Investigation of meiotic organelle checkpoint functions by *Drosophila* Myt1

by

Ramya Varadarajan

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Molecular Biology and Genetics

Department of Biological Sciences
University of Alberta

Abstract

Meiosis in eukaryotes includes a developmentally programmed pre-meiotic G2 phase arrest before the onset of MI division mediated by inhibitory phosphorylation of Cdk1. There are two inhibitory kinases, Wee1 and Myt1. Myt1 mediated Cyclin B-Cdk1 regulation serves as a conserved mechanism for maintaining pre-meiotic oocyte arrest in many animal models. Earlier reports from the Campbell lab, however, have shown that loss of Myt1 activity affected multiple aspects of *Drosophila* spermatogenesis resulting in male sterility. The conserved meiotic checkpoint function of Myt1 was hypothesized to account for *myt1* mutant male sterility, as *Drosophila* spermatocytes normally undergo a developmentally regulated pre-meiotic G2 phase arrest before MI. This possibility, however, has not been tested.

Here I show that loss of Myt1 activity neither affects the timing of pre-meiotic G2 phase arrest nor the overall coordination of G2/MI transition. Instead, the phenotypic analysis of *myt1* mutants indicated that Myt1 activity is required for structural integrity of a germline specific membranous cytoskeletal organelle called the fusome (or intercellular bridges). I found that inhibition of Cyclin A-Cdk1 during early spermatocyte development requires Myt1 activity to prevent fusomes from premature Cdk1 activation. Mis-regulation of Cyclin A-Cdk1 during spermatocyte development also perturbed premature centrioles dis-engagement, producing multipolar meiotic spindles resulting in aneuploidy of *myt1* meiocytes. I conclude that the role of Myt1 during pre-meiotic G2 phase arrest of male meiosis is to regulate discrete checkpoint mechanisms that are used to spatially and temporally coordinate cytoplasmic organelle behavior with the nuclear events of meiotic progression that are triggered by $Cdc25^{Twe}$-mediated Cdk1 activation, at G2/MI.

Preface

Chapter II of this thesis will be published as Ramya Varadarajan, Joseph Ayeni, Zhigang Jin, Ellen Homola and Shelagh D. Campbell, "Myt1 regulation of Cyclin A-Cdk1 links meiotic centrosome behaviour to ER integrity". I was responsible for the data collection and analysis as well as the manuscript composition. Joseph Ayeni partly contributed in acquiring data for Fig. 2-13. Zhigang Jin contributed by reporting the *myt1* mutant phenotypes. Ellen Homola assisted with standardizing the BrdU chase experiment. Shelagh Campbell is the supervisory author and is involved with manuscript composition.

The data presented in Chapter III were designed and collected by myself. The intention is to combine the data presented in this chapter with complementary data from the thesis of a previous Ph.D. student (Dr. Ayeni) and to submit this as a joint first-author manuscript.

Acknowledgments

I would like to express my sincere thanks to my supervisory Professor **Dr. Shelagh D Campbell**. You have been a wonderful mentor to me. I take this opportunity to acknowledge your continuous support and encouragement during the past six years and for allowing me to grow as an independent researcher. I am grateful to you for understanding my limitations and for bringing out the best in me. I also thank my committee members, **Dr. Gordon Chan, Dr. Sarah Hughes,** and **Dr. Martin Srayko** for providing realistic suggestions and for their guidance throughout my research journey. I would especially like to thank Dr. William Brook (University of Calgary) for offering his lab space and resources to perform four months of research work while I was in Calgary. Thanks to my lab members Dr. Ellen Homola, Dr. Joseph Ayeni and Dr. Zhigang Jin for their advice and comments in performing my thesis research. I thank Madhur Prashar, Eliza, Ammar and Erica for taking part in my thesis research while they were doing their summer internship in the lab. I specially thank Arlene Oatway in the microscopy facility, Biological sciences, Univ of Alberta for helping me with electron microscopy and Jin for providing fly food.

Special thanks to my long-term friend and beloved husband, Sabi, as you are an inspiration for doing research. I could recall our research collaboration from our first year undergrad spectrophotometry analysis done in physics laboratory, GCT. Wish to continue the research journey with you in future too. Words cannot express how grateful I am to my Amma, Appa and Akka for your unconditional love and the sacrifices that you have done for my well-being. Your prayers for me were what sustained me thus far. Your encouragement and support drove me to strive towards my goal. I thank the apple of my eye, my daughter Anjali, who arrived amidst my Ph.D. journey. Thanks sweetie for your cooperation and trust. Above all, thank God Almighty Christ, for giving me strength during the tough times and for lifting me up at every time my spirit felt down. “Your grace was sufficient for me” – 2 Corinthians 12:9.

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List of Abbreviations

A: Alanine

APC/C: anaphase promoting complex/cyclosome

ATM: Ataxia telangiectasia mutated

AurA-T288p: a phospho-specific T288 Aurora A antibody

Bam: Bags of Marbles

Bgcn: Benign gonial cell neoplasm

BicD: BicaudalD

BrdU: 5'-bromo-2'-deoxyuridine

CAK: Cdk1 activating kinase

cAMP: Cyclic adenosine 3', 5'-monophosphate

CKI: Cdk inhibitors

Cdks: Cyclin dependent kinases

cDNA: Complementary deoxy ribonucleic acid

E2F: Elongation factor-2

Egl: Egalitarian

EM: Electron microscopy

EMS: Ethyl methanesulfonate

ER: Endoplasmic reticulum

FACS: Fluorescence activated cell sorting

FLIP: Fluorescence loss in photobleaching

FRAP: Fluorescence recovery after photobleaching

G phase: Growth/Gap phase

GB: Gonioblast

GFP: Green fluorescent protein

GSC: Germline stem cells

GVBD: Germinal vesicle breakdown

Hts: Hu-li-tai-shao, an adducin-like protein

k Da: Kilo Dalton

M phase: Mitotic/Meiosis phase

MAPK: Mitogen activated protein kinase

Mos: Moloney murine sarcoma

MPF: Mitosis/Meiosis promoting factor

MPM2: mitotic/meiotic phosphoproteins monoclonal antibody 2

NEB: Nuclear envelop breakdown

p90(**RSK**): 90 kDa size ribosomal kinase protein

PCM: peri-centriolar matrix

PCR: Polymerase chain reaction

PH3: phosphorylated form of histone H3

PKA: Protein Kinase A

PI(3)K: phosphyatidylinositol-3-OH kinase

Plk: polo kinase

RB: retinoblastoma

RNAi: RNA interference

Sa: Spermatocyte arrest

SAC: spindle assembly checkpoint

SCF: Skip-Cullin-F-box

siRNA – Short interference ribonucleic acid

SOP: sensory organ precursor

S phase: Synthesis phase

SSC: Somatic stem cells

Stg: mitotic Cdc25 String

T: Threonine

TA: transit amplifications

Twe: meiotic Cdc25 Twine

VFP: Venus fluorescent protein

Y: Tyrosine

Chapter I

1. Introduction

"Therefore, I reasoned that study of the cell cycle responsible for the reproduction of cells was important and might even be illuminating about the nature of life" – Paul Nurse

Reproduction is an inherent behavior of living beings necessary to sustain life and avoid extinction. This phenomenon of life is observed from prokaryotes to multicellular eukaryotes. There are different methods of reproduction across species, but they can be classified into two types; asexual and sexual. Most prokaryotes and a few eukaryotes including certain fungi and plants reproduce asexually by undergoing binary fission, budding, sporulation, fragmentation or parthenogenesis to create genetically identical offspring. However, most eukaryotes including animals and plants reproduce sexually by fusing gametes of two parents, creating genetic diversity within their species. During the cell cycle, chromosomes and many different types of organelles are replicated during synthesis (S) phase so that they can be divided equally during mitotic (M) phase. In most somatic cell cycles these two events are separated by gap phases (G1 and G2) that are regulated by mechanisms that strictly monitor the readiness of cells to enter and exit S or M phases. These molecular mechanisms called "checkpoints" are meant to ensure that cells are ready to progress to the next stage of the cell cycle. For example, G2/M checkpoints that promote inhibitory phosphorylation of Cdk1 ensure that cells with damaged DNA do not enter mitosis and trigger apoptosis. Defects in such checkpoint mechanisms can lead to abnormal cell divisions resulting in deleterious conditions such as cancer.

1.1. Cdk regulation by inhibitory phosphorylation

Progression through different stages of the eukaryotic cell cycle is driven by activation and inactivation of conserved, **c**yclin-**d**ependent **k**inases collectively referred to as Cdks. My thesis concerns regulation of the major mitotic regulator Cdk1, which forms complexes with mitotic cyclins that include A and B-types. In eukaryotes, when Cdk1 is activated during early stages of M phase these kinases phosphorylate and modify the behavior of a vast array of proteins, triggering re-organization of almost every part of the cell. Cdk1 activity must therefore be restricted to M phase and not be present at other stages of the cells cycle. This is accomplished by several distinct mechanisms for regulating Cdk1 activity, including synthesis and destruction of mitotic cyclins, inhibitory and activating phosphorylation, and physical associations with Cdk inhibitors. Our laboratory studies the role that Cdk1 inhibitory phosphorylation by Wee1 and Myt1 kinases plays during the development of *Drosophila melanogaster*.

1.1.1 Cdk1 inhibitory kinases Wee1 and Myt1

Wee1 was first discovered in *S. pombe* as one regulator of a cell size checkpoint mechanism that operates during G2 phase of the cell cycle (Nurse and Thuriaux, 1980; Russell and Nurse, 1987) and was later shown to phosphorylate Cdk1 (Cdc2) on tyrosine residue 15 (Y15) (Gould and Nurse, 1989). Temperature-sensitive alleles of Wee1 caused premature mitotic entry without completing the G2 growth phase, resulting in small ('wee') daughter cells. Subsequently a second Wee1-type kinase called Mik1 was identified in a genetic screen for second-site mutations, with temperature-sensitive double mutants for *wee1* and *mik1* resulting in lethal mitotic catastrophe (Lundgren *et al.*, 1991). Although loss of Mik1 alone has no

phenotype, subsequent studies showed it acts redundantly with Wee1 in regulating Cdk1 activity during S phase (Rhind and Russell, 2001).

My work focuses on regulation of Cdk1 in *Drosophila* by a Wee1-related inhibitory kinase called Myt1, one of two conserved Wee1-related kinases found specifically in metazoan (multicellular) eukaryotic organisms. Myt1 was first identified in *Xenopus* oocyte membrane extracts as a dual-specificity membrane-associated protein kinase capable of inhibiting Cdk1 by both T14 and Y15 phosphorylation (Kornbluth *et al.*, 1994). Based on sequence similarities between conserved regions of the kinase domain in *S. pombe* Wee1, Mik1 and human Wee1, PCR primers designed to amplify Wee1-related sequences in complementary cDNA from *Xenopus* oocyte extracts were used to identify the Myt1 coding sequence, which stands for Membrane-bound tyrosine (Y) and threonine (T) kinase (Mueller *et al.*, 1995). Subsequent bioinformatics studies indicated that Myt1 kinases evolved specifically in metazoans (Matsuura and Wang, 1996; Booher *et al.*, 1997; Fattaey and Booher, 1997). Human Myt1 is 46% identical to the *Xenopus* homolog and specifically localizes to Golgi and Endoplasmic reticulum (ER) membranes through poorly characterized interactions involving a C-terminal trans-membrane hydrophobic domain (Liu *et al.*, 1997). *In vitro* experiments have also demonstrated that human Myt1 specifically inhibits Cdk1, not Cdk2, which functions during S phase (Booher *et al.*, 1997).

In vitro experiments in human somatic cells using either Myt1 cDNA overexpression or siRNA knockdown have implicated Myt1 in regulating the G2/M transition (Liu *et al.*, 1999a; Wells *et al.*, 1999). Flow cytometry (FACS) assays on Hela cells that over-express Myt1 revealed increased G2 phase cells in the population, indicating that Myt1 overexpression prolonged G2 phase to delay mitotic entry. Curiously, over-expression of a

kinase-inactive Myt1 (N238A) could also prolong G2 phase arrest in this assay, suggesting that catalytic activity of Myt1 does not fully account for the observed mitotic delay. Over-expression of Myt1 lacking a putative Cyclin B interaction motif exhibited no G2 prolongation however, revealing that a physical interaction between Myt1 and Cyclin B-Cdk1 was necessary for G2 phase arrest (Liu *et al.*, 1999a; Wells *et al.*, 1999). Based on these results, physical tethering of Cdk1 complexes to Myt1 has been proposed as a novel mechanism for limiting cytoplasmic and nuclear trafficking of Cdk1 complex to delay mitotic progression. The overexpression of dMyt1 in cultured *Drosophila* S2 cell lines caused a similar reduction in cell proliferation, indicating a block to mitotic progression (Cornwell *et al.*, 2002). Moreover, loss of Myt1 activity by RNAi- mediated knockdown also reduced Cdk1-T14 phosphorylation and accelerated cell proliferation.

Studies of G2/M timing using siRNA mediated knockdown of Wee1 and Myt1 activity in HeLa cells reached different conclusions about the respective roles of Myt1 and Wee1 (Nakajima *et al.*, 2008). Cell synchronized in S phase by thymidine block were transfected with siRNA against either Myt1 or Wee1 and PH3 immuno-labeling was used to quantify the mitotic index (number of mitotic cell/total number of cells) at set times after transfection. Wee1 siRNA transfected cells exhibited higher mitotic index and entered mitosis earlier than the controls, whereas Myt1-siRNA treated cell underwent mitosis at the same pace as the controls, indicating that Wee1 was primarily responsible for maintaining G2/M checkpoint arrest (Nakajima *et al.*, 2008). More recent studies of Wee1 or Myt1 siRNA depletion in HeLa cells using flow cytometry and time-lapse microscopic analysis also made similar findings (Chow and Poon, 2013). Although depletion of Myt1 did not appear to affect the timing of mitotic entry in these studies, subjecting Myt1-siRNA treated cells to ionization

radiation abruptly accelerated mitotic entry with no G2 arrest to repair the induced DNA damage (Chow and Poon, 2013). These observations indicate that regulation by Wee1 and Myt1 are both critical for responding to overwhelming DNA damage. Although loss of function studies of mitotic cells does not support a requirement for Myt1 in timing G2 phase arrest or mitotic entry, more recent studies of Myt1-siRNA treated cells revealed acceleration into G2/M (Villeneuve *et al.*, 2013). These controversial observations may reflect potential technical limitations due to procedures used to synchronize *in vitro* somatic cells for cell cycle checkpoint investigations.

1.1.2 Checkpoint regulation of the cell cycle

The concept of a cell cycle checkpoint was first proposed in 1989 by Hartwell & Weinert as a mechanism for 'ensuring the order of cell cycle events' (Hartwell and Weinert, 1989). Three major cell cycle checkpoints are used to regulate most eukaryotic cells (Fig.1-1). The G1/ S checkpoint determines the timing of DNA synthesis phase and is regulated by cross talk between transcriptional regulators: E2F and retinoblastoma (Rb) protein (Duronio and Xiong, 2013). Physical interaction of Rb with E2F inhibits S phase entry. When the cell is "ready" to proliferate (subject to nutritional and other signals), G1-specific Cdks phosphorylate and inhibit Rb, which in turn relieves E2F suppression. Activated E2F then promotes the synthesis of Cyclin E and other regulators needed for S phase progression and DNA replication (Duronio and O'Farrell 1995; Ikeda *et al.* 1996; Duronio, 2012). When cells encounter defects in DNA replication or DNA repair, the p53 tumor suppressor protein triggers transcription of other regulators to suppress S/G2 progression. DNA damage also induces the genotoxic stress-responsive kinases ATM and ATR, which stimulate check kinases 1/2 (Chk1

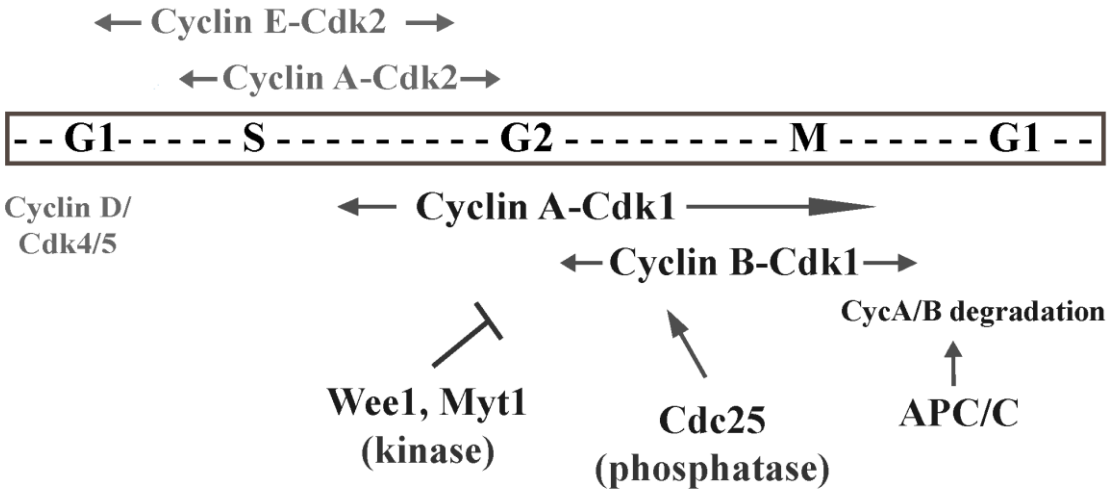

Fig. 1-1: Cdks, Cyclins and regulation of cell cycle

This diagram illustrates different Cyclin-CDK complexes in most eukaryotes, and their approximate times of activity during the cell cycle. During G2 to M phase, mitotic Cdk1 (Cyclin A/B bound) activity is regulated by phosphorylation dependent mechanism. Wee1 and Myt1 kinases inhibit Cdk1 activity, and are opposed by Cdc25 family phosphatases that promote mitotic progression. APC/cyclosome mediated activity degrades mitotic cyclins, and facilitates mitotic exit. Growth factor induced signals reinitiate cell division through CyclinD-Cdk4/5 activity. Cyclin E/A bound Cdk2 mediate DNA replication during S phase.

and Chk2) mediated checkpoint responses to establish a G2/M checkpoint that restricts initiation of mitosis. Activation of Chk1/2 inhibits Cdc25 phosphatase activity and activates Wee1-related kinases (Wee1 and Myt1), resulting in phospho-inhibition of Cyclin A-Cdk1 and Cyclin B-Cdk1 complexes (Abraham, 2001; Taylor and Stark, 2001). Completion of DNA replication and repair then permit the G2/M checkpoint to relax and allow Cdc25-mediated Cdk1 activation, initiating a bi-stable, feed back mechanism that further activates (Cdc25) and inhibits the inhibitors (Wee1/Myt1) to trigger full conversion of inhibited pools of Cyclin B- Cdk1(van Vugt and Yaffe, 2010). This rapid rise in Cyclin B- Cdk1activity triggers the G2/M transition by initiating early mitotic events in both the nucleus and cytoplasm (Ohi and Gould, 1999). A critical mitotic process that requires high Cyclin B-Cdk1 activity is associated with mitotic spindle assembly. Upon entry into mitosis, the spindle assembly checkpoint (SAC) ensures the correct segregation of chromosomes by monitoring proper kinetochore-spindle attachment and spindle tension during metaphase-anaphase transition (Hoyt *et al.* 1991; Irniger *et al.* 1995; Gorr *et al.* 2005; Bolanos-Garcia 2014). The anaphase promoting complex/cyclosome (APC/C) also plays a central role in regulating this checkpoint by Cyclin B and securin degradation, which in turn inactivates Cyclin B-Cdk1 and activate separase, respectively. Regulation of these two key events further allows the mitotic progression by disassembling cohesin ring around the sister chromatids enabling their segregation.

1.1.3 Wee1 and Myt1 serve functionally distinct roles in *Drosophila* development

Drosophila contains a single version of each type of Cdk1 inhibitory kinase: Wee1 and Myt1

(Price *et al.*, 2002; Cornwell *et al.*, 2002; Price *et al.*, 2002). Mutant alleles of *Drosophila* Wee1 and Myt1 were generated in the Campbell lab using ethyl methanesulfonate (EMS) (Price *et al.*, 2000; Price *et al.*, 2002; Stumpff *et al.*, 2004) and zygotic mutants for the wee^{ES1} and $myt1^{R6}$ alleles used in my thesis were characterized as homozygous and viable. Loss of function for both Wee1 and Myt1 (double mutant) caused lethality (personal communication with Dr. Campbell), however, providing evidence that these kinases serve partially redundant functions as well as the specialized developmental roles. Their developmental roles are outlined in the next section describing phenotypic characterization of each of these mutants at different stages of *Drosophila* development.

Drosophila Wee1 was first identified by complementation of a temperature-sensitive *wee1/mik1* double mutant strain of *S. pombe* with the cDNA of dWee1(Campbell *et al.*, 1995). Several loss-of-function alleles of *wee1* were subsequently recovered in a genetic screen for maternal-effect lethality, demonstrating that dWee1 kinase has an essential role during the rapid syncytial nuclear cycles of early embryogenesis (Price *et al.*, 2000). Live analysis of the timing of mitotic divisions in maternal-effect *wee1* mutant embryos showed that interphase length did not get progressively longer during cycles 10-13 as normal embryos do (Stumpff *et al.*, 2004). These and similar observations made with *grp* (dChk1) and *mei-41* (dATR) mutant embryos indicate that Wee1 activity is positively regulated by a conserved ATR/Chk1 checkpoint pathway that controls cellular responses to DNA replication and damage (Fogarty *et al.*, 1994; de Vries *et al.*, 2005). Additional defects including aberrant mitotic spindle assembly were also reported for *wee1* mutants, however the significance of this observation remains unclear (Stumpff *et al.*, 2004). In spite of the cellular abnormalities observed when maternal Wee1 activity was lacking, zygotic development of *wee1* mutants

appears normal, indicating that Myt1 can compensate for loss of Wee1 activity after embryonic cycle 14.

Loss of function *myt1* mutants were discovered in a genetic screen because of their bristle development defects involving thoracic and head macrochaetae with duplicated or missing/short shafts and/or duplicated sockets and were subsequently found to be male sterile (Jin *et al.*, 2005) (Jin *et al.*, 2008). Sensory organ precursor (SOP) cells undergo asymmetric divisions to produce different lineages resulting in the four terminally differentiated cell types that form the adult organ: socket, shaft, neuron and sheath. Shaft and socket cells secrete the external bristles and sockets, respectively, whereas the neuron and sheath lie beneath the cuticle and function in mechano-sensory signaling (Roegiers *et al.*, 2001; Furman and Bukharina, 2012). Loss of Myt1 appears to induce ectopic proliferation in SOP lineage by affecting the coordination of asymmetric division and cell fate determination during bristle formation (Audibert, A., S. Campbell and Gho, M, unpublished observations).

Experiments have also been performed in our laboratory to characterize the role of Myt1 in *Drosophila* other types of mitotic proliferating cells (Jin *et al.*, 2008). Larval imaginal disc cells exposed to ionizing radiation induce a rapid DNA-damage response including activation of a G2/M checkpoint that prevents mitosis and allows for DNA repair. This can be assayed by PH3-labeling to detect mitotic cells, which should be absent after exposure to DNA damage if the checkpoint is active (Brodsky *et al.*, 2000). In heterozygous and irradiated *wee* mutant discs there were no mitotic cells, revealing that Myt1 activity was sufficient for an effective G2/M checkpoint. In *myt1* mutant discs PH3 labeling was observed under these conditions, however, indicating that loss of Myt1 activity compromised the DNA damage G2 checkpoint (Jin *et al.*, 2008). My PhD thesis research concerns the other major

phenotype associated with loss of Myt1 activity, male sterility, with the main goal of my thesis being to understand the pre-meiotic role of Myt1 in G2-arrested spermatocytes.

1.1.4 Stability and activity of different Cdk1 phospho-isoforms

The Cdk1 kinase is not functional on its own, but only in association with a Cyclin A or Cyclin B to form a stable complex during G2 phase of the cell cycle (Chow *et al.*, 2011). Cyclin-bound Cdk1 activity is determined by three major phospho-modifications. Activating phosphorylation of the Cdk1-T161 residue located within the activation loop of the kinase domain is catalyzed by a nuclear kinase (CAK) that consists of Cdk7 and Cyclin H (Harper and Elledge, 1998). Cdk1 is also phosphorylated on the Y15 and T14 residues by the inhibitory kinases Wee1 and Myt1 (Parker *et al.*, 1992; Booher *et al.*, 1997). One commonly used method for studying the consequences of loss of Cdk1 inhibitory phosphorylation is to express Cdk1AF (T14AY15F) phospho-acceptor mutants that cannot be inhibited by Wee1/Myt1 but still contain T161p and so remain in a constitutively active state (Norbury *et al.*, 1991; Krek *et al.*, 1992; Su *et al.*, 1998). Expression of Cdk1 (T14A, Y15F) can bypass the G2/M checkpoint, by initiating a positive feedback loop that activates endogenous Cdk1 via activating Cdc25 phosphatases and inhibiting Wee1/Myt1 activity (Krek and Nigg, 1991).

A former PhD student in our lab (Dr. Ayeni) recently showed that expression of a Cdk1(Y15F) mutant in various cell types also effectively bypasses the G2/M checkpoint, but without inducing genomic instability like the Cdk1 (T14A,Y15F) mutant does (Ayeni *et al.*, 2014). This transgene therefore provides a useful tool for investigating mechanisms for regulating Cdk1 activity by inhibitory phosphorylation at different stages of development (Ayeni and Campbell, 2014)

Biochemical analysis of Cdk1 by western blotting using PSTAIRE antibodies (detecting a conserved epitope of Cdk1), reveal several distinct Cdk1 protein bands (electro mobility shift) corresponding to three different T14, Y15 and T161 phospho-isoforms (Edgar *et al.*, 1994). Furthermore, 2D-electrophoresis techniques revealed seven Cdk1 phospho-isoforms associated with Cyclin B (Coulonval *et al.*, 2011). Based on their experimental results, Coulonval *et al* proposed a model in which CyclinB-Cdk1 complex having no T161p is less stable (Coulonval *et al.*, 2011). T14 (not Y15p) phosphorylation of Cdk1 promoted T161 phosphorylation, leading them to conclude that Myt1 phosphorylation of T14 could indirectly facilitate Cdk1 stability and functionality (Coulonval *et al.*, 2011). Similarly, in *Drosophila*, Dr. Ayeni in our lab noticed that the transgenic Cdk1 T14A mutant protein was deficient in T161 phosphorylation. Having just T14 and T161 phosphorylation was not sufficient to fully inhibit Cdk1, as the Cdk1 complex containing this combination with no Y15p was partially active (Ayeni *et al.*, 2014). These results indicated that Y15 phosphorylation provides a key role in inhibitory regulation of Cdk1, however T14 phosphorylation is sufficient for protection from genome instability. Fig. 1-2 depicts a model that summarizes these ideas relating to the stability and activity of different Cdk1 phospho-isoforms as Cyclin B-bound complexes during G2 and M phases. Note, however, that Cyclin A-bound Cdk1 phospho-isoforms may be regulated differently from Cyclin B, as Cyclin A is synthesized earlier in the cell cycle during S phase.

1.2. Cell cycle arrest and Meiosis

Meiosis is a specialized cell cycle observed in sexually reproducing eukaryotic germ cells. The illustration in Fig.1-3 depicts the basic difference between mitosis and meiosis. Unlike

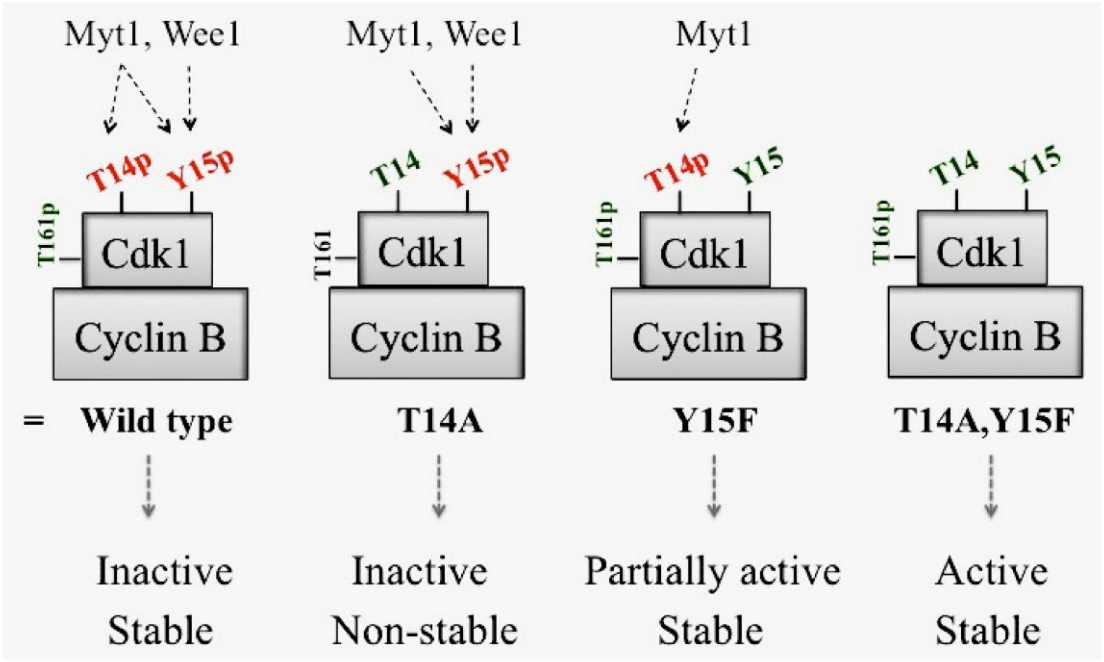

Fig. 1-2: Stability and activity of Cyclin B-Cdk1 based on its specific phospho-isoforms.

Cdk1 has three major phosphorylation sites: that includes T14, Y15 and T161 residues. T14 and Y15 are the inhibitory sites that are phosphorylated by Wee1 family kinases. During G2 phase, Myt1 targets T14 and both Wee1 and Myt1 can phosphorylate Y15. Phosphorylation of T161 is regulated by CAK (Cyclin H and Cdk7) activating complex that promote Cdk1 activation, yet in the presence of T14p and Y15p the complex remain inactive and stable. This is the scenario we noticed in the Cdk1 (WT) transgenic protein. Depending on the nuclear or cytoplasmic locations, Cdk1 can also exist in two other isoforms. The second possibility shows no Myt1 targeted T14 phosphorylation of Cdk1. Having no T14p (mimicked by T14A mutation) was found to lack T161p, therefore Cdk1 with Y15p phosphor-form alone is detected unstable therefore shows no activity. The third class of Cdk1 isoforms involves with no Y15 phosphorylation but has T14p and T161p. This combination of phosphorylation is mimicked by Cdk1 (Y15F) phospho-mutations, which are only inhabitable by Myt1. This phospho-isoform is found to be stable and partially active both *in vitro* and *in vivo* conditions. The fourth and completely active form of Cdk1 contains neither T14p or Y14p, but has only T161p. Cdk1(T14A, Y15F) phospho-mutants are equivalent to this active and stable Cdk1 isoform.

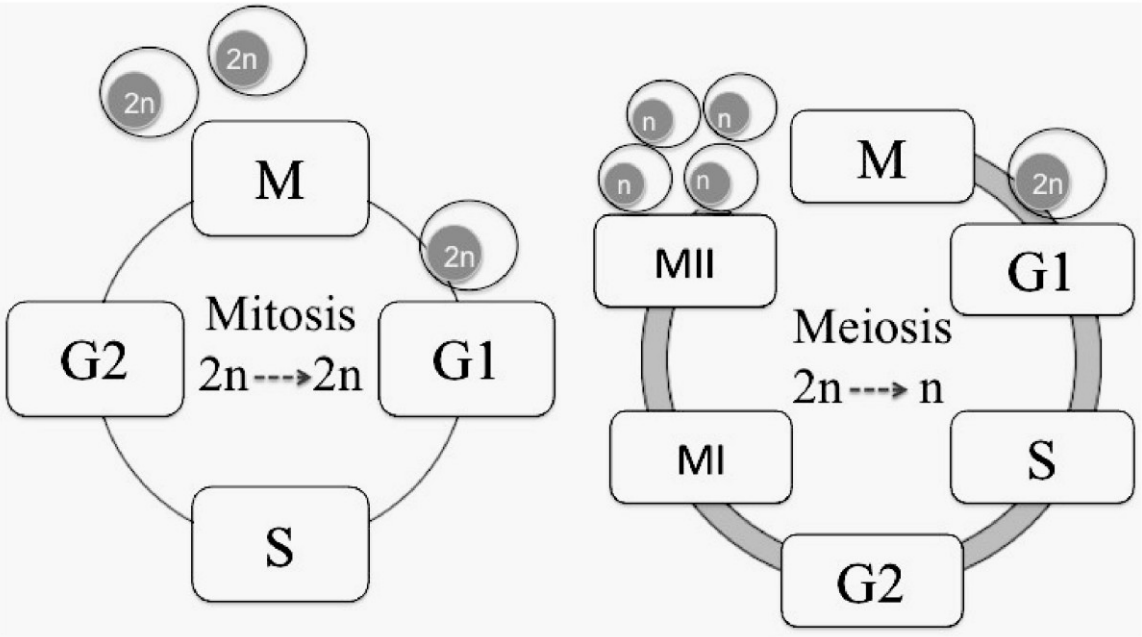

Fig. 1-3: Mitotic and meiotic cell division in a diploid organism

Mitosis includes a major interphase (G1, S, G2) and M phase. In a diploid organism, mitosis produces two identical daughter cells (2n) through DNA duplication and equal segregation of chromosomes during S and M phase, respectively. Meiosis also includes the interphase, but the M phase occurs at two steps. First meiotic division (MI) separates the homologous chromosomes; therefore reduce the number of chromosomes by half in each daughter cell (n). During the second division (MII), each daughter cell segregates the duplicated chromatids to the poles and produces two more daughter cells. At the end of two consecutive meiotic divisions four haploids are produced.

mitosis where each daughter cell receives a diploid complement of genetic information, meiosis involves two consecutive M phases with no intervening S phase. The resulting gametes therefore receive only half the genetic information of their parents. During fertilization, fusion of the haploid male and female gametes restores the diploid complement of information to the zygote. During meiosis, pre-meiotic S phase followed by G2 phase precedes the two specialized meiotic divisions (MI and MII).

During prophase of MI in most organisms, crossing over and homologous recombination promotes the formation of new recombinant chromosomes establishing genetic variations in the offspring compared to their parents. MI consists of separating recombinant homologous chromosomes, thereby reducing the ploidy. MII, which is similar to mitosis, segregates the chromatids of each chromosome. Depending on the sex of the parent, the specialized haploid products of meiosis are either sperm or eggs (oocytes).

1.2.1 Pre-meiotic G2 phase arrest

During oogenesis, immature oocytes typically undergo a period of "pre-meiotic arrest" that can vary in length from days to decades, depending on the species (Whitaker, 1996; Von Stetina and Orr-Weaver, 2011). Immature oocytes remain in pre-meiotic quiescence until encountering a hormonal stimuli or intrinsic signal that triggers maturation and meiotic resumption (Channing *et al.*, 1978; Zhang and Xia, 2012; Holt *et al.*, 2013). A second meiotic arrest also commonly occurs during metaphase-II, often synchronized with fertilization. Pre-meiotic arrest is thought to be crucial for proper synthesis and accumulation of maternal transcripts, proteins and nutrients needed for early zygotic development (Perry and Verlhac, 2008; Von Stetina and Orr-Weaver, 2011). A similar period of pre-meiotic arrest is

commonly observed during male meiosis, with primary spermatocytes undergoing a brief (2 to 15 days) "G2-like" prophase I arrest that facilitates the synthesis of essential factors required for post-meiotic development (Cantu *et al.*, 1981; Lin *et al.*, 1996; Perezgasga *et al.*, 2004).

