the spindle (green) and DNA (red) morphology of hand-dissected stage 14 oocytes. (G, H) are wildtype oocytes, whereas (I, J) are *Cyclin B^{-}; HsCycB* oocytes. (I) is undergoing precocious anaphase I involving chromosome missegregation. (K-L) depict FISH- (red) and DNA- (green) staining of stage 14 oocytes. (K, L) show wildtype oocytes. (M-S) show *Cyclin B^{-}; HsCycB* oocytes. (M, N) are similar to wildtype, whereas (O-S) are undergoing chromosome missegregation. All scale bars are 10 um.

metaphase I arrest (**Figure 13J**). The remaining 3 displayed a precocious anaphase I phenotype (**Figure 13I**), in which the DNA was unevenly distributed along the meiosis I spindle. This DNA distribution suggested that a fraction of the mutant oocytes were undergoing chromosome missegregation. Although a small number of dissected oocytes were observed, the phenotypes they exhibited corresponded with what was observed by DNA staining alone. Thus, it seems that Cyclin B is not necessary to progress to metaphase I, but it may be required to maintain the arrest and coordinate proper chromosome segregation during anaphase I.

Fluorescent in-situ hybridization (FISH) was performed to determine whether chromosome missegregation was occurring within the mutant stage 14 oocytes. The FISH probe was specific for a 359 bp centromeric repeat in the X-chromosome. In wildtype oocytes (n=114), three types of staining patterns were observed, all of which appeared normal (**Figure 13K,L**). The most common phenotype was one DNA dot associated with two FISH dots (**Figure 13L**). The two less common phenotypes were one DNA dot associated with one FISH dot or two DNA dots associated with one FISH dot each (**Figure 13K**). The mutant oocytes showed a variety of staining patterns. 81% of mutant oocytes were normal (n=158). As seen in the wildtype oocytes, the most common staining pattern was one DNA dot associated with two FISH dots (**Figure 3M**), whereas one DNA dot with one FISH dot or two DNA dots with two FISH dots were present at lower frequencies (**Figure 13N**). However, 19% of the examined oocytes exhibited staining patterns typical of chromosome missegregation (**Figure 13O-S**). The most common abnormal phenotype observed was two unequal DNA dots with only one

FISH dot (**Figure 13P**), indicating that both X-chromosomes had segregated to the same spindle pole. More complex phenotypes included three DNA dots associated with only one FISH dot or two FISH dots (**Figure 13 O,Q-R**). Occasionally four DNA dots with one or two FISH dots were also observed (**Figure 13S**). All of these phenotypes indicated that a fraction of *Cyclin B^{-}; HsCycB* oocytes underwent chromosome missegregation.

Timed egg collections were performed to determine whether meiosis could be successfully completed in the absence of Cyclin B. These timed collections were performed by crossing mutant flies with sterile males; therefore, the resulting eggs were unfertilized. Cyclin B levels in unfertilized mutant eggs were reduced to 1.5-4% of wildtype levels (**Figure 16A**). For the timed collections, three different egg collection time intervals were used: 0-20 minutes, 20-40 minutes, and 40-60 minutes (**Figure 14** and **Table 10**).

Figure 14A shows wildtype meiosis II spindles observed in the 0-20 minute collections; the two spindles are symmetrical and associated with equal amounts of DNA. During this time interval, approximately 40% of wildtype eggs were in meiosis, whereas the other 60% had formed polar bodies (**Table 10**). However, all mutant eggs observed were in meiosis (**Table 10**). The phenotypes of these eggs confirmed that chromosome missegregation occurred during meiosis I because the resulting meiosis II spindles were attached to unequal amounts of DNA (**Figure 14B-D''**). The spindle morphology of the meiosis II spindles was also abnormal. The spindles were often kinked and spindle fibers were often seen flanking the main spindles; therefore, it

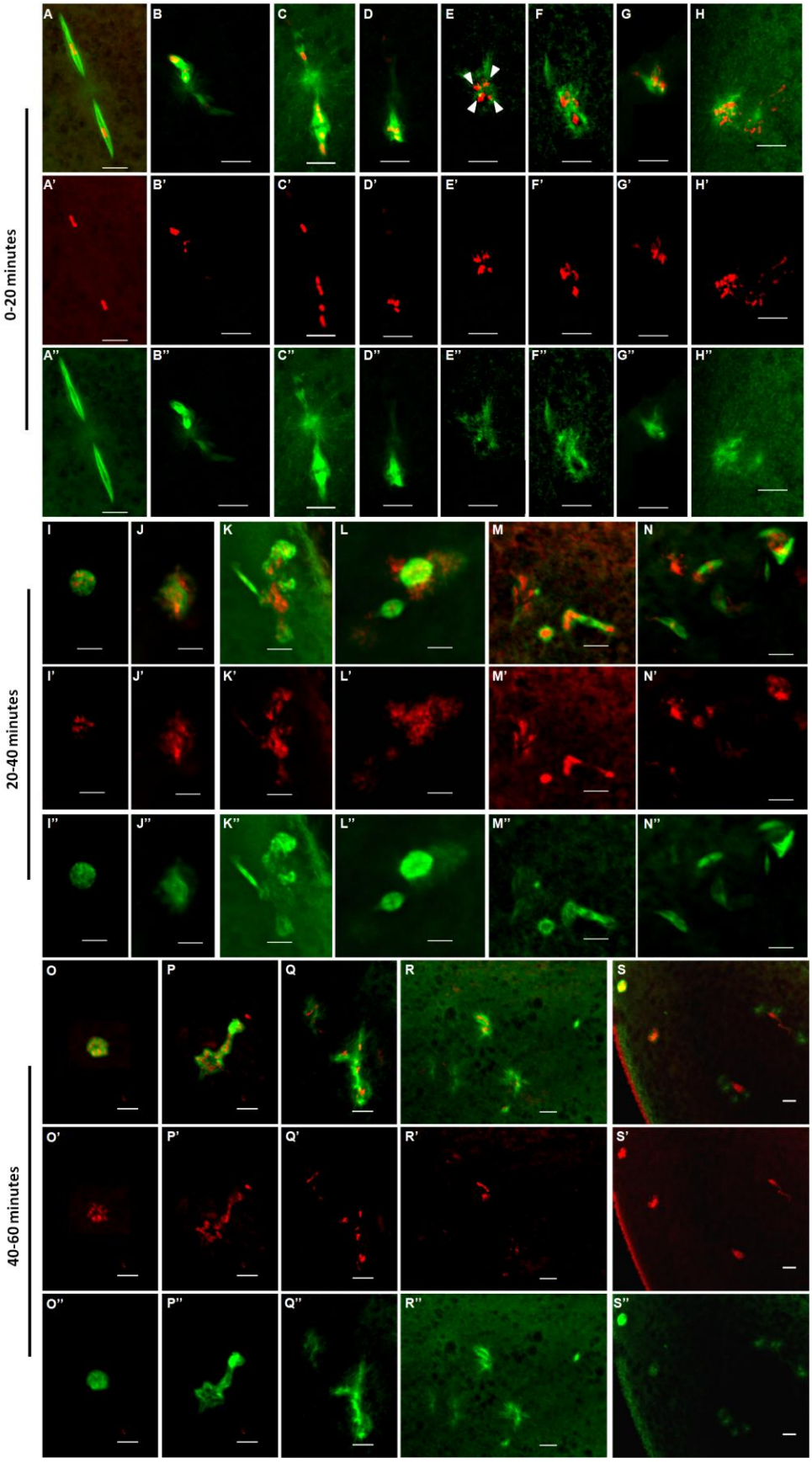
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Figure 14 – Cyclin B is required for proper chromosome segregation during meiosis II and proper completion of meiosis. DNA is red and microtubules are green. The collection times are indicated in the figure above on the left side. Unfertilized eggs were collected from wildtype or *Cyclin B^{-}; HsCycB* flies kept at 29°C. (A-A'') show a wildtype egg in metaphase II. (B-H'') show the contents of *Cyclin B^{-}; HsCycB* eggs. (B-D'') are clearly in meiosis II; the meiosis II spindles in these mutants are asymmetrical and associated with different amounts of DNA. (E-F'') show four meiotic products (arrowheads in E'), which indicate completion telophase II. In (G-H''), the meiotic products are undergoing DNA replication. (I-I'', O-O'') depicts a polar body in a wildtype egg in the later collections; the polar body marks successful completion of meiosis. (J-N'', P-S'') show *Cyclin B^{-}; HsCycB* mutants, which do not contain any discernible polar bodies. It seems that parts of the DNA fall away from the main replicating DNA mass and join with spindles (K-N'', P-S''). All scale bars represent 10 um.

	Metaphase I	Anaphase I	Metaphase II	Anaphase II	Telophase II Complete	In Meiosis But Not Decipherable	Polar Body	N
wildtype (0-20 min)	0%	0%	3%	0%	28%	9%	60%	35
wildtype (20-40 min)	0%	0%	0%	0%	5%	5%	90%	40
wildtype (40-60 min)	0%	0%	0%	0%	0%	0%	100%	49
***Cyclin B^{-}; HsCycB* (0-20 min)**	0%	0%	0%	24%	24%	52%	0%	35
***Cyclin B^{-}; HsCycB* (20-40 min)**	0%	0%	0%	0%	0%	100%	0%	32
***Cyclin B^{-}; HsCycB* (40-60 min)**	0%	0%	0%	0%	0%	100%	0%	28

Table 10 – Summary of developmental stages observed in timed collections of *Cyclin B^{-}; HsCycB* eggs. All eggs were unfertilized and collected from flies kept at 29°C. Genotypes of flies are indicated on the left. N represents the total number of eggs counted.

appeared as if the spindle fibers were not properly bundled together (**Figure 14B-B''**). The meiosis II spindles were asymmetrical due to association with unequal amounts of DNA; the spindle with more DNA also contained more microtubules (**Figure 14B-D''**). The DNA staining pattern of the meiosis II spindles indicated that chromosome nondisjunction was occurring during anaphase II (**Figure 14B-D''**). In the absence of Cyclin B, at least some mutant eggs completed telophase II and formed four meiotic products, which were surrounded by shells of microtubules (**Figure 14E-F''** and **Table 10**).

Later collections (20-40 minutes and 40-60 minutes) yielded similar results (**Table 10**). While wildtype eggs completed meiosis by forming polar bodies (**Figure 14I-I'', O-O''** and **Table 10**), none of the mutant eggs completed meiosis (**Figure 14J-N'',P-S''** and **Table 10**). All mutant eggs failed to form polar bodies and female pronuclei. Furthermore, many of these products failed to arrest after meiosis was complete; instead, they underwent repeated rounds of DNA replication as indicated by FISH (**Figure 15**) (see below). It seems that as the DNA mass(es) increased, parts of the DNA fell off the main mass(es) and associated with spindles. The size and morphology of the resulting spindles structures varied; some were long and thin while others were broader and more rounded (**Figure 14J-N'', S-S''**). Often times, both types of spindles were found within the same egg. Some eggs also contained spindles that were difficult to categorize (**Figure 14K-K'',P-R''**). Generally, the number of spindles within the egg increased as the egg aged. The amount of DNA and its position on each spindle also varied. Some spindles had DNA along their entire lengths, while others had DNA mostly at the spindle

equator. Some spindles also had large amounts of DNA associated with their poles (**Figure 14N-N''**). Free DNA without any spindles was also frequently observed. In some eggs, the intensity of DNA signal indicated that DNA replication was occurring.

FISH was performed to determine whether DNA replication was taking place within the *Cyclin B^{-}; HsCycB* eggs and also to determine whether chromosome segregation was successful during meiosis II (**Figure 15**). Wild type eggs all contained polar bodies associated with 4 FISH dots, each representing segregated X-chromosomes (**Figure 15A-A''**). Early mutant eggs that had just completed telophase II also contained four FISH dots, indicating that chromosome segregation was taking place (**Figure 15B-C''**). However, it is important to note that very few eggs were observed at this early stage. Also, the distribution of DNA could not be examined to determine whether each of the four FISH dots were associated with equal amounts of DNA. Nonetheless, it is highly likely that the FISH dots were not associated with equal amounts of DNA since chromosome missegregation occurs during meiosis II. Older eggs contained more than four FISH dots (**Figure 15D-H''**). Therefore, in addition to regulating chromosome segregation and spindle assembly during meiosis II, it seems that Cyclin B also keeps the meiotic products from entering S-phase.

Fertilized eggs (0-4 hrs) were also examined more closely. Early wildtype fertilized eggs undergo synchronous syncytial mitoses in which nuclei divide in a syncitium; cells are formed later in development. These divisions were not observed in fertilized mutant eggs probably because they failed to form a female pronucleus.

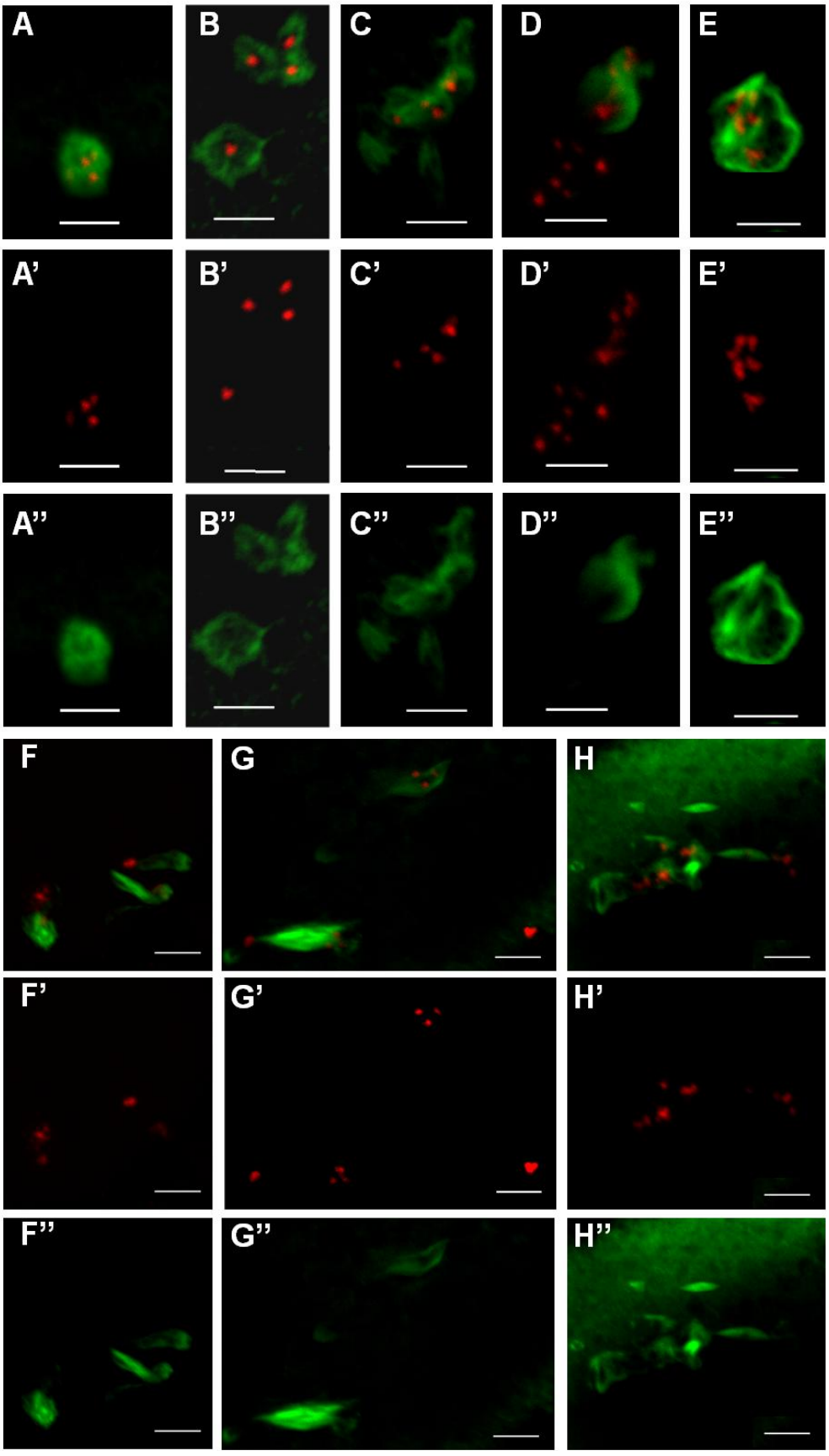
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Figure 15 - Meiotic products of *Cyclin B*$^{-}$; *HsCycB* eggs undergo DNA replication. FISH probe, specific for the centromeric region of the X chromosome, is shown in red, whereas microtubules are shown in green. All eggs were unfertilized and collected for 0-1 hours from flies kept at 29°C. (A-A') depict a wildtype polar body with four X-chromosome signals. (B-H'') depict *Cyclin B*$^{-}$; *HsCycB* eggs. (B-C'') are most similar to wildtype since they contains four X-chromosome dots. All the other eggs contain more than four X-chromosome signals, indicating that DNA replication in taking place within the eggs. (F-H'') show older eggs with multiple spindles, each associated with different numbers of X-chromosomes. All scale bars are 10 um.

Regardless, the presence of a female pronucleus is not required for mitotic entry because the male pronucleus has the capacity to divide and produce a haploid embryo. However, this phenomenon was also not seen in mutant embryos, indicating that Cyclin B-CDK1 serves some function in meiosis that also promotes mitotic entry after completion of meiosis. Like the unfertilized mutant eggs, the fertilized eggs also contained multiple, abnormal, DNA-associated spindles. Multiple paternal-origin centrosomes were also observed. A previous study has shown that knocking down all three mitotic Cyclins in the early embryo using RNAi causes nuclear arrest, but centrosomes still continue to divide (McCleland and O'Farrell, 2008). However, in this case, it seems that the absence of Cyclin B alone can promote centrosome divisions in the absence of nuclear divisions.

An attempt was made to obtain a more severe phenotype by shifting *Cyclin B^{-}; HsCycB* flies from 29°C to 18°C. The rationale behind this was that the *HsCycB* gene would provide Cyclin B at 29°C to promote oogenesis, and when shifted to a lower temperature (18°C), any Cyclin B present in the ovaries, even trace amounts, would be depleted, resulting in a stronger phenotype. However, when flies were shifted to 18°C, egg laying was severely affected. Several attempts were made to collect eggs, but the flies failed to lay a single egg in four hour collection times. Therefore, temperature shifts were not used in the experiments.

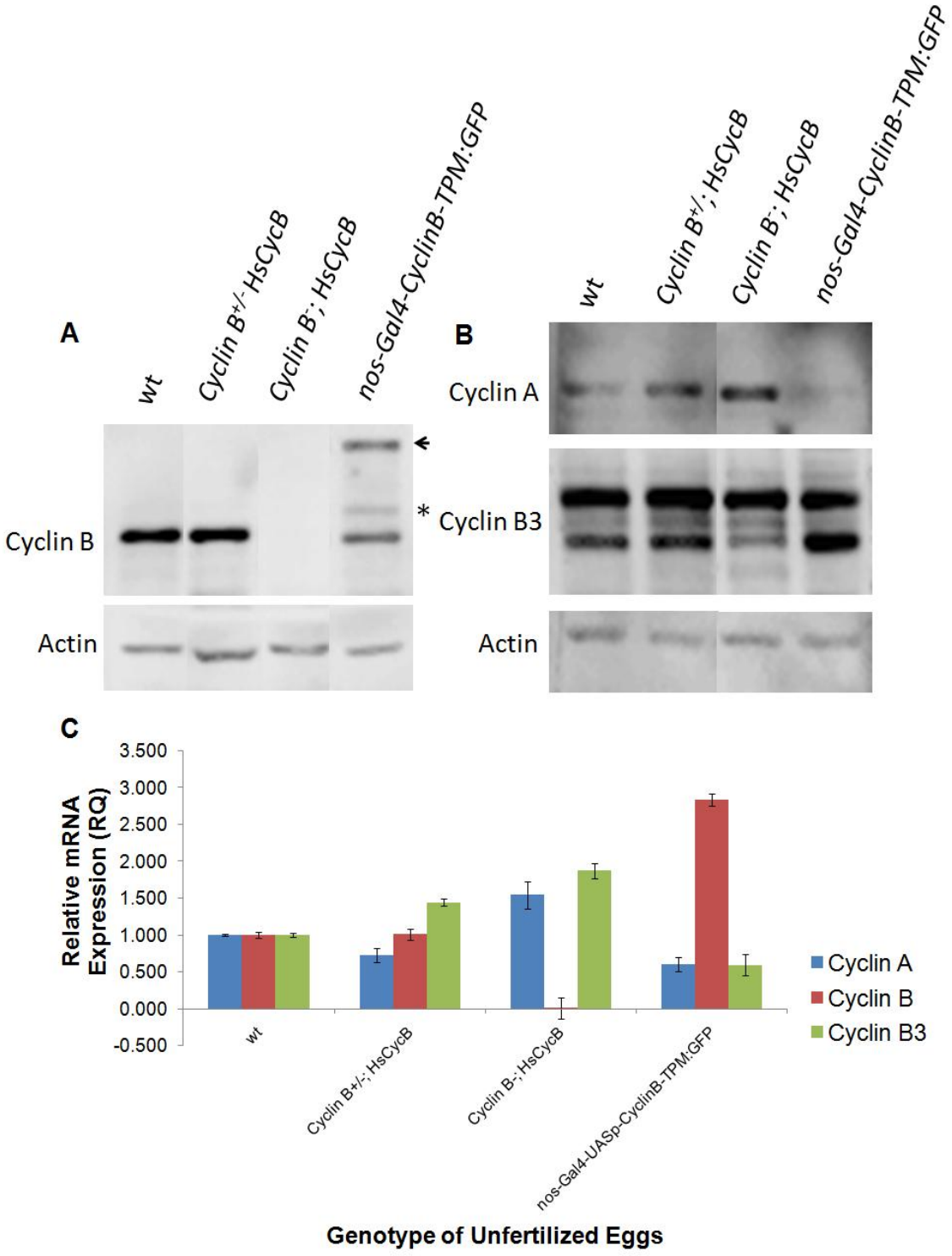
3.7 Cyclin B-CDK1 may promote Cyclin A degradation during meiosis

In mitosis, Cyclin-CDK1 complexes regulate APC/C activity through phosphorylation. Thus, it may be that Cyclin/CDK1 complexes also affect APC/C activity

during meiosis. To investigate this possibility, the levels of APC/C targets, Cyclins A and B3, were examined in unfertilized *Cyclin B^{-}; HsCycB* eggs (0-2 hours collections). Cyclin A levels were very low in wildtype eggs. In mutant eggs, however, the levels of Cyclin A were elevated by up to 5-fold (**Figure 16B**). The expression of a D-box mutant form of Cyclin B (Cyclin B-TPM:GFP) caused a slight decrease in Cyclin A levels (**Figure 16B**). Cyclin B3 levels were not significantly altered in mutant eggs (**Figure 16B**), although protein levels were difficult to compare due to the multiple phosphorylation states of Cyclin B3.

Cyclin A levels were also examined in *Cyclin B^{-}; HsCycB* stage 14 oocytes to determine whether Cyclin B affects Cyclin A protein levels before or after metaphase I. Interestingly, Cyclin A levels were not significantly altered in the mutant oocytes (**Figure 13B**), indicating that Cyclin B most likely affects Cyclin A levels after the metaphase I arrest is released.