1.2.2 Role of Myt1 in G2/M checkpoint mechanisms that coordinate pre-meiotic arrest

G2 phase checkpoint mechanisms delay mitotic entry by inhibiting the activity of MPF (mitosis promoting factor), consisting of a complex between the Cdk1 kinase and mitotic Cyclin B (Tuck *et al.*, 2013). Extensive studies in *Xenopus* oocytes revealed that pre-meiotic 'G2 like' arrest is coordinated by multiple molecular mechanisms controlling a balance between the opposing enzymatic activities of Cdk1 inhibitory kinases (Wee1-related family) and activating phosphatases (Cdc25). In metazoans, Wee1-related kinases have diversified into nuclear Wee1 (and in some species, Wee2) kinases that phosphorylate Cdk1 on tyrosine residue 15 (Y15), (Parker *et al.*, 1992) and cytoplasmic, membrane-bound Myt1 kinases that target the adjacent threonine 14 (T14) as well as Y15 residues (Ohsumi *et al.*, 1994; Booher *et al.*, 1997; Kishimoto, 2003; Hormanseder *et al.*, 2013).

In many organisms including *Xenopus* and *C. elegans*, Myt1 has been identified as the primary Cdk1 inhibitory kinase responsible for 'G2 like' prophase-I arrest during female meiosis, whereas Wee1 appears to be dispensable (Furuno *et al.*, 2003; Burrows *et al.*, 2006). For example, in *Xenopus* during meiotic resumption, induction of steroid hormonal signals decrease the levels of cyclic adenosine 3′, 5′-monophosphate (cAMP) or Ca^{2+} that in turn reduces protein kinase A (PKA). Activation of MAPK (mitogen activated protein kinase) signaling induces Cdc25 phosphatase activity. Cdc25 removes the T14 and Y15 inhibitory

phosphates from Cdk1, activating CyclinB-Cdk1 (MPF) to trigger MI. Active Cyclin B- Cdk1 then phosphorylates multiple substrates both in nucleus and cytoplasm, promoting nuclear envelope (NEB) or germinal vesicle breakdown (GVBD), chromosome condensation and spindle assembly (Ohsumi *et al.*, 1994; Nebreda and Ferby, 2000). Although mechanisms regulating oocyte maturation appear relatively conserved in invertebrate animal models such as *C .elegans* and *D. melanogaster,* the intrinsic stimuli inducing oocyte maturation varies in different organisms (Miller *et al.*, 2001; Morris and Spradling, 2012; Kim *et al.*, 2013).

In experiments using *Xenopus* immature pre-meiotic oocytes, depletion of Myt1 from G2 phase arrested oocytes by antibody injection resulted in abrupt meiotic resumption that was independent of hormonal stimuli (Mueller *et al.*, 1995). In contrast, Wee1 was undetectable in these immature oocytes and ectopic Wee1 expression resulted in an ectopic S phase between MI and MII (Nakajo *et al.*, 2000). These observations were interpreted as showing that Myt1 was the primary inhibitory kinase regulating Cdk1 during the 'G2 like' pre-meiotic arrest. Although Wee1 was dispensable for oocyte arrest during meiosis it accumulated after oocyte maturation or fertilization, indicating Wee1 was required for post meiotic or early embryonic development (Murakami and Vande Woude, 1998; Walter *et al.*, 2000).

1.2.3 Mechanisms for regulating Myt1 during oogenesis in other model systems

Upon hormonal (progesterone) stimuli, oocytes initiate translational signals to synthesize Mos (Moloney murine sarcoma) that activates MAPK signaling pathway. MAPK/MEK induces its downstream target p90 (RSK) to phosphorylate Myt1 and down regulates its function, which in turn releases CyclinB-Cdk1 from inactivation (Palmer *et al.*, 1998). In addition to Myt1

inhibition, MAPK also promotes Cdc25 activation, therefore facilitating a positive feedback loop that further down-regulates Myt1 activity (Palmer and Nebreda, 2000). Myt1 has also been identified as a direct target of the Mos kinase, as ectopic Mos expression induced oocyte maturation through inhibition of Myt1 independent of MAPK activity (Peter *et al.*, 2002; Priyadarshini *et al.*, 2009). Synthesis of RINGO, which is a non-Cyclin Cdk1 and Cdk2-associated partner protein, was also identified to physically interact and inhibit Myt1 activity through phospho-dependent regulation (Ruiz *et al.*, 2008). More recently this RINGO/Cdk1 and Myt1 interaction was proposed to be a prerequisite for p90 (RSK) to inhibit Myt1 (Ruiz *et al.*, 2010). In addition to these regulatory mechanisms, *Xenopus* Myt1 has also been demonstrated to autophosphorylate few serine residues to reach a hyper-phosphorylated state during oocyte maturation (Kristjansdottir *et al.,* 2006). Another mechanism associated with meiotic resumption involves a critical balance between Cyclin B synthesis and Myt1 inhibition. Raising Cyclin B translation can independently inactivate Myt1 activity without a requirement for Mos/MAPK/p90 (RSK) signaling (Gaffre *et al.*, 2011) . After the meiotic resumption, activation of *Xenopus* Plx1 was reported to enhance Cdc25 activity and inhibit Myt1 to promote meiotic progression (Inoue and Sagata, 2005), however regulation of Cdc25 appeared to have no role in Cyclin B- Cdk1 amplification before MI (Karaiskou *et al.*, 2004). Collectively, evidence exists that multiple mechanisms are used to coordinate Myt1 down-regulation with Cyclin B synthesis and activation of Cdk1 at the G2/MI stage of meiotic maturation (Fig. 1-4).

A. pectinifera (starfish), oocytes also undergo primary G2-like arrest, however the downstream response of the extracellular hormonal signal is independent of translational regulation (Kishimoto, 2011). Instead, induction occurs through a G-protein mediated

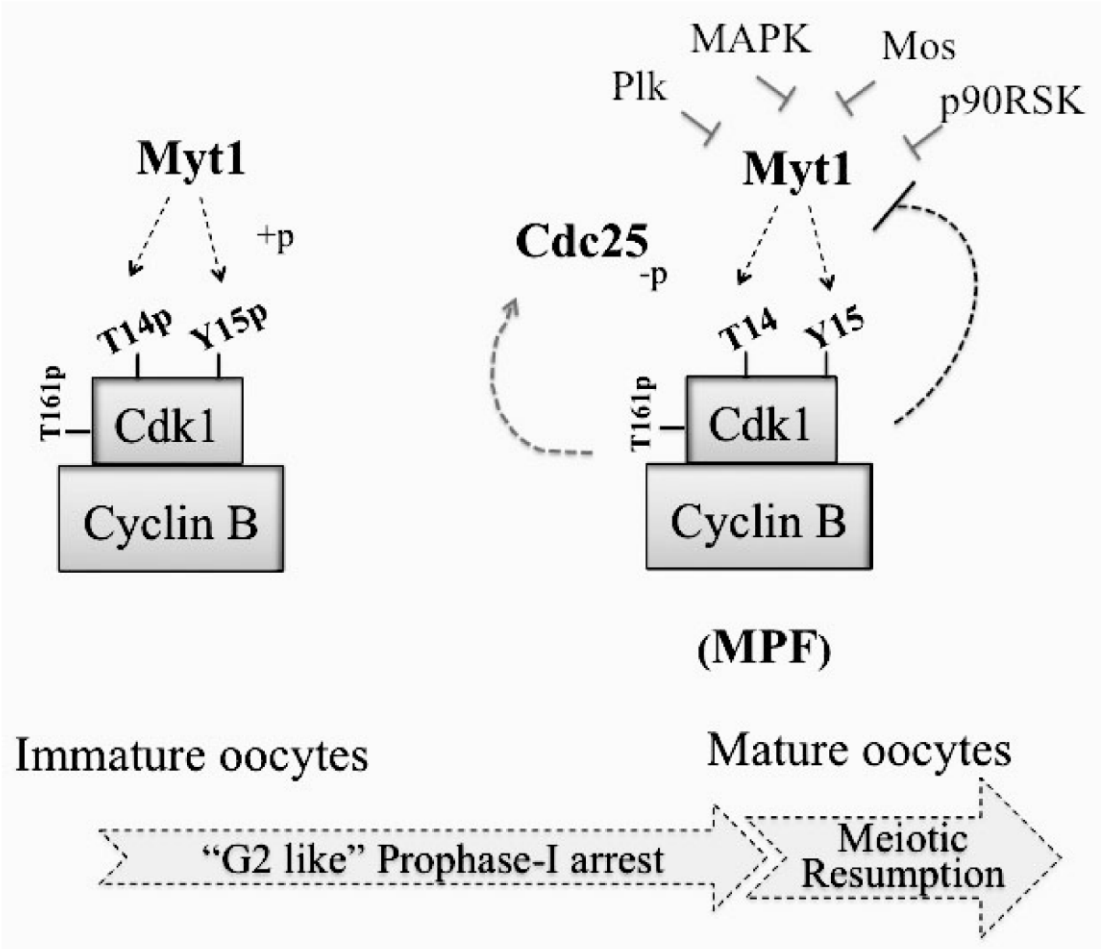

Fig. 1-4: Regulation of G2-M controls meiotic arrest and resumption

Myt1 is a dual kinase that inhibits Cdk1 by phosphorylating its T14 and Y15 residues. This phosphorylation dependent inactivation of Cyclin B-Cdk1 maintains the G2-like prophase-I arrest in immature oocytes. Upon external stimuli induced by either hormonal or sperm protein associated signals, the immature oocytes resume meiosis through regulations that activate the Cyclin B-Cdk1(MPF). Major meiotic regulators including Mos, MAPK and its related p90(RSK), Plk family kinases down regulate Myt1 by phosphorylation dependent manner to inactivate its function, therefore release Cyclin B-Cdk1 from inhibition. Cdc25 phosphatase is a positive regulator of Cdk1 that removes the inhibitory phosphates from T14 and Y15 sites. Active pool of Cdk1 complex triggers bistable positive feedback loop mechanism through which the Myt1 is further inhibited and the Cdc25 is activated. Down regulation of Myt1 further promote auto phosphorylation and remain inactive during the meiotic progression.

phosphatidylinositol-3-OH kinase (PI(3)K) pathway that activates Akt signaling. Acting like Mos and p90 (RSK) in *Xenopus*, Akt phosphorylates and inhibits Myt1 during oocyte maturation (Okumura *et al.*, 2002). In this system however, Plk has a role in down-regulating Myt1, but not in activating Cdc25 (Okano-Uchida *et al.*, 2003).

In *C.elegans*, the Myt1 ortholog WEE 1.3 also serves a conserved role in pre-meiotic oocyte arrest, with *in vivo* depletion by RNA interference inducing precocious resumption of meiotic progression (Burrows *et al.*, 2006). Abnormal chromosomal coalescence and meiotic spindle assembly, followed by expression of post meiotic fertilization markers, accompanied premature entry into MI. The molecular mechanisms regulating Myt1 and Cdk1 activity in *C. elegans* have recently been extended to identify potential interacting proteins that function with Myt1 during oocyte maturation (Allen *et al.*, 2014).

In mouse oocytes, morpholino injection experiments suggested a dual requirement for germ line-specific Wee1B and Myt1 to maintain oocyte prophase I arrest (Oh *et al.*, 2010). Appropriate sub-cellular localization of Wee1B (nuclear) and Myt1 (cytoplasm) was critical for proper MPF activation. Unlike the *Xenopus* system, Mos mediated regulation of Myt1 was dispensable for inducing meiotic maturation; instead alternative cAMP and Ca2+ signaling mechanisms were shown to primarily regulate it. This mechanism is well conserved in most mammalian animal model systems including human. (Avazeri *et al.*, 2003; Ajduk *et al.*, 2008).

1.2.4 *Drosophila* oocyte maturation

In *Drosophila melanogaster*, the external stimuli responsible for regulating oocyte maturation differ substantially from mammalian and other non-vertebrate systems, with ecdysone and

cyclooxygenase (COX) proposed as signals to induce the meiotic resumption (Von Stetina *et al.*, 2008; Tootle *et al.*, 2011). Neither Wee1 nor Myt1 appeared to play an essential role in prophase I arrest, since both *wee1* and *myt1* mutant females appear to undergo normal oocyte development (E. Homola and S. Campbell, unpublished). Nonetheless, a delay in nuclear envelope breakdown observed in temperature sensitive *cdk1 (cdc2)* and *twe* (meiotic Cdc25) mutants revealed a requirement for Cdk1 activation for normal timing of meiotic resumption (Alphey *et al.*, 1992; Courtot *et al.*, 1992; Sigrist *et al.*, 1995; Baker and Fuller, 2007). Another important regulator is the Greatwall kinase, which has been proposed to down-regulate Polo to limit Cdk1 activation before MI during oogenesis (Archambault *et al.*, 2007). Also unlike other model systems, the second meiotic arrest during *Drosophila* oogenesis occurs in metaphase I, not in MII, and requires re-inactivation of Cyclin B-Cdk (Von Stetina and Orr-Weaver, 2011). A specialized adaptor APC^{CORT}, which is a part of APC/C protein degradation complex, mediates Cyclin B degradation to regulate second arrest (Chu *et al.*, 2001; Kronja *et al.*, 2014). Although loss of Myt1 has no apparent effect on female germline development, it does play a role in regulating mitotic proliferation of the associated somatic follicle cells (Jin *et al.*, 2005). These observations suggest that regulation of Cyclin A/B-Cdk1 for mediating *Drosophila* oocyte meiotic resumption may be driven by a Myt1 independent mechanism.

1.2.5 Role of Myt1 in *C. elegans* pre-meiotic spermatocyte G2 phase arrest

The first Myt1 mutants recovered in any organism were *C. elegans* dominant negative *wee1.3* alleles (originally called *spe* mutants) that perturbed spermatocyte development by preventing MI entry, resulting in self-sterile hermaphrodites lacking sperm but did not perturb pre-

meiotic oocyte arrest (Doniach, 1986). The mutations corresponded to six different modifications in the C-terminal region of the *wee1.3* gene, which was discovered in a screen for intragenic loss-of-function suppressors of the original mutants (Lamitina and L'Hernault, 2002).

1.2.6 Myt1 is also essential for male meiosis during *Drosophila* development

A former graduate student in the Campbell lab (Dr. Zhigang Jin) generated the first null allele of *Drosophila* Myt1 and published the original characterization of its role in male and female gametogenesis (Jin *et al.*, 2005). The *myt1* mutants are male sterile but female fertile, however mitotic proliferation defects were observed in germline-associated somatic cells in both sexes. During spermatogenesis, two terminally differentiated somatic cells called cyst cells encapsulate each germ-line cyst as it undergoes 4 synchronous mitotic amplification divisions. Using antibodies against phosphorylated histone H3 at S10 (PH3) as a mitotic/meiotic marker, Jin showed that these somatic cyst cells undergo ectopic mitotic proliferation and *myt1* mutant gonial cells also often undergo extra mitotic divisions before differentiating into 16-cell cysts of spermatocytes. The mutant spermatocytes then undergo both MI and MII divisions, however the sizes of nuclei and nebernkern (mitochondrial derivatives) in the differentiating spermatids appeared abnormal, suggesting that the male sterility was a consequence of aneuploidy. Moreover, although spermatid differentiation appeared fairly normal the seminal vesicle appeared empty of mature sperms. Collectively, these results revealed that Myt1 is involved in multiple aspects of male meiosis in *Drosophila*. The exact role of Myt1 during pre-meiotic G2 phase of spermatocyte development had not yet been determined, however.

1.2.7 *Drosophila* spermatogenesis as a model system for studying Myt1 function

Drosophila spermatogenesis is a powerful model system for addressing many basic biological questions, as it comprises many different developmental and cellular processes in a linear progression (Fig. 1-5). Spermatogenesis can be broadly classified into three major processes according to the order of germline development: differentiation and proliferation of the gonial stem cells, meiotic maturation of spermatocytes and spermatid remodeling and terminal differentiation. These three processes are spatially organized along the length of the testes, providing a good opportunity to visualize the entire process within an intact tissue. Developing male larva contain spherical testes containing germ cells developing up to the spermatocyte stage whereas the newly eclosed adult has elongated tubular testes accommodating lengthy sperms.

The germline and somatic stem cells (GSC and SSC) are located within the hub cell niche at the tip of the testes. Each GSC is encapsulated within a pair of somatic SSC. These stem cells divide asymmetrically to each produce one self renewable stem cell, which remains attached to the hub, and a differentiated daughter cell that differentiates as a gonioblast and moves away from the niche (Kiger *et al.*, 2001). The gonioblasts then initiate four subsequent transit amplification (TA) mitotic divisions, with incomplete cytokinesis (Gonczy *et al.*, 1997). The cysts of dividing 2, 4 and 8-cell spermatogonia can be detected by increased expression of the product of the *bag-of-marbles* (*bam*) gene (Insco *et al.*, 2009). Bam is a regulatory protein that helps specify the number of spermatogonial TA divisions along with *benign gonial cell neoplasm* (*bgcn*) and other cell cycle regulators (Fuller, 1998). After the 4th division, a drop in Bam levels triggers the onset of expression for differentiation regulators such as *always early* and *spermatocyte arrest* (*Sa*) that restrict further TA divisions and

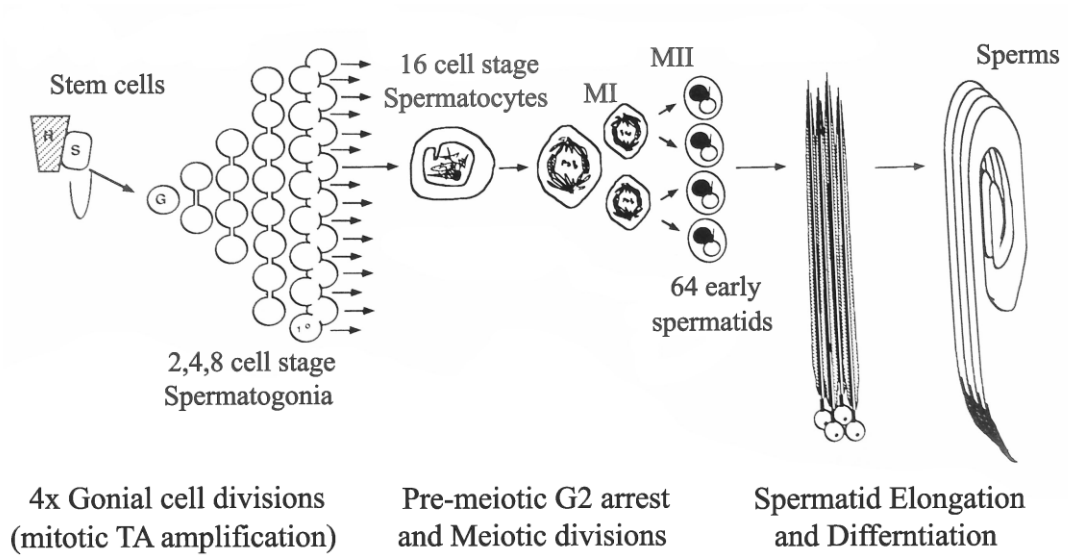

Fig. 1-5: *Drosophila* spermatogenesis model system

Dr. Fuller published original version of this illustration in "The development of Drosophila melanogaster" book (Bate 1994). I have obtained permission from Dr. Fuller to modify and use in the original image. This figure has been simplified and modified here for describing *Drosophila* spermatogenesis process. The first of the three main processes indicates the stem cell differentiation and proliferation. The germline stem cells (S) are attached to the hub (H) cells that are located at the tip of the testes. During stem cell division, the germline daughter stem cell, which is closer to the hub self renews and the distal cell, differentiates into gonioblast (G). Gonioblast cells undergo four consecutive mitotic divisions with an incomplete cytokinesis (IC). These transit-amplifying cells are identified as 2,4 or 8 spermatogonia cysts inter-connected through fusomes that are formed by the remnant IC components. The second section of this process shows the meiotic progression. During the fourth TA division, the 8-stage spermatogonia cyst divides and produces 16 cell primary spermatocytes. After the pre-meiotic S phase, spermatocytes enter 90 hour long G2 phase during which the cells grow (25 fold in volume) and undergo enormous cellular and chromosomal modifications in six stages (S1 to S6) before MI. The two consecutive MI and MII result in 64-cell stage spermatid cyst. Each early spermatid containing a single nucleus with an equal size nebenkern (dark) elongate and remodel to form sperm.

specify spermatocyte differentiation, respectively (Fuller, 1998). The 16 cell spermatocytes undergo pre-meiotic S phase followed by a prolonged (~90 hours) G2 phase arrest during which time they grow rapidly while transcribing and translating many new regulators required for post-meiotic development, increasing in volume by roughly 25 fold (Brink, 1968; Schafer *et al.*, 1995; White-Cooper *et al.*, 1998). Spermatocyte maturation is then induced by developmentally regulated expression of essential mitotic/meiotic regulators such as $Cdc25^{Twe}$ and Cyclin B, which trigger activation of Cdk1 and G2/MI progression during the onset of the first meiotic division (Baker and Fuller, 2007). MI is immediately followed by MII with no intertwining S phase, resulting in production of cysts with 64 secondary haploid meiocytes. These cells undergo spermatid differentiation in two steps (Tokuyasu, 1975; Hackstein, 1991). First, onion stage spermatids re-organize their mitochondrial structures to produce a single circular nebenkern similar in size to the nucleus. The Golgi-derived acroblast and nucleus are polarized to one end of the cell to organize the future sperm head, whereas the nebenkern and centrioles elongate to establish the axoneme for the sperm tail. These elongated cells then undergo individualization, where they strip off membranes and other cellular components from the cells to form functional sperm, which are then transported to seminal vesicles for storage until they are ejaculated. These diverse cellular processes make this system unique among other developmental models in *Drosophila*.

Previous studies of *Drosophila* Myt1 have shown that it regulates multiple aspects of male meiosis, however the role of Myt1 during pre-meiotic arrest had not yet been well characterized when I joined the lab. Characteristics of the system described above make it an ideal model for studying Myt1 functions during pre-meiotic arrest and examining sub-cellular

structures in the huge spermatocytes. I therefore took opportunity to examine how does Myt1 regulate *Drosophila* male meiosis.

1.3. The concept of an organelle checkpoint

During the cell cycle, replication and segregation of all cellular components in the nucleus and cytoplasm are accomplished in a coordinated fashion (Fagarasanu *et al.*, 2010). Other than DNA damage response, a mis-regulation of specific sub-cellular organelle contributing a blockage in cell cycle progression is defined as an "organelle checkpoint". One well-studied example is the Golgi checkpoint that accesses the G2 phase Golgi fragmentation, as inhibition in Golgi-disassembly prevents mitosis (Lowe and Barr, 2007; Sutterlin *et al.* 2002). Another example is the 'mito-checkpoint' that induces cell cycle arrest through p53-mediated mechanisms in response to mitochondrial damage (Singh, 2006; Kulawiec *et al.*, 2009; Minocherhomji *et al.*, 2012). The spindle assembly checkpoint mechanism that regulates the metaphase to anaphase transition by monitoring chromosome-spindle attachments (Gorbsky, 2015).

1.3.1 Organelle checkpoint functions of Myt1

Active Cdk1 recognizes multiple targets both in the nucleus and cytoplasm to mediate mitotic progression. Nuclear localization and export signal (NLS and NES) motifs of Wee1 influence the nuclear Cdk1 activity, whereas Golgi and ER localized Myt1 protects these cytoplasmic structures from precocious Cdk1 activity through organelle checkpoint regulation (Oh *et al.*, 2010). In mammalian cells, individual Golgi cisternae are organized through interlinking membranous tubules to form characteristic ribbon structures (Warren and Malhotra, 1998;

Klumperman, 2011). During the G2/M transition, these ribbon structures are unlinked through Plk3 (Polo kinase 3) and Cdk1 targeted phosphorylation of Golgi proteins including GRASP65, GM130 promotes unstacking of the Golgi cisternae (Persico *et al.*, 2009; Wei and Seemann, 2010). In HeLa cells, depletion of Myt1 by siRNA caused premature Golgi disassembly in G2 phase cells and MEK (MAPK related)-mediated inhibition of Myt1 induced a similar effect, directly implicating Myt1 in the regulation of this organelle checkpoint (Villeneuve *et al.*, 2013). Interestingly, Myt1 depletion also perturbed reassembly of the Golgi and ER network in telophase or post mitotic cells and this effect was suppressed by manipulating Cyclin B levels through APC/C proteasome activity (Nakajima *et al.*, 2008). Exactly how these mechanisms are coordinated with mitotic exit has not yet been clearly demonstrated, however.

In many eukaryotes, including *Drosophila*, organization of the Golgi into higher order ribbon structures is not prevalent; instead, Golgi stacks are dispersed throughout the cytoplasm (Yano *et al.*, 2005; Kondylis and Rabouille, 2009). In pre-meiotic spermatocytes, Golgi consists of individual stacks (Yasuno *et al.*, 2013). Based on the studies reported in other systems, the mechanisms unlinking paired Golgi stacks appeared to be independent of Cdk1 mediated mechanisms, but are shown to be regulated by cAMP-Rac and Abl/enabled pathways (Kondylis *et al.*, 2007; Kannan *et al.*, 2014). In *Drosophila* S2 cultured cells however, paired Golgi stacks linked through membrane tubules resembling mammalian Golgi ribbons were observed by electron microscopic analysis (Kondylis *et al.*, 2007). dMyt1 depletion by RNAi knockdown in these S2 cells perturbed these structures during mitosis (Cornwell *et al.*, 2002). Thus, evidence from different experimental systems has

implicated Myt1 in organelle checkpoint mechanism that regulate higher order Golgi ribbon disassembly and re-assembly during mitosis.

1.3.2 Myt1-mediated organelle checkpoints deployed during mitotic exit and G1 phase

The cyclin-dependent kinase Cdk1 serves as a master regulator of mitosis and meiosis during the cell cycle in most eukaryotic organisms, whereas G1 and S phase Cdk functions are provided by related kinases (Cdk4/6 and Cdk2, respectively). *S. cerevisiae* has a single Cdk called Cdc28, which is functionally equivalent to Cdk1 and also has other roles in combination with six different types of S/M specific Cyclins, Cln1 to Cln6 (Enserink and Kolodner, 2010). Similarly, in mammalian systems, Cdk1 forms functional complexes with multiple Cyclins that can drive the entire cell cycle in the absence of most other Cdks (Santamaria *et al.*, 2007). Inappropriate activation of Cdk1 is dangerous, as it can trigger apoptosis or genome instability that can lead to cancer (Castedo *et al.*, 2002). Alzheimer's disease provides another example, where aberrant activity of Cdk1 in post-mitotic (terminally differentiated) neurons has been proposed to cause ectopic expression of cell cycle regulatory proteins and result in neuron degeneration (Vincent *et al.*, 1997; Becker and Bonni, 2004; Potapova *et al.*, 2009). Given these potential hazards, it is clearly necessary to inhibit Cdk1 activity once cells exit mitosis or meiosis.

APC/^{Cdc20} mediated Cyclin B degradation is one key mechanism used for inactivating Cdk1 to exit mitosis (Clute and Pines, 1999; van Zon *et al.*, 2010). The Cdc20 activator of APC/C acts as a substrate-recruiting subunit for directing proteolytic degradation (Kimata *et al.*, 2008). Despite the essential function of Cdc20 for degrading Cyclin B and securin, RNAi depletion in mammalian cell lines did not prevent mitotic exit, but only delayed the process

(Chow *et al.*, 2011). Similar observations with co-depletion of Cdc20 and a related regulator Cdh1 suggested that post-mitotic Cdk1 inactivation might not solely be dependent on APC/C function. Interestingly, in Cdc20-depleted cells the levels of Cdk1 inhibitory phosphorylation (T14p and Y15p) were higher than normal and pharmacological inhibition of proteosome activity using MG132 or expression of non-degradable Cyclin B also elevated levels of Cdk1 T14p and Y15p (Chow *et al.*, 2011). Collectively these findings suggest that Wee1-related kinases (Wee1 and Myt1) can inactivate Cdk1 during mitotic exit, at least under conditions where APC/C regulation is compromised (Potapova *et al.*, 2009). How this relates mechanistically to the pre-mitotic Golgi and ER organelle checkpoint functions proposed for Myt1 remains to be determined, however.

Two different mechanisms can down-regulate Wee1 and Myt1 activity during the initiation of mitosis. Hyper-phosphorylation of Myt1 reduces its catalytic activity (Kristjansdottir *et al.*, 2006; Ruiz *et al.*, 2010), whereas phosphorylation of Wee1 targets it for proteosome-mediated degradation (Smith *et al.*, 2007). Under these circumstances, Myt1 would be the only Cdk1 inhibitory kinase potentially available at the stage when post-mitotic inactivation of Cdk1 needs to be established. Previous work from the Campbell lab showed that terminally differentiated *Drosophila* male germline-associated somatic cyst cells require Myt1 to prevent ectopic mitotic divisions, by an unknown mechanism (Jin *et al.*, 2005). It remains unclear how Myt1 mediated Cdk1 inactivation during G2 phase relates to its proposed role in reassembly of the Golgi and ER structures, however.

1.3.3 Fusome biogenesis and *Drosophila* germline development

The fusome is a germ-line specific cytoplasmic membranous structure that inter-connects cells through intercellular bridges. Verbindungsbrücken (1886) first described the fusome structures as a "bridging connection" that could be observed in spermatocytes of many insects. Sutton (1890) described these structures as "incomplete separation in the halves" in grasshopper testes (*Brachstola magna*). Next, Giardina (1901) found fusomes during beetle oogenesis. Later, Davis (1908) noted "intercellular bridges" connecting secondary spermatogonia via filamentous spindles, which persisted after incomplete division. Similar structures are seen in most insect germ-line cells (Carlson, 1988) and have also been described in *Xenopus*, implying that meiotic fusomes may be widely conserved in vertebrates and invertebrates (Huynh, 2005).

In *Drosophila*, fusomes were first identified in the female germ-line and described as mitotic residues fusing two cells during the cystocyte divisions. (Spradling, 1993; Lin *et al.*, 1994; Lin *et al.*, 1994). Similar structures were subsequently noticed in male germline cysts (Hime *et al.*, 1996). The gonial cells, which undergo the four mitotic cycles, remain connected after incomplete cytokinesis through remnant mid-body structures stabilized by actin and membrane-enriched ring canals (McKearin, 1997). Spectrosomes are spherical shaped membranous clusters located on each germ cell. During cytokinesis, these structures elongate and branch out to form fusomes. Although the basic features of fusome biogenesis appear similar in male and female germ cells, during the mitotic to meiotic transition the structures appear to remodel differently. The illustration shown in Fig.1-6 represents the behavior of fusomes in male and female gametogenesis.

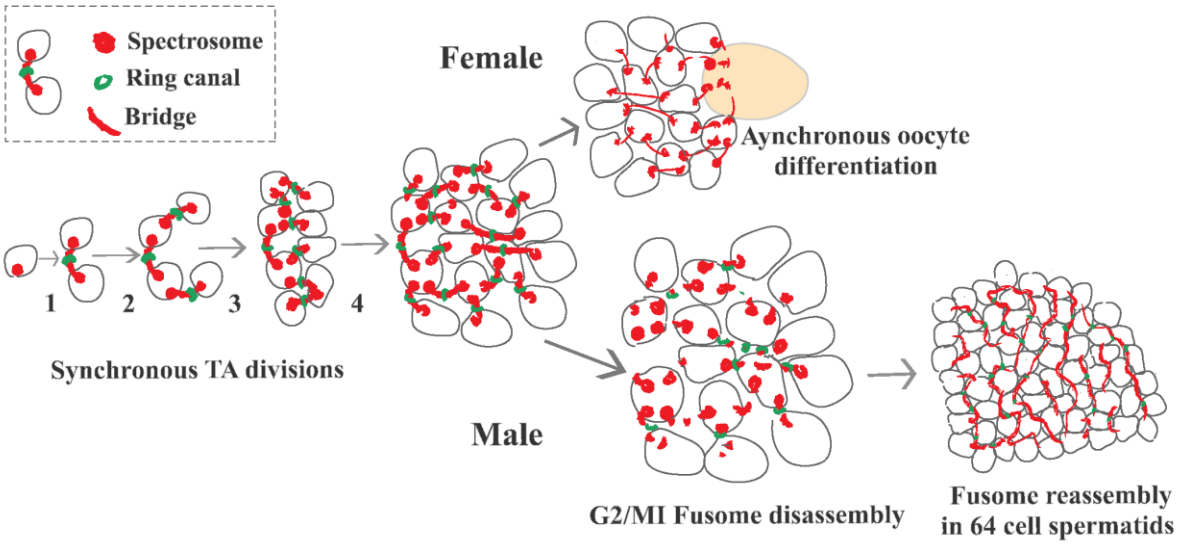

Fig. 1-6: Fusomes of female and male germline cells

Differentiated germline stem cells (GSC) inherit spherical shaped (red dot) spectrosome. During four synchronous transit amplification divisions, the spectrosome of each cell elongates through a ring canal (green ring) forming a bridge between daughter and mother cells. During the 4^{th} mitotic division, the female germ cells divide asynchronously producing 15 nurse cells and 1 oocyte. Female fusomes degenerate prior to oocyte maturation. Male germline cells continue to divide synchronously, yet briefly disassemble fusomes during MI onset and reassemble them when they differentiate into 64-stage spermatids.

Fusomes are composed of three different types of structural components: secretory vesicles, membrane skeletal proteins and a microtubule-actin based network (de Cuevas *et al.*, 1997). Based on electron microscopy observations by Telfer in 1975, fusomes were identified as distinctive cytoplasmic bridges containing no ribosomes, but small vesicles and mitochondria structures. Fusomes appear to be composed of ER derived components, based on the observations that expression of ER proteins with characteristic KDEL motifs localize to fusomes (Snapp *et al.*, 2004). Experiments using Fluorescence Recovery after Photo bleaching (FRAP) and Fluorescence Loss in Photo bleaching (FLIP) provided evidence that the fusome consists of a continuous ER network through which all cells are interconnected. Sharing a common ER connection could therefore provide a way of facilitating inter-communication between cells to synchronize their development. During female meiosis, ER connectivity is lost as fusomes disintegrate when 16 cells in a cyst begin to differentiate asynchronously to form one oocyte and 15 nurse cells (Snapp *et al.*, 2004). Unlike female fusomes, male fusomes do not disintegrate after the 4^{th} mitotic division, but the fusomes and the ring canals appeared modified during the mitotic to meiotic transition (Eikenes *et al.*, 2013). These modified fusome structures continue to elongate during spermatocyte maturation and growth, but undergo a brief partial dis-assembly during the onset of MI. The fusome branches are then reassembled after completion of the meiotic divisions and they further elongate, persisting throughout spermatid differentiation (Hime *et al.*, 1996).