Although Cyclin A protein levels were elevated in *Cyclin B^{-}; HsCycB* mutants eggs, it was unclear whether this was due to increased transcription, translation, or protein stability. To narrow down these possibilities, the mRNA levels of Cyclin A were examined using Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR). In unfertilized mutant eggs (0-2 hours), *Cyclin A* mRNA levels were 1.5- to 1.9-fold higher than in wildtype (**Figure 16C**); however, these fold changes were below the detection limit of the RT-PCR Cycler and were not considered significant. Furthermore, Cyclin A mRNA levels were unaltered in *Cyclin B$^{+/-}$* heterozygous eggs and in eggs expressing the D-box mutant form of Cyclin B. Therefore, it appears that Cyclin B does not regulate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Cyclin B+/-; HsCycB
Cyclin B-; HsCycB
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Cyclin B+/-; HsCycB
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Relative mRNA Expression (RQ)
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Cyclin B-; HsCycB
nos-Gal4-UASp-CyclinB-TPM:GFP
Cyclin A
Cyclin B
Cyclin B3
Genotype of Unfertilized Eggs

Figure 16 – Cyclin A proteins levels are increased in *Cyclin B*$^{-}$; *HsCycB* mutants. Western blots in (A, B) were done on unfertilized eggs collected for 0-2 hours at 29°C. Blots were probed for Cyclin A, Cyclin B, or Cyclin B3. Actin was used as a loading control. (A) shows that Cyclin B levels are drastically reduced in *Cyclin B*$^{-}$*; HsCycB.* The arrow points to GFP-tagged D-box mutant form of Cyclin B, which is only present in *nos-Gal4:VP16-UASp-CyclinB-TPM:GFP* eggs. * indicates a non-specific band. (B) shows that Cyclin A levels are increased in *Cyclin B*$^{-}$; *HsCycB* mutants, whereas Cyclin B3 are unaffected. (C) depicts the relative expression levels of *Cyclin A*, *Cyclin B*, and *Cyclin B3* (compared to wildtype). The mRNA levels of *Cyclins A* and *B3* are not significantly altered in *Cyclin B*$^{-}$*; HsCycB* mutants, whereas the mRNA levels of *Cyclin B* are drastically reduced. In *nos-Gal4:VP16-UASp-CyclinB-TPM:GFP* flies, which overexpress non-degradable Cyclin B, *Cyclin B* mRNA levels are elevated due to transgene expression; however the levels of *Cyclin A* and *B3* are not significantly reduced. Changes of 2-fold or greater were considered significant.

Cyclin A mRNA levels. This leaves open the possibilities that Cyclin B-CDK1 may act on either Cyclin A protein stability or on Cyclin A protein translation; further studies are required to test these possibilities. Since Cyclin A levels increased dramatically after the metaphase I arrest was released, it is most likely that Cyclin B-CDK1 acts on Cyclin A protein levels after the egg is activated. However, Cyclin B-CDK1 may also regulate Cyclin A protein levels in late metaphase I since *CyclinB; HsCycB* oocytes show chromosome nondisjunction defects at this stage, and embryos expressing non-degradable Cyclin A also exhibit defective meiosis I.

3.8 Cyclin B degradation is required for proper completion of meiosis

Gain-of-function studies were used to examine the importance of Cyclin B destruction during meiosis. In previous studies, a D-box mutant form Cyclin B (CyclinB-TPM:GFP) was overexpressed in the germline of wildtype flies. This resulted in a variable meiotic arrest phenotype; the variability may have been due to the presence of endogenous Cyclin B (Swan and Schupbach, 2007). To obtain a more consistent arrest phenotype, CyclinB-TPM:GFP was expressed in a *Cyclin B* mutant background at 29°C. The resulting flies had normal-sized ovaries, but laid very few eggs. Embryos collected from these flies did not exhibit a consistent meiotic arrest, but were inviable nonetheless (**Figure 17**). Interestingly, the phenotypes of these mutants were even more varied than those of wildtype eggs expressing Cyclin B-TPM:GFP. When Cyclin B-TPM:GFP was overexpressed in wildtype embryos, divisions beyond the first mitosis were never observed (Swan and Schupbach, 2007). However, when Cyclin B-TPM:GFP was overexpressed in a *Cyclin B*$^{-}$ background, many embryos progressed beyond the first

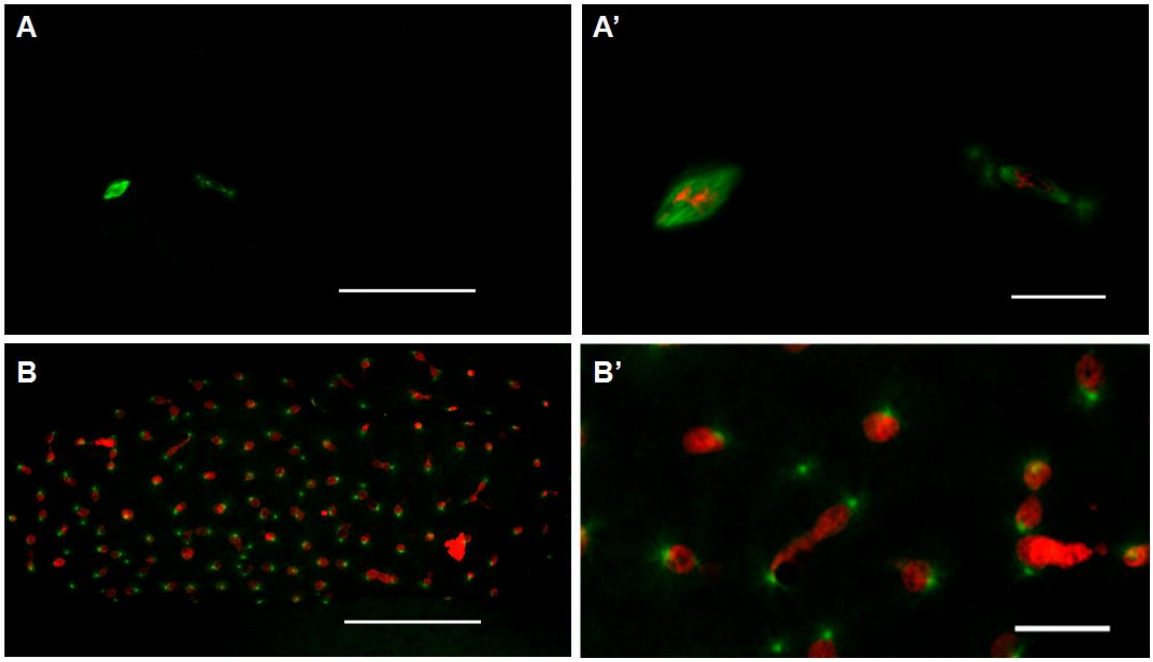

Figure 17 – Overexpressing non-degradable Cyclin B in a *Cyclin B*$^{-}$ background does not produce a consistent meiotic arrest. DNA is shown in red and microtubules are shown in green. Eggs were collected for 0-3 hours from wildtype or *CyclinB*$^{-}$*;nos-Gal4:VP16-UASp-CyclinB-TPM:GFP* flies kept at 29°C. Right panels show magnified views of left panels. (A-A') depicts an early *Cyclin B*$^{-}$ embryo expressing Cyclin B-TPM:GFP; the embryo contains a meiotic spindle (left) and a mitotic spindle (right). (B-B') shows a further developed embryo in interphase. Thus, expressing non-degradable Cyclin B in a *Cyclin B*$^{-}$ background does not produce a consistent meiotic phenotype. Scale bars in (A,B) are 100 um, while scale bars in (A', B') are 20 um.

mitotic division. Very few embryos were examined due to the reduced egg laying capacity of the flies. Older embryos contained unevenly spaced nuclei, some connected via chromosome bridges; large clumps of DNA were also observed within certain embryos (**Figure 17B**). This was very similar to the phenotype seen in embryos overexpressing non-degradable Cyclin A. Thus, overexpressing non-degradable Cyclin B in a *Cyclin B*$^{-}$ background did not produce a more consistent arrest phenotype.

3.9 Redundancy of Cyclin proteins during meiosis cannot be tested using *Cyclin* mutants

Cyclins A, B, and B3 play redundant roles in mitosis; if one is knocked down, the others can usually compensate. The exception to this is Cyclin A because *Cyclin A* mutants die as embryos. Because of this, *Cyclin A Cyclin B* and *Cyclin A Cyclin B3* double mutants also die, as do *Cyclin A Cyclin B Cyclin B3* triple mutants. Although *Cyclin B* and *Cyclin B3* single mutants survive to adulthood, *Cyclin B Cyclin B3* double mutants die as embryos (Knoblich and Lehner, 1993; Jacobs *et al.*, 1998). Since the *Cyclin A* mutant could not be rescued, double mutants involving *Cyclin A* were not generated. However, an attempt was made to test the redundancy of Cyclins B and B3 during meiosis. This was done by introducing a *HsCycB* transgene into the *Cyclin B Cyclin B3* double mutant background. The purpose of this transgene was to provide Cyclin B to the embryo to rescue the early divisions. However, heat-shocks (at 29°C and 37°C) failed to produce any double mutant progeny, indicating that the *HsCycB* transgene did not produce enough Cyclin B to rescue the mutants.

Flies null for one gene and heterozygous for the other gene were also examined.

Mutants null for *Cyclin B* and heterozygous for *Cyclin A* did not survive (*CyclinB^{-}; HsCycB, Cyclin A$^{+/-}$*). Mutants null for *Cyclin B* and heterozygous for *Cyclin B3* failed to develop ovaries in the presence of *HsCycB* at 29°C (*CyclinB^{-}; HsCycB,CyclinB3$^{+/-}$*). This phenotype could not be rescued by periodic heat-shocks at 37°C. Thus, it seems that Cyclin B and Cyclin B3 play a redundant role in germline stem cell formation or maintenance since *CyclinB^{-}; HsCycB* flies can develop ovaries at 29°C and *CyclinB^{-}; HsCycB,CyclinB3$^{+/-}$* flies cannot. This means that when Cyclin B is knocked down (not completely absent), a certain level of Cyclin B3 is required to promote the GSC divisions.

Mutants null for *Cyclin B3* and heterozygous for *Cyclin B* were also examined (*CyclinB$^{+/-}$; HsCycB,CyclinB3^{-}*). These flies developed ovaries and could lay eggs that did not hatch. However, the frequency of flies with this genotype (from large scale crosses) was very low; thus, egg collections were not attempted.

To overcome the problem of embryonic lethality in mutants, shRNAs against the *Cyclin* genes were designed and cloned into the Valium22 vector, which allows for RNAi in the germline. Two shRNA sequences were designed for each *Cyclin* gene: one targeting a region in the 3'UTR and the other targeting an internal coding sequence. The resulting transgenic flies will be used to examine redundancy of Cyclins in meiosis.

3.10 Making early oogenesis *Gal4* drivers

As mentioned previously, oogenesis consists of two general stages. The first stage involves mitotic divisions that give rise to the oocyte. The second stage involves the process of meiosis. There are certain genes that function both during mitosis and meiosis, although they may serve different functions in each process. Null mutations in

such genes may interfere with the germline stem cell divisions that give rise to the oocyte. If this is the case, certain null mutations may never give rise to an oocyte, hampering any efforts to deduce the meiotic functions of these genes. This is the case with *Cyclin B* and possibly also *Cyclin A*. Cyclin B is required for the GSC divisions that give rise to the oocyte; therefore, examination of meiosis in the absence of Cyclin B requires the first stage of oogenesis to be rescued. The *HsCycB* meets this requirement, but in the early stages of these experiments it was not known that *HsCycB* would selectively rescue the first stage of oogenesis, allowing examination of meiosis in the absence of Cyclin B. Thus, an alternative approach to rescue the first stage of oogenesis was also attempted. This approach involved the construction of an early oogenesis *Gal4* driver.

Benign gonial cell neoplasm (*Bgcn*) is a gene that is involved in the GSC divisions which give rise to the cystoblast, which in turn divides several times and gives rise to the oocyte. The *Bgcn* gene is specifically expressed in the GSCs and the cystoblast and is not expressed during meiosis (Ohlstein *et al.*, 2000). Because of its specificity of expression during the first stage of oogenesis, the regulatory elements of this gene were used to make an early oogenesis *Gal4* driver (**Figure 18A**). The promoter region of *Bgcn* is currently unknown, therefore, an 878 bp region, which contained approximately 500 bp of the *Bgcn* gene (5' end), 100 bp of the gene directly upstream of *Bgcn* (*Tbph*), and the genomic region in between these two genes, was cloned at the 5' end of the *Gal4:VP16* sequence. A 463 bp region, which contained approximately 300 bp of the *Bgcn* gene (3' end), 100 bp of the downstream gene *Glass bottom boat* (*Gbb)* and the genomic region

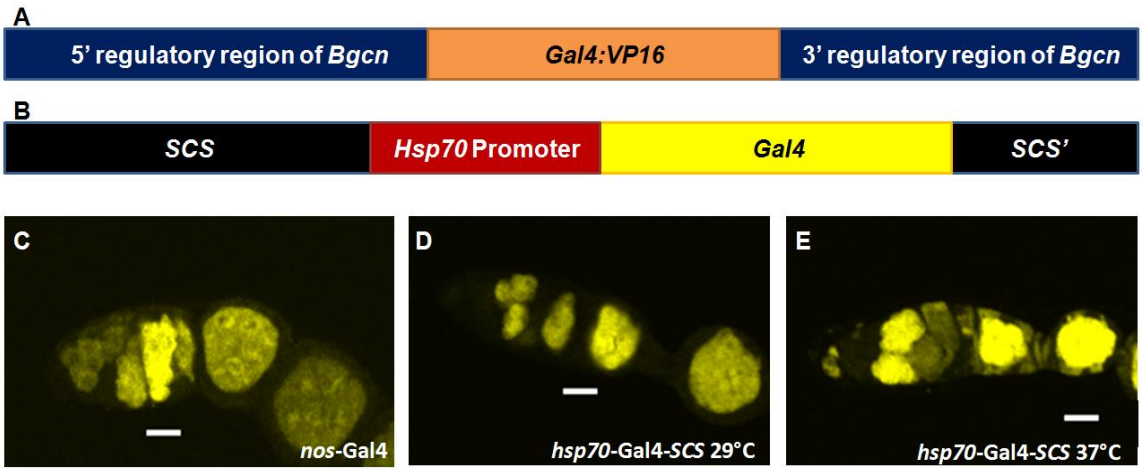

Figure 18 – *bgcn-Gal4:VP16* driver does not express in the germline, whereas the *hsp70-Gal4-SCS* driver does. (A) shows the structure of the *bgcn-Gal4:VP16* driver. (B) shows the structure of the *hsp70-Gal4-SCS* driver. For testing purposes, flies containing the *hsp70-Gal4-SCS* driver were crossed with flies containing the reporter construct *UASp-LacZ*. The resulting progeny were subjected to heat-shocks at 29°C or 37°C. Ovaries of these flies were stained for β-galactosidase (depicted in yellow). The driver *nos-Gal4:VP16* was used as a control since it expresses at high levels in the germarium (A). (B) shows expression of the *hsp70-Gal4-SCS* driver at 29°C. (C) shows expression of the *hsp70-Gal4-SCS* driver at 37°C. (C-E) are oriented with germarium to the left. Scale bars are 10 um.

between these two genes, was cloned at the 3' end of the *Gal4:VP16* sequence. Sequencing results revealed that the sequence of this transgene was satisfactory, except for the 3' region, which was missing two C's that were present in the template sequence. However, it was unlikely that such a minor sequence difference would render the transgene dysfunctional. The transgene was introduced into embryos to generate stable transgenic flies. The *bgcn-Gal4:VP16* flies were crossed with flies containing the reporter genes *UASp-GFP* or *UASp-LacZ* to test for transgene expression in the germarium. However, no transgene expression was detected in the germarium, indicating that the transgene was dysfunctional (see Discussion).

A heat-shock-inducible *GAL4* transgene was also created (**Figure 18B**) because currently available *hs-Gal4* transgenic flies do not strongly express Gal4 in the germline. Such a transgene would provide a means to rescue the early oogenesis requirement for genes such as *Cyclin B*. The transgene was cloned between Scs and Scs' insulator elements to prevent leaky expression in the absence of heat-shock (due to nearby enhancer elements). The cloning procedure is described in Materials and Methods. To test the *hsp70-Gal4-SCS* transgene, flies were crossed with flies containing *UASp-LacZ*. The progeny were subjected to heat-shocks at 29°C or 37°C and expression of β-galactosidase in the germarium was examined via immunostaining. Strong expression was observed in the germline at both temperatures, but expression was more robust at 37°C (**Figure 18D,E**). However, β-galactosidase expression could not be clearly detected in the region containing GSCs and cystoblasts; thus, further testing is required to assess whether such a driver could rescue early oogenesis.

4.0 DISCUSSION

Cyclin-CDK1 complexes are the chief regulators of mitosis. Events such as mitotic entry, NEBD, and chromosome condensation are triggered by phosphorylation of specific Cyclin-CDK1 substrates. Nucleus reformation, chromosome decondensation, and mitotic exit rely on dephosphorylation of these substrates. Since Cyclin-CDK1 complexes mediate key events during mitosis, they may also do the same during meiosis. This study explored this possibility by examining the roles of Cyclin-CDK1 complexes in Drosophila female meiosis.

4.1 The localizations of Cyclins A and B suggest that they may not be involved in NEBD

Localization studies revealed that Cyclin A localizes to the spectrosome and to the fusome in the germarium, where it regulates germline stem cell divisions and cystocyte divisions, respectively (Lilly *et al.*, 2008). Similarly, studies also revealed that Cyclin B is expressed in germline stem cells, where it coordinates GSC divisions (Wang and Lin, 2005). Interestingly, Venus:Cyclin A also localized to the centrosomes or microtubule organizing centres (MTOC) of early oocytes. The oocyte MTOC nucleates an array of microtubules that extend into the nurse cells. This microtubule array aids in establishing the anterior/posterior and dorsal/ventral axes of the oocyte by localizing mRNAs of certain proteins to specific regions of the oocyte. Initially (stages 1-6) the MTOC is found at the posterior end of the oocyte, near the nucleus. During stages 7-8 of oogenesis, the MTOC and the nucleus migrate to the anterior end of the oocyte (Megraw and Kaufman, 2000). Venus:Cyclin A localization followed a similar pattern. During the early stages, Venus:Cyclin A was found at discrete bodies near the nucleus at

the posterior end of the oocyte; and during stages 7-8, Venus:Cyclin A was found at the anterior end, again near the oocyte nucleus (**Figure 1**). However, studies examining endogenous Cyclin A localization in Drosophila ovaries failed to detect centrosomal Cyclin A localization (Lilly *et al.*, 2000). Furthermore, Cyclin A protein levels are downregulated during the prophase I arrest via protein degradation and translational repression; this prevents premature entry into prometaphase I (Vardy et al., 2009; Sugimura and Lilly, 2006). Therefore, the observed centrosomal localization may be an artifact of Cyclin A overexpression during a stage when it is not ordinarily present. Alternatively, it may be that ovaries require to be fixed in a certain manner to preserve centrosomes, which is why immunostaining failed to detect endogenous Cyclin A on centrosomes (Lilly *et al.*, 2000). It may be that Cyclin A is sequestered to the MTOC during prophase I, where it promotes phosphorylation of certain proteins, such as those involved in microtubule nucleation or MTOC migration. The centrosomal Cyclin A would be protected from degradation, whereas the cytoplasmic pool would be degraded by the APC/C; this would prevent premature entry into prometaphase I. In support of this, studies conducted in Xenopus egg extracts have shown that Cyclin A regulates the microtubule nucleating activity of centrosomes (Buendia *et al.*, 1992). Furthermore, Xenopus and mammalian Cyclin A has a centrosomal localization signal that directs it to the centrosome (Pascreau *et al.*, 2010). Therefore, it may be useful to re-examine the localization of endogenous Cyclin A during oogenesis by using fixation procedures that properly preserve the MTOC.

Venus-Cyclin B also exhibited a specific localization pattern during oogenesis.

This fusion protein was found to localize around the oocyte DNA in stage 14 oocytes, which are arrested in metaphase I. The region around the oocyte DNA contains overlapping microtubules and is referred to as the spindle midzone; thus, Cyclin B localizes to the meiotic spindle midzone. Such a localization pattern has been reported previously (Swan and Schupbach, 2007). Furthermore, Cyclin B also localizes to the spindle midzone during metaphase II. Thus, based on its localization pattern it seems that Cyclin B may be involved in spindle assembly, chromosome alignment, and chromosome segregation during meiosis I and meiosis II. This speculation is supported by the findings of this study. Oocytes lacking Cyclin B have deformed spindles (during meiosis II) and exhibit defects in both chromosome alignment (during meiosis II) and chromosome segregation (during meiosis I and II).

During prophase of Drosophila mitosis, both Cyclins A and B localize to the nucleus to mediate NEBD (Whitfield *et al.*, 1990; Stiffler *et al.*, 1999). A similar phenomenon is expected during meiosis since *Twine* mutants, which lack the meiotic Cdc25 complex, show a significant delay in the timing of NEBD, implying that Cyclin-CDK1 complexes direct NEBD during meiosis (Von Stetina *et al.*, 2008). However, this study revealed that both Cyclins A and B do not localize to the oocyte nucleus prior to NEBD. Furthermore, the timing of NEBD is unaffected in *Cyclin B*$^{-}$; *HsCycB* mutants. Therefore, Cyclin B-CDK1 is not required for NEBD, although this does not eliminate the possibility that Cyclin B may be involved in NEBD in a redundant manner. The Cyclin A localization pattern suggests that Cyclin A-CDK1 is not directly involved in NEBD; but this does not eliminate the possibility that Cyclin A-CDK1 phosphorylates proteins in the

cytoplasm, which then localize to the nucleus and promote NEBD. Unfortunately, this possibility could not be tested since *Cyclin A* mutants die as embryos. Another possibility is that Cyclin B3-CDK1 is the primary complex that regulates NEBD during meiosis. Confirmation of this requires examination of Cyclin B3 localization during meiosis and examination of NEBD timing in *Cyclin B3* mutants.

4.2 Examination of meiosis in *Cyclin A* mutants, *Cyclin* double mutants, and *Cyclin* triple mutants

Cyclin A mutants die as early embryos; thus examination of meiosis in the absence of Cyclin A requires embryos to be rescued. One to four copies of *HsCycA* failed to rescue *Cyclin A* mutants under heat-shock conditions. This may have been due to two reasons. It is possible that the *HsCycA* transgenes failed to produce enough Cyclin A to rescue the mutant embryos. Alternatively, it may be that embryos must be at a certain stage of development for rescue to be successful. For example, it may be that embryos heat-shocked during mitosis 14 or 15 of embryonic development would have produced enough Cyclin A at the appropriate time to rescue mitosis 16 and subsequent development, whereas those heat-shocked earlier or later would not be rescued. Therefore, it may have been useful to take the age of embryos into consideration when conducting the heat-shocks. However, there is no guarantee that this method would have rescued the mutant embryos.