Fig.1-7 illustrates the major fusomes components reported in male fusomes. Both in male and female, membrane skeletal proteins including the *Drosophila* adducin-like homolog hu-li-tai shao (Hts) and alpha-spectrin were shown to be an integral part of fusomes (de Cuevas *et al.*, 1996; Petrella *et al.*, 2007). Both of these proteins are thought to tether to actin,

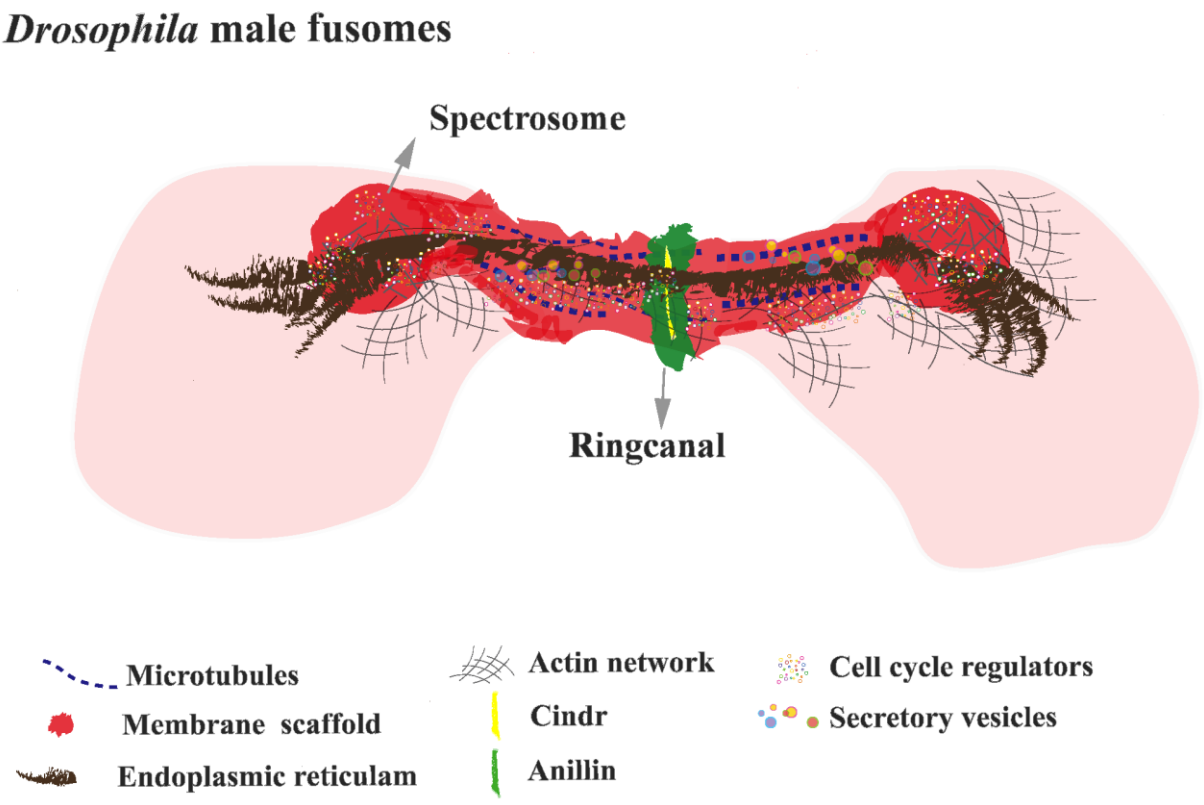

Fig. 1-7: *Drosophila* male fusome components

This illustration depicts the major components of spectrosome/fusomes reported in early (S1-2) spermatocyte development. Fusome is composed of three major structural components including Hts and spectrin rich membrane scaffold, actin and microtubule network. Fusomes also include endo-membranes that are derived from endoplasmic reticulum (ER). These structures are specifically detected during early stage of germ cell development and they appear to remodel from mid-late stage of development. The ring canals of male fusomes constitute Cindr, Anillin and Tyrosine phosphor-substrates. Fusomes also include secretory vesicles and cell cycle regulators as part of mediating molecular transportation and mitotic/meiotic coordination.

forming an actin-membrane cytoskeleton. Loss of function *hts* mutants was reported to have no fusome vesicles in germ-line cysts, and result in the absence of oocyte differentiation in females (Yue and Spradling, 1992). In male *hts* mutants, no evidence of fusomes was found and spermatocytes contained an abnormal number of centrosomes and often formed aberrant monopolar and multipolar meiotic spindles (Wilson, 2005). These observations revealed that proper membrane integrity was required for normal fusome function.

Microtubules are a third major constituent of fusomes, as well as microtubule-associated motor proteins including dynein and its regulator Lis1, Orbit and its related Mast, BicD (BicaudalD) and Egalitarian (Egl) associate with cystocyte fusomes (Liu *et al.*, 1999b; Bolivar *et al.*, 2001; Mathe *et al.*, 2003; Wehr *et al.*, 2006). In addition to the three major structural constituents, fusomes also include transiently associated cell cycle regulators such as Cyclin A, Cyclin E, Cul1 proteolytic complex and polarity specifying Par1 and Par5 (Lilly *et al.*, 2000; Cox *et al.*, 2001a; Huynh *et al.*, 2001; Ohlmeyer and Schupbach, 2003; Lighthouse *et al.*, 2008). Although these proteins are associated with germ-cell development, they are not specifically required for fusome biogenesis. Many new proteins are also identified and shown to associate with fusomes in recent literature indicating that fusomes are composed of a complex assembly regulating multiple aspects of germ cell development (Lighthouse *et al.*, 2008).

Phenotypic analysis has shown that fusomes facilitate oocyte determination. Asymmetric distribution of fusomes during four rounds of cystocyte division serves as a critical factor for determining the oocyte (Fichelson and Huynh, 2007). One out of the 16 cystocytes (the first cell), which has maximum number of four fusome branches, becomes an oocyte and the remainder differentiates into nurse cells (Megraw and Kaufman, 2000).

Fusome connections are thought to facilitate molecular transportation by motor protein-mediated cargo activity to supply mRNA and other essential factors to the oocyte (Wilson, 1999; Roper and Brown, 2004; Pokrywka *et al.*, 2009). Fusome-localized Par1 has also been implicated in microtubule dependent polarization of the oocyte and transportation of its determinants from anterior to the posterior region (Cox *et al.*, 2001a; Vaccari and Ephrussi, 2002). Proximity of the centrosome to the spectrosome may also provide a polarity cue for fusome orientation (Megraw and Kaufman, 2000). Another key requirement of oocyte determination is associated with centrosome inactivation in nurse cells followed by its migration into the posterior end through fusome-mediated transport (Megraw and Kaufman, 2000; Bolivar *et al.*, 2001; Huynh *et al.*, 2001).

The fusome is also thought to play a role in female germ cell cycle synchrony and in controlling the number of transit amplification divisions to precisely four (Snapp *et al.*, 2004; Fichelson and Huynh, 2007). Co-ordination of cell cycle synchrony is thought to be mediated by Cyclin A within the fusome (Lilly *et al.*, 2000; Ohlmeyer and Schupbach, 2003). Cyclin A is one of the G2 phase Cyclin that forms a complex with Cdk1 to regulate G2/M transition. Requirement of Cyclin A on fusome and its relevant mechanism of regulating cell cycle synchrony are not understood yet, however the phosphorylated form of Cyclin E (pCyclin E) is also localized on fusomes along with Cul1 and 19S-S1 proteosome complexes. Encore recruits the proteolytic complex to target pCyclinE and Cyclin A for degradation. This degradation mediated regulatory mechanism was shown to restrict the number of mitotic division to four, as failing to execute this mechanism resulted in extra mitotic divisions.

During female meiosis, only one of the 16 germ cells in each cyst differentiates into an oocyte, while the remaining cells differentiate into nurse cells (Deng and Lin, 2001; Fichelson

and Huynh, 2007). In males, all 16 cells in each cyst synchronously differentiate into spermatocytes and arrest in pre-meiotic G2 phase for 90 hr before synchronously undergoing the two meiotic divisions (MI and MII). Fusomes have been proposed to help synchronize cell cycle behavior of germline cysts based on three reasons: 1) cell cycle regulators such as Cyclin A accumulate on fusomes in both mitotic and pre-meiotic cells, 2) fusomes are enriched for ER-derived proteins linking all of the cells in each cyst, meaning that effectively a common ER could be used to coordinate cell cycle progression by trafficking and protein modification 3) fusome disruption results in asynchronous mitotic divisions in the female germline (de Cuevas and Spradling, 1998; Cox *et al.*, 2001b; Deng and Lin, 2001; Huynh and St Johnston, 2004; Fichelson and Huynh, 2007).

In both male and female *Drosophila* germline development fusome-related defects are linked to infertility (Lighthouse *et al.*, 2008), demonstrating that this organelle serves an essential role in gametogenesis. In general, it has been proposed that the fusome serves as a dynamic scaffold for mediating interactions and trafficking of cell cycle regulators such as Cyclin A and cytoskeletal proteins involved in spindle assembly and cytokinesis as a mechanism for synchronizing cell division. The molecular mechanisms involving this proposed interaction of these proteins with fusomes to coordinate cystocyte behavior are not yet understood, however.

1.3.4 Centrosome behavior during *Drosophila* spermatogenesis

Centrosomes are the primary microtubule-organizing center in animal cells (MTOC) and were first discovered in 1887. Boveri named these cytoplasmic organelles centrosomes and their core components as centrioles, describing them as “the true division organ of the cell,

mediates the nuclear and cellular division" (Scheer, 2014). In addition to their role in mitotic spindle assembly, centrosomes are also involved in cell polarity, adhesion and motility (Tang and Marshall, 2012). Centrioles, also assemble into axonemes to provide motility to cilia, flagella and sperm tail in certain cell types (Kierszenbaum, 2002; Mottier-Pavie and Megraw, 2009; Riparbelli *et al.*, 2009).

Each centrosome is composed of a pair of centrioles surrounded by peri-centriolar matrix (PCM) composed of numerous regulatory and structural proteins that orchestrate centrosome behavior (Ou *et al.*, 2004; Luders, 2012; Woodruff *et al.*, 2014). *Drosophila* spermatogenesis has been an excellent developmental system for studying centrosome behavior, because centrioles undergo enormous changes in size and morphology during spermatocyte maturation and spermatid differentiation can be studied genetically with mutants (Riparbelli and Callaini, 2011). Centriole biogenesis is coordinated with cell cycle progression, with duplication and dis-engagement synchronized with DNA replication and chromosome segregation, respectively (Fig.1-8). Similar to humans (Habedanck *et al.*, 2005; Kleylein-Sohn *et al.*, 2007), regulation of centriole replication in *Drosophila* appears to be mediated by Polo-like kinase 4 (Plk4 or SAK) and its associated regulators, with increased Plk4 activity resulting in supernumerary centrioles (Bettencourt-Dias *et al.*, 2005). During S phase, Plk4 recruits a protein called Sas4 and modifies PCM proteins to allow accumulation of two other proteins (Ana2 and Sas6) specifically at the proximal end of the mother centriole (Stevens *et al.*, 2010; Levine and Holland, 2014; Moyer *et al.*, 2015). Sas6 appears to organize the early cartwheel structure of the pro-centriole, which then elongates orthogonally to form the daughter centriole (Fong *et al.*, 2014). Engagement between the mother and daughter centrioles could therefore play a role in restricting the number of centrioles to four

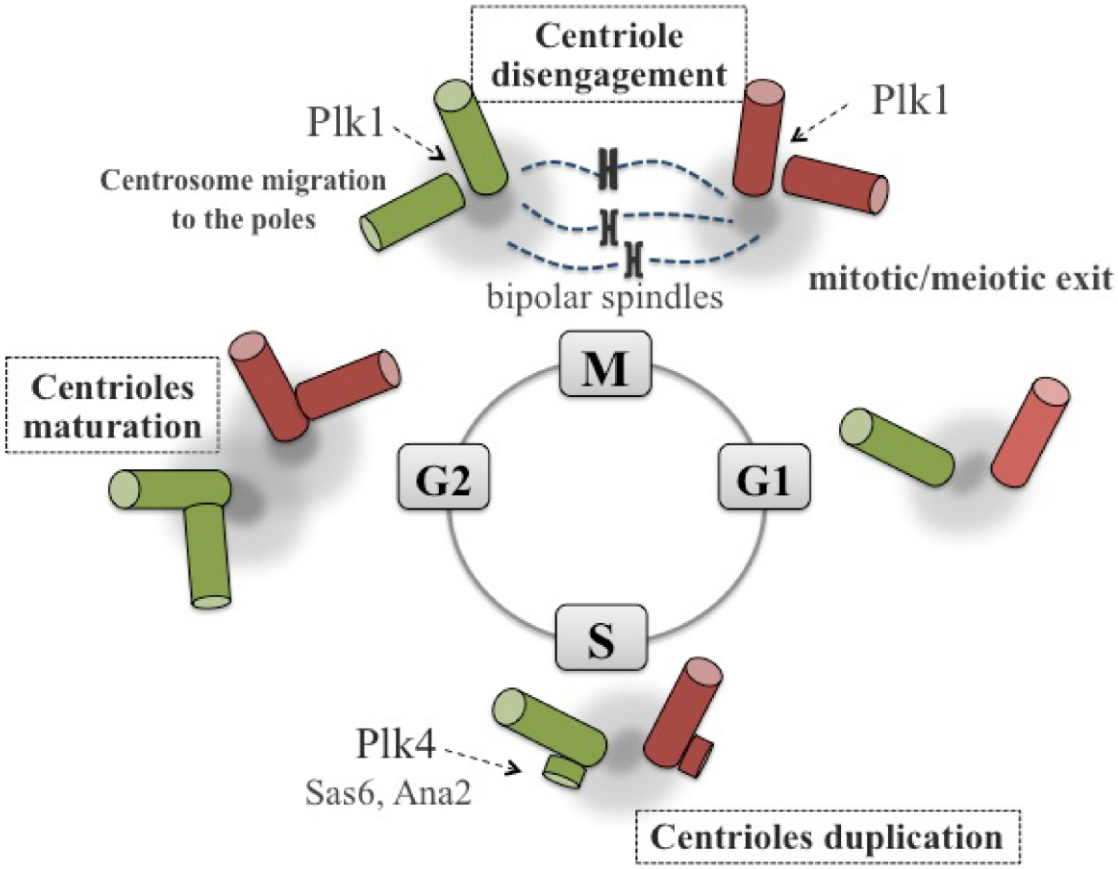

Fig. 1-8: The centrosome cycle

Centrosomes undergo three main processes (mentioned in the text box) during cell cycle. G1 cells inherit a single disengaged centrosome having two individual centrioles (red and green) that are located with in the peri-centriolar matrix (PCM- grey shade). Plk4 mediated regulations assemble a procentriole at the proximal end of each existing (mother) centrioles. The newly formed daughter centrioles remain engaged with the mother centrioles and gradually elongate throughout S and G2 phase. During the G2/M transition, each mature centrosome containing a pair of orthogonal centrioles migrate to the opposite end of the cell to form mitotic spindles. Plk1 mediated regulations dis-engage mother and daughter centrioles during ana-telo phase transition, when chromatids are also segregated.

(two per centrosome) per cell and serve as an intrinsic mechanism for preventing inappropriate amplification of centrioles (Chen and Megraw, 2014; Firat-Karalar and Stearns, 2014; Kim *et al.*, 2014; Fu *et al.*, 2015). This is important because centriole amplification can lead to formation of multipolar spindles and chromosome segregation defects (Nash and Bowerman, 2004; Nigg, 2006; Tsou and Stearns, 2006; Gottardo *et al.*, 2014). During mitotic exit, mitotic kinase Plk1 and the APC/C mediated degradation pathway can induce centriole disengagement, by licensing nucleation of a new pro-centriole (Hatano and Sluder, 2012; Riparbelli *et al.*, 2014). Collectively Plk4/Sas6/Ana2/Sas4 and Plk1 regulate centriole duplication and engagement in *Drosophila* spermatocytes.

During the prolonged pre-meiotic G2 phase arrest of *Drosophila* spermatogenesis, centriole pairs (mother and daughter) elongate proportionally in each spermatocyte to form the future basal body in the spermatids (Gottardo *et al.*, 2013; Riparbelli *et al.*, 2013). Centriole length increases from 0.9 to 2.6 microns in these cells, significantly longer than centrioles in somatic cells (Riparbelli *et al.*, 2012). These enormous centrioles form a characteristic V shaped structure at late stages of spermatocyte maturation. During G2/MI progression, centrosome migration inwards from the plasma membrane to the nuclear envelope coincides with chromosome condensation, while centrosomes separate and migrate to each pole of the cell where they nucleate spindle microtubules. Mother-daughter centriole disengagement is coordinated with homologous chromosome dis-junction during anaphase of MI during *Drosophila* male meiosis. In telophase I, each daughter cell receives two disengaged centrioles capable of immediately forming the second meiotic spindle for MII division (Rodrigues-Martins *et al.*, 2008; Blachon *et al.*, 2009). By late telophase I or prophase II stages the meiocytes (32 cell stage) lack S phase regulators, therefore these

disengaged centrioles are not licensed for replication. Upon segregation of sister chromatids in Meiosis II, the daughter or secondary meiocytes (64 cell stage) each receive a haploid genome along with a single centriole that differentiate into spermatids with a centriole near the nucleus that organizes a basal body to form the axoneme of the sperm tail. The illustration shown in Fig.1-9 depicts the sequence of events undertaken by centrosomes and centrioles throughout *Drosophila* male meiosis.

The molecular mechanisms used to coordinate centriole dis-engagement with cell division are currently under debate, as more than one pathway appears to regulate this process (Mardin and Schiebel, 2012; Sluder, 2013; Fry, 2015). Studies of mitotic mammalian cells suggested that Plk1-mediated separase degradation promoted centriole dis-engagement when cohesin is degraded to facilitate sister-chromatid separation (Schockel *et al.*, 2011; Lee and Rhee, 2012). Although Plk1 mediated dis-engagement appear to be a conserved mechanism, cohesin degradation does not seem to account with the data in most animal models (Inanc *et al.*, 2010; Cabral *et al.*, 2013; Oliveira and Nasmyth, 2013). In *C. elegans* and human, APC/C mediated degradation of phosphorylated Sas6 appears to be important for disengaging the centrioles (Cunha-Ferreira *et al.*, 2009; Zitouni *et al.*, 2014). The upstream regulators that promote phospho-dependent degradation of Sas6 have not been identified, however. How these proposed mechanisms regulate centriole-engagement and disengagement during *Drosophila* male meiosis has not yet been fully described, however.

In my thesis research I have studied many of the outstanding issues previously raised by studies of *Drosophila* male meiosis. Using transgenic reporters and immuno-labeling to phenotypically analyze *myt1* loss of function mutants, my goal has been to understand why

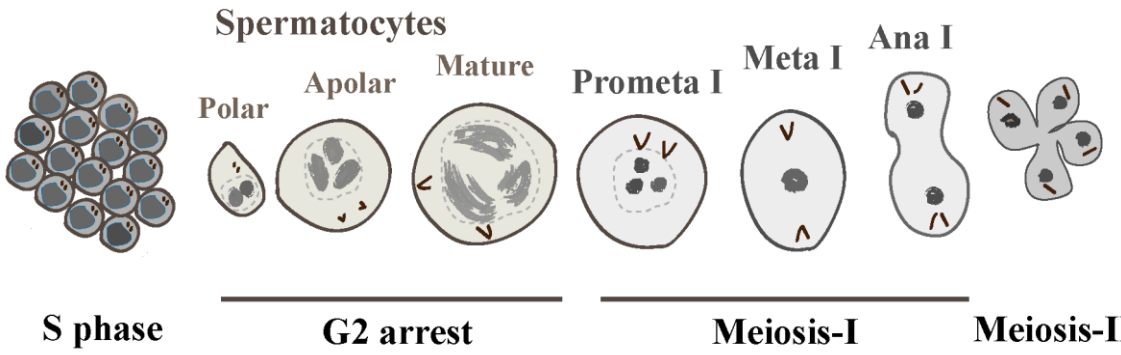

Fig. 1-9: Meiotic centrosome behavior during *Drosophila* spermatogenesis

Post-mitotic germ cells produce 16-cell cyst, and during pre-meiotic S phase their centrioles duplicate. Centrioles elongate ten fold of its original size during the prolonged G2 phase and are noticed like a V shaped (paired centrioles) structure in the mature spermatocyte. They remain close to the plasma membrane until the late spermatocyte stage and migrate inwards to the nucleus during prometaI phase. Mature centrosomes continue to move to the poles forming the meiotic bipolar spindles. During the anaphaseI transition, the V shaped centriole pair at each pole dis-engages preparing for another meiotic division. After MII, four interconnected secondary spermatocytes are produced, and each receives a single centriole. The spermatid centriole differentiates into a basal body to form the axoneme of the sperm.

these mutants were male sterile. By characterizing defects in fusome organization and centriole disengagement in these *myt1* mutant spermatocytes I was able to identify two distinct 'organelle checkpoint' functions for Myt1 that have not been previously described. Based on the developmental timing of the defects, I proposed that regulation of Cyclin A/Cdk1 was the target of Myt1 for these checkpoint mechanisms and by manipulating Cyclin A levels and known regulators of Cyclin A-associated Cdk1 I was able to successfully test this hypothesis. The results reported in this thesis therefore shed new light on the role of Myt1 kinase in coordinating nuclear and cytoplasmic events as spermatocytes transition from pre-meiotic G2 phase arrest into MI phase.

1.4 References

1. Abraham, RT. 2001. “Cell cycle checkpoint signaling through the ATM and ATR kinases.” *Genes Dev* 15(17): 2177–96.
2. Ajduk, A, A Malagocki, and M Maleszewski. 2008. “Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca2+ oscillations.” *Reprod Biol* 8(1): 3–22.
3. Allen, AK, JE Nesmith, and A Golden. 2014. “An RNAi-based suppressor screen identifies interactors of the Myt1 ortholog of Caenorhabditis elegans.” *G3 (Bethesda)* 4(12): 2329–43.
4. Alphey, L, J Jimenez, H White-Cooper, I Dawson, P Nurse, and DM Glover. 1992. “twine, a cdc25 homolog that functions in the male and female germline of Drosophila.” *Cell* 69(6): 977–88.
5. Archambault, V, X Zhao, H White-Cooper, AT Carpenter, and DM Glover. 2007. “Mutations in Drosophila Greatwall/Scant reveal its roles in mitosis and meiosis and interdependence with Polo kinase.” *PLoS Genet* 3(11): e200.
6. Avazeri, N, AM Courtot, A Pesty, and B Lefevre. 2003. “Meiosis resumption, calcium-sensitive period, and PLC-beta1 relocation into the nucleus in the mouse oocyte.” *Cell Signal* 15(11): 1003–10.
7. Ayeni, JO, and SD Campbell. 2014. “”Ready, set, go”: checkpoint regulation by Cdk1 inhibitory phosphorylation.” *Fly (Austin)* 8(3): 140–47.
8. Ayeni, JO, R Varadarajan, O Mukherjee, DT Stuart, F Sprenger, M Srayko, and SD Campbell. 2014. “Dual phosphorylation of cdk1 coordinates cell proliferation with key developmental processes in Drosophila.” *Genetics* 196(1): 197–210.

9. Baker, CC, and MT Fuller. 2007. “Translational control of meiotic cell cycle progression and spermatid differentiation in male germ cells by a novel eIF4G homolog.” *Development* 134(15): 2863–69.

10. Bate, Michael. 1994. *The Development of Drosophila Melanogaster*. Cold Spring Harbor Laboratory Pr.

11. Becker, EB, and A Bonni. 2004. “Cell cycle regulation of neuronal apoptosis in development and disease.” *Prog Neurobiol* 72(1): 1–25.

12. Bettencourt-Dias, M, A Rodrigues-Martins, L Carpenter, M Riparbelli, L Lehmann, MK Gatt, N Carmo, F Balloux, G Callaini, and DM Glover. 2005. “SAK/PLK4 is required for centriole duplication and flagella development.” *Curr Biol* 15(24): 2199–207.

13. Blachon, S, X Cai, KA Roberts, K Yang, A Polyanovsky, A Church, and T Avidor-Reiss. 2009. “A proximal centriole-like structure is present in Drosophila spermatids and can serve as a model to study centriole duplication.” *Genetics* 182(1): 133–44.

14. Bolanos-Garcia, VM. 2014. “Formation of multiprotein assemblies in the nucleus: the spindle assembly checkpoint.” *Int Rev Cell Mol Biol* 307: 151–74.

15. Bolivar, J, JR Huynh, H Lopez-Schier, C Gonzalez, D St Johnston, and A Gonzalez-Reyes. 2001. “Centrosome migration into the Drosophila oocyte is independent of BicD and egl, and of the organisation of the microtubule cytoskeleton.” *Development* 128(10): 1889–97.

16. Booher, RN, PS Holman, and A Fattaey. 1997. “Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity.” *J Biol Chem* 272(35): 22300–06.

17. Brink, NG. 1968. “Protein synthesis during spermatogenesis in Drosophila melanogaster.” *Mutat Res* 5(1): 192–94.

18. Brodsky, MH, JJ Sekelsky, G Tsang, RS Hawley, and GM Rubin. 2000. "mus304 encodes a novel DNA damage checkpoint protein required during Drosophila development." *Genes Dev* 14(6): 666–78.

19. Burrows, AE, BK Sceurman, ME Kosinski, CT Richie, PL Sadler, JM Schumacher, and A Golden. 2006. "The C. elegans Myt1 ortholog is required for the proper timing of oocyte maturation." *Development* 133(4): 697–709.

20. Cabral, G, SS Sans, CR Cowan, and A Dammermann. 2013. "Multiple mechanisms contribute to centriole separation in C. elegans." *Curr Biol* 23(14): 1380–87.

21. Campbell, SD, F Sprenger, BA Edgar, and PH O'Farrell. 1995. "Drosophila Wee1 kinase rescues fission yeast from mitotic catastrophe and phosphorylates Drosophila Cdc2 in vitro." *Mol Biol Cell* 6(10): 1333–47.

22. Cantu, JM, F Rivas, P Hernandez-Jauregui, M Diaz, V Cortes-Gallegos, G Vaca, A Velazquez, and B Ibarra. 1981. "Meiotic arrest at first spermatocyte level: a new inherited infertility disorder." *Hum Genet* 59(4): 380–85.

23. Castedo, M, JL Perfettini, T Roumier, and G Kroemer. 2002. "Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe." *Cell Death Differ* 9(12): 1287–93.

24. Channing, CP, T Hillensjo, and FW Schaerf. 1978. "Hormonal control of oocyte meiosis, ovulation and luteinization in mammals." *Clin Endocrinol Metab* 7(3): 601–24.

25. Chen, JV, and TL Megraw. 2014. "Mother centrioles do a cartwheel to produce just one daughter." *Dev Cell* 30(2): 111–12.

26. Chow, JP, and RY Poon. 2013. "The CDK1 inhibitory kinase MYT1 in DNA damage checkpoint recovery." *Oncogene* 32(40): 4778–88.

27. Chow, JP, RY Poon, and HT Ma. 2011. "Inhibitory phosphorylation of cyclin-dependent kinase 1 as a compensatory mechanism for mitosis exit." *Mol Cell Biol* 31(7): 1478–91.

28. Chu, T, G Henrion, V Haegeli, and S Strickland. 2001. "Cortex, a Drosophila gene required to complete oocyte meiosis, is a member of the Cdc20/fizzy protein family." *Genesis* 29(3): 141–52.

29. Clute, P, and J Pines. 1999. "Temporal and spatial control of cyclin B1 destruction in metaphase." *Nat Cell Biol* 1(2): 82–87.

30. Cornwell, WD, PJ Kaminski, and JR Jackson. 2002. "Identification of Drosophila Myt1 kinase and its role in Golgi during mitosis." *Cell Signal* 14(5): 467–76.

31. Coulonval, K, H Kooken, and PP Roger. 2011. "Coupling of T161 and T14 phosphorylations protects cyclin B-CDK1 from premature activation." *Mol Biol Cell* 22(21): 3971–85.

32. Courtot, C, C Fankhauser, V Simanis, and CF Lehner. 1992. "The Drosophila cdc25 homolog twine is required for meiosis." *Development* 116(2): 405–16.

33. Cox, DN, B Lu, TQ Sun, LT Williams, and YN Jan. 2001a. "Drosophila par-1 is required for oocyte differentiation and microtubule organization." *Curr Biol* 11(2): 75–87.

34. Cox, DN, SA Seyfried, LY Jan, and YN Jan. 2001b. "Bazooka and atypical protein kinase C are required to regulate oocyte differentiation in the Drosophila ovary." *Proc Natl Acad Sci U S A* 98(25): 14475–80.

35. Cunha-Ferreira, I, A Rodrigues-Martins, I Bento, M Riparbelli, W Zhang, E Laue, G Callaini, DM Glover, and M Bettencourt-Dias. 2009. "The SCF/Slimb ubiquitin ligase

limits centrosome amplification through degradation of SAK/PLK4." *Curr Biol* 19(1): 43–49.

36. de Cuevas, M, JK Lee, and AC Spradling. 1996. "alpha-spectrin is required for germline cell division and differentiation in the Drosophila ovary." *Development* 122(12): 3959–68.

37. de Cuevas, M, MA Lilly, and AC Spradling. 1997. "Germline cyst formation in Drosophila." *Annu Rev Genet* 31: 405–28.

38. de Cuevas, M, and AC Spradling. 1998. "Morphogenesis of the Drosophila fusome and its implications for oocyte specification." *Development* 125(15): 2781–89.

39. de Vries, HI, L Uyetake, W Lemstra, JF Brunsting, TT Su, HH Kampinga, and OC Sibon. 2005. "Grp/DChk1 is required for G2-M checkpoint activation in Drosophila S2 cells, whereas Dmnk/DChk2 is dispensable." *J Cell Sci* 118(Pt 9): 1833–42.

40. Deng, W, and H Lin. 2001. "Asymmetric germ cell division and oocyte determination during Drosophila oogenesis." *Int Rev Cytol* 203: 93–138.

41. Doniach, T. 1986. "Activity of the sex-determining gene tra-2 is modulated to allow spermatogenesis in the C. elegans hermaphrodite." *Genetics* 114(1): 53–76.

42. Duronio, RJ. 2012. "Developing S-phase control." *Genes Dev* 26(8): 746–50.

43. Duronio, RJ, and Y Xiong. 2013. "Signaling pathways that control cell proliferation." *Cold Spring Harb Perspect Biol* 5(3): a008904.

44. Duronio, Robert J, and Patrick H O'Farrell. 1995. "Developmental control of the G1 to S transition in Drosophila: cyclin Eis a limiting downstream target of E2F." *Genes & Development* 9(12): 1456–68.

45. Edgar, BA, F Sprenger, RJ Duronio, P Leopold, and PH O'Farrell. 1994. "Distinct

molecular mechanism regulate cell cycle timing at successive stages of Drosophila embryogenesis." *Genes Dev* 8(4): 440–52.

46. Eikenes, AH, A Brech, H Stenmark, and K Haglund. 2013. "Spatiotemporal control of Cindr at ring canals during incomplete cytokinesis in the Drosophila male germline." *Dev Biol* 377(1): 9–20.

47. Enserink, JM, and RD Kolodner. 2010. "An overview of Cdk1-controlled targets and processes." *Cell Div* 5: 11.

48. Fagarasanu, A, FD Mast, B Knoblach, and RA Rachubinski. 2010. "Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast." *Nat Rev Mol Cell Biol* 11(9): 644–54.

49. Fattaey, A, and RN Booher. 1997. "Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14." *Prog Cell Cycle Res* 3: 233–40.

50. Fichelson, P, and JR Huynh. 2007. "Asymmetric divisions of germline cells." *Prog Mol Subcell Biol* 45: 97–120.

51. Firat-Karalar, EN, and T Stearns. 2014. "The centriole duplication cycle." *Philos Trans R Soc Lond B Biol Sci* 369(1650):

52. Fogarty, P, RF Kalpin, and W Sullivan. 1994. "The Drosophila maternal-effect mutation grapes causes a metaphase arrest at nuclear cycle 13." *Development* 120(8): 2131–42.

53. Fong, CS, M Kim, TT Yang, JC Liao, and MF Tsou. 2014. "SAS-6 assembly templated by the lumen of cartwheel-less centrioles precedes centriole duplication." *Dev Cell* 30(2): 238–45.

54. Fry, AM. 2015. "Solving the centriole disengagement puzzle." *Nat Cell Biol* 17(1): 3–5.

55. Fu, J, IM Hagan, and DM Glover. 2015. "The centrosome and its duplication cycle."

Cold Spring Harb Perspect Biol 7(2): a015800.

56. Fuller, MT. 1998. "Genetic control of cell proliferation and differentiation in Drosophila spermatogenesis." *Semin Cell Dev Biol* 9(4): 433–44.

57. Furman, DP, and TA Bukharina. 2012. "Morphogenesis of Drosophila melanogaster macrochaetes: cell fate determination for bristle organ." *J Stem Cells* 7(1): 19–41.

58. Furuno, N, A Kawasaki, and N Sagata. 2003. "Expression of cell-cycle regulators during Xenopus oogenesis." *Gene Expr Patterns* 3(2): 165–68.

59. Gaffre, M, A Martoriati, N Belhachemi, JP Chambon, E Houliston, C Jessus, and A Karaiskou. 2011. "A critical balance between Cyclin B synthesis and Myt1 activity controls meiosis entry in Xenopus oocytes." *Development* 138(17): 3735–44.

60. Gonczy, P, E Matunis, and S DiNardo. 1997. "bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis." *Development* 124(21): 4361–71.

61. Gorbsky, GJ. 2015. "The spindle checkpoint and chromosome segregation in meiosis." *FEBS J* 282(13): 2458–74.

62. Gorr, IH, D Boos, and O Stemmann. 2005. "Mutual inhibition of separase and Cdk1 by two-step complex formation." *Mol Cell* 19(1): 135–41.

63. Gottardo, M, G Callaini, and MG Riparbelli. 2013. "The cilium-like region of the Drosophila spermatocyte: an emerging flagellum?" *J Cell Sci* 126(Pt 23): 5441–52.

64. Gottardo, M, G Callaini, and MG Riparbelli. 2014. "Procentriole assembly without centriole disengagement - a paradox of male gametogenesis." *J Cell Sci* 127(Pt 16): 3434–39.

65. Gould, KL, and P Nurse. 1989. "Tyrosine phosphorylation of the fission yeast cdc2+

protein kinase regulates entry into mitosis." *Nature* 342(6245): 39–45.

66. Habedanck, R, YD Stierhof, CJ Wilkinson, and EA Nigg. 2005. "The Polo kinase Plk4 functions in centriole duplication." *Nat Cell Biol* 7(11): 1140–46.
67. Hackstein, JH. 1991. "Spermatogenesis in Drosophila. A genetic approach to cellular and subcellular differentiation." *Eur J Cell Biol* 56(2): 151–69.
68. Harper, JW, and SJ Elledge. 1998. "The role of Cdk7 in CAK function, a retro-retrospective." *Genes Dev* 12(3): 285–89.
69. Hartwell, LH, and TA Weinert. 1989. "Checkpoints: controls that ensure the order of cell cycle events." *Science* 246(4930): 629–34.
70. Hatano, T, and G Sluder. 2012. "The interrelationship between APC/C and Plk1 activities in centriole disengagement." *Biol Open* 1(11): 1153–60.
71. Hime, GR, JA Brill, and MT Fuller. 1996. "Assembly of ring canals in the male germ line from structural components of the contractile ring." *J Cell Sci* 109 (Pt 12): 2779–88.
72. Holt, JE, SI Lane, and KT Jones. 2013. "The control of meiotic maturation in mammalian oocytes." *Curr Top Dev Biol* 102: 207–26.
73. Hormanseder, E, T Tischer, and TU Mayer. 2013. "Modulation of cell cycle control during oocyte-to-embryo transitions." *EMBO J* 32(16): 2191–203.
74. Hoyt, MA, L Totis, and BT Roberts. 1991. "S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function." *Cell* 66(3): 507–17.
75. Huynh, JR, JM Shulman, R Benton, and D St Johnston. 2001. "PAR-1 is required for the maintenance of oocyte fate in Drosophila." *Development* 128(7): 1201–09.
76. Huynh, JR, and D St Johnston. 2004. "The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte." *Curr Biol* 14(11): R438–49.

77. Ikeda, MA, L Jakoi, and JR Nevins. 1996. “A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation.” *Proc Natl Acad Sci U S A* 93(8): 3215–20.

78. Inanc, B, H Dodson, and CG Morrison. 2010. “A centrosome-autonomous signal that involves centriole disengagement permits centrosome duplication in G2 phase after DNA damage.” *Mol Biol Cell* 21(22): 3866–77.

79. Inoue, D, and N Sagata. 2005. “The Polo-like kinase Plx1 interacts with and inhibits Myt1 after fertilization of Xenopus eggs.” *EMBO J* 24(5): 1057–67.

80. Insco, ML, A Leon, CH Tam, DM McKearin, and MT Fuller. 2009. “Accumulation of a differentiation regulator specifies transit amplifying division number in an adult stem cell lineage.” *Proc Natl Acad Sci U S A* 106(52): 22311–16.

81. Irniger, S, S Piatti, C Michaelis, and K Nasmyth. 1995. “Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast.” *Cell* 81(2): 269–78.

82. Jin, Z, E Homola, S Tiong, and SD Campbell. 2008. “Drosophila myt1 is the major cdk1 inhibitory kinase for wing imaginal disc development.” *Genetics* 180(4): 2123–33.

83. Jin, Z, EM Homola, P Goldbach, Y Choi, JA Brill, and SD Campbell. 2005. “Drosophila Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis.” *Development* 132(18): 4075–85.

84. Kannan, R, I Kuzina, S Wincovitch, SH Nowotarski, and E Giniger. 2014. “The Abl/enabled signaling pathway regulates Golgi architecture in Drosophila photoreceptor neurons.” *Mol Biol Cell* 25(19): 2993–3005.

85. Karaiskou, A, AC Lepretre, G Pahlavan, D Du Pasquier, R Ozon, and C Jessus. 2004.

"Polo-like kinase confers MPF autoamplification competence to growing Xenopus oocytes." *Development* 131(7): 1543–52.