The other methods attempted made use of the *UAS*/*Gal4* system. The *UASt-Cyclin A* transgene in combination with the *actin-Gal4* driver failed to rescue the

embryos to adulthood because it was expressed at very high levels in the larvae; all flies died as pharate adults due to constitutive Cyclin A-CDK1 activity. Since Cyclin A-CDK1 inhibits the APC/$C^{Cdh1/Fzr}$, it is likely that this defect was due to accumulation of certain APC/C substrates (Reber *et al.*, 2006). On the other hand, the *UASp-Cyclin A* transgenes, in combination with the *da-Gal4* driver, were expressed at lower levels in mitotic cells and could rescue the mutant embryos to adulthood. Unexpectedly, the *da-Gal4* driver also caused Cyclin A expression in the ovaries, meaning that the rescued flies overexpressed Cyclin A during meiosis. Therefore, while rescue of *Cyclin* A^{-} embryos is possible, it requires a suitable *UAS/Gal4* combination that provides Cyclin A during the somatic cell mitoses, but does not allow expression of Cyclin A during meiosis. Currently, there are no Gal4 drivers that meet this requirement.

Similarly, the rescue of *Cyclin B Cyclin B3* double mutants probably failed due to inadequate *HsCycB* expression in the embryo or the inability to express at the right time during embryogenesis (i.e. too early or too late). Thus, the heat-shock method is ineffective at rescuing embryogenesis. To overcome this problem, two short hairpin RNAs have been designed against each Cyclin gene. One shRNA targets the 3'UTR region of the mRNA, whereas the other targets an internal coding sequence. These shRNAs will be used to specifically knock down single and multiple *Cyclin* genes to examine Cyclin function and Cyclin redundancy during meiosis. This technique will bypass the need to rescue the *Cyclin A* or double mutant embryos. Furthermore, expression of the shRNAs will be restricted to the meiotic egg chambers by using *Gal4* drivers such as *mat67-Gal4*. The shRNAs that target the 3'UTR regions open up

possibilities for interesting experiments, such as expressing altered versions of Cyclins in *Cyclin* knockdown backgrounds.

4.3 Cyclin A degradation is required for proper chromosome segregation during meiosis, timely completion of meiosis, and proper embryonic development

Studies in Drosophila embryos have shown that stabilized Cyclin A causes a significant delay in metaphase of mitosis (Sigrist *et al.*, 1995; Jacobs *et al.*, 2001). This implies that Cyclin A degradation occurs during prometaphase and is required for timely completion of mitosis. The delay in metaphase also suggests that Cyclin A-CDK1 may phosphorylate and inhibit Separase, which is a protease that cleaves Cohesin complexes during the metaphase-to-anaphase transition (Stemmann et al., 2001). Thus, the metaphase delay may be due to inhibition of Separase activity by ΔCyclin A-CDK1. This study tested the consequences of stabilized Cyclin A overexpression during meiosis.

When Venus-tagged ΔCyclin A was expressed during meiosis, oogenesis was slowed due to defects in the nurse cell endocycles. Studies have shown that Cyclin A-CDK1 inhibits the activity of APC/$C^{Cdh1/Fzy}$. This allows Cyclins to accumulate and drive entry into mitosis (Reber *et al.*, 2006). Furthermore, when the APC/C is dysfunctional due to mutations in *Morula*, nurse cells exit the endocycle and enter mitosis due to accumulation of Cyclins (Kashevsky *et al.*, 2002). Therefore, it seems that non-degradable Cyclin A inhibits APC/$C^{Cdh1/Fzr}$ in the nurse cells, resulting in Cyclin protein accumulation and entry into mitosis. Alternatively, the stabilized Cyclin A may directly promote entry into mitosis. Although oogenesis is defective, stage 14 oocytes are still

produced.

Oocytes expressing non-degradable Cyclin A undergo defective meiosis I and II. During meiosis II, each meiotic spindle is associated with different amounts of DNA, which suggests that chromosome missegregation took place during anaphase I. Furthermore, the DNA distribution on the meiosis II spindle is abnormal. The majority of eggs have DNA at the spindle equators and also at the outermost spindle poles and/or at the spindle pole body. These distributions suggest that chromosome missegregation occurs during meiosis II. Furthermore, the spindle morphology of the meiosis II spindles is somewhat abnormal. The spindles appear slightly less organized than in wildtype. Sometimes small DNA-associated spindle structures are observed, which appear to have broken off the main meiotic spindle. The spindle pole body is also defective in eggs expressing stabilized Cyclin A. Some eggs have a prominent spindle pole body that is associated with DNA, while others either do not have a spindle pole body or have a dispersed spindle pole body.

During meiosis, the meiosis I spindle undergoes a rearrangement to form the meiosis II spindle without any intervening spindle disassembly. Once the chromosomes have separated in anaphase I, the meiosis I spindle elongates. A spindle pole body, which contains a MTOC, forms at the centre of the elongated spindle. The microtubules on each side of the spindle pole body form meiosis II spindles (Endow and Komma, 1998). If chromosome missegregation does occur during meiosis I, anaphase I spindles should contain DNA dispersed along their lengths (this was observed). Thus, the region where the spindle pole body forms may contain DNA; this may explain why some

meiosis II spindles have DNA associated with their spindle pole bodies. To confirm that there is a defect in either alignment of chromosomes during metaphase I or segregation of chromosomes during anaphase I, it may be useful to closely examine stage 14 oocytes for metaphase I defects. It may also be worthwhile to examine activated oocytes for defects in later stages of meiosis I. Therefore, it seems that Cyclin A needs to be degraded before anaphase I to allow proper chromosome segregation. In agreement with this, studies have shown that Cyclin A protein is degraded during late prometaphase I (Vardy *et al.*, 2009). Therefore, it seems that Cyclin A degradation during late prometaphase I allows dephosphorylation of proteins that would otherwise interfere with normal chromosome segregation.

During meiosis II, non-degradable Cyclin A affects spindle morphology. The two meiosis II spindles are usually different sizes; this may be due to association with unequal amounts of DNA. There also appears to be a slight defect in microtubule bundling as evidenced by faint spindle fibers observed around the meiotic spindle and also due to the presence of small spindle structures (which contain DNA) that dissociate from the main spindle. Thus, it seems that Cyclin A-CDK1 may phosphorylate proteins that promote spindle instability. Defects were also observed at the spindle pole body, which contains the MTOC. The meiosis II eggs that had DNA at their spindle pole bodies also had very prominent spindle pole bodies. This may be because microtubules usually associate with DNA. However, the spindles that did not have DNA at the spindle pole body region were either missing spindle pole bodies or had dispersed-looking spindle pole bodies. Some of the phenotypes observed in later collections implied that the

spindles that had no detectable spindle pole bodies separated from one another to form two spindle structures, which appeared to arrest in metaphase or anaphase of meiosis II. Therefore, it seems that Cyclin A-CDK1 interferes with normal spindle pole body formation and maintenance. This is an interesting result because it implies that Cyclin A-CDK1 may localize to the meiosis II MTOC, in addition to the MTOC found in prophase I oocytes. To test this possibility, it may be useful to examine the localization of Cyclin A during meiosis II.

Later collections and FISH results revealed that meiosis is eventually completed in some Venus:ΔCyclin A eggs. Therefore, non-degradable Cyclin A does not prevent polar body formation. The eggs that fail to complete meiosis often contain two to four spindle structures, which may arise from the small spindle structures that dissociate from the meiosis II spindle and/or from the two meiosis II spindles separating from one another due to spindle pole body defects.

Venus:ΔCyclin A also caused defects during early embryogenesis. In a wildtype fertilized egg, early embryogenesis is characterized by nuclear divisions that occur in a syncitium. These nuclear divisions are synchronized and involve oscillations between S-phase and M-phase. The syncytial mitoses are controlled by maternally-derived gene products deposited in the egg during oogenesis. After the 13^{th} mitosis, the nuclei are sorted into cells; subsequent development is controlled by zygotic gene activity and involves asynchronous mitoses (Lee and Orr-Weaver, 2003). Of the few Venus:ΔCyclin A eggs that enter mitosis, the majority show defective syncytial mitoses characterized by asynchrony. These embryos contain chromatin bridges, nuclei at different stages of

mitosis, nuclei devoid of centrosomes, and large clumps of DNA. The defective embryos do not contain discernible polar bodies. Instead, they contain clumps of DNA, which resemble meiotic products that have undergone DNA replication. The chromatin bridges may arise from defects in chromosome segregation, possibly due to inhibition of Separase via Cyclin A-CDK1. The asynchronous mitoses are difficult to comprehend; it seems that constitutive ΔCyclin A-CDK1 activity interferes with the timing of mitotic entry during the syncytial mitoses. The DNA replication phenotype is unexpected. In Drosophila, Cyclin A is not thought to play an important role in DNA replication. Nonetheless, it has been shown that ectopic Cyclin A expression can induce premature entry into S-phase, which promotes DNA replication (Sprenger *et al.*, 1997). Although Cyclin A-CDK1 can induce entry into S-phase, it also prevents pre-replication complexes from reassembling on replication origins. Because of this, embryos expressing stabilized Cyclin A may undergo one round of DNA replication, but they should not undergo multiple rounds. Therefore, the DNA replication phenotype observed in the presence of ΔCyclin A requires further investigation.

Mushroom body defect (*Mud*) mutants display similar phenotypes during meiosis II and embryogenesis as embryos expressing stabilized Cyclin A. Mud is a coiled-coil protein that localizes to the spectrosomes and fusomes during the GSC and cystocyte divisions, respectively, and to the MTOC during meiosis II. Mutations in *Mud* cause female sterility due to defective spindle pole bodies, which are either absent or dispersed. This defect causes the two meiotic spindles to become separated from one another. Furthermore, embryonic development involves asynchronous mitoses

characterized by nuclei at different stages of mitosis, nuclei devoid of centrosomes, and large clumps of DNA that arise from DNA replication (Yu *et al.*, 2006). Therefore, it seems that the Cyclin A has the opposite function of Mud. It is possible that Venus:ΔCyclin A-CDK1 inhibits the activity of Mud, meaning that some of the phenotypes observed in embryos expressing stabilized Cyclin A might be due to loss of Mud activity. This is supported by the fact that the protein sequence of Mud contains a possible CDK1 phosphorylation site (T2183). Further studies are required to investigate this possibility. One way to explore the relationship between these two proteins may be by overexpressing Mud and Venus:ΔCyclin A together to determine if the Venus:ΔCyclin A phenotype is rescued.

Another point of interest is that the non-degradable Cyclin A causes a slight delay in meiosis II. Furthermore, most of the meiosis II spindles observed in the eggs expressing Venus:ΔCyclin A are found in a metaphase II state (even though some also have DNA at the spindle pole bodies and/or outer spindle poles). Therefore, there appears to be a metaphase II delay during meiosis; this may be due to inhibition of Separase activity. Thus, the possibility that Cyclin A-CDK1 phosphorylates and inhibits Separase during meiosis remains open for further investigation. Separase activity is also regulated by inhibitory binding by Pim (Securin). Studies in the Swan lab have shown that majority of embryos expressing non-degradable Pim complete meiosis, but meiotic progression is slow and characterized by defects, such as chromosome nondisjunction and abnormal spindle morphology (Batiha and Swan, unpublished data). Thus, Securin stabilization or constitutive Cyclin A-CDK1 activation alone do not cause a strong

metaphase arrest phenotype. Therefore, it is possible that both Securin activity and Cyclin A-CDK1 activity cooperatively regulate chromosome segregation during meiosis. This possibility is currently being tested.

4.4 Cyclin B is required for proper chromosome segregation during meiosis I and II, and also for prevention of polyploidy after the completion of meiosis

The localization pattern of Cyclin B during meiosis suggests that it is involved in processes such as chromosome alignment, chromosome segregation and spindle assembly. The phenotypes seen in the *Cyclin B^{-}; HsCycB* mutants support this speculation.

This study found that Cyclin B-CDK1 activity is not required for progression to metaphase I. However, it seems that Cyclin B-CDK1 plays an important role in maintaining this arrest until ovulation occurs since *Cyclin B^{-}; HsCycB* stage 14 oocytes exhibit precocious anaphase I characterized by chromosome missegregation; therefore, this indicates that Cyclin B is required for proper chromosome segregation during meiosis I. Furthermore, the resulting meiosis II spindle is misshapen and asymmetrical. Usually one spindle is more prominent and contains majority of the DNA, whereas the other spindle is barely visible and has very little DNA. The spindles themselves are sometimes kinked and not as neat as wildtype meiosis II spindles, indicating that Cyclin B-CDK1 is needed for proper spindle organization during meiosis II. Previous studies examining the effect of Cyclin B dose on spindle dynamics (in embryos) have shown that lowering Cyclin B dose results in longer, less dynamic spindles (Stiffler *et al.*, 1999).

Therefore, the spindle defects seen in the mutants may be due to increased microtubule stability.

Cyclin B^{-}; *HsCycB* mutant eggs also undergo chromosome missegregation during anaphase II. This produces four unequal meiotic products, which fail to form a normal polar body. A similar anaphase defect is seen when Cyclin B is knocked down in early embryos using RNAi. Knocking down Cyclin B in the embryo causes precocious anaphase characterized by chromosome missegregation. This raises the possibility that Cyclin B may be required for the formation of stable kinetochore-spindle attachments before the onset of anaphase (McCleland *et al.*, 2009). This may also be true for meiosis. Overall, the results of this study indicate that Cyclin B is also required for proper meiotic completion and exit. Thus, it is possible that Cyclin B-CDK1 complexes activate proteins that establish stable spindle-kinetochore attachments during metaphase I, proteins that maintain stable spindle-kinetochore attachments until the metaphase I arrest is released, proteins that regulate chromosome segregation during meiosis I and II, proteins involved in spindle organization during meiosis II, proteins that establish stable spindle-kinetochore attachments during meiosis II, and/or proteins that form normal polar bodies.

Intriguingly, the meiotic products in *Cyclin B*$^{-}$*; HsCycB* eggs undergo DNA replication and form multiple DNA-associated spindle structures. This phenotype is somewhat similar to what is seen in *PNG*, *GNU*, or *PLU* mutants. PNG, GNU, and PLU are proteins that form a trimeric complex with kinase activity. This complex inhibits DNA replication until egg fertilization. In *PNG*, *GNU*, or *PLU* mutants, the meiotic products fail

to arrest; instead, they enter S-phase and undergo multiple rounds of DNA replication to form large, polyploid nuclei. The levels of Cyclins A and B are downregulated in these mutants. Thus, it seems that low levels of Cyclin-CDK1 activity promote replication origin relicensing, which results in DNA replication (Fenger *et al.*, 2000). Interestingly, depletion of the B-type Cyclin of *Schizosaccharomyces pombe* also causes DNA rereplication (Hayles *et al*., 1994). Therefore, it seems that one of the roles of Cyclin B-CDK1 is to prevent DNA replication until egg fertilization, perhaps by interfering with reloading of origin of replication complexes.

Another interesting phenotype observed in *Cyclin B^{-}; HsCycB* mutants is the unregulated proliferation of centrosomes. This phenotype is also seen when all three mitotic Cyclins are knocked down in the early embryo using RNAi. Knocking down all three Cyclins prevents the embryonic nuclei from entering mitosis, but centrosomes continue to divide independent of the nuclei (McCleland and O'Farrell, 2008). Furthermore, this same phenotype is observed in *PNG*, *GNU*, or *PLU* mutants, in which levels of Cyclins A and B are reduced (Fenger *et al.*, 2000). This is because the PNG kinase is involved in translational activation of *Cyclin A* and *Cyclin B* mRNAs (Vardy *et al.*, 2009; Vardy and Orr-Weaver, 2007). Mitotic entry also does not occur in *Cyclin B^{-}; HsCycB* mutants. This may be because these mutants do not form a normal female pronucleus or the cellular environment does not favour mitotic divisions. However, centrosomes still continue to divide. Therefore, it seems that Cyclin B-CDK1 is needed for mitotic entry and for coupling of nuclear division cycles to centrosome division cycles during the early embryonic divisions.

4.5 Non-degradable Cyclin B does not produce a consistent meiotic arrest

Expression of non-degradable Cyclin B in a wildtype background produces a variable phenotype. Most embryos arrest in different stages of meiosis, whereas some arrest after the first mitosis (Swan and Schupbach, 2007). Therefore, it is difficult to determine exactly when Cyclin B needs to be degraded during meiosis. This variability may have been due to the presence endogenous Cyclin B. However, when non-degradable Cyclin B is expressed in the *Cyclin B*$^{-}$ background, greater variability is observed. Embryos arrested in meiosis are observed, but embryos that progress beyond the first mitotic division are also seen. It may be that the variability seen in the *Cyclin B*$^{-}$ background is due to an alteration in APC/C substrate ubiquitination. In the presence of endogenous Cyclin B, the APC/C is expected to ubiquitinate specific substrates at a certain level. However, when Cyclin B is absent, the APC/C may cause increased degradation of specific substrates; therefore, the observed phenotype may be due to knockdown of multiple proteins. Another possibility is that there are higher overall levels of Cyclin B in wildtype embryos that overexpress non-degradable Cyclin B than in *Cyclin B*$^{-}$ embryos that overexpress non-degradable Cyclin B; therefore, it may be that higher Cyclin B protein levels produce a more consistent arrest phenotype.

4.6 Cyclin A-CDK1 does not regulate APC/C activity during meiosis, whereas Cyclin B-CDK1 does

Cyclin-CDK1 complexes regulate the activity of APC/C during mitosis. APC/C$^{Cdc20/Fzy}$ activity is positively regulated by phosphorylation whereas APC/C$^{Cdh1/Fzr}$

activity is negatively regulated (Kramer et al., 2000). The possibility that Cyclin-CDK1 complexes might regulate APC/C activity during meiosis was also tested in this study. This was done by examining the levels of APC/C substrates in the presence of non-degradable Cyclin A, in the presence of non-degradable Cyclin B, and in the absence of Cyclin B. The levels of APC/C substrates were unaffected in the presence of Venus-ΔCyclin A, implying that Cyclin A-CDK1 does not regulate APC/C activity during meiosis. However, the levels of Cyclin A were elevated by 5-fold in *Cyclin B*$^{-}$; *HsCycB* mutants, implying the Cyclin B-CDK1 somehow regulates Cyclin A protein levels during meiosis. Non-degradable Cyclin B also caused a slight decrease in Cyclin A levels. Furthermore, *Cyclin A* mRNA levels were not significantly altered in the *Cyclin B*$^{-}$; *HsCycB* mutants. Therefore, it seems that *Cyclin B*$^{-}$; *HsCycB* positively regulates APC/C activity to promote Cyclin A degradation during meiosis. It may be useful to determine whether Cyclin B exerts a similar effect on other APC/C substrates as well.

The phenotypes seen in *Cyclin B*$^{-}$; *HsCycB* mutants and in the presence of Venus:ΔCyclin A have several similarities. They both involve chromosome missegregation during meiosis I and meiosis II, implying that these defects may be due to high levels of Cyclin A-CDK1 activity. Previous studies have shown that Cyclin A is present at low levels during the metaphase I arrest, but *Cyclin A* mRNA undergoes polyadenylation at this stage; this promotes Cyclin A accumulation after the metaphase I arrest is released (Vardy *et al.*, 2009). *Cyclin B* mRNA also undergoes polyadenylation during the metaphase I arrest; this corresponds with an increase in Cyclin B levels during the metaphase I arrest. Thus it seems that Cyclin B protein accumulates before Cyclin A

protein even though their mRNAs are polyadenylated at the same stage. Therefore, Cyclin B-CDK1 may activate APC/C as soon as Cyclin B starts to accumulate; this would prevent inappropriate Cyclin A accumulation during late metaphase I and anaphase I.

Since the transition from meiosis I to meiosis II can take place in the absence of Cyclin B, it is possible that Cyclin B protein levels are down-regulated after chromosomes have segregated during meiosis I. If this is the case, Cyclin A protein levels can rise. Cyclin A-CDK1 may then aid in the meiosis I to meiosis II transition possibly by localizing to the MTOC and regulating the activity of proteins such as Mud. Cyclin A-CDK1 may also aid in aligning chromosomes at the spindle equators (since most eggs expressing stabilized Cyclin A had metaphase II-like spindles). During early meiosis II, Cyclin B levels may start rising again. Cyclin B-CDK1 may then activate the APC/C (APC/C^{Cort}) to promote Cyclin A degradation during prometaphase II (Vardy *et al.*, 2009; Swan *et al.*, 2005); this would promote proper and timely chromosome segregation during anaphase II. Cyclin B3-CDK1 may also play a role in the regulation of Cyclin A and Cyclin B protein stability since the levels of Cyclins A and B are elevated in *Cyclin B3* mutant eggs and reduced in eggs expressing stabilized Cyclin B3 (Bourouh and Swan, unpublished data). However, the post-transcriptional regulation of Cyclin B3 needs to be examined to better understand the stages during which Cyclin B3 may promote Cyclin degradation.

Although this study suggests that Cyclin B-CDK1 promotes Cyclin A degradation via the APC/C, further studies are still required to confirm direct activation of APC/C by Cyclin B-CDK1. Furthermore, Cyclin protein levels are controlled in various ways during

meiosis, including those involving mRNA stability, mRNA masking, mRNA translational activation, and protein degradation (Vardy and Orr-Weaver, 2007; Vardy *et al.*, 2009). Therefore, Cyclin-CDK1 complexes may regulate expression of fellow Cyclins by acting on any or many of these pathways. Further studies are also required to rule out other modes of Cyclin regulation via Cyclin-CDK1 complexes.

4.7 Development of genetic tools to examine meiotic function of essential genes

Bgcn is expressed in early oogenesis, during which mitotic divisions give rise to the oocyte (Ohlstein *et al.*, 2000). This gene is not expressed during meiosis. Therefore, the regulatory elements of this gene were used to make a *Gal4* driver that would have the potential to rescue the early mitotic divisions that give rise to the oocyte in certain null backgrounds. For example, *Cyclin B* mutants fail to form and maintain GSCs; therefore, to study meiosis in the *Cyclin B* mutant background, the GSC divisions need to be rescued. A driver such as *bgcn-Gal4:VP16* (in combination with *UASp-Cyclin B* transgenes) could theoretically rescue the early oogenesis requirement for Cyclin B.

However the constructed *bgcn-Gal4:VP16* driver was not expressed in the Drosophila germline. This was most likely due to missing regulatory elements in the 5' promoter region or the 3' region. Although the 3' end was missing two C's, it is unlikely that this would affect the expression of this driver. The most likely possibility is that the 5' promoter region used to make the driver does not contain the entire promoter sequence of *Bgcn* or is missing key enhancer elements that aid in gene expression. In a recent study, *Bgcn* mutants were rescued by a *Bgcn* transgene (*bgcn*P-Bgcn:GFP) that

was expressed using a 2000 bp putative *Bgcn* promoter (Li *et al.*, 2009). Thus, it may be that the promoter region of *Bgcn* is much larger than the 878 bp fragment used to generate the *bgcn-Gal4:VP16* driver. To reconstruct this driver, it may be useful to inquire about the putative promoter region used by the authors of the *Bgcn* study (Li *et al.*, 2009).Unlike the *bgcn-Gal4:VP16* driver, the *hsp70-Gal4-SCS* transgene may serve as an early oogenesis driver since it is well-expressed in the germarium under heat-shock conditions.