86. Kierszenbaum, AL. 2002. "Sperm axoneme: a tale of tubulin posttranslation diversity." *Mol Reprod Dev* 62(1): 1–3.

87. Kiger, AA, DL Jones, C Schulz, MB Rogers, and MT Fuller. 2001. "Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue." *Science* 294(5551): 2542–45.

88. Kim, M, CS Fong, and MF Tsou. 2014. "Centriole duplication: when PLK4 meets Ana2/STIL." *Curr Biol* 24(21): R1046–48.

89. Kim, S, C Spike, and D Greenstein. 2013. "Control of oocyte growth and meiotic maturation in Caenorhabditis elegans." *Adv Exp Med Biol* 757: 277–320.

90. Kimata, Y, JE Baxter, AM Fry, and H Yamano. 2008. "A role for the Fizzy/Cdc20 family of proteins in activation of the APC/C distinct from substrate recruitment." *Mol Cell* 32(4): 576–83.

91. Kishimoto, T. 2003. "Cell-cycle control during meiotic maturation." *Curr Opin Cell Biol* 15(6): 654–63.

92. Kishimoto, T. 2011. "A primer on meiotic resumption in starfish oocytes: the proposed signaling pathway triggered by maturation-inducing hormone." *Mol Reprod Dev* 78(10-11): 704–07.

93. Kleylein-Sohn, J, J Westendorf, M Le Clech, R Habedanck, YD Stierhof, and EA Nigg. 2007. "Plk4-induced centriole biogenesis in human cells." *Dev Cell* 13(2): 190–202.

94. Klumperman, J. 2011. "Architecture of the mammalian Golgi." *Cold Spring Harb Perspect Biol* 3(7)

95. Kondylis, V, and C Rabouille. 2009. “The Golgi apparatus: lessons from Drosophila.” *FEBS Lett* 583(23): 3827–38.

96. Kondylis, V, HE van Nispen tot Pannerden, B Herpers, F Friggi-Grelin, and C Rabouille. 2007. “The golgi comprises a paired stack that is separated at G2 by modulation of the actin cytoskeleton through Abi and Scar/WAVE.” *Dev Cell* 12(6): 901–15.

97. Kornbluth, S, B Sebastian, T Hunter, and J Newport. 1994. “Membrane localization of the kinase which phosphorylates p34cdc2 on threonine 14.” *Mol Biol Cell* 5(3): 273–82.

98. Krek, W, J Marks, N Schmitz, EA Nigg, and V Simanis. 1992. “Vertebrate p34cdc2 phosphorylation site mutants: effects upon cell cycle progression in the fission yeast Schizosaccharomyces pombe.” *J Cell Sci* 102 (Pt 1): 43–53.

99. Krek, W, and EA Nigg. 1991. “Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates.” *EMBO J* 10(11): 3331–41.

100. Kristjansdottir, K, A Safi, C Shah, and J Rudolph. 2006. “Autophosphorylation of Ser66 on Xenopus Myt1 is a prerequisite for meiotic inactivation of Myt1.” *Cell Cycle* 5(4): 421–27.

101. Kronja, I, ZJ Whitfield, B Yuan, K Dzeyk, J Kirkpatrick, J Krijgsveld, and TL Orr-Weaver. 2014. “Quantitative proteomics reveals the dynamics of protein changes during Drosophila oocyte maturation and the oocyte-to-embryo transition.” *Proc Natl Acad Sci U S A* 111(45): 16023–28.

102. Kulawiec, M, V Ayyasamy, and KK Singh. 2009. “p53 regulates mtDNA copy number and mitocheckpoint pathway.” *J Carcinog* 8: 8.

103. Lamitina, ST, and SW L'Hernault. 2002. "Dominant mutations in the Caenorhabditis elegans Myt1 ortholog wee-1.3 reveal a novel domain that controls M-phase entry during spermatogenesis." *Development* 129(21): 5009–18.

104. Lee, K, and K Rhee. 2012. "Separase-dependent cleavage of pericentrin B is necessary and sufficient for centriole disengagement during mitosis." *Cell Cycle* 11(13): 2476–85.

105. Levine, MS, and AJ Holland. 2014. "Polo-like kinase 4 shapes up." *Structure* 22(8): 1071–73.

106. Lighthouse, DV, M Buszczak, and AC Spradling. 2008. "New components of the Drosophila fusome suggest it plays novel roles in signaling and transport." *Dev Biol* 317(1): 59–71.

107. Lilly, MA, M de Cuevas, and AC Spradling. 2000. "Cyclin A associates with the fusome during germline cyst formation in the Drosophila ovary." *Dev Biol* 218(1): 53–63.

108. Lin, H, L Yue, and AC Spradling. 1994. "The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation." *Development* 120(4): 947–56.

109. Lin, TY, S Viswanathan, C Wood, PG Wilson, N Wolf, and MT Fuller. 1996. "Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in Drosophila males." *Development* 122(4): 1331–41.

110. Liu, F, C Rothblum-Oviatt, CE Ryan, and H Piwnica-Worms. 1999a. "Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes." *Mol Cell Biol* 19(7): 5113–23.

111. Liu, F, JJ Stanton, Z Wu, and H Piwnica-Worms. 1997. "The human Myt1 kinase

preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex." *Mol Cell Biol* 17(2): 571–83.

112. Liu, Z, T Xie, and R Steward. 1999b. "Lis1, the Drosophila homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation." *Development* 126(20): 4477–88.
113. Lowe, M, and FA Barr. 2007. "Inheritance and biogenesis of organelles in the secretory pathway." *Nat Rev Mol Cell Biol* 8(6): 429–39.
114. Luders, J. 2012. "The amorphous pericentriolar cloud takes shape." *Nat Cell Biol* 14(11): 1126–28.
115. Lundgren, K, N Walworth, R Booher, M Dembski, M Kirschner, and D Beach. 1991. "mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2." *Cell* 64(6): 1111–22.
116. Mardin, BR, and E Schiebel. 2012. "Breaking the ties that bind: new advances in centrosome biology." *J Cell Biol* 197(1): 11–18.
117. Mathe, E, YH Inoue, W Palframan, G Brown, and DM Glover. 2003. "Orbit/Mast, the CLASP orthologue of Drosophila, is required for asymmetric stem cell and cystocyte divisions and development of the polarised microtubule network that interconnects oocyte and nurse cells during oogenesis." *Development* 130(5): 901–15.
118. Matsuura, I, and JH Wang. 1996. "Demonstration of cyclin-dependent kinase inhibitory serine/threonine kinase in bovine thymus." *J Biol Chem* 271(10): 5443–50.
119. McKearin, D. 1997. "The Drosophila fusome, organelle biogenesis and germ cell differentiation: if you build it." *Bioessays* 19(2): 147–52.
120. Megraw, TL, and TC Kaufman. 2000. "The centrosome in Drosophila oocyte

development." *Curr Top Dev Biol* 49: 385–407.

121. Miller, MA, VQ Nguyen, MH Lee, M Kosinski, T Schedl, RM Caprioli, and D Greenstein. 2001. "A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation." *Science* 291(5511): 2144–47.

122. Minocherhomji, S, TO Tollefsbol, and KK Singh. 2012. "Mitochondrial regulation of epigenetics and its role in human diseases." *Epigenetics* 7(4): 326–34.

123. Morris, LX, and AC Spradling. 2012. "Steroid signaling within Drosophila ovarian epithelial cells sex-specifically modulates early germ cell development and meiotic entry." *PLoS One* 7(10): e46109.

124. Mottier-Pavie, V, and TL Megraw. 2009. "Drosophila bld10 is a centriolar protein that regulates centriole, basal body, and motile cilium assembly." *Mol Biol Cell* 20(10): 2605–14.

125. Moyer, TC, KM Clutario, BG Lambrus, V Daggubati, and AJ Holland. 2015. "Binding of STIL to Plk4 activates kinase activity to promote centriole assembly." *J Cell Biol* 209(6): 863–78.

126. Mueller, PR, TR Coleman, A Kumagai, and WG Dunphy. 1995. "Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15." *Science* 270(5233): 86–90.

127. Murakami, MS, and GF Vande Woude. 1998. "Analysis of the early embryonic cell cycles of Xenopus; regulation of cell cycle length by Xe-wee1 and Mos." *Development* 125(2): 237–48.

128. Nakajima, H, S Yonemura, M Murata, N Nakamura, H Piwnica-Worms, and E Nishida. 2008. "Myt1 protein kinase is essential for Golgi and ER assembly during mitotic exit."

J Cell Biol 181(1): 89–103.

129. Nakajo, N, S Yoshitome, J Iwashita, M Iida, K Uto, S Ueno, K Okamoto, and N Sagata. 2000. "Absence of Wee1 ensures the meiotic cell cycle in Xenopus oocytes." *Genes Dev* 14(3): 328–38.
130. Nash, B, and B Bowerman. 2004. "Uncoiling centriole duplication." *Nat Cell Biol* 6(7): 573–75.
131. Nebreda, AR, and I Ferby. 2000. "Regulation of the meiotic cell cycle in oocytes." *Curr Opin Cell Biol* 12(6): 666–75.
132. Nigg, EA. 2006. "Cell biology: a licence for duplication." *Nature* 442(7105): 874–75.
133. Norbury, C, J Blow, and P Nurse. 1991. "Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates." *EMBO J* 10(11): 3321–29.
134. Nurse, P, and P Thuriaux. 1980. "Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe." *Genetics* 96(3): 627–37.
135. Oh, JS, SJ Han, and M Conti. 2010. "Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption." *J Cell Biol* 188(2): 199–207.
136. Ohi, R, and KL Gould. 1999. "Regulating the onset of mitosis." *Curr Opin Cell Biol* 11(2): 267–73.
137. Ohlmeyer, JT, and T Schupbach. 2003. "Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during Drosophila oogenesis." *Development* 130(25): 6339–49.
138. Ohsumi, K, W Sawada, and T Kishimoto. 1994. "Meiosis-specific cell cycle regulation in maturing Xenopus oocytes." *J Cell Sci* 107 (Pt 11): 3005–13.
139. Okano-Uchida, T, E Okumura, M Iwashita, H Yoshida, K Tachibana, and T Kishimoto.

2003. “Distinct regulators for Plk1 activation in starfish meiotic and early embryonic cycles.” *EMBO J* 22(20): 5633–42.

140. Okumura, E, T Fukuhara, H Yoshida, S Hanada Si, R Kozutsumi, M Mori, K Tachibana, and T Kishimoto. 2002. “Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition.” *Nat Cell Biol* 4(2): 111–16.
141. Oliveira, RA, and K Nasmyth. 2013. “Cohesin cleavage is insufficient for centriole disengagement in Drosophila.” *Curr Biol* 23(14): R601–03.
142. Ou, Y, M Zhang, and JB Rattner. 2004. “The centrosome: The centriole-PCM coalition.” *Cell Motil Cytoskeleton* 57(1): 1–7.
143. Palmer, A, AC Gavin, and AR Nebreda. 1998. “A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1.” *EMBO J* 17(17): 5037–47.
144. Palmer, A, and AR Nebreda. 2000. “The activation of MAP kinase and p34cdc2/cyclin B during the meiotic maturation of Xenopus oocytes.” *Prog Cell Cycle Res* 4: 131–43.
145. Parker, LL, S Atherton-Fessler, and H Piwnica-Worms. 1992. “p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15.” *Proc Natl Acad Sci U S A* 89(7): 2917–21.
146. Perezgasga, L, J Jiang, B Jr Bolival, M Hiller, E Benson, MT Fuller, and H White-Cooper. 2004. “Regulation of transcription of meiotic cell cycle and terminal differentiation genes by the testis-specific Zn-finger protein matotopetli.” *Development* 131(8): 1691–702.
147. Perry, AC, and MH Verlhac. 2008. “Second meiotic arrest and exit in frogs and mice.” *EMBO Rep* 9(3): 246–51.

148. Persico, A, RI Cervigni, ML Barretta, and A Colanzi. 2009. “Mitotic inheritance of the Golgi complex.” *FEBS Lett* 583(23): 3857–62.

149. Peter, M, JC Labbe, M Doree, and E Mandart. 2002. “A new role for Mos in Xenopus oocyte maturation: targeting Myt1 independently of MAPK.” *Development* 129(9): 2129–39.

150. Petrella, LN, T Smith-Leiker, and L Cooley. 2007. “The Ovhts polyprotein is cleaved to produce fusome and ring canal proteins required for Drosophila oogenesis.” *Development* 134(4): 703–12.

151. Pokrywka, NJ, A Payne-Tobin, KM Raley-Susman, and S Swartzman. 2009. “Microtubules, the ER and Exu: new associations revealed by analysis of mini spindles mutations.” *Mech Dev* 126(5-6): 289–300.

152. Potapova, TA, JR Daum, KS Byrd, and GJ Gorbsky. 2009. “Fine tuning the cell cycle: activation of the Cdk1 inhibitory phosphorylation pathway during mitotic exit.” *Mol Biol Cell* 20(6): 1737–48.

153. Price, D, S Rabinovitch, PH O’Farrell, and SD Campbell. 2000. “Drosophila wee1 has an essential role in the nuclear divisions of early embryogenesis.” *Genetics* 155(1): 159–66.

154. Price, DM, Z Jin, S Rabinovitch, and SD Campbell. 2002. “Ectopic expression of the Drosophila Cdk1 inhibitory kinases, Wee1 and Myt1, interferes with the second mitotic wave and disrupts pattern formation during eye development.” *Genetics* 161(2): 721–31.

155. Priyadarshini, A, D Basu, AK Navneet, A Bhattacharya, S Bhattacharya, S Maitra, and S Bhattacharya. 2009. “Activation of both Mos and Cdc25 is required for G2-M transition in perch oocyte.” *Mol Reprod Dev* 76(3): 289–300.

156. Rhind, N, and P Russell. 2001. “Roles of the mitotic inhibitors Wee1 and Mik1 in the G(2) DNA damage and replication checkpoints.” *Mol Cell Biol* 21(5): 1499–508.

157. Riparbelli, MG, OA Cabrera, G Callaini, and TL Megraw. 2013. “Unique properties of Drosophila spermatocyte primary cilia.” *Biol Open* 2(11): 1137–47.

158. Riparbelli, MG, and G Callaini. 2011. “Male gametogenesis without centrioles.” *Dev Biol* 349(2): 427–39.

159. Riparbelli, MG, G Callaini, and TL Megraw. 2012. “Assembly and persistence of primary cilia in dividing Drosophila spermatocytes.” *Dev Cell* 23(2): 425–32.

160. Riparbelli, MG, R Dallai, D Mercati, Y Bu, and G Callaini. 2009. “Centriole symmetry: a big tale from small organisms.” *Cell Motil Cytoskeleton* 66(12): 1100–05.

161. Riparbelli, MG, M Gottardo, DM Glover, and G Callaini. 2014. “Inhibition of Polo kinase by BI2536 affects centriole separation during Drosophila male meiosis.” *Cell Cycle* 13(13): 2064–72.

162. Rodrigues-Martins, A, M Riparbelli, G Callaini, DM Glover, and M Bettencourt-Dias. 2008. “From centriole biogenesis to cellular function: centrioles are essential for cell division at critical developmental stages.” *Cell Cycle* 7(1): 11–16.

163. Roegiers, F, S Younger-Shepherd, LY Jan, and YN Jan. 2001. “Two types of asymmetric divisions in the Drosophila sensory organ precursor cell lineage.” *Nat Cell Biol* 3(1): 58–67.

164. Roper, K, and NH Brown. 2004. “A spectraplakin is enriched on the fusome and organizes microtubules during oocyte specification in Drosophila.” *Curr Biol* 14(2): 99–110.

165. Ruiz, EJ, T Hunt, and AR Nebreda. 2008. “Meiotic inactivation of Xenopus Myt1 by

CDK/XRINGO, but not CDK/cyclin, via site-specific phosphorylation." *Mol Cell* 32(2): 210–20.

166. Ruiz, EJ, M Vilar, and AR Nebreda. 2010. "A two-step inactivation mechanism of Myt1 ensures CDK1/cyclin B activation and meiosis I entry." *Curr Biol* 20(8): 717–23.

167. Russell, P, and P Nurse. 1987. "The mitotic inducer nim1+ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis." *Cell* 49(4): 569–76.

168. Santamaria, D, C Barriere, A Cerqueira, S Hunt, C Tardy, K Newton, JF Caceres, P Dubus, M Malumbres, and M Barbacid. 2007. "Cdk1 is sufficient to drive the mammalian cell cycle." *Nature* 448(7155): 811–15.

169. Schafer, M, K Nayernia, W Engel, and U Schafer. 1995. "Translational control in spermatogenesis." *Dev Biol* 172(2): 344–52.

170. Scheer, U. 2014. "Historical roots of centrosome research: discovery of Boveri's microscope slides in Wurzburg." *Philos Trans R Soc Lond B Biol Sci* 369(1650):

171. Schockel, L, M Mockel, B Mayer, D Boos, and O Stemmann. 2011. "Cleavage of cohesin rings coordinates the separation of centrioles and chromatids." *Nat Cell Biol* 13(8): 966–72.

172. Sigrist, S, G Ried, and CF Lehner. 1995. "Dmcdc2 kinase is required for both meiotic divisions during Drosophila spermatogenesis and is activated by the Twine/cdc25 phosphatase." *Mech Dev* 53(2): 247–60.

173. Singh, KK. 2006. "Mitochondria damage checkpoint, aging, and cancer." *Ann N Y Acad Sci* 1067: 182–90.

174. Sluder, G. 2013. "Centriole engagement: it's not just cohesin any more." *Curr Biol*

23(15): R659–60.

175. Smith, A, S Simanski, M Fallahi, and NG Ayad. 2007. “Redundant ubiquitin ligase activities regulate wee1 degradation and mitotic entry.” *Cell Cycle* 6(22): 2795–99.

176. Snapp, EL, T Iida, D Frescas, J Lippincott-Schwartz, and MA Lilly. 2004. “The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts.” *Mol Biol Cell* 15(10): 4512–21.

177. Spradling, AC. 1993. “Germline cysts: communes that work.” *Cell* 72(5): 649–51.

178. Stevens, NR, H Roque, and JW Raff. 2010. “DSas-6 and Ana2 coassemble into tubules to promote centriole duplication and engagement.” *Dev Cell* 19(6): 913–19.

179. Stumpff, J, T Duncan, E Homola, SD Campbell, and TT Su. 2004. “Drosophila Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis.” *Curr Biol* 14(23): 2143–48.

180. Su, TT, SD Campbell, and PH O’Farrell. 1998. “The cell cycle program in germ cells of the Drosophila embryo.” *Dev Biol* 196(2): 160–70.

181. Sutterlin, C, P Hsu, A Mallabiabarrena, and V Malhotra. 2002. “Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells.” *Cell* 109(3): 359–69.

182. Tang, N, and WF Marshall. 2012. “Centrosome positioning in vertebrate development.” *J Cell Sci* 125(Pt 21): 4951–61.

183. Taylor, WR, and GR Stark. 2001. “Regulation of the G2/M transition by p53.” *Oncogene* 20(15): 1803–15.

184. Tokuyasu, KT. 1975. “Dynamics of spermiogenesis in Drosophila melanogaster. VI. Significance of “onion” nebenkern formation.” *J Ultrastruct Res* 53(1): 93–112.

185. Tootle, TL, D Williams, A Hubb, R Frederick, and A Spradling. 2011. "Drosophila eggshell production: identification of new genes and coordination by Pxt." *PLoS One* 6(5): e19943.

186. Tsou, MF, and T Stearns. 2006. "Controlling centrosome number: licenses and blocks." *Curr Opin Cell Biol* 18(1): 74–78.

187. Tuck, C, T Zhang, T Potapova, M Malumbres, and B Novak. 2013. "Robust mitotic entry is ensured by a latching switch." *Biol Open* 2(9): 924–31.

188. Vaccari, T, and A Ephrussi. 2002. "The fusome and microtubules enrich Par-1 in the oocyte, where it effects polarization in conjunction with Par-3, BicD, Egl, and dynein." *Curr Biol* 12(17): 1524–28.

189. van Vugt, MA, and MB Yaffe. 2010. "Cell cycle re-entry mechanisms after DNA damage checkpoints: giving it some gas to shut off the breaks!" *Cell Cycle* 9(11): 2097–101.

190. van Zon, W, J Ogink, B ter Riet, RH Medema, H te Riele, and RM Wolthuis. 2010. "The APC/C recruits cyclin B1-Cdk1-Cks in prometaphase before D box recognition to control mitotic exit." *J Cell Biol* 190(4): 587–602.

191. Villeneuve, J, M Scarpa, M Ortega-Bellido, and V Malhotra. 2013. "MEK1 inactivates Myt1 to regulate Golgi membrane fragmentation and mitotic entry in mammalian cells." *EMBO J* 32(1): 72–85.

192. Vincent, I, G Jicha, M Rosado, and DW Dickson. 1997. "Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain." *J Neurosci* 17(10): 3588–98.

193. Von Stetina, JR, and TL Orr-Weaver. 2011. "Developmental control of oocyte

maturation and egg activation in metazoan models." *Cold Spring Harb Perspect Biol* 3(10): a005553.

194. Von Stetina, JR, S Tranguch, SK Dey, LA Lee, B Cha, and D Drummond-Barbosa. 2008. "alpha-Endosulfine is a conserved protein required for oocyte meiotic maturation in Drosophila." *Development* 135(22): 3697–706.
195. Walter, SA, SN Guadagno, and JE Jr Ferrell. 2000. "Activation of Wee1 by p42 MAPK in vitro and in cycling xenopus egg extracts." *Mol Biol Cell* 11(3): 887–96.
196. Warren, G, and V Malhotra. 1998. "The organisation of the Golgi apparatus." *Curr Opin Cell Biol* 10(4): 493–98.
197. Wehr, K, A Swan, and T Schupbach. 2006. "Deadlock, a novel protein of Drosophila, is required for germline maintenance, fusome morphogenesis and axial patterning in oogenesis and associates with centrosomes in the early embryo." *Dev Biol* 294(2): 406–17.
198. Wei, JH, and J Seemann. 2010. "Unraveling the Golgi ribbon." *Traffic* 11(11): 1391–400.
199. Wells, NJ, N Watanabe, T Tokusumi, W Jiang, MA Verdecia, and T Hunter. 1999. "The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression." *J Cell Sci* 112 (Pt 19): 3361–71.
200. Whitaker, M. 1996. "Control of meiotic arrest." *Rev Reprod* 1(2): 127–35.
201. White-Cooper, H, MA Schafer, LS Alphey, and MT Fuller. 1998. "Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid differentiation with meiosis I in Drosophila." *Development* 125(1): 125–34.
202. Wilson, PG. 1999. "BimC motor protein KLP61F cycles between mitotic spindles and

fusomes in Drosophila germ cells." *Curr Biol* 9(16): 923–26.

203. Wilson, PG. 2005. "Centrosome inheritance in the male germ line of Drosophila requires hu-li tai-shao function." *Cell Biol Int* 29(5): 360–69.
204. Woodruff, JB, O Wueseke, and AA Hyman. 2014. "Pericentriolar material structure and dynamics." *Philos Trans R Soc Lond B Biol Sci* 369(1650):
205. Yano, H, M Yamamoto-Hino, M Abe, R Kuwahara, S Haraguchi, I Kusaka, W Awano, A Kinoshita-Toyoda, H Toyoda, and S Goto. 2005. "Distinct functional units of the Golgi complex in Drosophila cells." *Proc Natl Acad Sci U S A* 102(38): 13467–72.
206. Yasuno, Y, J Kawano, YH Inoue, and MT Yamamoto. 2013. "Distribution and morphological changes of the Golgi apparatus during Drosophila spermatogenesis." *Dev Growth Differ* 55(6): 635–47.
207. Yue, L, and AC Spradling. 1992. "hu-li tai shao, a gene required for ring canal formation during Drosophila oogenesis, encodes a homolog of adducin." *Genes Dev* 6(12B): 2443–54.
208. Zhang, M, and G Xia. 2012. "Hormonal control of mammalian oocyte meiosis at diplotene stage." *Cell Mol Life Sci* 69(8): 1279–88.
209. Zitouni, S, C Nabais, SC Jana, A Guerrero, and M Bettencourt-Dias. 2014. "Polo-like kinases: structural variations lead to multiple functions." *Nat Rev Mol Cell Biol* 15(7): 433–52.

Chapter II

Myt1 regulation of CycA-Cdk1 coordinates novel G2 checkpoints in *Drosophila* spermatocytes

2.1 Introduction

Pre-meiotic G2 arrest mediated by inhibitory phosphorylation of Cdk1 is a conserved mechanism for coordinating oocyte maturation and meiosis in many organisms. In vertebrates, this 'G2-like' meiotic arrest can maintain oocytes in prophase-1 from days to decades (Solc *et al.*, 2010; Von Stetina and Orr-Weaver, 2011; Adhikari and Liu, 2014). Myt1 is a metazoan-specific Cdk1 inhibitory kinase that serves a specialized role during pre-meiotic arrest in many organisms (Mueller *et al.*, 1995; Ruiz *et al.*, 2010; Gaffre *et al.*, 2011). The meiotic function of Myt1 was first identified in *X. laevis* (Kornbluth *et al.*, 1994) and *A. pectinifera* oocytes (Kishimoto, 2011), in which steroid hormones inactivate Myt1 activity, terminate G2 phase arrest and trigger meiotic divisions (Duckworth *et al.*, 2002; Kishimoto, 2003; Holt *et al.*, 2013). In *C. elegans,* the Myt1 homolog Wee1.3 performs a similar role as loss of function causes precocious oocyte maturation (Burrows *et al.*, 2006), triggers early embryonic mitotic divisions, and results in fertilization defects (Allen *et al.*, 2014). Myt1 therefore serves a conserved role during pre-meiotic G2 phase arrest.

Myt1 over-expression delays progression of somatic cells into mitosis (Liu *et al.*, 1999; Wells *et al.*, 1999; Price *et al.*, 2002). A physical interaction and mitotic CyclinB1 is proposed to facilitate Cdk1 inhibition by a kinase independent tethering mechanism that sequesters CyclinB1-Cdk1 in the cytoplasm, prevents nuclear trafficking and delays mitotic initiation (Wells *et al.*, 1999; Liu *et al.*, 1999). siRNA-mediated knockdown of Myt1 in cultured cells disrupts Golgi and endoplasmic reticulum (ER) dynamics, suggesting that Myt1

regulates an organelle checkpoint that coordinates the behavior of these cytoplasmic membranes with mitotic progression (Cornwell *et al.*, 2002; Nakajima *et al.*, 2008; Villeneuve *et al.*, 2013). These pre-mitotic checkpoint mechanisms of Myt1 have not yet been well studied in any developmental context, however.

Loss of *myt1* function in *Drosophila melanogaster* is associated with mitotic proliferation defects in both oogenesis and spermatogenesis (Jin *et al.*, 2005). In spite of similar mitotic defects in both males and females, only the *myt1* mutant males are sterile. One key difference is that *Drosophila* spermatocytes undergo pre-meiotic arrest in G2 phase, whereas oocytes arrest in metaphase (Orr-Weaver, 1995). This suggests that a specialized role for Myt1 in developmentally programmed G2 phase arrest could cause *myt1* mutant male sterility. *Drosophila* spermatogenesis is an excellent developmental model for studying the role of Myt1 during meiosis (Fig. 2-1A). Germline stem cell divisions produce differentiated gonioblasts (GB) that undergo four rounds of transit amplification (TA) divisions with incomplete cytokinesis, resulting in 16-cell cysts. The cells are connected by endoplasmic reticulum (ER)-derived organelles called fusomes that pass through mid-body-derived actin-based ring canal remnants (Hime *et al.*, 1996; de Cuevas *et al.*, 1997; Eikenes *et al.*, 2013). These cells differentiate into spermatocytes that pass rapidly through early stages of the cell cycle before a prolonged G2 phase arrest of approximately 90 hours. During this arrested stage of development the 16 cells in each cyst grow rapidly and increase ~25 fold in volume. Throughout spermatocyte maturation the cells remain connected by fusomes, until MI when the structure disassembles. After two consecutive meiotic divisions (MI/MII), fusomes then re-assemble as spermatid differentiation begins (Hime *et al.*, 1996). Although fusomes are essential for male meiosis and thought to coordinate cell division and facilitate molecular

transport (de Cuevas *et al.*, 1997; McKearin, 1997), the mechanisms regulating fusome re-modeling remain unclear.

In this study I employed a combination of live analysis, genetics and biochemistry to investigate the role of Myt1 during pre-meiotic G2 phase in *Drosophila* primary spermatocytes. I found that Myt1 regulation of Cdk1-Cyclin A was required for the structural integrity of fusomes and maintenance of centriole engagement throughout pre-meiotic G2 phase. These novel organelle checkpoint functions of Myt1 are necessary for proper spatial-temporal coordination of cytoplasmic and nuclear events during meiosis and explain why *myt1* mutant males are sterile.

2.2 Materials and Methods

2.2.1 Generation of Myt1 transgenic lines

D.melanogaster Myt1 wildtype (WT) cDNA was initially subcloned into the pCaspeR transposon vector (Pirrotta, 1988). From this construct, Myt1 gene was amplified using Pfx50™ DNA Polymerase (Catalogue # 12355-012, Invitrogen) using the following primer combination: dMyt Fwd-CACCATGGAAAAGCATCATCG and dMyt Rev-TCACTCGTCGTCATATTC CAGGA. The amplified DNA was purified and sub-cloned into a pENTR vector based on the directional TOPO cloning protocol recommended by the product manual (Catalogue # K2400-20, Invitrogen). Myt1 WT was further cloned in to two destination vectors using the Gateway cloning system. I used an N' terminal fluorescent protein tagged UASp vector (Brand and Perrimon, 1993) for Gal4 inducible transgenic expression and testes-specific Tubulin beta3 (tv3) promoter (Wong *et al.*, 2005) containing vector to express the transgenes in spermatocytes. Similarly I also individually cloned Cdk1

(WT), Cdk1(T14A), Cdk1(Y15F), Cdk1(T14A, Y15F) (Ayeni *et al.*, 2014) constructs into the tv3 GFP tagged vector. Each of these constructs was used to generate transgenic flies using P-element mediated transformations (Best gene). To initially characterize Myt1 (WT) transgenes, I tested for complementation of a visible *myt1* mutant defect (Table 3.1). Loss of Myt1 results in thoracic macrochaetae (bristle/shaft) defects (Jin *et al.*, 2008), so I expressed UASp promoter-linked EGFP-Myt1(WT) in the sensory organ lineage of *myt1* mutants using *neuralized Gal-4* (Yeh *et al.*, 2000). EGFP-Myt1(WT) fully rescued the *myt1* mutant bristle phenotype (100% normal bristles, not shown), confirming that the fusion protein is fully functional *in vivo*.

2.2.2 Fly stocks

To express transgenic proteins in late spermatogonia and mature spermatocytes I used *bam-Gal4* (McKearin and Spradling, 1990; Chen and McKearin, 2003) and *topi-Gal4* (Raychaudhuri *et al.*, 2012) stocks, respectively. I also used stocks carrying the mutant alleles for $myt1^{R6}$ (Campbell Lab), rux^{8} (Thomas *et al.*, 1994), wee^{ES1} (Campbell Lab) and *twe* (Alphey *et al.*, 1992; Courtot *et al.*, 1992). I used the following transgenes to drive ectopic expression; *UASp::VFP Myt1(N229A)* (Campbell Lab), *UASp::EGFP-KDEL* (Snapp *et al.*, 2004), *UASp::Rux* (Gonczy *et al.*, 1994), $UASp::CyclinA^{TRiP}$ (Harvard Medical school) $UASp::CyclinA^{siRNA}$ and $UASp::CyclinB^{siRNA}$(KK library stocks)

2.2.3 BrdU pulse chase assay

1-day-old heterozygous $myt1^{R6}$/+ and $myt1^{R6}$ mutant males were collected and starved for 8 hours and 4 hours respectively. The $myt1^{R6}$ males were sensitive to 8 hours of starvation; therefore we reduced their starvation time to half as compared to their controls. After starvation, the flies were fed with a solution of 10 mM 5-bromo-2-deoxyuridine (BrdU,

Invitrogen) diluted in 10% grape juice for a 15-minute pulse. Fed flies with pink abdomens were transferred to fresh vials containing normal media and incubated subsequently at 25 C. At defined intervals after the BrdU pulse, testes from 10 flies were dissected and squashed in PBS, then fixed for immuno-labeling as described below. To detect BrdU incorporation, the squashed tissues were treated with 2.2 N Hydrochloric acid (HCl) and 2 M Borax before incubating in primary antibodies.

2.2.4 Immunocytochemistry

One to two day old adult males were used for the spermatocyte immuno-labeling experiments. The testes were dissected in 1X phosphate buffer saline (PBS) and then transferred to a coverslip having a drop of the same buffer. The tip of the testes was carefully teased apart and a poly lysine coated glass slide was used to gently squash out the spermatocytes. The slide was then snap frozen with liquid nitrogen to remove the cover slip. The slides were stored in 95% ethanol until they were ready for next step. Frozen tissue squashes were fixed in 4% paraformaldehyde (PFA) at room temperature for 7-10 minutes and then permeabilized in a drop of 0.3% Triton X-100, 0.3% Sodium deoxycholate in PBS buffer for 10 minutes. After a brief wash with a drop of 0.1% Triton X-100 in PBS buffer (PBT), the slides were incubated in blocking buffer consisting of 5% bovine serum albumin (BSA) in PBT (PBTB) for 1 hour. The slides were then incubated in the appropriate dilution of primary antibodies overnight, at 4 C. To visualize the microtubules and centrosomes, the snap frozen tissue squashes were pre-fixed using 100% methanol for 5 minutes, transferred to cold Acetone for 1-2 minutes and then incubated in PBS containing 0.5% Acetic acid and 1% Triton X- 100 for 10 minutes. The tissues were then briefly washed with PBT and blocked in 5% PBTB for one hour. We noticed that the microtubule/ centrosome specific fixation condition quenches the GFP

fluorescent signals of the transgenic fusion proteins. The following primary antibodies were used at these concentrations: mouse anti-BrdU (DSHB, Developmental Studies Hybridoma Bank; 1:20), rabbit anti Lava lamp (Sisson *et al.*, 2000) (received from Sullivan lab; 1:100) to label Golgi structures, guinea pig anti-SA (1:500, from M.T. Fuller), mouse anti-Hts (DSHB; 1:5) and mouse anti-alpha-spectrin (DSHB; 1:500) to label fusomes, rabbit anti-Anillin (Field and Alberts, 1995) (1: 300) for labeling ring canals, mouse gamma tubulin GTU-88 (Sigma; 1:100), rabbit anti- AurA-T228P (abcam; 1:500) to label centrosome, rabbit anti-Sas6 (1:500, gift from Dr. Raff, Habedanck *et al.*, 2005) for centriole labels, mouse anti-Cyclin A (Lehner and O'Farrell, 1989) (DSHB; 1:10). After primary antibody labeling, the slides were washed and incubated with conjugated secondary antibodies (Alexa-488 and Alexa-568, Molecular Probes) at 1:1000 dilution. DNA was labeled using 8.3 μg/ml Hoechst 33342. The slides were mounted and the images were acquired using a Zeiss Axioplan microscope, equipped with a CCD camera. The Z-stack images were deconvolved and processed with Volocity image processing software.

2.2.5 Western blot analysis

Eight to twenty testes (depending on the antibodies being used) were dissected in 1x PBS (supplemented with 2 mM Sodium Orthovanadate and 10 mM Sodium Fluoride) and then frozen at -20 C until ready to use. The tissues were lysed with SDS-PAGE sample buffer and boiled for 7-10 minutes before loading on polyacrylamide gels. The testes extracts were separated on 10% SDS-PAGE gels and the proteins were transferred to Hybond P membrane (Amersham). Protein blots were blocked in 5% BSA+TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) before incubation in primary antibodies overnight at 4 C. Phospho-specific Cdk1 antibodies obtained from Cell Signaling were used at the following

concentrations: rabbit anti-Cdk1-T14p (1:1000) and rabbit anti-Cdk1-Y15p (1:5000). Transgenic expression of EGFP-Myt1 (WT) was analyzed using mouse anti-GFP antibodies (Clontech; 1: 5000). Labeled proteins were detected using anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Amersham) and the enzymatic chemi-luminescence signals were developed using an ECL Plus/prime kit (GE Healthcare).

2.2.6 Male Sterility Assay

I used 15-30 individual males to analyze each genotype. One to two days old single male from the appropriate genotypes was crossed with 3 age-matched *yw* virgin female flies. After 4 days, the parents were tipped over to new vials to generate a second brood. I counted the number of adult progeny from each vial as a measure of male fertility. This strategy of performing the sterility assay was adapted from the earlier studies (Gonczy *et al.*, 1994). A cross between $myt1^{R6}$/+ heterozygous control males with *yw* virgin females was used as positive control.

2.2.7 TEM Analysis

Testis samples for transmission electron microscopy (TEM) were prepared in collaboration with Dr. Lacramioara Fabian and Dr. Julie Brill from University of Toronto). Methods used in testis sample preparation were adapted from (Tokuyasu *et al*., 1972). According to the protocol mentioned in Tokuyasu, 1972, the testes were dissected in chilled glutaraldehyde (2% in 0.1 M phosphate buffer, pH 7.4) and fixed for two hours at 4 C. They were rinsed thrice with cold PBS buffer at 30-minute intervals and post-fixed at 4C for two hours in 2% OsO (in PBS). The samples were dehydrated in graded concentrations of ethanol (5%, 10%, 20% and 50%) and allowed to reach room temperature during the final 100% ethanol treatment. After appropriate steps in propylene oxide, they were left in Epon 812 overnight

under vacuum. Before polymerization at 60 C under vacuum, the testes were often cut into three (A-C) segments. I used the head segment of the testis (A) to analyze spermatocyte fusomes. These segments were micro sectioned to thin (100-200 nm) section using Ultra-microtome for TEM sectioning (Reichert UltraCut E). Thin sections were stained with 2% uranyl acetate and lead citrate and the sectioned were examined in Philips / FEI (Morgagni) Transmission Electron Microscope with Gatan Digital (CCD) camera. Thick sections (0.5 micron) were always examined in light microscopes to relate the testis organization. 1% methylene blue dye was used to label the thick sections.

2.3 Results

2.3.1 Timing of the G2/MI transition is unaffected in *myt1* mutant spermatocytes

In most animal models, Myt1 is required to maintain immature oocytes in pre-meiotic G2 phase-arrest by inhibitory phosphorylation of Cdk1 (Palmer *et al.*, 1998; Nebreda and Ferby, 2000; Okumura *et al.*, 2002). Depletion of Myt1 in such oocytes triggers precocious meiotic events such as nuclear envelope break down (GVBD), chromosome condensation and meiotic spindle formation (Burrows *et al.*, 2006). In *Drosophila*, loss of Myt1 activity results in mitotic proliferation defects and male sterility (Jin *et al.*, 2005). To investigate if loss of Myt1 activity caused premature G2/MI progression, I examined the temporal coordination of meiotic events by comparing heterozygous control and *myt1* mutant spermatocytes for nuclear envelope breakdown and chromosome condensation with antibodies against lamin DmO and phospho(S10)-histone H3 (PH3), respectively (Fig.2-1B). The *myt1/+* control spermatocytes retained an intact nuclear lamina until late prophase I and disassembled at prometaphase I, as staged by appearance of PH3 labeling as condensed chromosomes coalesced together. In *myt1*

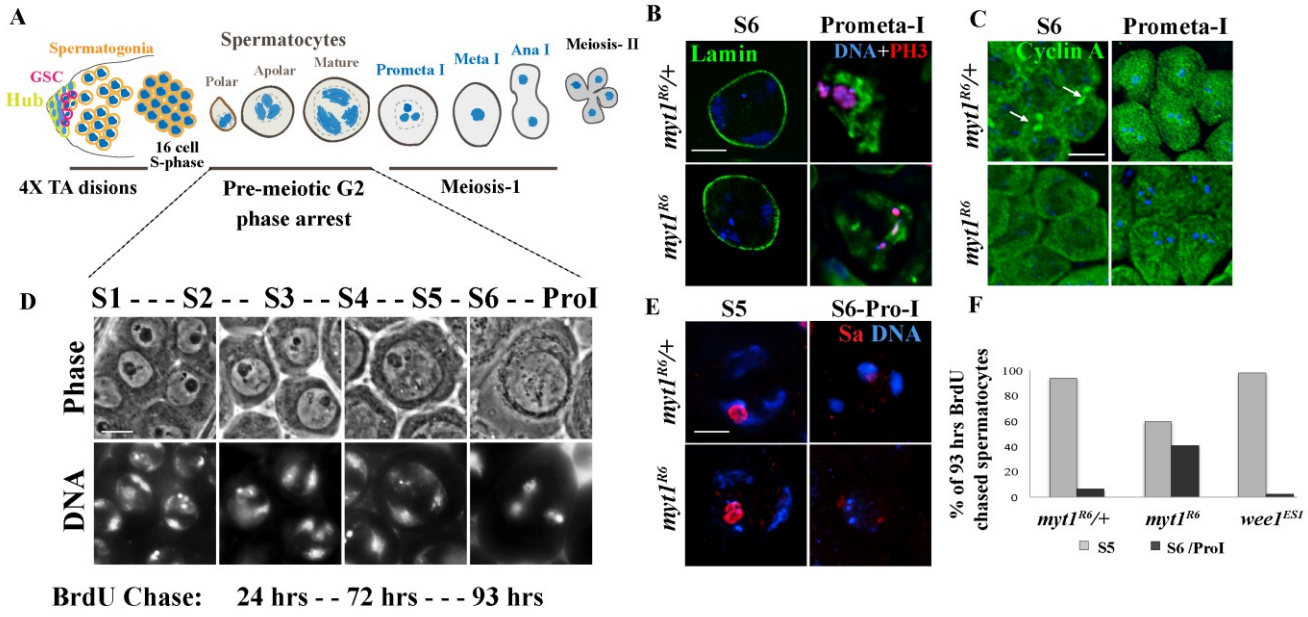

Fig. 2-1: Co-ordination of G2/MI transition is unperturbed in *myt1* spermatocytes

(A) Schematics of *Drosophila* spermatogenesis process. A single spermatocyte, rather than the 16 cysts has been shown at each stage of pre-meiotic G2 phase arrest due to space occupancy. The chromosome morphology is represented in blue color. (B) The spermatocytes are co-labeled with lamin (green) and PH3 (red) antibodies. The blue indicates the Hoechst DNA label, scale bar-10 microns. Both *myt1*/+ and *myt1* mutant mature spermatocytes show an intact nuclear lamina and the structure appear broken down during prometaphase-I, the stage in which the PH3 labels are detected. (C) The mature spermatocytes are labeled with Cyclin A (green) antibodies and DNA dye (blue), scale bar-16 microns. In both *myt1*/+ and *myt1* mutant S6 spermatocyte, the Cyclin A is detected primarily in cytoplasm and the signals appear nuclear in prometaphase-I stage. Arrows in *myt1*/+ mature spermatocytes indicate the Cyclin A enrichment at the spectrosome structures, which is absent in *myt1* mutants. (D) The phase and DNA labels indicate the cellular and chromosome morphology of the live S1 to S6 stage spermatocytes. The timing of spermatocyte development is indicated based on BrdU pulse chase detections at 24, 72 and 93 hours. Scale bar-10 microns (E) The G2/MI spermatocytes are indicated with nucleolar labels using Sa (red) antibodies and the DNA is in blue. Scale bar-10 microns (F) Comparison of the development stage of *myt1* and *wee* mutant spermatocytes with *myt1*/+ control detected at 93 hour BrdU pulse chase time point.

mutant spermatocytes, PH3 labeling and nuclear envelope breakdown appeared similar to the controls (Fig.2-1B), however appearance of the condensed chromosomes looked abnormal. I also used Cyclin A translocation into the nucleus as a marker of the G2/MI transition. In *myt1/+* controls, Cyclin A was primarily cytoplasmic but enriched at the spectrosome (arrow) in S6 stage spermatocytes (Fig.2-1C), however at S6-Prophase I the Cyclin A translocated into the nucleus. In the *myt1* mutant S6 spermatocytes, I cytoplasmic Cyclin A appeared normal, however, spectrosome enrichment was not observed. Cyclin A translocation and chromosome condensation were observed normal in *myt1* mutants however.

I undertook an *in vivo* BrdU labeling experiment to determine whether *myt1* mutant spermatocytes remained arrested in G2 phase for the normal 90 hours. Starved 1-2 day old male flies were fed BrdU for 15 minutes (see Materials and Methods), so that post-mitotic 16 cell spermatocyte cysts that incorporated BrdU during pre-meiotic S phase could be detected with antibodies. Heterozygous control and *myt1* spermatocytes were fixed over the normal 93 hour period of G2 phase arrest, and staged with established cytological criteria based on chromosome and cellular morphology (Cenci *et al.*, 1994). The *myt1/+* controls examined at 24, 72 and 93 hours post-BrdU pulse corresponded to polar (S1-2), apolar (S3-4) and mature (S5-6) stage G2 phase spermatocytes, respectively (Fig. 2-1D). The G2/MI transition was indicated by fragmentation of the nucleolus and chromosome condensation (S6-ProI, Fig. 2-1D). I also examined nucleolar breakdown using Sa (Spermatocyte Arrest) immuno-labeling and stained chromosomes with Hoechst (Fig. 2-1E). In *myt1/+* controls, S5 stage spermatocytes exhibited intact nucleoli with three major chromosome compartments in the nucleus. In S6-ProI stage controls, the nucleoli were fragmented and chromosome compartments appeared condensed, indicating the G2/MI transition. These characteristics of

the G2/MI transition appeared normal in the immuno-labeled *myt1* mutant spermatocytes (Fig. 2-1D, E).

Based on conditions established in the BrdU chase experiment, I detected the G2/MI transition at 93 hours after the BrdU pulse in both the *myt1/+* controls and *myt1* mutants. In three independent experiments I observed that 98% of the *myt1/+* controls had intact nucleolar structures (~98%, arrowheads in Fig. 2-2 B), with DNA morphology characteristic of S5 spermatocytes at 93 hour as shown in Fig. 1D-F and Fig. S1A-C. At the same time point, 60% of BrdU-labeled *myt1* mutant spermatocytes appeared indistinguishable from *myt1/+* controls with intact nucleolar morphology (Fig. 2-1F; arrowheads, Fig. 2-2E) characteristic of the S5 stage (Fig. 2-2D-F), however 40% had fragmented nucleoli characteristic of S6-ProI stage spermatocytes (asterisk, Fig. 2-2E). These results therefore revealed a subtle defect in the timing of nucleolus fragmentation in *myt1* mutant spermatocytes. Although the timing of *myt1* mutant spermatocyte development appeared relatively normal, the BrdU-labeled chromosomes appeared fragmented, suggesting that they were more sensitive to the HCl treatment required for BrdU detection (arrows, Fig. 2-2F). Chromosome abnormalities were previously noted in *myt1* mutant imaginal cells and were correlated with defects in the DNA damage cell cycle checkpoint (Jin *et al.*, 2008).

At 100 hour in the *myt1/+* controls, labeled cysts with more than 16 cells were first observed, demonstrating that MI cell division occurred between 93 and 100 hours after the BrdU pulse (Fig. 2-2J). The 111-hour time point revealed ~64 cell BrdU-labeled *myt1/+* cysts, indicating completion of MII (Fig. 2-2K). In *myt1* mutants, the 100 hour and 111 hour post-BrdU pulse samples also had 32 and 64 cell labeled cysts (Fig. 2-2J, K). This demonstrates that the temporal coordination of both meiotic divisions is normal in *myt1*

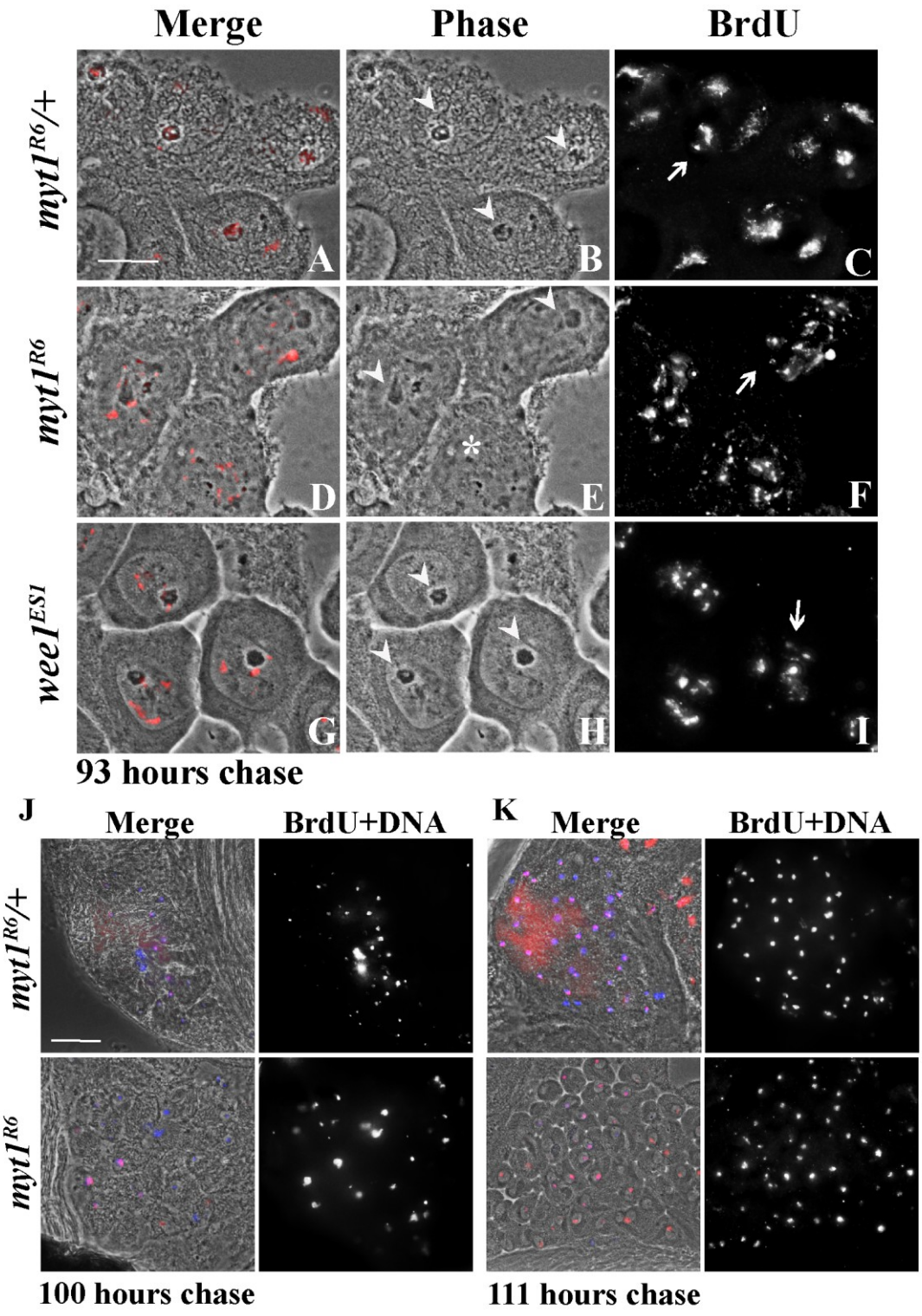

Fig. 2-2: Timing of spermatocytes arrest is unperturbed in *myt1* and *wee*^{ES1}

(A-I) 93 hour BrdU chase corresponds to S5-S6 stage of spermatocytes. S5 spermatocytes exhibit characteristic intact nucleoli (arrowhead-B,E,H) and the S6-ProI cells show fragmented nucleoli (asterisks). The chromosome organization is shown with BrdU immuno-labeled signals (arrows-C,F,I). (J) In *myt1*/+control testes, BrdU signals detected at 100 hour chase time point shows >16 or ~32 condensed chromosomes per cyst. The *myt1* mutants show the similar number of chromosomes per cyst. DNA is labeled in blue. (K) In 111 hour chase time point, the BrdU signal is detected in cyst containing ~ 64 (more than 32) condensed chromosomes. The count of 32 and 64 cell per cyst is not accurate due to semi-mount testes sample preparation to visualize the entire cells in the cyst (n=2). Scale bar -10 microns.

mutants. I also examined *wee1* mutants that lack the partially redundant Cdk1 inhibitory kinase, but found no apparent differences between the *wee1* mutants and controls (Fig. 2-2G-I). These results showed that *myt1* mutant spermatocytes undergo normal (~90 hour) pre-meiotic G2 phase arrest.

2.3.2 Loss of Myt1 causes pre-meiotic centriole disengagement and multipolar spindles

Male sterility in *myt1* mutants has been correlated with aneuploidy (Jin *et al.*, 2005), however, the basis for this phenotype is not clear. Therefore, I examined whether defects in meiotic spindle organization could account for this phenotype. For this experiment I labeled the meiotic spindles and centrosomes with antibodies against β-tubulin and centrosomin (Cnn), as shown in Fig. 2-3A. In *myt1/+* controls, prometaphase-I spermatocytes exhibit bipolar spindles and a pair of centrosomes at each pole. In the *myt1* mutant spermatocytes, however, I observed multipolar spindles, typically with four distinct Cnn foci. These *myt1* mutant spindle defects were completely rescued by a GFP tagged-Myt1 transgene expressed with a spermatocyte-specific tubulin promoter, *tv3*. From these results I concluded that loss of Myt1 activity resulted in abnormal segregation of chromosomes and aneuploidy because of defects in meiotic spindle organization.

To further examine how loss of Myt1 affected pre-meiotic centrosome behavior I labeled pre-meiotic centrosomes using antibodies against a mitotic form of phosphorylated Aurora A (AurA- T288p; Cell Signaling) and Hoechst to label DNA (Fig. 2-3B). In *myt1/+* controls, 100% of apolar stage S3-4 spermatocytes had a pair of closely-associated centrosomes (n= 75). In *myt1* mutants, 70.2 % of stage S3-4 spermatocytes had more than two AurA-T288p labeled foci (n= 191, Fig. 2-3C). In mature S5-6 stage *myt1/+* spermatocytes,

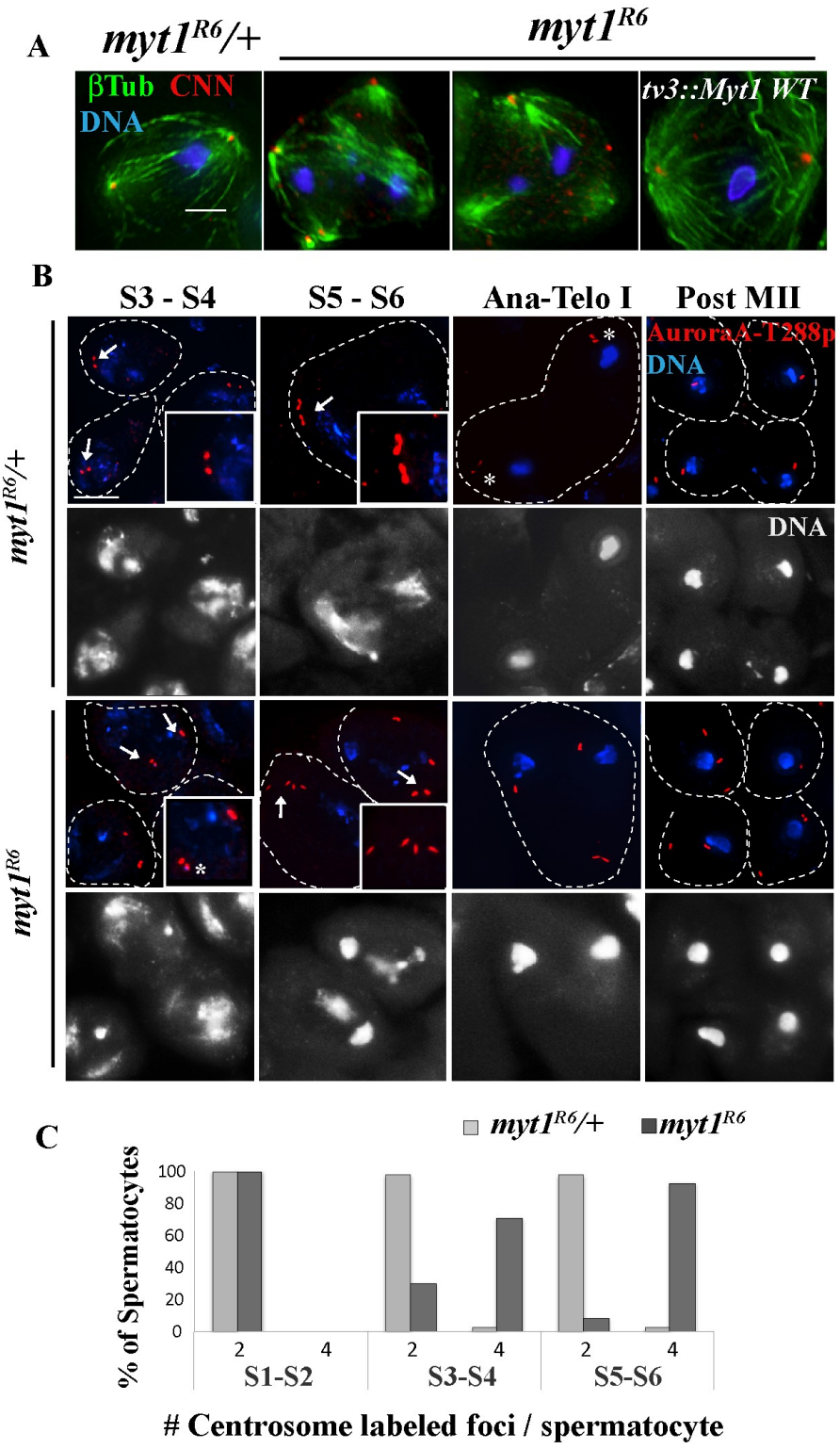

Fig. 2-3: *myt1* mutant spermatocytes exhibit abnormal meiotic spindles and centrioles

(A) The meiotic spindle is shown with β–Tubulin (green) and centrosomin (Cnn, red) co-labeling in prometaphase-I spermatocytes, scale bar- 8 microns. The Cnn antibodies represent peri-centriolar matrix and they also appear to show non-specific background signals. DNA is labeled in blue. (B) The pre-meiotic G2 phase centrosomes in S3 to S6 spermatocyte are labeled with AurA-T288p isoform (Red) antibodies and DNA is labeled in blue. S3-S6 stage spermatocytes exhibit two foci per cell (inset) representing the duplicated centrosomes. In *myt1* mutants, S3-S6 stage centrosomes are detected as four foci structures (arrows indicating the inset), instead of two centrosomes. In mutant S3-S4 stage of spermatocyte, the pair of closely associated foci for each centrosome appearing like a centrosome splitting (indicated by asterisks) resembles the centriole dis-engagement (indicated by asterisks), which is normally detected during ana-telo phase transition of MI. The *myt1*/+ control, the post meiotic cells receive one-centriole per cell, where as the mutants show more than one (2 or 3) centrioles per cell, Scale bar-10 microns. (C) Graph indicates the percentage of spermatocytes exhibiting either 2 or 4 centriole labels in early (S1-S2), mid (S3-S4) and late (S5-S6) stage of spermatocytes. S1-S2 spermatocytes in both *myt1*/+ controls and *myt1* mutants exhibit 2 centrioles per cell. In *myt1* mutant S3-S4 stage, 70.2 % (n= 191) of the cells contained the spitting centrosomes (asterisks). In S5-S6 stage, 92.2 % (n= 192) of the spermatocytes exhibited 4 elongated centrioles per cell.

most of the mother and daughter centrioles of each centrosome were oriented orthogonally and elongated (up to 2 microns), exhibiting a characteristic V shaped structure (n=223). In *myt1* mutants I usually observed four (but never more) widely separated foci, each corresponding in size to an elongated control centriole (~ 2 microns, n=192). Centriole disengagement was not normally observed until late anaphase of meiosis I in *myt1*/+ controls (Fig. 2-3B, marked by asterisks) so that by completion of MII each spermatid received one centriole. In *myt1* mutant spermatocytes, I observed that centriole disengagement occurred as early as mid G2 phase (S3-4), resulting in multipolar spindles and abnormal segregation of disengaged centrioles in anaphase-I spermatocytes. As a consequence, the *myt1* secondary meiocytes received abnormal number of centrioles (Fig. A-4, Appendix A)

In order to determine the mechanism of the *myt1* mutant centriole engagement, cells were co-labeled with Sas6 and γTubulin antibodies. Sas6 is a proximal centriole protein and *sas6* mutants exhibit premature centriole disengagement in primary spermatocytes (Stevens *et al.*, 2010). In S3-4 stage spermatocytes, Sas6 was detected in both *myt1*/+ controls and *myt1* mutants. In S6-Prophase-I spermatocyte *myt*/+ controls, Sas6 localized proximally on each centriole of the V shaped centrosomes however centriole-localized Sas6 was not detected in *myt1* mutant S5-6 spermatocytes (Fig. 2-4), suggesting that a failure to recruit or stabilize Sas6 association may underlie centriole disengagement in *myt1* mutants. These results show that Myt1 is essential for normal centrosome behavior during spermatocyte maturation, and provide an explanation for the chromosome and centriole segregation defects that underlie the male sterility of *myt1* mutants (Jin *et al.*, 2005).

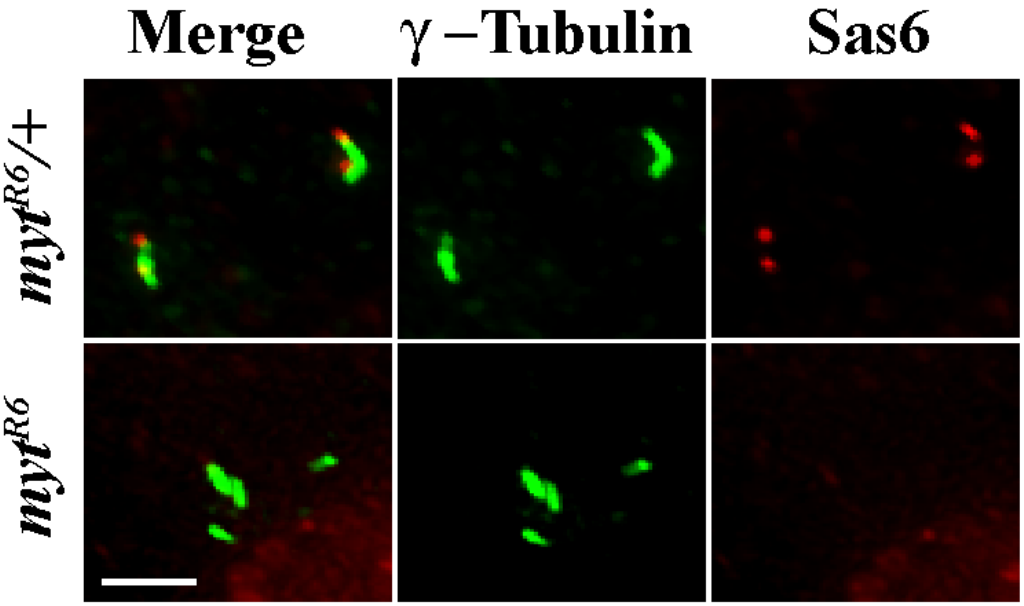

Fig. 2-4: myt1 mature meiotic centrioles lack Sas6 association.

The late S5-6 stage spermatocytes are co-labeled using γ-Tubulin (GTU-88, green) and Sas-6 (red) antibodies. Scale bar-8 microns.

2.3.3 Loss of Myt1 activity disrupts spermatocyte fusomes

Studies in *Drosophila* S2 cells and mammalian HeLa cells have identified a role for Myt1 in Golgi and endoplasmic reticulum (ER) disassembly and re-assembly as cells enter and exit mitosis (Cornwell *et al.*, 2002; Nakajima *et al.*, 2008; Villeneuve *et al.*, 2013). At most stages of *Drosophila* development, however, the Golgi is comprised of simple stacks quite unlike the ribbon structures observed in cultured cells (Kondylis and Rabouille, 2009). In *myt1/+* controls, stage S5-6 spermatocyte labeled with antibodies against a peripheral Golgi protein called Lava lamp (Sisson *et al.*, 2000) showed ring-shaped structures in the cytoplasm (Fig. 2-5A, inset). The mean number of labeled Golgi structures was 16 per S6 spermatocyte (n=74, S.D 2.8). Stage S6 *myt1* mutant spermatocytes had similar numbers of Lva-labeled structures (n= 85, mean=15, S.D 3.2) with no evidence of premature meiotic Golgi fragmentation (Fig.2-5B). Golgi fragmentation normally occurs during late prometaphase-I (Fig.2-5C), and the structure re-assembles at exit from MI and MII to organize the acroblast in spermatids (Belloni *et al.*, 2012; Yasuno *et al.*, 2013). I observed similar Golgi dynamics in *myt1* mutant spermatocytes during stages S6, MI and MII (Fig. 2-5C). These results indicate that Myt1 is not required for meiotic regulation of simple Golgi structures that are characteristic of most stages of *Drosophila* development.

In both male and female *Drosophila* meiosis, germline-specific ER-derived structures called spectrosomes and fusomes play essential roles (Lin *et al.*, 1994; Lin and Spradling, 1995; de Cuevas *et al.*, 1997; McKearin, 1997; Wilson, 2005). I used antibodies against the Adducin-related membrane protein Hts (Yue *et al.*, 1992; Zaccai and Lipshitz, 1996) to label spectrosomes and branched fusomes in whole mount testes throughout spermatogenesis (Fig. 2-6). In this experiment, testes were co-labeled for a nucleolar protein called Spermatocyte

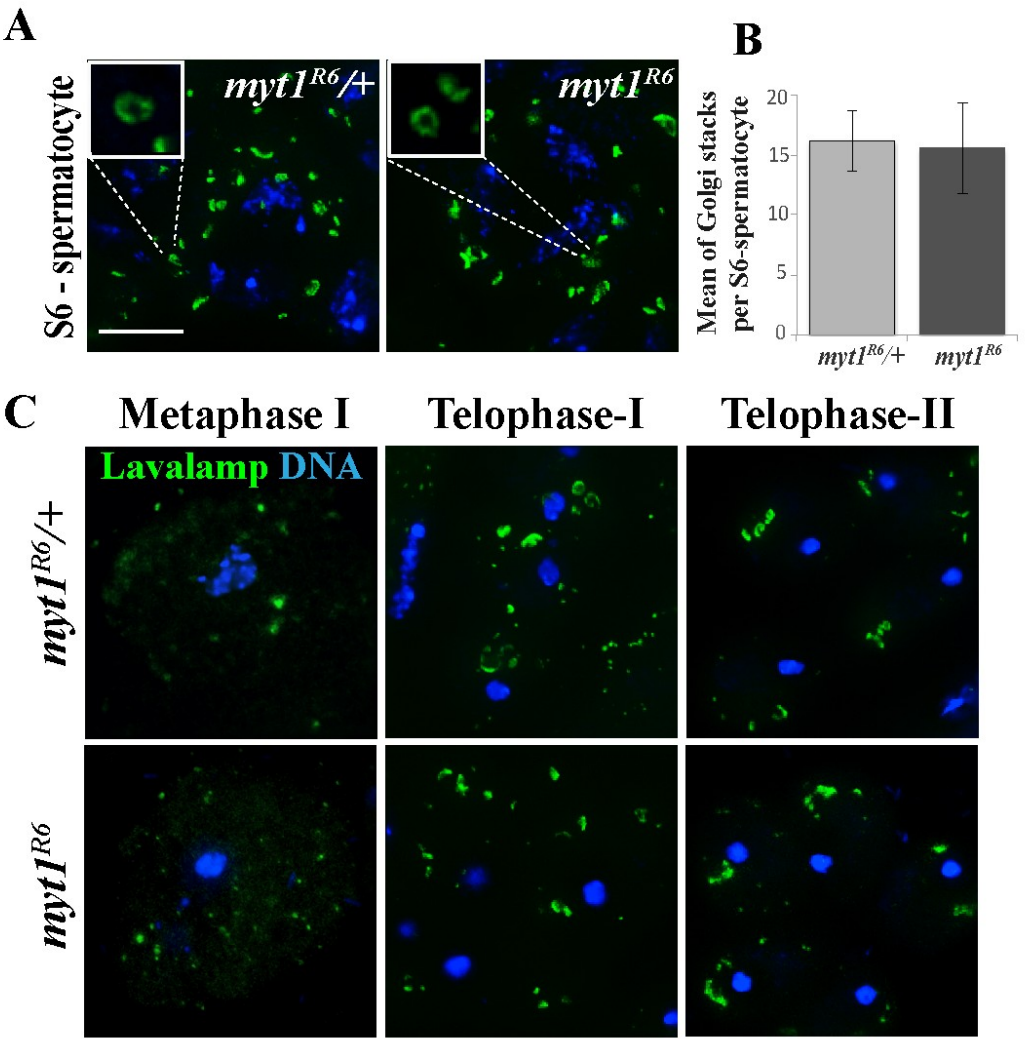

Fig. 2-5: Golgi dynamics appear normal in *myt1* mutant meiocytes

(A, C) Golgi is labeled with Lava lamp antibodies (green). In S5-S6 stage of spermatocytes, the individual "ring shaped" Golgi stacks are shown in the insets. (B) Graph represents the number of Golgi structures counted per spermatocyte at the S6 stage. The quantification is done in merged images. The error bar indicates the standard deviation of the mean (C) Golgi disassembly and the reassembly after each meiotic exit are shown in metaphaseI and telo phases, respectively. Scale bar-10 microns.

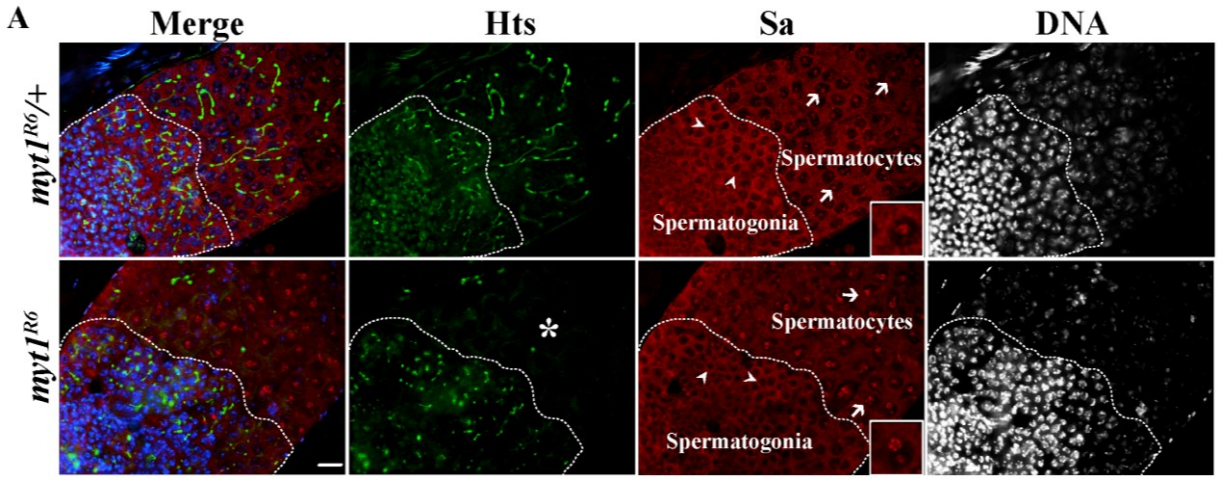

Fig. 2-6: Fusomes are disrupted in *myt1* mutant spermatocytes but not in spermatogonia.

(A) The whole-mount testes are co-labeled with Hts (green) and Sa (red) antibodies. Hts and Sa labels indicate the fusomes and the characteristic nucleoli structures (inset) of G2 phase arrested spermatocytes, respectively. In control and *myt1* mutants, the spermatogonia cysts located close to the testes tip show normal fusomes. The *myt1* mutant spermatocytes exhibit no fusomes (asterisks). Scale bar – 16 microns.

arrest (Sa, from M.T. Fuller) to distinguish spermatogonial cysts from G2 arrested spermatocytes (Fig. 2-6). In *myt1/+* controls, spermatogonia and spermatocyte cysts had branched fusome structures that interconnected the cells. These structures appeared normal in the *myt1* mutant spermatogonia, however Hts-labeling was almost undetectable in spermatocytes (Fig. 2-6). Disappearance of Hts-labeled fusomes was detected as early as spermatocyte stages S1-2 (Fig. 2-7). Although no Hts signals were detected in *myt1* spermatocyte fusomes, the endogenous levels of total Hts protein remained as normal as *myt1/+* control (Fig. A-5) indicating abnormalities in fusome composition. I also examined phenotypes using anti α– Spectrin (membrane-associated protein) antibodies, which also revealed a similar fusome defect in *myt1* mutant spermatocytes.