The best alternative to the *UAS*/Gal4 system may be the use of RNAi (shRNAs) in the Drosophila germline. This technique allows knockdown of specific genes during meiosis, therefore bypassing the need to rescue embryonic or early oogenesis defects. Therefore, RNAi is a promising approach for examining meiosis in the absence of essential gene products such as Cyclin A. It is also an option for examining redundancy of genes such as those that encode the mitotic Cyclins.

4.8 Conclusion

This study demonstrated that Cyclin A-CDK1 and Cyclin B-CDK1 play important roles during meiosis. Constitutive Cyclin A-CDK1 activity has serious consequences during meiosis and early embryonic development. It causes defects such as chromosome missegregation, abnormal spindle formation, compromised embryonic development, and embryonic death. Uncontrolled Cyclin A-CDK1 activity also promotes replication of meiotic products. Therefore, proper regulation of Cyclin A-CDK1 activity is essential for normal meiotic progression and subsequent embryonic development.

This study also shows that constitutive inactivation of Cyclin B-CDK1 causes

severe defects during meiosis. In the absence of Cyclin B, oocytes fail to maintain a stable metaphase I arrest and undergo precocious anaphase I, which is characterized by chromosome missegregation. This defect may be caused by a failure to establish or maintain stable kinetochore-microtubule attachments during metaphase I. In the absence of Cyclin B-CDK1 activity, meiosis II spindles are abnormal and anaphase II also involves defects in chromosome segregation. The resulting meiotic products fail to produce a female pronucleus; instead they undergo repeated DNA replication. Embryonic development is never initiated after fertilization; thus, all resulting eggs are inviable. Furthermore, Cyclin A levels are elevated in eggs of *Cyclin B*$^{-}$; *HsCycB* mutants, suggesting that Cyclin B-CDK1 may regulate Cyclin A protein levels, possibly by activating the APC/C. Therefore, this study demonstrates that proper regulation of Cyclin-CDK1 activity is essential for normal meiotic progression and subsequent embryonic development.

This study can also help understand the basic aspects of human meiosis regulation. In humans, both meiosis I and meiosis II are regulated by Cyclin-CDK1 complexes, implying that proper regulation of Cyclin-CDK1 activity is critical for normal meiosis completion and embryonic development. Diseases such as Down's Syndrome result from chromosome segregation errors that occur during meiosis I (Jones, 2007). This study showed that constitutive Cyclin A-CDK1 activation and constitutive Cyclin B-CDK1 inactivation cause chromosome nondisjunction during meiosis I. Furthermore, aneuploidy seen in human embryos is thought to arise from gradual loss of Cohesin complexes from the chromosome arms (Jones, 2007). Interestingly, studies in Xenopus

and mammals have shown that Cyclin-CDK1 complexes can promote the removal of Cohesin complexes from the arms of sister chromatids during mitosis (Nigg, 2001). Based on this, it is possible that deregulation of Cyclin-CDK1 activity may contribute to defective human meiosis. Thus, the results of this study may provide a basis for understanding and further exploring the mechanisms that cause infertility and birth defects in humans.

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APPENDIX A: List of Plasmids

	Plasmid Name	Additional Notes	Location
1	FzrΔd		Plasmids-1-RD
2	Fzr cDNA RE20929		Plasmids-1-RD
3	pSK CycB GFP	Could not PCR amplify *Cyclin B* from this vector.	Plasmids-1-RD
4	LD44443	*Cyclin A* cDNA	Plasmids-1-RD
5	pBI		Plasmids-1-RD
6	pBSKS+	pBlueScript	Plasmids-1-RD
7	pTiger-GFP		Plasmids-1-RD
8	1093	UASp-Venus Gateway Destination Vector	Plasmids-1-RD
9	1077	UASp-eGFP Gateway Destination Vector	Plasmids-1-RD
10	UASp-NterProtA	Gateway Destination Vector	Plasmids-1-RD
11	UASp-CterProtA	Gateway Destination Vector	Plasmids-1-RD
12	ActSC-Gal4	Gateway Destination Vector	Plasmids-1-RD
13	Hsp70SCS	pELBA	Plasmids-1-RD
14	F89	*Gal4*-containing vector from Dr. Andrea Brand's lab	Plasmids-1-RD
15	Valium 22	ShRNA Expression Vector	Plasmids-1-RD
16	Cyclin A-pENTRY		Plasmids-1-RD
17	UASp-Venus-Cyclin A		Plasmids-1-RD
18	UASp-ProtA-Cyclin A		Plasmids-1-RD
19	UASp-FLAG-Cyclin A		Plasmids-1-RD
20	dCyclin A-pENTRY		Plasmids-1-RD
21	UASp-Venus-dCyclin A		Plasmids-1-RD
22	Cyclin B-pENTRY		Plasmids-1-RD
23	UASp-Venus-Cyclin B		Plasmids-1-RD
24	UASp-ProtA-Cyclin B		Plasmids-1-RD
25	UASp-FLAG-Cyclin B		Plasmids-1-RD
26	Hsp70Gal4SCS-pattB		Plasmids-1-RD
27	3'-bgcn pBS	3' region of *Bgcn* in pBS	Plasmids-1-RD
28	Gal4-3'bgcn pBS	*Gal4:VP16* and 3'region of *Bgcn* in pBS	Plasmids-1-RD
29	5'-Gal4-3' pBS	5' region of *Bgcn*, *Gal4:VP16*, and 3' region of *Bgcn* in pBS	Plasmids-1-RD
30	bgcn-Gal4 pBI		Plasmids-1-RD
31	wt Fzr pBS		Plasmids-1-RD
32	wt Fzr pTiger-GFP		Plasmids-1-RD
33	Δd Fzr pBS		Plasmids-1-RD

34	Δd Fzr pTiger-GFP		Plasmids-1-RD
35	CycA 1503 Val22	shRNA sequence targeting position 1503 of *Cyclin A* mRNA	Plasmids-1-RD
36	CycA UTR Val22	shRNA sequence targeting 3' UTR of *Cyclin A* mRNA	Plasmids-1-RD
37	CycB 1289 Val22	shRNA sequence targeting position 1289 of *Cyclin B* mRNA	Plasmids-1-RD
38	CycB UTR Val22	shRNA sequence targeting 3' UTR of *Cyclin B* mRNA	Plasmids-1-RD
39	CycB3 196 Val22	shRNA sequence targeting position 196 of *Cyclin B3* mRNA	Plasmids-1-RD
40	CycB3 UTR Val22	shRNA sequence targeting 3' UTR of *Cyclin B3* mRNA	Plasmids-1-RD

APPENDIX B: List of Primers

	Primer Name	Primer Sequence	Primer Purpose	Additional Notes	Location
1	rp49 (rpL32) For a	CGTGAAGAAGCGCACCAAG CAC	qRT-PCR		Primers-1-RD
2	rp49 (rpL32) Rev a	GCGCCATTTGTGCGACAGCT TAG	qRT-PCR		Primers-1-RD
3	rp49 (rpL32) For b	AGCTGTCGCACAAATGGCG CAA	qRT-PCR	Produces non-specific band	Primers-1-RD
4	rp49 (rpL32) Rev b	GGGTGCGCTTGTTCGATCCG T	qRT-PCR	Produces non-specific band	Primers-1-RD
5	Cyclin A For	TCAGCGTGGGCACTGAAAC GG	qRT-PCR		Primers-1-RD
6	Cyclin A Rev	GGGCGATGTTTCTTCTCGCT CTCC	qRT-PCR		Primers-1-RD
7	Cyclin B For	GCCGAGGACGAGCACCATA CG	qRT-PCR		Primers-1-RD
8	Cyclin B Rev	GTGAGGCAGCTGCAATCTCC GA	qRT-PCR		Primers-1-RD
9	Cyclin B3 For	TTGCCGCCGTCAAGCCAGTT	qRT-PCR		Primers-1-RD
10	Cyclin B3 Rev	CGCGCCGCCTCAAAGTTTCC	qRT-PCR		Primers-1-RD
11	Cyclin A gDNA For	GGAAGTCGGACCCTGTCGC AC	qRT-PCR		Primers-1-RD
12	Cyclin A gDNA Rev	CGGACGCAGACAGACAAGC GT	qRT-PCR		Primers-1-RD
13	Cyclin A (1508) Top	ctagcagtCTCCCTAGTCGACA AAGAGAAtagttatattcaagcat aTTCTCTTTGTCGACTAGGG AGgcg	shRNA cloning		Primers-1-RD
14	Cyclin A (1508) Bottom	aattcgcCTCCCTAGTCGACAA AGAGAAtatgcttgaatataacta TTCTCTTTGTCGACTAGGGA Gactg	shRNA cloning		Primers-1-RD
15	Cyclin A (3'UTR) Top	ctagcagtCCCGTTGCAAGTGC CGGACAAtagttatattcaagcat aTTGTCCGGCACTTGCAACG GGgcg	shRNA cloning		Primers-1-RD
16	Cyclin A (3'UTR) Bottom	aattcgcCCCGTTGCAAGTGCC GGACAAtatgcttgaatataacta TTGTCCGGCACTTGCAACGG	shRNA cloning		Primers-1-RD

		Gactg			
17	Cyclin B (1289) Top	ctagcagtCCGACCAAGACCACTGTAGAAtagttatattcaagcataTTCTACAGTGGTCTTGGTCGGgcg	shRNA cloning		Primers-1-RD
18	Cyclin B (1289) Bottom	aattcgcCCGACCAAGACCACTGTAGAAtatgcttgaatataactaTTCTACAGTGGTCTTGGTCGGactg	shRNA cloning		Primers-1-RD
19	Cyclin B (3'UTR) Top	ctagcagtCCGGTAAGACTAGGAACCGAAtagttatattcaagcataTTCGGTTCCTAGTCTTACCGGgcg	shRNA cloning		Primers-1-RD
20	Cyclin B (3'UTR) Bottom	aattcgcCCGGTAAGACTAGGAACCGAAtatgcttgaatataactaTTCGGTTCCTAGTCTTACCGGactg	shRNA cloning		Primers-1-RD
21	Cyclin B3 (196) Top	ctagcagtTAGCGAACGACTCAAGTTCTAtagttatattcaagcataTAGAACTTGAGTCGTTCGCTAgcg	shRNA cloning		Primers-1-RD
22	Cyclin B3 (196) Bottom	aattcgcTAGCGAACGACTCAAGTTCTAtatgcttgaatataactaTAGAACTTGAGTCGTTCGCTAactg	shRNA cloning		Primers-1-RD
23	Cyclin B3 (3'UTR) Top	ctagcagtCACAATCAATCTAAACTCGAAtagttatattcaagcataTTCGAGTTTAGATTGATTGTGgcg	shRNA cloning		Primers-1-RD
24	Cyclin B3 (3'UTR) Bottom	aattcgcCACAATCAATCTAAACTCGAAtatgcttgaatataactaTTCGAGTTTAGATTGATTGTGactg	shRNA cloning		Primers-1-RD
25	attB1 - cyclin A	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCAGTTTC	Cloning Cyclin A into pDONR221	Contains full attB sequence	Primers-1-RD
26	attB2 - cyclin A	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAAAACTTATAAAA	Cloning Cyclin A into pDONR221	Contains full attB sequence	Primers-1-RD
27	attB1 - cyclin A d1-53	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGCAACAACAAT	Cloning ΔCyclin A into pDONR221	Contains full attB sequence	Primers-1-RD
28	CycB attb1 for	AAAGCAGGCTTAATGGTGGGCACAACACTGAAAATG	Cloning Cyclin B into pDONR221	Contains partial attB sequence	Primers-1-RD