The fusomes pass through actin-based ring canals that result from incomplete cytokinesis during germline mitotic divisions (Hime *et al.*, 1996). I used Anillin and Hts antibodies to co-label ring canals and fusomes (Oegema *et al.*, 2000), in spermatogonia (Fig. 2-8A, inset) and spermatocytes (Fig. 2-8B,C, arrows). In *myt1* mutant spermatogonia, ring canals appeared normal (Fig.2-8A, inset), however fewer ring canals were detected in *myt1* mutant spermatocytes (Fig. 2-8B,C, arrowhead). Collectively these results reveal two possibilities in which Myt1 could affect the fusomes: loss of Myt1 may interfere with the localization of certain fusome components including Hts, spectrin or Cyclin A to these structures. Alternatively, it may disrupt the fusome *per se* causing loss of these structures. To analyze these possibilities, I examined fusomes of *myt1* mutant spermatocytes using transmission electron microscopic analysis. Testes samples were prepared (Materials and Methods) in collaboration with Dr. Brill and Dr. Fabian (Univ of Toronto). The samples were micro sectioned (1 to 0.5 micron) and examined in collaboration with Arlene Oatway from

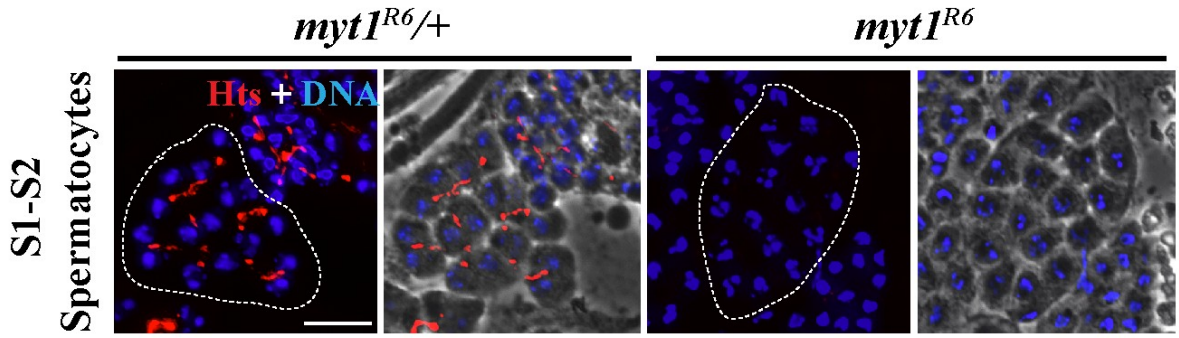

Fig. 2-7: *myt1* mutant fusome defects are detected as early as S1-S2 stage of spermatocyte.

(A) Early spermatocytes are identified based on the relative size of the 16-cells and their brightly stained DNA labels (blue). Hts labeled fusomes (red) in *myt1/+* early stage spermatocytes are encircled, whereas in *myt1* mutants the structures are undetectable, scale bar -10 microns.

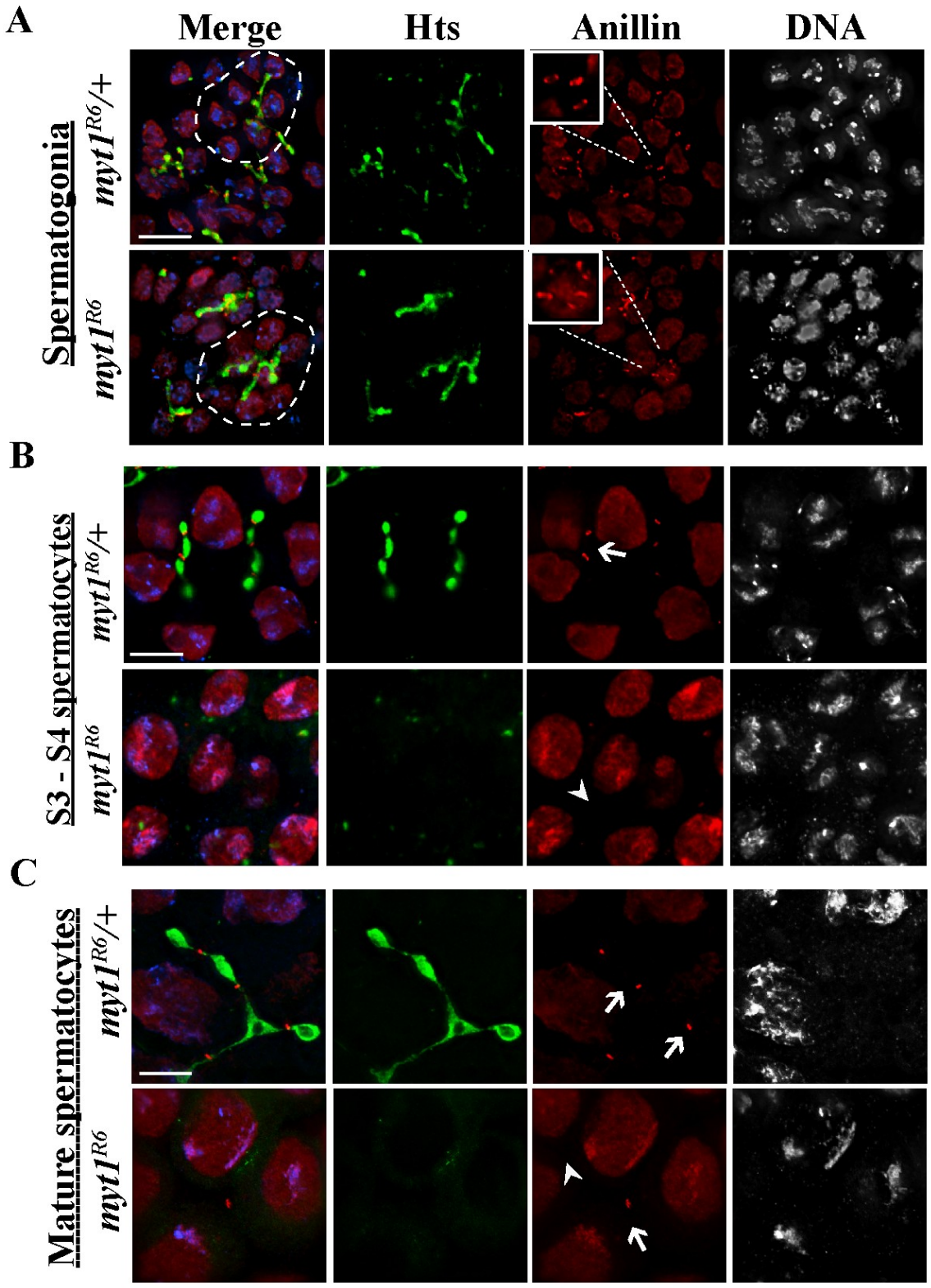

Fig. 2-8: *myt1* mutant spermatocytes exhibit only fewer ring canals.

The germ line specific inter-cellular bridges are labeled with fusome (Hts, Green) and ring canal (Anillin, red) specific markers. (A) The 8 cell spermatogonia cysts (circled) in control and *myt1* mutants show normal fusomes and ring canals. The insets indicate the group of ring canals magnified from the same cyst. (B,C) The 16 cell spermatocytes of the *myt1/+* control show the inter-cellular bridges connected through the ring canals (indicated by the arrows). In *myt1* spermatocytes, the Hts labels are undetectable with a lack few ring canals (indicated by the arrowheads), scale bar-10 microns.

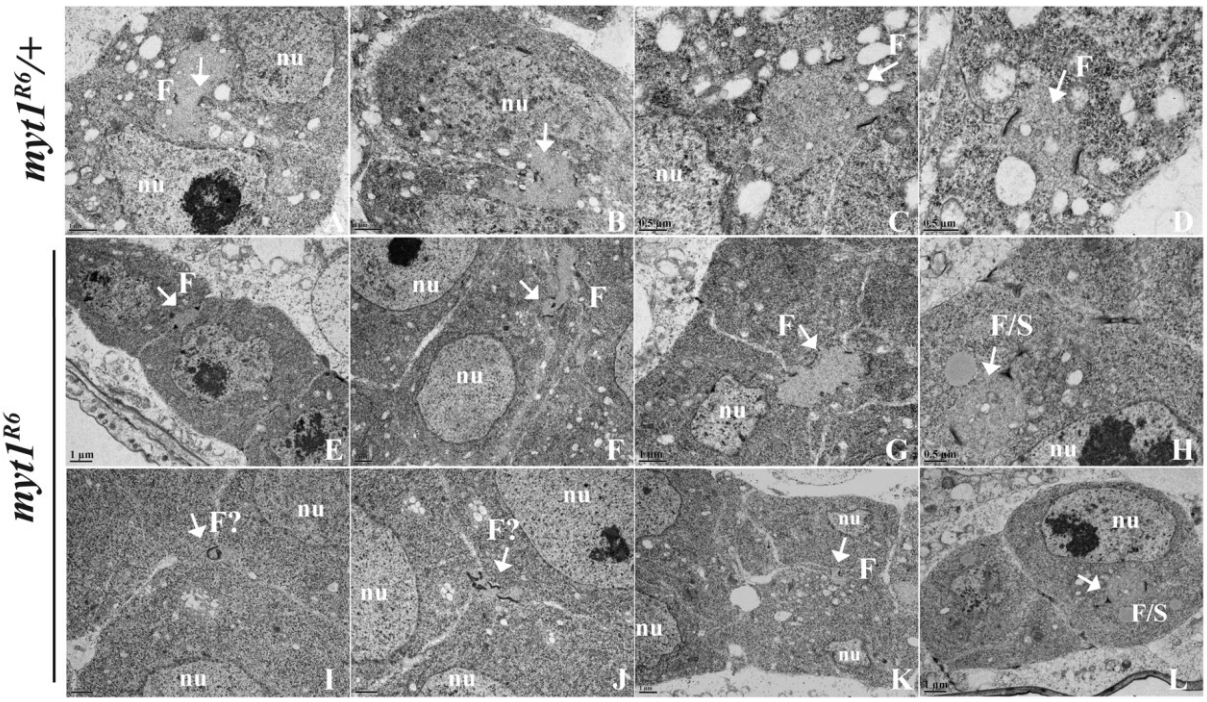

Fig. 2-9: TEM analysis of fusomes in control and *myt1* mutant spermatocytes

Nucleus (n) and fusome (F)/spectrosome (S)(arrows) are indicated. Distinctive electron rich dark spots in the nucleus indicate the characteristic nucleolus structures of spermatocytes (A-D) *myt1/+* control spermatocytes. Intercellular bridge or fusomes (arrow) are identified based on the smooth cytoplasmic junction between two spermatocytes. Scale bar in A, B- 1 microns, C,D-0.5 microns. (E-L) *myt1* mutant control spermatocyte. E-G *myt1* mutant fusomes look normal and they appear similar to the controls shown in A, B. (H) Cross-section of spermatocyte shows spectrosome like structures located with in the cell, scale-0.5 micron, and its corresponding zoom out image (L, scale bar 1 microns) is shown in the bottom row. (I-K) Spermatocytes from *myt1* mutants are shown inter-connected through fusomes, however the structures appear thin and narrower. Scale bar-1 microns.

Biological sciences microscopy facility (Univ of Alberta). The spermatocytes were identified based on their characteristic nucleolus cluster that was appeared as a dark electron-enriched granular structure within the nucleus (Fig. 2-9). In *myt1/+* control spermatocytes fusomes (F) were identified as a smooth cytoplasmic structures inter-connecting spermatocytes (Fig. 2-9A-D). Two dark parallel lines (arrows) of inter-cellular bridges appear to indicate the margin/edge of the fusomes. In *myt1* mutant spermatocytes, the remnants of fusome/spectrosomes and ring canals were found (Fig. 2-9E-L), however, they appeared mostly distorted and narrower compared to the controls. These results indicated that loss of Myt1 disrupted fusome integrity and stability; therefore fail to retain Hts/spectrin containing branches.

2.3.4 Transgenic GFP-Myt1 localizes to ER and Golgi-like structures and rescues *myt1* mutant defects

Myt1 associates with ER and Golgi membranes in other systems, so I examined whether *Drosophila* Myt1 kinase localized similarly in male germ cells (Liu *et al.*, 1997). Antibodies against *Drosophila* Myt1 are not available, so I made tagged transgenic strains of Gal4-inducible *EGFP-Myt1* and stage 3-4 spermatocyte-specific *tv3-GFP-Myt1*). Live-cell analysis using *bam-Gal4* induced EGFP-Myt1 showed localization to membranous cytoplasmic structures that appeared to inter-connect early-polarized stage S1-2 spermatocytes (Fig. 2-10A, insets and arrowheads). In fixed cells, these EGFP-Myt1 labeled connections partially co-localized with Hts-labeled fusomes (Fig. 2-11A), suggesting that Myt1-labeled ER membranes might be closely associated with fusomes during early spermatocyte development. Live analysis of *tv3-GFP-Myt1* expressed in apolar (S3-4) and mature (S5-6)

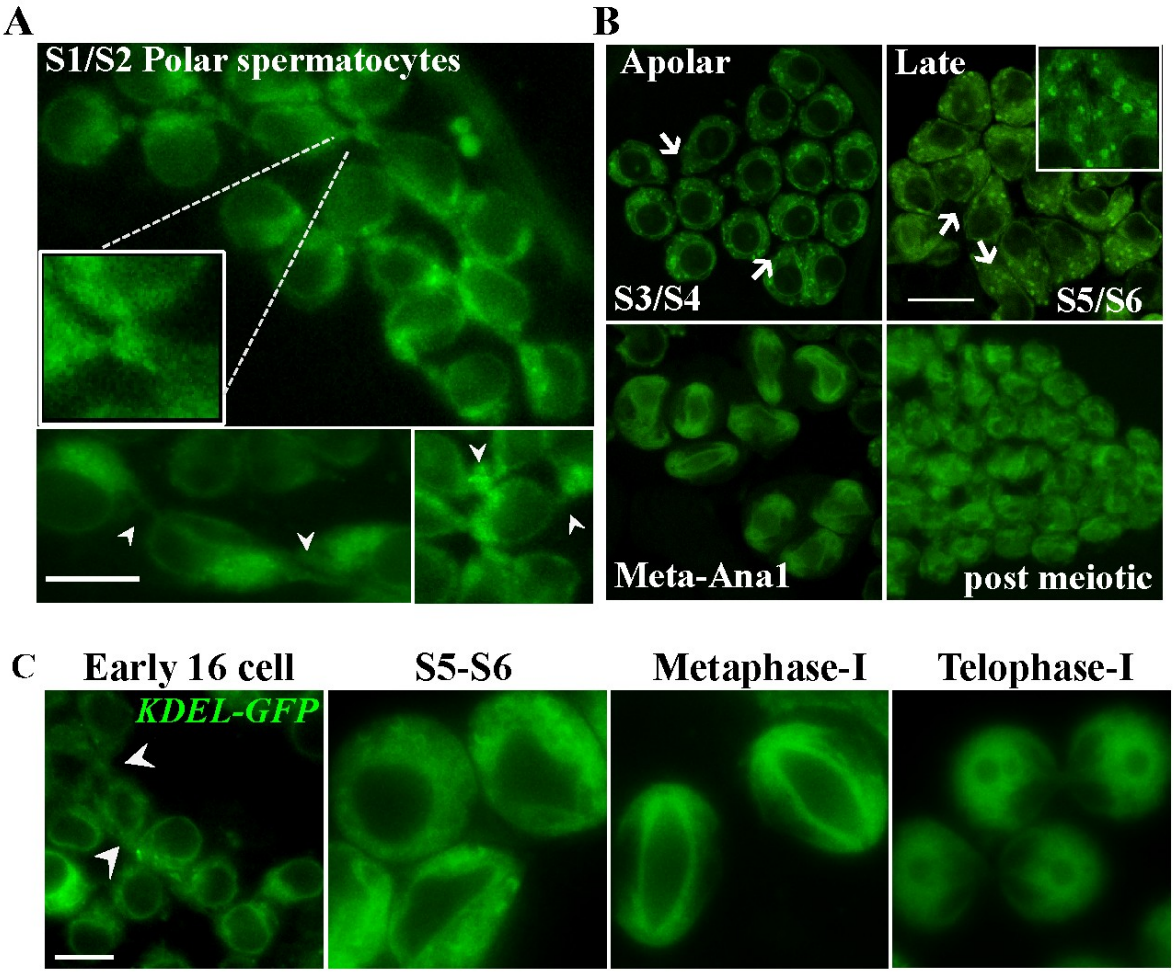

Fig. 2-10: Live analysis of GFP-Myt1 expression and its localization in spermatocyte.

(A) In polar (early) 16 cell spermatocytes, EGFP Myt1 is primarily detected in the cytoplasm and appears enriched at the ER derived inter-cellular junctions (indicated by the arrowhead and inset), scale bar - 10 microns. (B) In apolar (S3/S4) to mature (S5/S6) spermatocytes, GFP Myt1 is detected mostly in the Golgi (inset) and the cytoplasmic ER structures compared to the inter-cellular bridges (indicated by the arrows). During the metaphase I-anaphase I transition, the membranous GFP-Myt1 exhibits meiotic ER sheath in association with the spindle matrix. The post meiotic cells show the stable GFP-Myt1 protein, scale bar - 16 microns. (C) Transgenic expression of EGFP-KDEL labels ER dynamics in pre-meiotic spermatocytes, scale bar-10 microns.

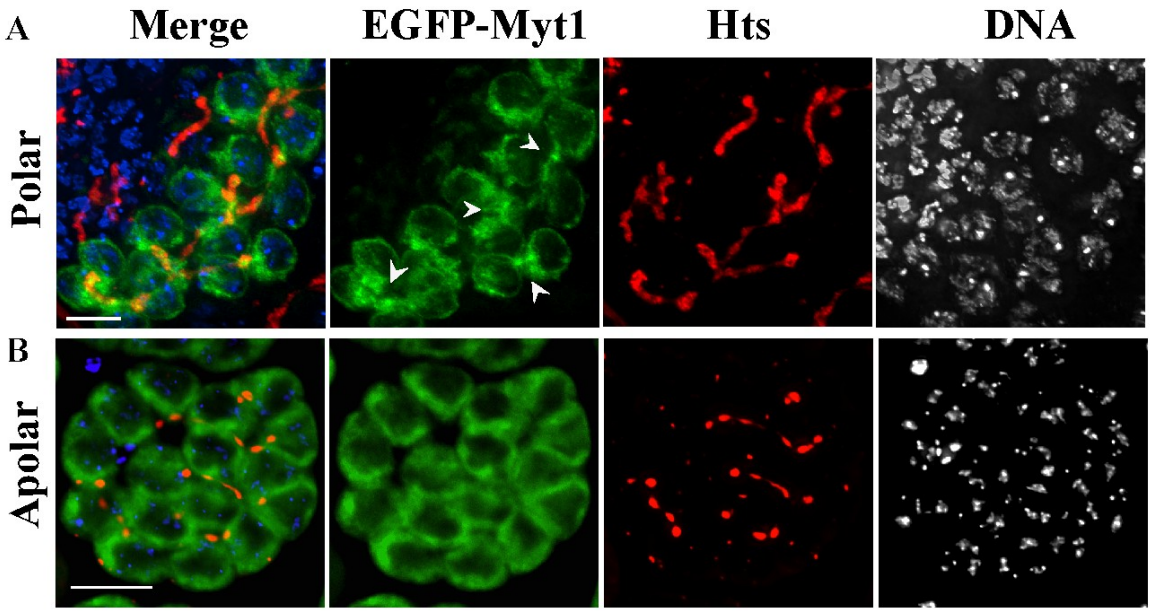

Fig. 2-11: ER-fusome remodeling during polar to apolar spermatocyte transition.

(A) Fixed polar spermatocytes expressing EGFP-Myt1 (Green) are co-labeled with Hts fusome marker (red). EGFP-Myt1 signals are enriched at the inter-cellular junctions (arrowheads) and they appear to partially co-localize with Hts labeled fusomes, scale bar-10 microns (B) Apolar spermatocytes expressing GFP-Myt1 show a reduced or absent GFP-Myt1 overlap with the Hts labeled fusomes, scale bar - 16 microns.

spermatocytes also revealed localization at perinuclear structures that appeared contiguous with these inter-cellular junctions (Fig. 2-10B, arrows). *tv3-GFP-Myt1* co-labeled with Hts to mark the fusome also showed similar localization in fixed cells (Fig. 2-11B). By late-stage S5-6 spermatocytes, labeled structures similar in size, number and morphology to Lva-labeled Golgi were observed (Fig. 2-10B, inset). An established ER marker, called EGFP-tagged KDEL, showed similar localization in early and late spermatocytes (Fig. 2-10C) that were imaged live. Tagged Myt1 is therefore a useful marker for analyzing ER and Golgi membrane dynamics during early spermatocyte maturation.

In mammalian cells, ER membranes form a sliding sheath around spindle microtubules during mitosis (McCullough and Lucocq, 2005). GFP-Myt1 (Fig. 2-10A) and EGFP-KDEL (Fig. 2-10C) also associate with meiotic spindles, implying a conserved structural relationship during *Drosophila* male meiosis. Cytoplasmic GFP-Myt1 was also observed in 64-cell cysts (Fig. 2-10B), presumably associated with remnant ER structures left after meiosis. Collectively, these results show that tagged Myt1 associates with ER-derived structures throughout meiosis. The co-localization of Myt1 and Hts in early polar 16-cell spermatocytes corresponds temporally to the stage when the fusomes first become susceptible to loss of Myt1 activity (Fig. 2-6).

To test the biochemical activity of the tagged Myt1 proteins, I expressed transgenes in *myt1* mutant germ cells using *Gal4* inducible and *tv3*-promoter driven expression to compare testes protein extracts on western blots (Fig.2-12A). Since Myt1 is a dual-specificity kinase, protein blots were probed with phospho-specific antibodies that recognize Cdk1-T14p and Cdk1-Y15p inhibitory isoforms. In *myt1/+* controls, both Cdk1 phospho-isoforms were observed (lane 1), whereas Cdk1-T14p was absent in *myt1* mutant (lane 2). This was

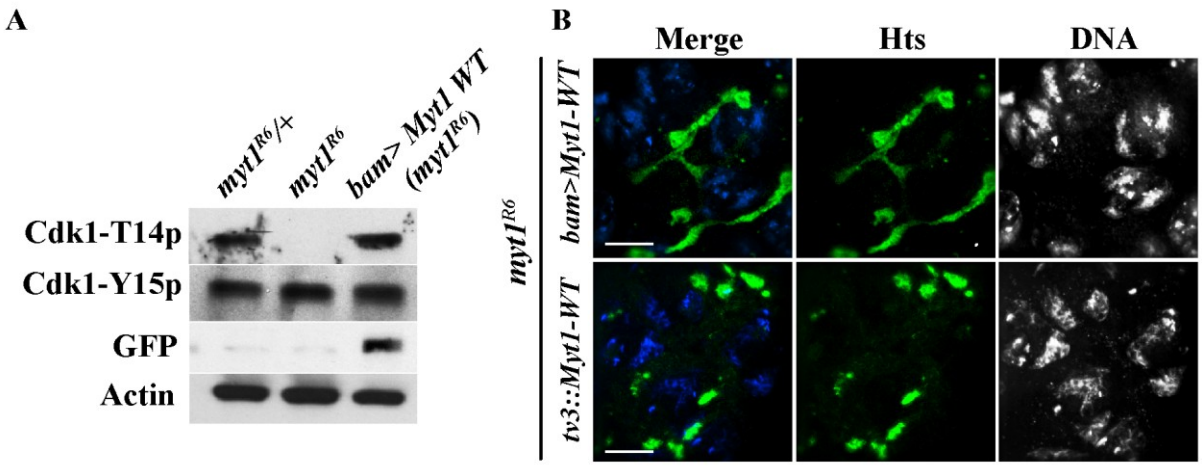

Fig. 2-12: Transgene expression of Myt1 in early spermatocyte restores *myt1* mutant fusomes

(A) Immuno-blot of testes extracts probed sequentially with phospho-specific antibodies against the endogenous Cdk1- T14p and Cdk1-Y15p. The blot was stripped between each reprobing. Molecular weight of the endogenous Cdk1 isoforms is around 34 kDa and the Actin is 47 kDa. Transgenic expression of 92 kDa size GFP-Myt1(WT) fusion protein was detected using GFP antibodies. (B) The Hts immuno-labeling in *myt1* spermatocytes expressing *bamGal4 >Myt1(WT)* reveals complete restoration of fusome branches, whereas the *tv3:: Myt1(WT)* only partially rescues these structures. The DNA labels represent the S3-S4 stage of *myt1* spermatocytes. Scale bar -10 microns.

expected, since dual-specificity Myt1 phosphorylates both T14 and Y15 residues of Cdk1 whereas Wee1 only phosphorylates the Y15 residue (Jin *et al.*, 2008). *bam-Gal4* and *tv3*-induced expression of tagged-Myt1 (WT) in *myt1* mutant late spermatogonia and spermatocytes, respectively (lanes 3 in Fig. 2-12A, lane 3 in Fig 3-1B) showed complementation of the Cdk1 T14 phosphorylation defect.

Using *bam-Gal4* expression of tagged Myt1 and immuno-labeling with Hts to mark fusomes, I observed that EGFP-Myt1(WT) fully rescued *myt1* mutant fusome spermatocyte defects (Fig. 2-12B) and restored male fertility (Table 2.1). In contrast, *tv3*-induced GFP-Myt1 (WT) later in spermatocyte development only partially rescued the fusome defect, assessed by Hts labeling (Fig. 2-12B). Partial rescue was also observed when EGFP-Myt1 was induced with *topi-Gal4*, another late spermatocyte driver (Raychaudhuri *et al.*, 2012) (data not shown). These results show that transgenic Myt1 expressed in early (S1/S2, polar) spermatocytes rescues the fusome structure in *myt1* mutants throughout pre-meiotic G2 phase arrest and the timing corresponded to when ER-localized Myt1 appeared to be closely associated with the fusome.

2.3.5 Expression of Cdk1(Y15F) disrupts pre-meiotic fusome organization and centriole engagement

Inhibitory phosphorylation of cyclin-dependent kinase 1 (Cdk1) is the only known function of Myt1 kinase (Fattaey and Booher, 1997; Booher *et al.*, 1997; Ayeni and Campbell, 2014). Mitotic Cyclin B is not expressed until late in spermatocyte maturation long after fusome defects are detectable in *myt1* mutants (White-Cooper *et al.*, 1998), however Cyclin A is expressed early in spermatocyte development and also enriched on fusomes (Lilly *et al.*,

2000). I used GFP-tagged Cdk1 transgenes induced with a *tv3* spermatocyte promoter at stage 3-4 to determine if Cdk1 also localized to fusomes. Indeed, Cdk1(WT)::GFP fusion proteins localized to spectrosomes and fusomes in both spermatocytes (Fig. 2-13) and spermatogonia (shown Fig. 3-8A). The tagged Cdk1 presumably exists in a complex with Cyclin A that localizes to fusomes. In *myt1* mutants, neither Cdk1(WT)::GFP nor Cyclin A localization to fusomes could be detected (Fig.2-14).

The simplest explanation of these results would be that Myt1 inhibits fusome-localized Cyclin A/Cdk1 complexes during spermatocyte maturation. To test this idea, in collaboration with Dr. Ayeni in our lab, I used the partially inhibitable phospho-acceptor mutants Cdk1(Y15F)-GFP, to disrupt Cdk1 regulation. Similar constructs used for studies of imaginal wing discs and larval neuroblasts showed that Cdk1(Y15F) expression can bypass developmental and DNA damage-induced G2 phase checkpoint arrest (Ayeni *et al.*, 2014) as effectively as the more widely used non-inhibitable Cdk1(T14A, Y15F), but without causing chromosome instability. If Myt1 normally regulates Cyclin A/Cdk1 during pre-meiotic G2 phase arrest, then expression of Cdk1(Y15F)-GFP would be expected to bypass this mechanism and phenocopy *myt1* mutant defects. As predicted, *tv3*-driven Cdk1(Y15F)-GFP expression in an otherwise wild type background caused fusome defects, though not as extreme as *myt1* mutants (Fig. 2-13B). These results therefore support the idea that regulation of Cyclin A/Cdk1 activity by Myt1-mediated inhibitory phosphorylation serves as a "fusome checkpoint" for maintaining the stability of these structures during the prolonged pre-meiotic G2 phase arrest.

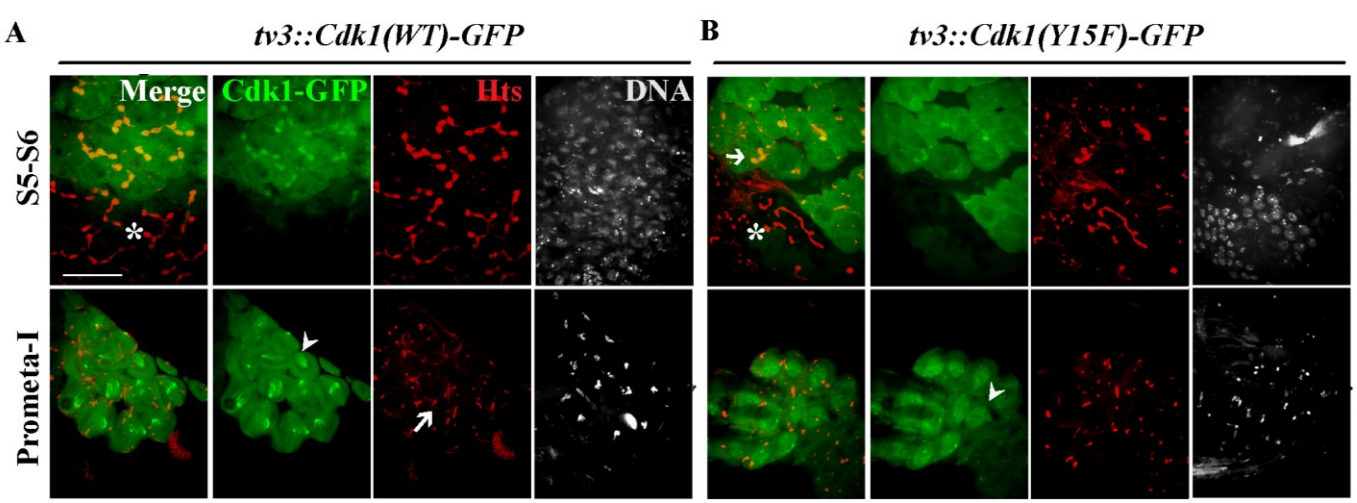

Fig. 2-13: Ectopic expression of Cdk1 GFP (Y15F) partially bypasses Myt1 fusome checkpoints

This data was produced in collaboration with Dr. Ayeni. I contributed by generating the transgenes (*tv3* lines) used in this figure and Dr.Ayeni performed the immuno-labeling experiment. (A) Transgenic expression of tv3::Cdk1(WT)-GFP co-labels with the Hts labeled fusomes. The tv3 promoter driven expression is detected from late stage of S5-S6 stage spermatocytes. Note that the early stage 16-spermatocytes (indicated by asterisks) show no GFP signal. During prometaphase of MI, when Cdk1-GFP translocate in to the nucleus (arrowhead), the Hts labeled fusomes appear dis-assembled (arrow). (B) Transgenic expression of *tv3::Cdk1(Y15F) GFP* triggers premature fusome-disassembly in S5-S6 spermatocytes. Note the early stage of spermatocytes (indicated by asterisks), where the Cdk1 (Y15F) GFP is not expressed, the Hts labeled fusomes appear normal. Although the temporal co-ordination of fusome disassembly is disrupted, the nuclear translocation of Cdk1 (Y15F) GFP is detected only in prometaphase-I, scale bar -16 microns.

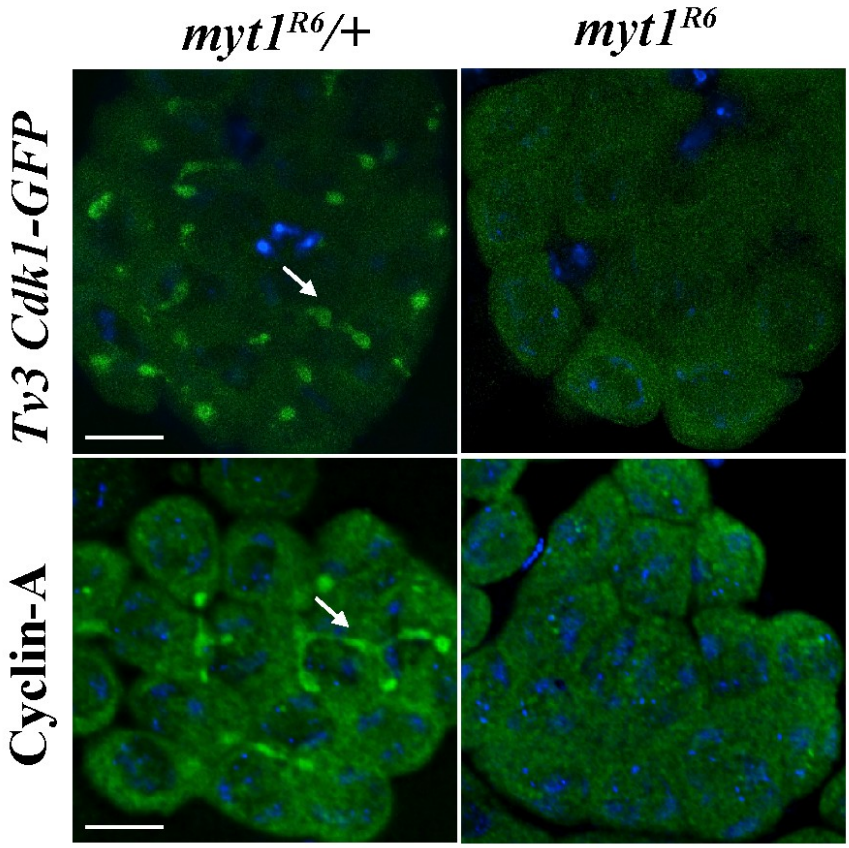

Fig. 2-14: Fusome localized Cdk1 and CyclinA are undetectable in *myt1* spermatocytes.

In *myt1*/+ spermatocytes, the Cdk1(WT)-GFP and the Cyclin A immuno-labels revealed a localization pattern consistent with the fusome (arrows). In *myt1* mutants, neither of these shows any fusome localization, scale bar -16 microns.

Fusomes normally disassemble during late prometaphase of meiosis I, as shown in *myt1/+* controls with Hts immuno-labeling (red, Fig. 2-13A). This stage is also marked by translocation of Cdk1(WT)::GFP signal into the nucleus (green, arrow, Fig. 2-13A), a phenomena also linked to mitotic progression (Gavet and Pines, 2010). Although partial fusome disassembly occurs prematurely when Cdk1(Y15F) is expressed, translocation of the fusion protein into the nucleus occured at prometaphase-I as in the controls, indicating that the fusome checkpoint and G2/M activation of Cdk1 could be temporally uncoupled.

2.3.6 Down regulation of Cyclin A suppresses *myt1* mutant defects

To test our hypothesis that meiotic defects caused by loss of Myt1 activity reflect failure to inhibit Cyclin A-Cdk1, I reduced endogenous Cyclin A by *bam-Gal4*-driven RNA interference. I used western blots to confirm that *bam*-driven $CycA^{siRNA}$ was reducing Cyclin A levels (Fig. 2-15A). The antibodies detected two bands as previously reported (Lehner and O'Farrell, 1990), a 56 kDa band that was significantly reduced in $bam>CycA^{siRNA}$ testes samples whereas a 70 kDa band was not (Fig. 2-15A). These results showed that Cyclin A levels corresponding to the 56 kDa band were reduced by RNA interference. Examination of these $bam>CycA^{siRNA}$ spermatocytes by immunofluorescence also showed Cyclin A localization to the fusome was undetectable compared to *myt1/+* control spermatocytes (Fig. 2-15B). Cyclin A immunofluorescence was unaffected in $bam>CycA^{siRNA}$ spermatogonia, however, showing that this effect was temporally restricted.

I observed that *bam*-driven expression of $CycA^{siRNA}$ resulted in spermatocytes that failed to execute the metaphase-anaphase I transition before differentiating into 16-cell onion stage spermatids, unlike *myt1/+* control spermatocytes that progressed through M1 and MII

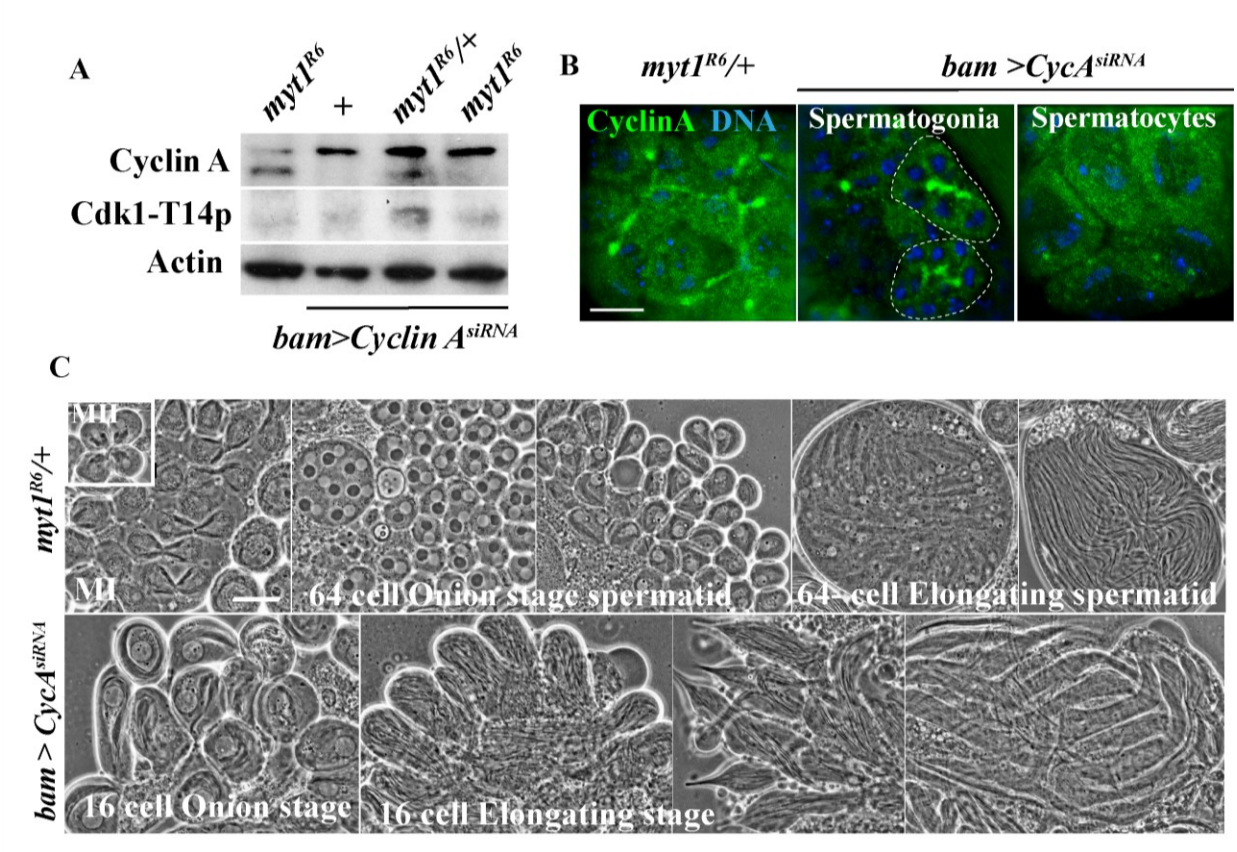

Fig. 2-15: CyclinA knockdown prevents MI division

(A) Immuno-blot was probed sequentially with Cyclin A and Cdk1- T14p antibodies. Note that immuno-detection of endogenous Cyclin A exhibited a doublet (Lehner and O'Farrell, 1990). The top band of the doublet corresponds to an undetermined 70 kDa protein, whereas the bottom 56 kDa size band represent the endogenous Cyclin A. (B) Spermatocytes are labeled with Cyclin A (green) antibodies and the DNA is in blue, scale bar -10 microns. The fusome localized Cyclin A signals appeared normal in *bamGal4 > CycA*^{siRNA} spermatogonial, while being undetectable in spermatocytes, scale bar -10 microns (C)The control *myt1/+* exhibits the normal execution MI and MII followed by spermatid remodeling in the post meiotic 64 cell cyst. The Cyclin A knockdown using *bamGal4 >Cyclin A*^{siRNA} expression arrests 16-spermatocytes in prometaphase-I and prevents the meiotic divisions. The arrested spermatocytes undergo spermatid like differentiation process, however. Scale bar-10 microns.

before differentiating (Fig. 2-15C). However, RNAi depletion of Cyclin A later in spermatocyte maturation using *topi-gal4,* or RNAi against Cyclin B with either early (*bam-Gal4)* or late (*topi-Gal4*) had no effect on MI and the meiotic progression appeared as normal as the control. These results showed that Cyclin A was essential for MI progression, but dispensable for spermatid differentiation. Loss of meiotic $Cdc25^{twe}$ causes a phenotype that is similar to early spermatocyte depletion of Cyclin A (Alphey *et al.*, 1992; Lin *et al.*, 1996), suggesting that $Cdc25^{Twe}$ -dependent activation of Cyclin A-Cdk1 may be required for MI onset during male meiosis.

Next I examined the effect of Cyclin A depletion on *myt1* mutant defects. I observed that Cyclin A depletion fully rescued the *myt1* mutant fusome defects (compare Fig. 2-8B with Fig. 2-16A), supporting the idea that Myt1 regulation of Cyclin A-Cdk1 is required for maintaining normal fusome structure during pre-meiotic G2 phase arrest. I also examined Roughex (Rux) a known *Drosophila* inhibitor of Cyclin A-Cdk1 (Thomas *et al.*, 1994; Foley *et al.*, 1999). Previous studies of *rux* mutants and Rux over-expression demonstrated that Rux regulates CyclinA-Cdk1 during meiosis II (Gonczy *et al.*, 1994). Ectopic expression of Rux driven by *bam-Gal4* in *myt1* mutant spermatocytes partially rescued both the fusome defect (Fig. 2-16A) and male sterility (Table 2.1). As a control, I examined whether *bam*-driven expression of Rux in a *myt1* mutant background affected inhibitory phosphorylation of Cdk1 (Fig. 2-16C). I observed that *bam*-driven expression of Rux resulted in lower levels of Cdk1-Y15p compared to *myt1* mutants alone, whereas the absence of Cdk1-T14p observed in *myt1* mutants was not affected. Moreover, rux^{8} mutant spermatocytes labeled with Hts had normal fusomes throughout spermatocyte development (Fig. 2-17). I examined fusomes of wee^{ES1} mutant spermatocytes, since Wee1 was also known to regulate Cdk1 through Y15

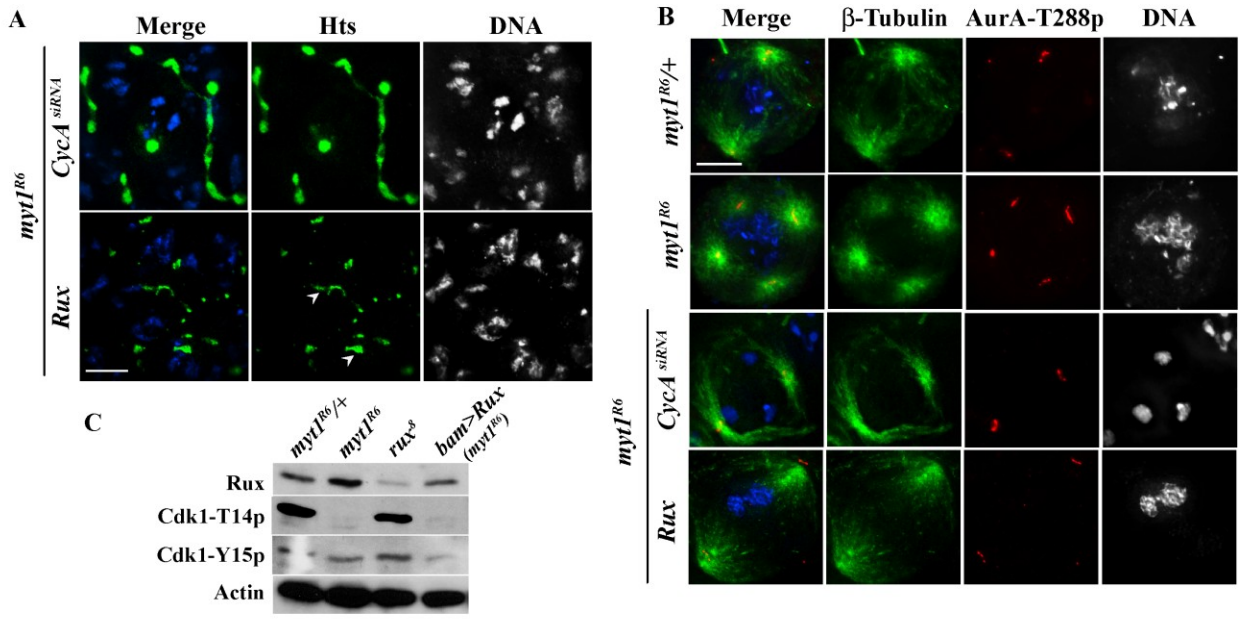

Fig. 2-16: Down regulation of CyclinA suppresses *myt1* meiotic organelle defects.

(A) The *myt1* mutant spermatocytes are labeled with Hts antibodies (green). Depletion of Cyclin A by *bamGal4 > CycA*^{siRNA} restores *myt1* mutant fusome defects. Ectopic expression of Rux by *bam-Gal4* partially suppresses the mutant fusome defect. Arrowheads indicate the remnant fusome structures. (B) The meiotic spindles labeled with β–Tubulin (green) antibodies and AurA-T288p isoform antibodies (red) restore the *myt1* mutant multipolar spindles both in *CycA*^{siRNA} or Rux mediated CyclinA knockdown conditions, scale bar -10 microns. (C) Immuno-blot was probed sequentially with Rux, Cdk1- T14p and Cdk1- Y15p antibodies. Actin was probed for loading control.

Table 2-1: Rescue of *myt1* mutant male sterility

Degree of Fertility	***myt1*^{R6}/+**	***myt1*^{R6}**	***bamGal> Myt1WT***	***topiGal> Myt1WT***	***bamGal > Rux***	***bamGal4 >CycA*^{siRNA}**
Fertile (> 20)	30	0	17	13	3	0
Semi-Fertile (< 20)	0	0	1*	2*	6	0
Sterile (= 0)	0	30	2	5	23	25
# of Male (n)	30	30	20	20	32	25

Fertility Assay: Single test male was crossed with 3-5 *yw* virgin females in an individual vial at 25C incubator. The first brood progenies from this cross were counted. Fertility of the single test males was scored based on the following criteria; progenies count over 20 flies (fertile), less than 20(semi-fertile) and no progenies (sterile). In most case all the fertile males produced over 100 progenies/cross. The gray area in this table indicates the mentioned transgene expression in *myt1*^{R6} background.
* Indicates the exceptional case, where a single male does not fit with the most observed categories of the corresponding genotype.

phosphorylation (Fig.2-17). Hts labeled fusomes appeared normal in wee^{ES1}, however. These results indicate that neither Rux nor Wee1 can substitute for the function of Myt1 in the fusome checkpoint. Nonetheless, these results showing that ectopic Rux can suppress *myt1* fusome and sterility defects support our hypothesis that Myt1 regulation of Cyclin A/Cdk1 is specifically required for the fusome checkpoint in pre-meiotic spermatocytes.

I examined how Myt1 regulation of CyclinA-Cdk1 affected centriole engagement by immuno-labeling MI meiotic spindles with antibodies against β–Tubulin and a mitotic AurA-T288p isoform. I observed *myt1*/+ and *myt1* mutant spermatocytes with bipolar and tetra-polar spindles, respectively (Fig. 2-16B). Early down-regulation of Cyclin A, by either *bam*-driven $CycA^{siRNA}$ knockdown or ectopic *Rux* expression completely rescued the *myt1* mutant centriole engagement defect (Fig. 2-16B). In spite of this rescue, bipolar spindle assembly did not occur in these Cyclin A-depleted cells, supporting our earlier conclusion that Cyclin A function is essential for MI progression (Fig. 2-15C). In contrast, ectopic expression of *Rux* rescues both the *myt1* mutant centriole disengagement and multipolar spindle defects, however (Fig.2-16B).

I also investigated whether Myt1 regulation of Cyclin B-Cdk1 had any role in meiotic progression by undertaking similar experiments to deplete Cyclin B in *myt1* mutant and control spermatocytes using $CycB^{siRNA}$ transgenes. *bam*-driven $CycB^{siRNA}$ expression had no detectable effect on either *myt1* mutant fusome or centriole engagement defects, however (Fig. 2-18A) Myt1 mutants have little or no reduction in Cyclin B protein levels relative to the controls (Fig. 2-18B), likely because Cyclin B does not accumulate until late stages of spermatocyte maturation and would therefore not be affected by *bam*-driven RNA interference. Although these results do not rule out a role for Myt1 regulation of Cyclin B-

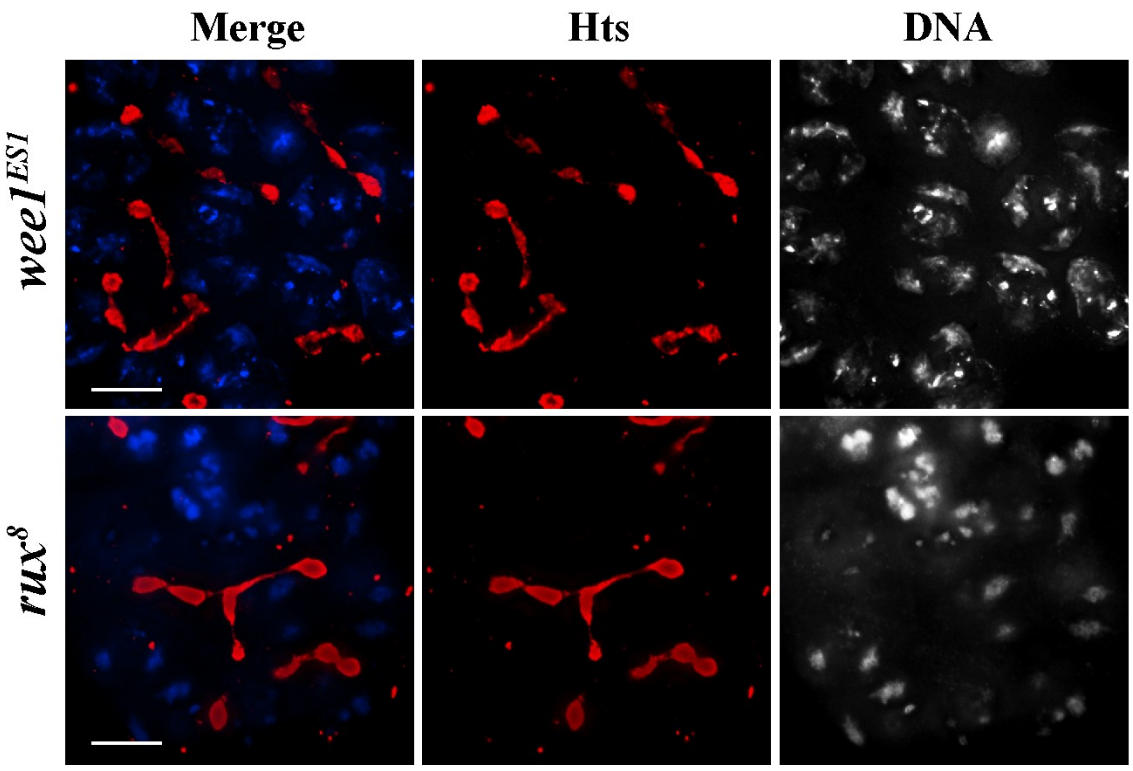

Fig. 2-17: *wee*^{ES1} and *rux*^{8} spermatocytes have normal fusomes.

Hts labeled fusome structures appeared normal in both *wee*^{ES1} and *rux*^{8} spermatocytes. Scale bar -10 microns.

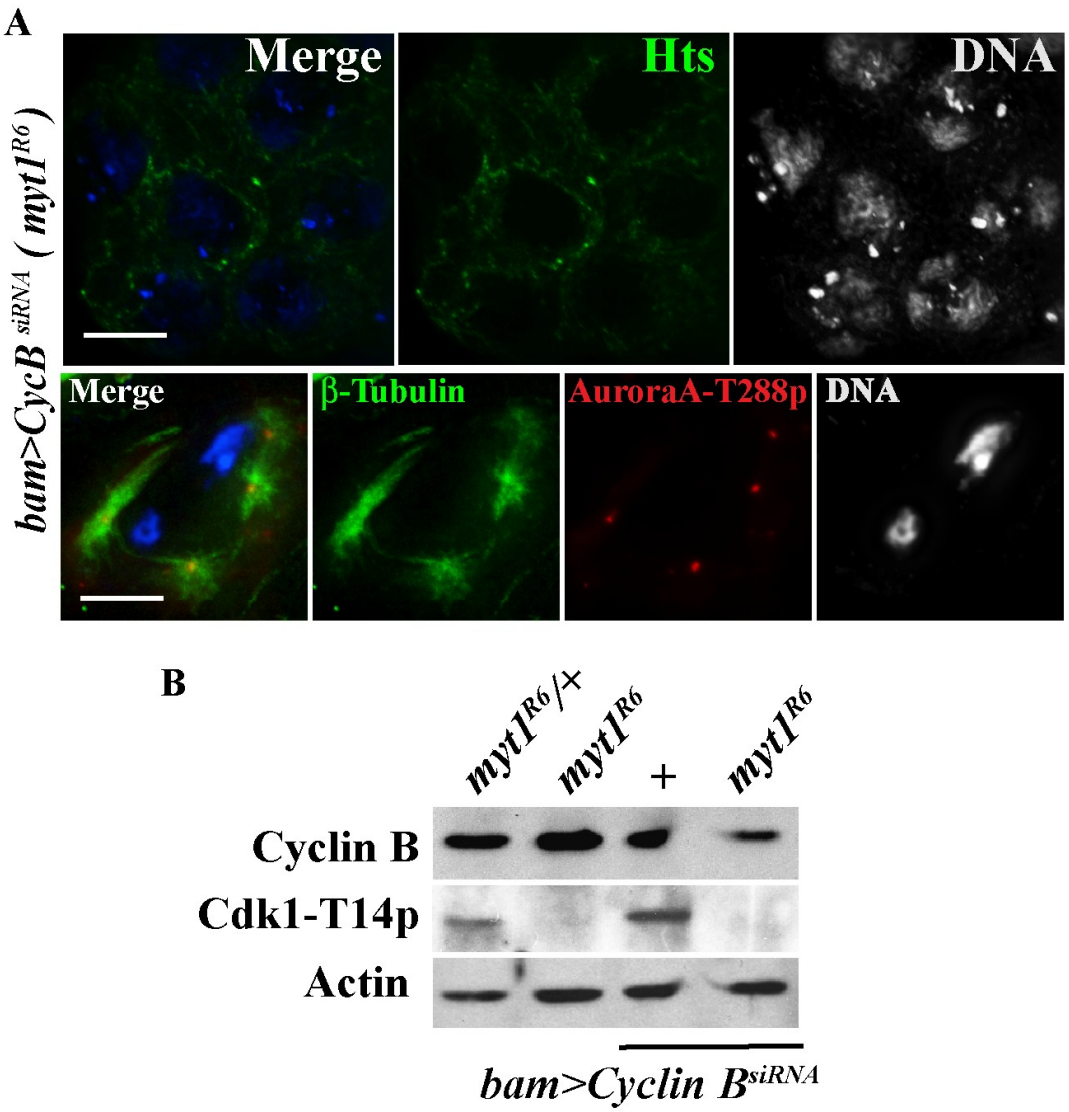

Fig. 2-18: Myt1 meiotic organelle checkpoint is independent of Cyclin B-Cdk1 regulation.

(A) Fusomes are labeled using Hts antibodies (green). (B) Meiotic spindles are labeled with β–Tubulin (green) and AurA-T288 phospho isoform (red). The *bamGal4 > CycB*^{siRNA} expression in *myt1* mutant spermatocytes neither rescued the *myt1* mutant fusomes nor meiotic spindles defects, scale bar -10 microns (B) Immuno-blot was probed sequentially with Cyclin B, Cdk1-T14p antibodies and actin antibodies.

Cdk1 in mature spermatocytes, regulation of Cyclin A-Cdk1 appears to be the primary mechanism by which Myt1 controls the organelle checkpoint that protects fusomes and centrioles during the prolonged pre-meiotic G2 phase arrest.

2.4 Discussion

I investigated the role of Myt1 kinase during the prolonged pre-meiotic G2 phase arrest of *Drosophila* spermatogenesis. In this developmental system, canonical functions of Myt1 as inhibitor of Cyclin B-Cdk1 appear to be dispensable. Instead, Myt1 regulation of spatially localized Cyclin A-Cdk1 is required for fusome stability and centriole engagement during pre-meiotic G2 phase arrest. These findings reveal novel organelle checkpoint functions for Myt1 that are used for temporally coordinating cytoplasmic and nuclear-associated events during pre-meiotic G2 phase and G2/MI.

2.4.1 Myt1 regulation of Cyclin A-Cdk1 controls a fusome checkpoint in spermatocytes

In mammalian cells, Myt1-regulation of Cyclin B/Cdk1 prevents premature G2/M fragmentation of Golgi structures (Villeneuve *et al.*, 2013) and promotes Golgi/ER re-assembly during mitotic exit (Nakajima *et al.*, 2008). I showed that Myt1 activity does not serve a comparable role in regulating the simple Golgi stacks that are prevalent during *Drosophila* development (Kondylis and Rabouille, 2009). Instead, loss of Myt1 disrupts the ER-derived fusome in spermatocytes, leading us to conclude that Myt1 serves a novel organelle checkpoint function that ensures fusome stability during a prolonged pre-meiotic G2 phase. The *myt1* mutant fusome defects were suppressed by RNA interference against

Cyclin A and ectopic expression of the Cyclin A-specific inhibitor Rux, indicating that Cyclin A-Cdk1 is the target for this proposed Myt1 organelle checkpoint.

Although fusomes exist throughout spermatogenesis, loss of Myt1 only affected these structures in 16-cell pre-meiotic spermatocytes. I observed an association between the ER and the fusomes using tagged Myt1 and the ER marker KDEL that occurs shortly before the transition between polar and apolar stages of early spermatocyte development (Fig. 2-10). Modification of ER and fusome membranes in cystocytes exiting mitotic divisions (Snapp *et al.*, 2004) and dissociation of ring canal components such as Cintr and F-actin in early 16 cell spermatocytes have also been reported to accompany the mitotic-to-meiotic transition (Eikenes *et al.*, 2013; Hime *et al.*, 1996). Re-organization of membranous ER during the onset of mitosis has been reported to rely on Cyclin A-Cdk1 activity in *Drosophila* syncytial embryos, suggesting that the relevant substrates may be conserved ER-associated proteins (Bergman *et al.*, 2015). Our findings indicate that Myt1-mediated inhibitory phosphorylation of Cyclin A-Cdk1 serves a specialized role in maintaining these actively remodeling membrane cytoskeletal structures during pre-meiotic G2 phase.

2.4.2 Is there a connection between the Myt1 fusome checkpoint and meiotic centrosome behavior?

During *Drosophila* male meiosis, centrosome maturation, spindle assembly, chromosome condensation, fusome disassembly, chromosome segregation and centriole dis-engagement must be temporally and spatially coordinated. In *myt1* mutants, fusome disassembly and centriole dis-engagement occur prematurely although other meiotic events proceed with apparently normal developmental timing (Fig. 2-19). Fusome-defective *hts* (hypomorphic allele) mutants have also been reported to exhibit similar centrosome defects in

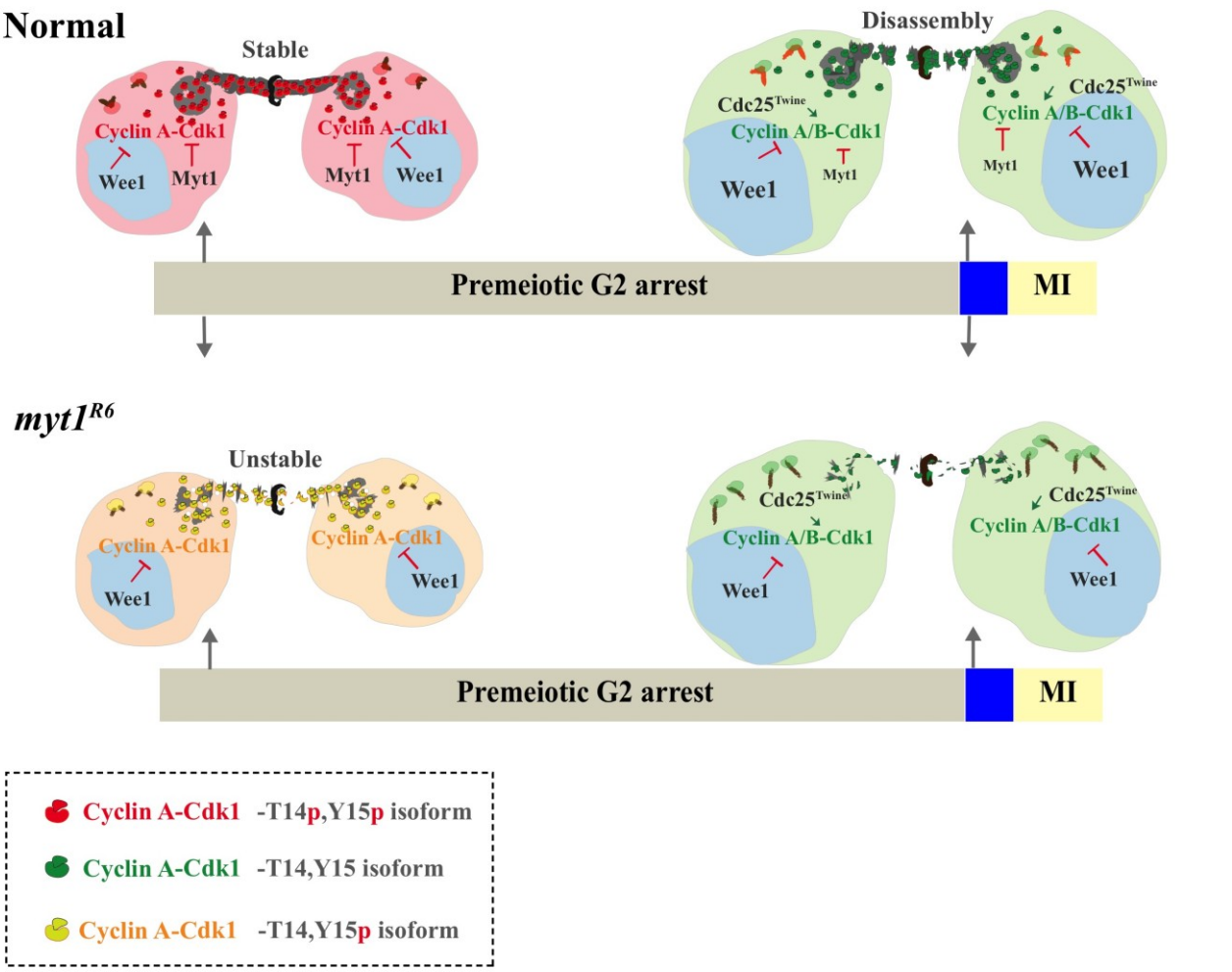

Fig. 2-19: Illustration of myt1 mutant pre-meiotic organelle checkpoint defects.

Illustration compares normal vs *myt1* mutant pre-meiotic checkpoint regulation. In normal meiosis, both Myt1 and Wee1 could inhibit Cyclin A-Cdk1 activity that could ensure a strict pre-meiotic organelle checkpoint to co-ordinate fusome integrity and centriole engagement. In *myt1* mutants, CyclinA-Cdk1 lack T14 phosphorylation due to the loss of Myt1 activity therefore the complex may not be completely inhibited. Partially active Cyclin A-Cdk1 therefore perturbs the pre-meiotic organelle checkpoint resulting in abrupt fusome disruption and centriole dis-engagement.

spermatocytes, suggesting possible mechanistic connections between these cytoplasmic organelles (Wilson, 2005). In HeLa cells, Cdk1 phosphorylation of the Hts-like Adducin-1 protein promotes binding to an actin-based motor protein to facilitate mitotic spindle assembly, whereas depletion of Adducin-1 activity results in multipolar spindles and chromosome segregation defects (Chan *et al.*, 2014). These observations suggest that the link between regulation of Cdk1 activity by Myt1 and Hts-actin mediated spindle assembly might be conserved. The Klp61F motor protein is another potential Cdk1 substrate relevant to the *Drosophila* spermatocyte fusome checkpoint as this microtubule-associated kinesin also localizes to fusomes in spermatocytes and has been defined as a target of Cdk1 activity in *Drosophila* syncytial embryos (Wilson, 1999; Sharp *et al.*, 1999). I propose a model suggesting that failure of Myt1 to regulate Cyclin A-Cdk1 activity during pre-meiotic G2 phase triggers premature centriole dis-engagement by targeting molecules that modify the centriolar matrix.

Is there any direct physical relationship between fusomes and centrosomes revealed by the *myt1* mutant phenotype? Our current model shown in Fig.2-20 predicts that fusomes serve as scaffolds for accumulating or tethering Myt1-inhibited CyclinA-Cdk1 complexes until G2/MI, when Cyclin B and $Cdc25^{Twe}$ are first expressed. $Cdc25^{Twe}$ is expected to dephosphorylate fusome-associated CyclinA-Cdk1 to trigger fusome disassembly, releasing active CyclinA-Cdk1 to coordinate early events of meiosis-I. In *myt1* mutants, premature fusome disassembly would be expected to release partially active (due to the absence of T14 phosphorylation) CyclinA-Cdk1 activity that perturbs G2 phase centriole engagement. During G1/S phase of cell cycle, Plk4/SAK mediated phosphorylation regulates Ana2 in recruiting Sas6 to the centrioles to facilitate duplication and engagement (Dzhindzhev *et al.*, 2014). At

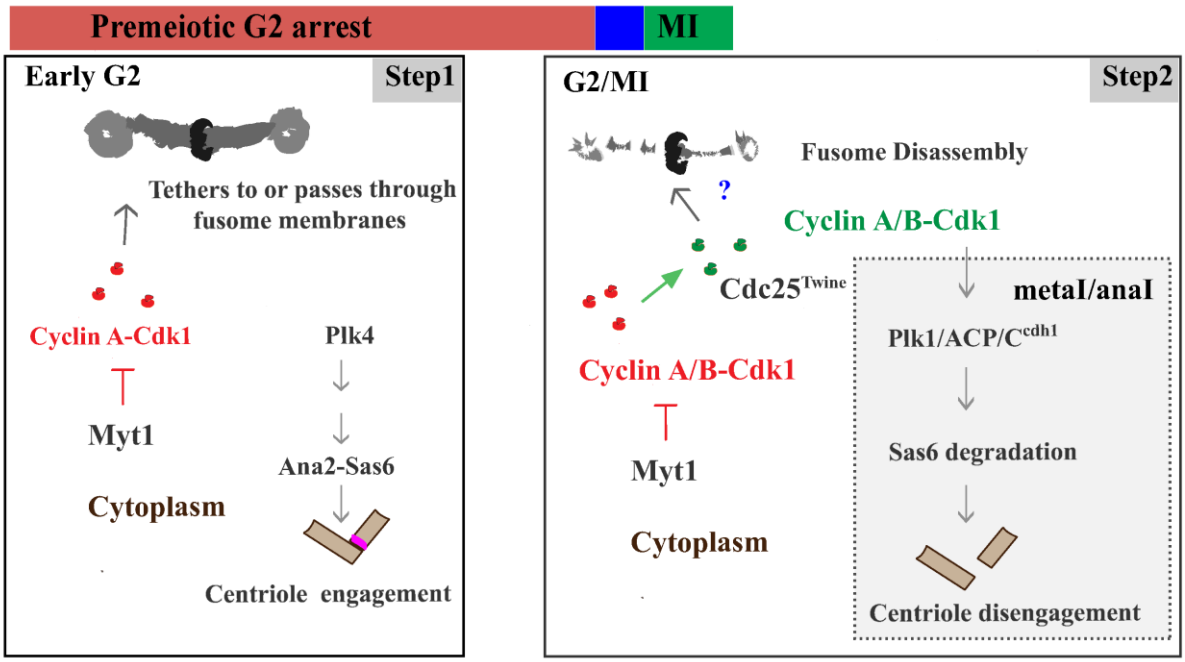

Fig. 2-20: Model: Two step regulation of Cdk1 during *Drosophila* meiosis.

During early G2 phase arrest inhibitory phosphorylation of Cyclin A-Cdk1 by Myt1 employs fusome checkpoint that prevents $Cdc25^{Twe}$ independent activation of Cdk1. This checkpoint function protects fusomes integrity and centriole engagement until the late prophaseI, when $Cdc25^{Twe}$ dependent Cdk1 activation is established. We propose that the fusome might serve as a platform where the inhibited Cyclin A-Cdk1 is tethered to or passed through intercellular connections. Failure to inhibit this complex by Myt1 could perturb the fusome structures by causing premature disassembly. Furthermore, partially activated Cdk1 (in the absence of $Cdc25^{Twe}$) could mis-regulate the co-ordination of mechanisms involving centriole engagement and dis-engagement.

anaphase onset, APC/C^{cdh1} driven proteolysis degrades Sas6 and causes the centrioles to disengage (Strnad *et al.*, 2007; Hatano and Sluder, 2012). In *myt1* mutant late spermatocytes (S6-ProI), prematurely dis-engaged centrioles showed no association with Sas6 (Fig. 2-4), suggesting a failure in regulation of either Sas6 recruitment or degradation. How Myt1 kinase contributes to mechanisms that normally regulate centriole engagement and dis-engagement during meiosis needs to be fully characterized.

2.4.3 Spatial-temporal regulation of Cyclin A-Cdk1 during pre-meiotic G2 phase arrest

During oocyte maturation in vertebrates, Myt1 inhibition of Cyclin B- Cdk1 (MPF, M-phase promoting factor) maintains G2 phase arrest until steroid-hormone triggers the activation of Cyclin B- Cdk1 by feedback amplification mechanisms that down-regulate Myt1 and activate Cdc25 phosphatases to remove inhibitory phosphates from Cdk1 (Schmitt and Nebreda, 2002; Oh *et al.*, 2010; Ruiz *et al.*, 2010). The rapid rise in Cdk1 activity coordinates germinal vesicle breakdown (GVBD) and subsequent meiotic events (Adhikari and Liu, 2014; Schmitt and Nebreda, 2002). *C. elegans* Myt1 (Wee-1.3) plays a similar role in regulating meiotic entry in oocytes (Burrows *et al.*, 2006). In *Drosophila*, BrdU pulse labeling of *myt1* and *wee1* mutants revealed that neither Myt1 nor Wee1 was solely responsible for preventing premature MI by a canonical "all-or-none" checkpoint mechanism. Instead, the major role of Myt1 during early and mid G2 phases of spermatocyte maturation appears to be inhibition of Cyclin A-Cdk1, which promotes fusome integrity and centriole engagement. Myt1 may also inhibit newly formed Cyclin B-Cdk1 complexes before $Cdc25^{Twe}$ is expressed to establish conditions for a Cdk1 positive feed back loop to execute the G2/MI transition by an "all-or-none" mechanism in late G2 phase (O'Farrell, 2001). We observed that $Cdc25^{Twe}$ independent meiotic functions appear to include fusome dis-assembly and centrosome maturation (R.