29	CycB attb2 rev	GAAAGCTGGGTACTATTTCC TCTGGCTCTGGCCCAC Bottom of Form	Cloning Cyclin B into pDONR221	Contains partial attB sequence	Primers-1-RD
30	fzr-Xba-rev	ACACTCTAGATTATCTGATA TTGGCAAACAGATTC	Cloning Fzr into pTIGER-GFP		Primers-1-RD
31	Fzr-Bam-for-2	ACACGGATCCATGTTTAGTC CCGAGTACGAGAAGC	Cloning Fzr into pTIGER-GFP		Primers-1-RD
32	bgcn-pr-forNot	TCTCGCGGCCGCCTTTAAGG CGGTGCCTGTCACC	Cloning bgcn-Gal4:VP16 into pBS		Primers-1-RD
33	bgcn-pr-revBam	TCTCGGATCCCATTTCTGCTA GCTTCAAACAGATTGAC	Cloning bgcn-Gal4:VP16 into pBS		Primers-1-RD
34	Gal4-forBam	TCTCGGATCCAAGCTACTGT CTTCTATCGAACAAGC	Cloning bgcn-Gal4:VP16 into pBS		Primers-1-RD
35	VP16Hind	TCTCAAGCTTCTACCCACCG TACTCGTCAATTCC	Cloning bgcn-Gal4:VP16 into pBS		Primers-1-RD
s	bgcn-3prime-forH3	TCTCAAGCTTTAAAGCCCGC TTCCTCGCGCATATTGC	Cloning bgcn-Gal4:VP16 into pBS		Primers-1-RD
37	bgcn-3prime-revKpn	TCTCGGTACCTGAATTCAAT TTACCACTTAGGC	Cloning bgcn-Gal4:VP16 into pBS		Primers-1-RD
38	Gal4 rev EcoRI	TCTCGAATTCTTACTCTTTTT TTGGGTTTGGTGG	Cloning Gal4 into pELBA		Primers-1-RD
39	Gal4 for EcoRI	TCTCGAATTCATGAAGCTAC TGTCTTCTATCGAA	Cloning Gal4 into pELBA		Primers-1-RD
40	Gal4 for 2217	TACCAAGAAGCACACCTTCG	Sequencing		Primers-1-RD
41	Gal4 for 745	GTTTACTTCAAAGTTATCTC	Sequencing		Primers-1-RD
42	Gal4 for 1302	GCATCATTGAAACAGCAAG G	Sequencing		Primers-1-RD
43	Gal4 rev 377	TGATGATGTCGCACTTATTC	Sequencing		Primers-1-RD
44	Fzr For 1	CGCAAGATCTCTCGCATTCC	Sequencing		Primers-1-

		C			RD
45	Fzr Rev 1	GCCTTTACAGCCGCCATATGC	Sequencing		Primers-1-RD
46	Fzr For 2	GATCGGTGTATCCGTTTCTGG	Sequencing		Primers-1-RD
47	Fzr Rev 2	ATTGTAGTCCTGCTTGGTGGG	Sequencing		Primers-1-RD
48	Venus For	GCTGCTGCCCGACAACCACT	Sequencing		Primers-1-RD
49	Cyclin A For 1	TCAACACCCATGTCCGTGAC	Sequencing		Primers-1-RD
50	Cyclin A Rev 1	GATGAAGACGTAAGCAGTCGG	Sequencing		Primers-1-RD
51	Cyclin A For 2	GCTGTACTTTGCGACATGCC	Sequencing		Primers-1-RD
52	Cyclin A Rev 2	CGAGAATGGAGCGGTCCACGG	Sequencing		Primers-1-RD
53	Cyclin B gDNA forward 1984	TCCGTGGACTACGAAATGGCC	Sequencing		Primers-1-RD
54	Cyclin B gDNA reverse 381	AGCACCGCAAGACTGATCCTC	Sequencing		Primers-1-RD
55	Cyclin B gDNA forward 709	GTCCCATCGCAGCGAAGGATG	Sequencing		Primers-1-RD
56	Cyclin B gDNA forward 1322	ACTCACTGGAAGTGAAAAAGG	Sequencing		Primers-1-RD
57	Cyclin B3 for 729	AAACTTTGAGGCGGCGCGACG	Sequencing		Primers-1-RD
58	Cyclin B3 rev 324	CTGGGCCGTTGGCTTTTTGCC	Sequencing		Primers-1-RD
59	Cyclin B3 for 1300	ATCCCGCTCTCGTACCGTTTC	Sequencing		Primers-1-RD

APPENDIX C: List of Fly Stocks

	Line	Chromosome	Genotype	Additional Comments	Location
1	CycB2;nosGal4	2;3	CycB2/CyO; nosGal4/TM6	For expressing non-degradable Cyclin B in *Cyclin B* mutant background	RD - 1
2	Cyclin B-TPM-GFP	3	CyO/S; UASp-CyclinBTPM-GFP	Rebalanced (stock at 18C is contaminated)	RD - 1
3	Df(2R)59AB; Cyclin B-TPM-GFP	2;3	Df(2F)59AB/CyO; UASp-CyclinBTPM-GFP/TM6	For expressing non-degradable Cyclin B in *Cyclin B* mutant background	RD - 1
4	CycB2	2	CycB2/CyO; TM6/Pr	Balanced	RD - 1
5	Df(2R)59AB	2	Df(2R)59AB/CyO; TM6/Pr	Balanced	RD - 1
6	CycB2; CycB3L6-HsCycB	2;3	CycB2/CyO; CycB3L6540-HsCycB/TM6		RD - 1
7	GFP	2	UASp-GFP/CyO	Reporter gene	RD - 1
8	GFP	X	UASp-GFP	Reporter gene	RD - 1
9	LacZ	X	UASp-LacZ	Reporter gene	RD - 1
10	HsCycA	2	HsCycA/HsCycA	New HsCycA from Lehner Lab - unbalanced (not in 18C stocks)	RD - 1
11	HsCycA	2	HsCycA/CyO; TM6/Pr	New HsCycA from Lehner Lab (not in 18C stocks)	RD - 1
12	CycAL7R1	3	CyO/S; CycAL7R1/TM6		RD - 1
13	CycAH17b	3	CyO/S; CycAH17b/TM6		RD - 1
14	HsCycA; CycAL7R1	2;3	HsCycA/CyO; CycAL7R1/TM6	For rescue of Cyclin A mutants	RD - 1
15	HsCycA; CycAH17b	2;3	HsCycA/CyO; CycAH17b/TM6	For rescue of Cyclin A mutants	RD - 1
16	Hsp70Gal4SCS; CycAL7R1	2;3	Hsp70Gal4SCS/CyO; CycAL7R1/TM6		RD - 1
17	Flag-Cyclin A; CycAH17b	2;3	UASp-Flag-Cyclin A/CyO;	For rescue of *Cyclin A* mutants	RD - 1

			CycAH17b/TM6		
18	Flag-Cyclin A; CycAL7R1	2;3	UASp-Flag-Cyclin A/CyO; CycAL7R1/TM6		RD - 1
19	CycAH17b-daGal4	3	CyO;S; CycAH17b-daGal4/TM6	For rescue of Cyclin A mutants	RD - 1
20	actGal4; CycAL7R1	2;3	actGal4/CyO; CycAL7R1/TM6	For rescue of Cyclin A mutants	RD - 2
21	UASt-Cyclin A; CycAH17b	2;3	UASt-Cyclin A/CyO; CycAH17b/TM6	For rescue of Cyclin A mutants	RD - 2
22	CycAH17b-HsCycA	3	CyO/S; CycAH17b-HsCycA/TM6	For rescue of Cyclin A mutants	RD - 2
23	CycAH17b-HsCycB	3	CyO/S; CycAH17b-HsCycB/TM6		RD - 2
24	Cyclin A	2	Sp/CyO; UASp-Cyclin A/TM3	From China	RD - 2
25	Venus-Cyclin A 15c	3	CyO/S; UASp-Venus-Cyclin A 15c/TM6		RD - 2
26	Venus-Cyclin A 21c	2	UASp-Venus-Cyclin A 21c; TM6/Pr		RD - 2
27	Venus-Cyclin A 21a	3	CyO/S; UASp-Venus-Cyclin A 21a/TM6	Expressed at high levels with mat67-Gal4	RD - 2
28	ΔCyclin A		Sp/CyO; UASp-ΔCyclin A/TM3	From China	RD - 2
29	Venus-ΔCyclin A 6d	2	UASp-Venus-ΔCyclin A 6d/CyO; TM6/Pr	Expressed at low levels with *mat67-Gal4*	RD - 2
30	Venus-ΔCyclin A 7e	2	UASp-Venus-ΔCyclin A 7e/CyO; TM6/Pr	Expressed at low levels with *mat67-Gal4*	RD - 2
31	Venus-ΔCyclin A 16f	3	CyO/S; UASp-Venus-ΔCyclin A 16f	Expressed at higher levels with *mat67-Gal4*	RD - 2
32	Venus-ΔCyclin A 2a	2	UASp-Venus-ΔCyclin A 2a; TM6/Pr	Expressed at high levels with *mat67-Gal4*	RD - 2
33	Venus-ΔCyclin A 16x	3	CyO/S; UASp-Venus-ΔCyclin A 16x	Expressed at very high levels with *mat67-Gal4*	RD - 2
34	Venus-Cyclin B 31a	2	UASp-Venus-Cyclin B 31a/CyO; TM6/Pr	Used for experiments	RD - 3
35	Venus-Cyclin B 31b	3	CyO/S; UASp-Venus-Cyclin B		RD - 3

			31b/TM6		
36	ProtA-Cyclin B 11f	3	CyO/S; UASp-ProtA-Cyclin B 11f		RD - 3
37	Flag-Cyclin B 11b	3	CyO/S; UASp-Flag-Cyclin B 11b/TM6		RD - 3
38	Flag-Cyclin A 37h	3	CyO/S; UASp-Flag-Cyclin A 37h/TM6		RD - 3
39	Flag-Cyclin A 37b	2	UASp-Flag-Cyclin A 37b/CyO; TM6/Pr	Used for experiments	RD - 3
40	Flag-Cyclin A 25c	X	UASp-Flag-Cyclin A 25c		RD - 3
41	Flag-Cyclin A 37j	X	UASp-Flag-Cyclin A 37j		RD - 3
42	Flag-Cyclin A 3f	2	UASp-Flag-Cyclin A 3f/CyO; TM6/Pr		RD - 3
43	Flag-Cyclin A 56d	X	UASp-Flag-Cyclin A 56d		RD - 3
44	ProtA-Cyclin A 10a	3	CyO/S; UASp-ProtA-Cyclin A 10a/TM6		RD - 3
45	ProtA-Cyclin A 30a	2	UASp-ProtA-Cyclin A 30a/CyO; TM6/Pr		RD - 3
46	ProtA-Cyclin A 40a	2	UASp-ProtA-Cyclin A 40a/CyO; TM6/Pr		RD - 3
47	ProtA-Cyclin A 40b	2	UASp-ProtA-Cyclin A 40b; TM6/Pr		RD - 3
48	ProtA-Cyclin A 30b	2	UASp-ProtA-Cyclin A 30b/ CyO; TM6/Pr		RD - 3
49	ProtA-Cyclin A 57d	3	CyO/S; UASp-ProtA-Cyclin A 57d/TM6		RD - 3
50	ProtA-Cyclin A 2x	2	UASp-ProtA-Cyclin A 2x/CyO; TM6/Pr		RD - 3
51	Hsp70Gal4SCS 4c	2	Hsp70Gal4SCS 4c/CyO; TM6/Pr		RD - 3
52	Hsp70Gal4SCS 4b	2	Hsp70Gal4SCS 4b/CyO; TM6/Pr	Used for experiments	RD - 3
53	Hsp70Gal4SCS 1a	3	CyO/S; Hsp70Gal4SCS 1a/TM6		RD - 3

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