Varadarajan and J. Ayeni, unpublished observation), whereas $Cdc25^{Twe}$ activity is required for formation of the meiotic spindle but not for other meiotic events such as chromosome condensation (White-Cooper *et al.*, 1993). I conclude that cell cycle progression during *Drosophila* male meiosis is regulated by at least two distinct mechanisms. During spermatocyte maturation, Myt1 regulation of Cyclin A-Cdk1 stabilizes fusomes and promotes centriole engagement to facilitate pre-meiotic arrest. Later, distinct mechanisms regulating the synthesis and activation of Cyclin B-Cdk1 by $Cdc25^{Twe}$ in mature spermatocytes are used to control the G2/MI transition itself. This more complex view of the cell cycle checkpoint mechanisms that regulate meiotic progression during *Drosophila* male meiosis may also be relevant to other systems.

2.5 Reference

1. Adhikari, D, and K Liu. 2014. “The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes.” *Mol Cell Endocrinol* 382(1): 480–87.
2. Allen, AK, JE Nesmith, and A Golden. 2014. “An RNAi-Based Suppressor Screen Identifies Interactors of the Myt1 Ortholog of Caenorhabditis elegans.” *G3 (Bethesda)*
3. Alphey, L, J Jimenez, H White-Cooper, I Dawson, P Nurse, and DM Glover. 1992. “twine, a cdc25 homolog that functions in the male and female germline of Drosophila.” *Cell* 69(6): 977–88.
4. Ayeni, JO, and SD Campbell. 2014. “”Ready, set, go”: checkpoint regulation by Cdk1 inhibitory phosphorylation.” *Fly (Austin)* 8(3): 140–47.
5. Ayeni, JO, R Varadarajan, O Mukherjee, DT Stuart, F Sprenger, M Srayko, and SD Campbell. 2014. “Dual phosphorylation of cdk1 coordinates cell proliferation with key developmental processes in Drosophila.” *Genetics* 196(1): 197–210.
6. Belloni, G, S Sechi, MG Riparbelli, MT Fuller, G Callaini, and MG Giansanti. 2012. “Mutations in Cog7 affect Golgi structure, meiotic cytokinesis and sperm development during Drosophila spermatogenesis.” *J Cell Sci* 125(Pt 22): 5441–52.
7. Bergman, ZJ, JD Mclaurin, AS Eritano, BM Johnson, AQ Sims, and B Riggs. 2015. “Spatial Reorganization of the Endoplasmic Reticulum during Mitosis Relies on Mitotic Kinase Cyclin A in the Early Drosophila Embryo.” *PLoS One* 10(2): e0117859.
8. Booher, RN, PS Holman, and A Fattaey. 1997. “Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity.” *J Biol Chem* 272(35): 22300–06.
9. Brand, AH, and N Perrimon. 1993. “Targeted gene expression as a means of altering

cell fates and generating dominant phenotypes." *Development* 118(2): 401–15.

10. Burrows, AE, BK Sceurman, ME Kosinski, CT Richie, PL Sadler, JM Schumacher, and A Golden. 2006. "The C. elegans Myt1 ortholog is required for the proper timing of oocyte maturation." *Development* 133(4): 697–709.
11. Cenci, G, S Bonaccorsi, C Pisano, F Verni, and M Gatti. 1994. "Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of Drosophila melanogaster spermatogenesis." *J Cell Sci* 107 (Pt 12): 3521–34.
12. Chan, PC, RY Hsu, CW Liu, CC Lai, and HC Chen. 2014. "Adducin-1 is essential for mitotic spindle assembly through its interaction with myosin-X." *J Cell Biol* 204(1): 19–28.
13. Chen, D, and DM McKearin. 2003. "A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell." *Development* 130(6): 1159–70.
14. Cornwell, WD, PJ Kaminski, and JR Jackson. 2002. "Identification of Drosophila Myt1 kinase and its role in Golgi during mitosis." *Cell Signal* 14(5): 467–76.
15. Courtot, C, C Fankhauser, V Simanis, and CF Lehner. 1992. "The Drosophila cdc25 homolog twine is required for meiosis." *Development* 116(2): 405–16.
16. de Cuevas, M, MA Lilly, and AC Spradling. 1997. "Germline cyst formation in Drosophila." *Annu Rev Genet* 31: 405–28.
17. Duckworth, BC, JS Weaver, and JV Ruderman. 2002. "G2 arrest in Xenopus oocytes depends on phosphorylation of cdc25 by protein kinase A." *Proc Natl Acad Sci U S A* 99(26): 16794–99.
18. Dzhindzhev, NS, G Tzolovsky, Z Lipinszki, S Schneider, R Lattao, J Fu, J Debski, M

Dadlez, and DM Glover. 2014. “Plk4 phosphorylates Ana2 to trigger Sas6 recruitment and procentriole formation.” *Curr Biol* 24(21): 2526–32.

19. Eikenes, AH, A Brech, H Stenmark, and K Haglund. 2013. “Spatiotemporal control of Cindr at ring canals during incomplete cytokinesis in the Drosophila male germline.” *Dev Biol* 377(1): 9–20.
20. Fattaey, A, and RN Booher. 1997. “Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14.” *Prog Cell Cycle Res* 3: 233–40.
21. Field, CM, and BM Alberts. 1995. “Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex.” *J Cell Biol* 131(1): 165–78.
22. Foley, E, PH O’Farrell, and F Sprenger. 1999. “Rux is a cyclin-dependent kinase inhibitor (CKI) specific for mitotic cyclin-Cdk complexes.” *Curr Biol* 9(23): 1392–402.
23. Gaffre, M, A Martoriati, N Belhachemi, JP Chambon, E Houliston, C Jessus, and A Karaiskou. 2011. “A critical balance between Cyclin B synthesis and Myt1 activity controls meiosis entry in Xenopus oocytes.” *Development* 138(17): 3735–44.
24. Gavet, O, and J Pines. 2010. “Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis.” *Dev Cell* 18(4): 533–43.
25. Gonczy, P, BJ Thomas, and S DiNardo. 1994. “roughex is a dose-dependent regulator of the second meiotic division during Drosophila spermatogenesis.” *Cell* 77(7): 1015–25.
26. Habedanck, R, YD Stierhof, CJ Wilkinson, and EA Nigg. 2005. “The Polo kinase Plk4 functions in centriole duplication.” *Nat Cell Biol* 7(11): 1140–46.
27. Hatano, T, and G Sluder. 2012. “The interrelationship between APC/C and Plk1 activities in centriole disengagement.” *Biol Open* 1(11): 1153–60.
28. Hime, GR, JA Brill, and MT Fuller. 1996. “Assembly of ring canals in the male germ

line from structural components of the contractile ring." *J Cell Sci* 109 (Pt 12): 2779–88.

29. Holt, JE, SI Lane, and KT Jones. 2013. "The control of meiotic maturation in mammalian oocytes." *Curr Top Dev Biol* 102: 207–26.
30. Jin, Z, E Homola, S Tiong, and SD Campbell. 2008. "Drosophila myt1 is the major cdk1 inhibitory kinase for wing imaginal disc development." *Genetics* 180(4): 2123–33.
31. Jin, Z, EM Homola, P Goldbach, Y Choi, JA Brill, and SD Campbell. 2005. "Drosophila Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis." *Development* 132(18): 4075–85.
32. Kishimoto, T. 2003. "Cell-cycle control during meiotic maturation." *Curr Opin Cell Biol* 15(6): 654–63.
33. Kishimoto, T. 2011. "A primer on meiotic resumption in starfish oocytes: the proposed signaling pathway triggered by maturation-inducing hormone." *Mol Reprod Dev* 78(10-11): 704–07.
34. Kondylis, V, and C Rabouille. 2009. "The Golgi apparatus: lessons from Drosophila." *FEBS Lett* 583(23): 3827–38.
35. Kornbluth, S, B Sebastian, T Hunter, and J Newport. 1994. "Membrane localization of the kinase which phosphorylates p34cdc2 on threonine 14." *Mol Biol Cell* 5(3): 273–82.
36. Lehner, CF, and PH O'Farrell. 1989. "Expression and function of Drosophila cyclin A during embryonic cell cycle progression." *Cell* 56(6): 957–68.
37. Lehner, CF, and PH O'Farrell. 1990. "The roles of Drosophila cyclins A and B in mitotic control." *Cell* 61(3): 535–47.
38. Lilly, MA, M de Cuevas, and AC Spradling. 2000. "Cyclin A associates with the fusome during germline cyst formation in the Drosophila ovary." *Dev Biol* 218(1): 53–

63.

39. Lin, H, and AC Spradling. 1995. “Fusome asymmetry and oocyte determination in Drosophila.” *Dev Genet* 16(1): 6–12.

40. Lin, H, L Yue, and AC Spradling. 1994. “The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation.” *Development* 120(4): 947–56.

41. Lin, TY, S Viswanathan, C Wood, PG Wilson, N Wolf, and MT Fuller. 1996. “Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in Drosophila males.” *Development* 122(4): 1331–41.

42. Liu, F, C Rothblum-Oviatt, CE Ryan, and H Piwnica-Worms. 1999. “Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes.” *Mol Cell Biol* 19(7): 5113–23.

43. Liu, F, JJ Stanton, Z Wu, and H Piwnica-Worms. 1997. “The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex.” *Mol Cell Biol* 17(2): 571–83.

44. McCullough, S, and J Lucocq. 2005. “Endoplasmic reticulum positioning and partitioning in mitotic HeLa cells.” *J Anat* 206(5): 415–25.

45. McKearin, D. 1997. “The Drosophila fusome, organelle biogenesis and germ cell differentiation: if you build it.” *Bioessays* 19(2): 147–52.

46. McKearin, DM, and AC Spradling. 1990. “bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis.” *Genes Dev* 4(12B): 2242–51.

47. Mueller, PR, TR Coleman, A Kumagai, WG Dunphy, WM Michael, and J Newport. 1995. “Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on

both threonine-14 and tyrosine-15." *Science* 282(5395): 86–90.

48. Nakajima, H, S Yonemura, M Murata, N Nakamura, H Piwnica-Worms, and E Nishida. 2008. "Myt1 protein kinase is essential for Golgi and ER assembly during mitotic exit." *J Cell Biol* 181(1): 89–103.

49. Nebreda, AR, and I Ferby. 2000. "Regulation of the meiotic cell cycle in oocytes." *Curr Opin Cell Biol* 12(6): 666–75.

50. O'Farrell, PH. 2001. "Triggering the all-or-nothing switch into mitosis." *Trends Cell Biol* 11(12): 512–19.

51. Oegema, K, MS Savoian, TJ Mitchison, and CM Field. 2000. "Functional analysis of a human homologue of the Drosophila actin binding protein anillin suggests a role in cytokinesis." *J Cell Biol* 150(3): 539–52.

52. Oh, JS, SJ Han, and M Conti. 2010. "Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption." *J Cell Biol* 188(2): 199–207.

53. Okumura, E, T Fukuhara, H Yoshida, S Hanada Si, R Kozutsumi, M Mori, K Tachibana, and T Kishimoto. 2002. "Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition." *Nat Cell Biol* 4(2): 111–16.

54. Orr-Weaver, TL. 1995. "Meiosis in Drosophila: seeing is believing." *Proc Natl Acad Sci U S A* 92(23): 10443–49.

55. Palmer, A, AC Gavin, and AR Nebreda. 1998. "A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1." *EMBO J* 17(17): 5037–47.

56. Pirrotta, V. 1988. "Vectors for P-mediated transformation in Drosophila."

Biotechnology 10: 437–56.

57. Price, DM, Z Jin, S Rabinovitch, and SD Campbell. 2002. "Ectopic expression of the Drosophila Cdk1 inhibitory kinases, Wee1 and Myt1, interferes with the second mitotic wave and disrupts pattern formation during eye development." *Genetics* 161(2): 721–31.
58. Raychaudhuri, N, R Dubruille, GA Orsi, HC Bagheri, B Loppin, and CF Lehner. 2012. "Transgenerational propagation and quantitative maintenance of paternal centromeres depends on Cid/Cenp-A presence in Drosophila sperm." *PLoS Biol* 10(12): e1001434.
59. Ruiz, EJ, M Vilar, and AR Nebreda. 2010. "A two-step inactivation mechanism of Myt1 ensures CDK1/cyclin B activation and meiosis I entry." *Curr Biol* 20(8): 717–23.
60. Schmitt, A, and AR Nebreda. 2002. "Signalling pathways in oocyte meiotic maturation." *J Cell Sci* 115(Pt 12): 2457–59.
61. Sharp, DJ, KL McDonald, HM Brown, HJ Matthies, C Walczak, RD Vale, TJ Mitchison, and JM Scholey. 1999. "The bipolar kinesin, KLP61F, cross-links microtubules within interpolar microtubule bundles of Drosophila embryonic mitotic spindles." *J Cell Biol* 144(1): 125–38.
62. Sisson, JC, C Field, R Ventura, A Royou, and W Sullivan. 2000. "Lava lamp, a novel peripheral golgi protein, is required for Drosophila melanogaster cellularization." *J Cell Biol* 151(4): 905–18.
63. Snapp, EL, T Iida, D Frescas, J Lippincott-Schwartz, and MA Lilly. 2004. "The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts." *Mol Biol Cell* 15(10): 4512–21.
64. Solc, P, RM Schultz, and J Motlik. 2010. "Prophase I arrest and progression to metaphase I in mouse oocytes: comparison of resumption of meiosis and recovery from

G2-arrest in somatic cells." *Mol Hum Reprod* 16(9): 654–64.

65. Stevens, NR, H Roque, and JW Raff. 2010. "DSas-6 and Ana2 coassemble into tubules to promote centriole duplication and engagement." *Dev Cell* 19(6): 913–19.

66. Strnad, P, S Leidel, T Vinogradova, U Euteneuer, A Khodjakov, and P Gonczy. 2007. "Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle." *Dev Cell* 13(2): 203–13.

67. Thomas, BJ, DA Gunning, J Cho, and L Zipursky. 1994. "Cell cycle progression in the developing Drosophila eye: roughex encodes a novel protein required for the establishment of G1." *Cell* 77(7): 1003–14.

68. Tokuyasu, KT, WJ Peacock, and RW Hardy. 1972. "Dynamics of spermiogenesis in Drosophila melanogaster. I. Individualization process." *Z Zellforsch Mikrosk Anat* 124(4): 479–506.

69. Villeneuve, J, M Scarpa, M Ortega-Bellido, and V Malhotra. 2013. "MEK1 inactivates Myt1 to regulate Golgi membrane fragmentation and mitotic entry in mammalian cells." *EMBO J* 32(1): 72–85.

70. Von Stetina, JR, and TL Orr-Weaver. 2011. "Developmental control of oocyte maturation and egg activation in metazoan models." *Cold Spring Harb Perspect Biol* 3(10): a005553.

71. Wells, NJ, N Watanabe, T Tokusumi, W Jiang, MA Verdecia, and T Hunter. 1999. "The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression." *J Cell Sci* 112 (Pt 19): 3361–71.

72. White-Cooper, H, MA Schafer, LS Alphey, and MT Fuller. 1998. "Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid

differentiation with meiosis I in Drosophila." *Development* 125(1): 125–34.

73. Wilson, PG. 1999. "BimC motor protein KLP61F cycles between mitotic spindles and fusomes in Drosophila germ cells." *Curr Biol* 9(16): 923–26.

74. Wilson, PG. 2005. "Centrosome inheritance in the male germ line of Drosophila requires hu-li tai-shao function." *Cell Biol Int* 29(5): 360–69.

75. Wong, R, I Hadjiyanni, HC Wei, G Polevoy, R McBride, KP Sem, and JA Brill. 2005. "PIP2 hydrolysis and calcium release are required for cytokinesis in Drosophila spermatocytes." *Curr Biol* 15(15): 1401–06.

76. Yasuno, Y, J Kawano, YH Inoue, and MT Yamamoto. 2013. "Distribution and morphological changes of the Golgi apparatus during Drosophila spermatogenesis." *Dev Growth Differ* 55(6): 635–47.

77. Yeh, E, L Zhou, N Rudzik, and GL Boulianne. 2000. "Neuralized functions cell autonomously to regulate Drosophila sense organ development." *EMBO J* 19(17): 4827–37.

78. Yue, L, AC Spradling, M Zaccai, and HD Lipshitz. 1992. "hu-li tai shao, a gene required for ring canal formation during Drosophila oogenesis, encodes a homolog of adducin.

79. Zaccai, M, and HD Lipshitz. 1996. "Role of Adducin-like (hu-li tai shao) mRNA and protein localization in regulating cytoskeletal structure and function during Drosophila Oogenesis and early embryogenesis." *Dev Genet* 19(3): 249–57.

Chapter III

Differential regulation of CyclinA-Cdk1 during *Drosophila* spermatocyte development

In this section I describe further experiments analyzing how inhibitory phosphorylation of Cdk1 by Myt1/Wee1 and Cdk1 activation by Cdc25 phosphatases are coordinated during male meiosis. The intention is to combine the data presented in this section with complementary data from the thesis of a previous PhD student (Dr. Ayeni) and to submit this as a joint first-author manuscript.

3.1 Introduction

The two major mitotic Cyclins A and B serve partially redundant but distinct functions during cell cycle progression during *Drosophila* development (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990; Knoblich and Lehner, 1993; Sprenger *et al.*, 1997; Dienemann and Sprenger, 2004; McCleland *et al.*, 2009; Bergman *et al.*, 2015). In other systems, Cyclin B-Cdk1 has been described as the key mitosis-promoting factor (maturation promoting factor: MPF) regulating the G2/M transition, whereas type-A Cyclins are required for additional functions during interphase (Strausfeld *et al.*, 1996; Sprenger *et al.*, 1997; Voronina *et al.*, 2003). In particular, Cyclin A association with Cdk2 has been implicated in regulation of DNA replication and S phase progression, whereas Cyclin A-Cdk1 is important for mitotic progression (Yang *et al.*, 1999; Fung *et al.*, 2007; De Boer *et al.*, 2008). Apart from its role in mitosis, Cyclin A also plays an essential function during meiosis, as classic experiments in *Xenopus* showed that injecting Cyclin A into 'G2-like' immature oocytes triggered the G2/MI transition through Cyclin B- Cdk1 activation, demonstrating functional redundancy with Cyclin B (Swenson *et al.*, 1986; Roy *et al.*, 1991; Furuno *et al.*, 1999). Similar observations

were reported for Cyclin A1 (but not A2) during murine spermatocyte development (Liu *et al.*, 1998; van der Meer *et al.*, 2004).

Despite the evidence that Cyclin A plays important roles in the execution of both mitosis and meiosis, we know surprisingly little about specific substrates for Cyclin A/Cdk1 and how its activity is regulated during the G2 phase, largely due to overlapping functions with Cyclin B. In *Drosophila,* Cyclin A is the only essential mitotic cyclin, as it can functionally substitute for loss of either Cyclin B or Cyclin B3 during mitosis (Lehner and O'Farrell, 1989a; Knoblich and Lehner, 1993). Spermatocyte-specific knockdown of Cyclin A experiments described in Chapter 2 have now revealed for the first time that Cyclin A is also essential during *Drosophila* male meiosis.

Drosophila spermatogenesis is an excellent model for addressing questions about Cyclin A-mediated cell cycle regulation, as this is the only mitotic cyclin expressed in spermatocytes during the prolonged (~90 hr) pre-meiotic G2 arrest period that precedes the synthesis of Cyclin B (and $Cdc25^{Twe}$) shortly before the end of this period (White-Cooper, 2010). As discussed in Chapter 2 (Fig. 2-13), endogenous Cyclin A as well as transgenic Cdk1(WT):VFP expressed using a *bam-Gal4* driver accumulate on fusomes throughout spermatocyte maturation, presumably as a Cyclin A/Cdk1:VFP complex. One key result from Chapter 2 was that Myt1 (an ER and Golgi-associated protein) appeared much more closely associated with fusomes very early in spermatocyte development, around the time of the pre-meiotic S/G2 phase transition. This brief association could be important to facilitate the catalytic activity of Myt1 to regulate CyclinA-Cdk1 required for the fusome checkpoint disrupted in early *myt1* mutant spermatocytes.

To test these ideas I generated a new transgenic *myt1* variant lacking catalytic activity to examine how manipulating enzymatic activity affected the fusome checkpoint. I also examined the effect of loss of putative interaction between Myt1and CyclinB-Cdk1 (shown in Appendix A), I also used genetic approaches to manipulate Wee1 expression and increase inhibitory phosphorylation of Cdk1 or by expressing $Cdc25^{Stg}$ to ectopically activate Cyclin A-Ckd1. This method of manipulating Cdk1 regulation revealed new insights into how Myt1 regulates fusome and centriole checkpoints during spermatocyte development.

3.2 Materials and Methods

3.2.1 Generation of Myt1 (N229A) variant

The active site of the *Drosophila* Myt1 catalytic domain is located between amino acids 220 and 232 (Jin *et al.*, 2008, #11563). This region is highly conserved among Myt1 kinases from humans, *X. laevis*, *A. pectinifera* and *C.elegans*. In humans, Asparagine 238 (N238) has been identified as a critical residue for Myt1 catalytic activity (Liu *et al.*, 1999), with mutation of this residue to an alanine (A) resulting in inactivation of catalytic functions. Based on sequence homology, residue N229 of *Drosophila Myt1* is predicted to correspond to the human counterpart of this residue (Fig. 3-1A). I therefore changed residue N229 to Alanine by site-directed mutagenesis using the following strategy. First, a dMyt1 cDNA was moved from a pCASPER vector to a pENTR vector using the Topo-cloning protocol (Invitrogen). The resulting pENTR-dMyt1 plasmid was used as a template with the following primers to introduce the desired N229A mutation. Forward-(5'GGACATTAAACTGGACGCCGT TCTGATCGGCGAG 3') and Reverse- (5'CTCGCCGATCAGAACGGCGTCCAGTTTAA TGTCC 3') were used for the PCR amplification with a Stratagene QuikChange II site-

directed mutagenesis kit (Agilent). Out of ten clones obtained two were identified to have the appropriate nucleotide change corresponding to a N229A replacement. DNA sequencing analysis indicting the nucleotide (codon) change corresponding to N229A mutation is shown in Appendix-B. The new Myt1 allele (N229A) was then moved into different destination vectors using the gateway cloning conditions.

3.2.2 Gateway cloning into *TV3-GFP* and *UASp-VFP* Vectors

In order to express Myt1(N229A) in *Drosophila* somatic and germline cells, I cloned the new variant from a pENTR vector into N-terminal GFP/VFP with UASp-VFP or *tv3* promoter (Wong *et al.*, 2005) destination vectors designed for Gal4-inducible or spermatocyte-specific expression, respectively. After sequencing to confirm their identity, these Myt1(N229A) constructs were microinjected into *Drosophila* embryos to generate transgenic lines (BestGene).

3.2.3 Fly stocks

To express transgenic proteins in late spermatogonia and mature spermatocytes I used *bam-Gal4* (McKearin and Spradling, 1990; Chen and McKearin, 2003) and *topi-Gal4* (Raychaudhuri *et al.*, 2012) stocks, respectively. I used *neuralized Gal-4* (Yeh *et al.*, 2000) to express in the sensory organ precursor cells. I also used stocks carrying the mutant alleles for $myt1^{R6}$ (Campbell Lab), wee^{ES1} (Campbell Lab) and *twe* (Alphey *et al.*, 1992b; Courtot *et al.*, 1992b). I used the following Myt1 transgenes for their ectopic expression: *UASp::EGFP Myt1(WT), UASp::VFP Myt1(N229A),Tv3::GFP Myt1(WT) and Tv3::GFP Myt1(N229A).* Cdk1 related transgenes are the following: $UASp::Cdk1^{TRiP}$ (Harvard Medical school), *UASp::Cdk1(WT) VFP, UASp::Cdk1(T14A) VFP, UASp::Cdk1(Y15F) VFP, UASp::Cdk1(T14A,Y15F) VFP, Tv3::Cdk1(WT) VFP, Tv3::Cdk1(T14A) VFP,*

Tv3::Cdk1(Y15F) VFP, Tv3::Cdk1(T14A,Y15F) VFP. Transgenes carrying *UASp::Wee1 VFP (Campbell lab), UASp::String* (KK library) were also used in this chapter.

3.2.4 Immunochemistry experiments

The immuno-labeling and western blot experiments were performed using identical methods described for Chapter 2. The following primary antibodies were used at the previously mentioned concentrations for fixed spermatocyte immuno labeling: mouse anti-Hts (DSHB; 1:5) to label fusomes, rabbit anti- Aur A-T228P (abcam; 1:500) to label centrosome, mouse anti-Cyclin A (Lehner and O'Farrell, 1989b) (DSHB; 1:10), mouse anti-MPM2 (Cell signaling, 1:500) to label phosphorylated Cdk1 substrates, rabbit anti-phosphorylated Histone H3-S10 (Upstate, PH3-1: 5000). For western blot analysis, Phospho-specific Cdk1 antibodies obtained from Cell Signaling were used at the following concentrations: rabbit anti-Cdk1-T14p (1:1000) and rabbit anti-Cdk1-Y15p (1:5000). mouse anti-MPM2 (Cell signaling, 1:5000) was used to detect phosphorylated Cdk1 substrates. Transgenic expression of VFP tagged Cdk1 phospho-isoforms was detected using mouse anti-GFP antibodies (Clontech; 1: 5000) and mouse anti-Actin (1:5000) was used for loading control

3.2.5. Scoring *myt1* mutant bristles

Frequency of bristle defect was estimated based on the appearance of the shaft (Fichelson *et al.*, 2005; Jin *et al.*, 2008). This data was generated based on the eight major macrochaetae bristles located on the scutum. Normal bristles have a single long shaft. *myt1* mutant bristle defects were classified into three categories: (1) short shafts that were noticed with distinctively reduced bristle length (2) no shaft indicates the absence of bristle (3) bristle that were duplication. An equal number of male and female flies was included in this data collection. Transgenes of Myt1(WT) , Myt1 (N229A) and *neuGal4* are genetically combined

with *myt1*^{R6} mutant allele to enable the expression of these transgenes in *myt1* mutant background. *UASp::EGFP-Myt1(WT)* and *UASp::VFP-Myt1(N229A)* were expressed in the sensory organ lineage of *myt1* mutants using *neuralized Gal-4* driver (Yeh *et al.*, 2000).

3.2.6 Male Sterility Assays

The strategy used for performing sterility assays was adapted from a published protocol (Gonczy *et al.*, 1994). Unless otherwise noted, 30 individual males were tested to analyze each genotype. 1 to 2 day old single male flies from the appropriate genotypes were crossed with 3 age matched *yw* virgin female flies. After 4 days, parents were tipped over to new vials to generate a second brood. We counted the number of adult progeny from each vial as a measure of male fertility. A cross between *myt1*^{R6}/+ heterozygous control males with *yw* virgin females was always included as positive control.

3.3 Results

3.3.1 Characterization of Myt1 (N229A) allele

Developmental consequences of loss of Myt1 including spermatocyte organelle defects (Chapter 2) and sensory organ bristle phenotypes were characterized using *myt1* null allele (Jin *et al.*, 2008). To specifically characterize the requirement of Myt1 kinase function, I generated kinase-inactive Myt1 by mutating the conserved asparagine amino acid (N229) that was identified as a putative residue for Myt1 catalytic activity (Liu *et al.*, 1999; Wells *et al.*, 1999). GFP tagged transgenic Myt1(N229A) was expressed using *Tv3* promoter variants in *myt1* mutant spermatocytes (*Tv3::GFP Myt1(N229A)/CyO; myt/myt*). I assayed catalytic activity towards endogenous Cdk1 by performing western-blot experiments with phospho-specific antibodies recognizing Cdk1-T14p and Cdk1-Y15p isoforms (Fig. 3-1B). Both

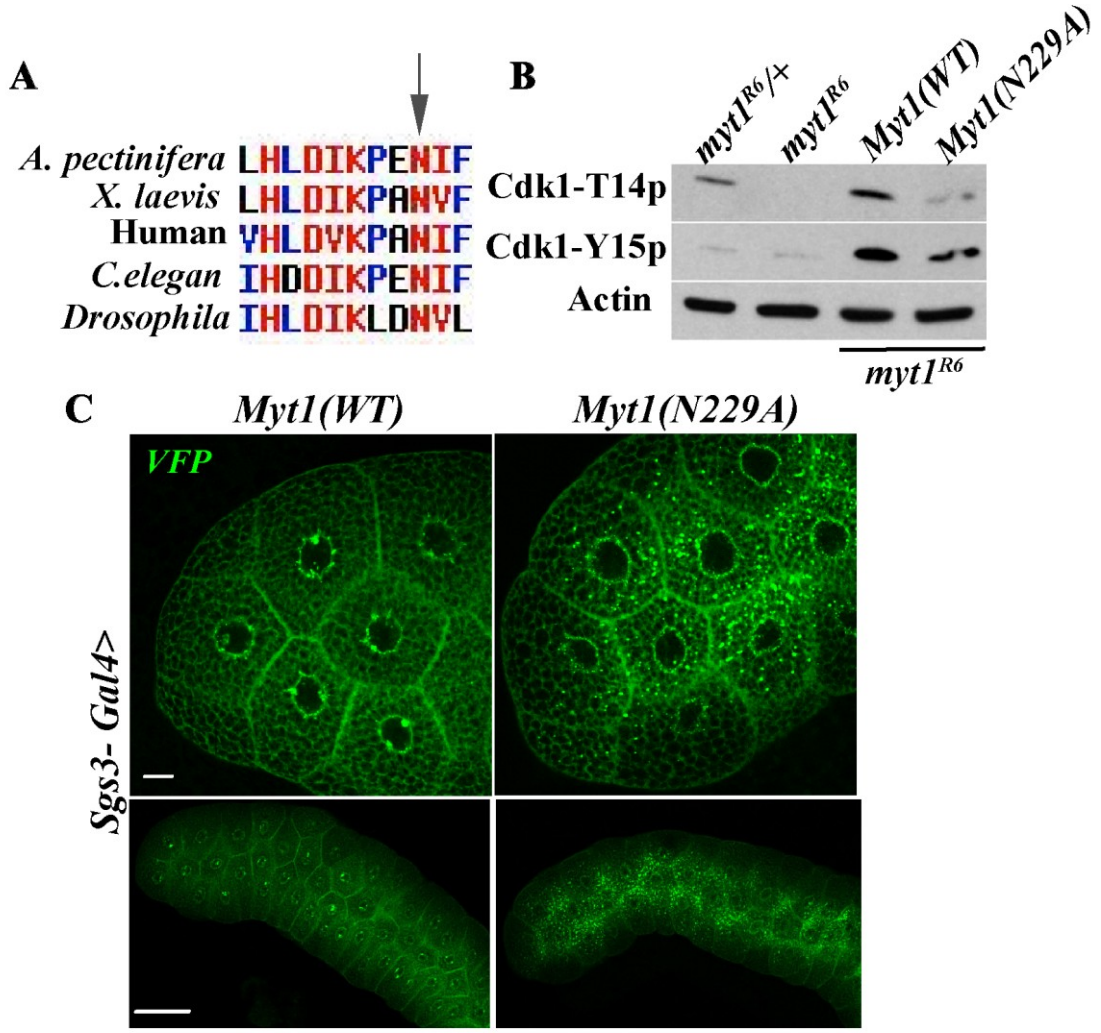

Fig. 3-1: Analysis of Myt1 kinase-inactive (N229) allele

(A) Myt1 kinase active domain is conserved across many metazoan animal models. The conserved Asparagine (N) residue (arrow) was mutated to generate kinase-inactive Myt1. (B) Western blot analysis of Myt1 catalytic activity shows normal levels of Cdk1 T14 and Y15 phosphorylation in *myt1*/+ control testes sample (lane 1). Cdk1-T14 phosphorylation indicates Myt1 mediated Cdk1 regulation that is absent in *myt1* mutant protein sample (lane 2). Cdk1-Y15 phospho-isoforms are detected in both *myt1*/+ and *myt1* (alone) samples, although the levels appear reduced. Transgenic expression of tv3 driven Myt1(WT) in *myt1* mutant spermatocytes restored Cdk1-T14 phosphorylation (lane 3). Myt1 with the N229A mutation shows reduced T14p levels (lane), as compare to the WT control (C) UASp-Gal4 inducible expression of the VFP tagged variants including wildtype (WT) or kinase inactive/hypomorph (N229A) are shown in Salivary gland cells using Sgs3 Gal4 driver. Scale bar-10 and 30 microns.

Wee1 and Myt1 kinases can phosphorylate Cdk1 on residue Y15 (Y15p), however Myt1 alone phosphorylates residue T14 (T14p). In lane 1 of Fig 3-1B, a *myt1*/+ testes extract serving as a positive control showed the presence of both Cdk1 T14 and Y15 phospho-isoforms. The homozygous *myt1* null mutants shown in lane 2 had no detectable Cdk1-T14p isoform however, confirming the loss of Myt1 kinase activity. Expression of transgenic Myt1(WT) in *myt1* mutant spermatocytes (lane 3) completely rescued this biochemical defect, whereas expression of the N229A catalytic mutant Myt1 variant in a *myt1* mutant background showed lower but detectable Cdk1-T14p and Y15p isoforms (lane 4). These results show that mutation of the N229 residue only reduced Myt1 kinase activity, but did not make the allele fully kinase-inactive. Myt1 (N229A) should therefore be considered as a hypomorphic allele.

3.3.2 Sub-cellular localization of Myt1(N229A)

Myt1 was originally described as an ER and Golgi membrane-bound kinase whose localization was mediated through a poorly conserved trans-membrane hydrophobic domain located between the kinase domain and the C-terminus of the protein (Liu *et al.*, 1997). To test whether or not the N229A mutation affected its ER or Golgi membrane association, I examined the sub-cellular localization of the transgenic proteins compared to GFP-Myt1(WT) controls in two tissues: fixed third instar larval salivary gland endo-replicating cells and live spermatocytes.

In salivary gland cells expressing EGFP-Myt1 (WT) using an *Sgs3-Gal4* driver (Fig. 3-1C) the tagged protein appeared as a reticular cytoplasmic network enriched at perinuclear foci. In cells expressing VFP-Myt1(N229A) however, cytoplasmic foci rather than perinuclear foci appeared to predominate. In glands imaged at lower objective (bottom panel)

the VFP-Myt1(N229A) appeared to be enriched at the site of cilia located near the lumen interface of the gland (J. Brill, personal communication). These endoreplicating salivary gland cells lack Cdk1 as well as mitotic CyclinA or B proteins (Sigrist and Lehner, 1997), so it is unclear what the significance of this localization is and whether differences in Myt1 catalytic activity would be expected to have an effect.

3.3.3 Functional complementation of *myt1* mutant defects by Myt1(N229A)

As a first test to functionally characterize the transgenes I tested for rescue of the *myt1* mutant bristle phenotype, involving abnormal shaft and socket structures (Fichelson *et al.*, 2005; Jin *et al.*, 2008). For this experiment I expressed Gal4-inducible *UASp*-EGFP-Myt1(WT) and VFP-Myt1(N229A) transgenes in *myt1* mutant sensory organ cells using the *neuralized Gal-4* driver. Expression of EGFP-Myt1(WT) fully rescued of the *myt1* mutant bristles, whereas VFP-Myt1(N229A) expression rescued only partially (Table 3.1). This result shows that VFP-Myt1(N229A) behaves as a hypomorphic allele with respect to sensory organ development, consistent with the reduced catalytic activity shown in Fig.3-1B.

I also analyzed EGFP-Myt1(WT) and VFP-Myt1(N229A) expressed during *Drosophila* embryogenesis. Observations (made by Dr. Homola, unpublished) showed that maternal expression of VFP-tagged wild-type Myt1 was lethal very early during embryonic development, showing that this stage was very sensitive to Myt1 activity. I performed a similar test with maternally expressed UASp-VFP-Myt1(N229A) using a strong maternally driver, nanos-Gal4 (Wang and Lehmann, 1991) and examined newly laid embryos stained with Hoechst 33258 to visualize DNA. As expected from earlier observations, *nos>EGFP*

Table 3-1: Frequency of bristle defect in *myt1* mutants

Genotype	Normal bristle	Short shaft	no shaft	2x shaft	Total bristle	% Defect	# Flies counted
myt1/+	260	0	3	9	272	4.4	34
myt1^{R6}	42	65	57	26	168	88.1	21
neuGal4>Myt1 WT(myt1^{R6})	349	0	6	21	376	7.2	47
neuGal4>Myt1 N229A(myt1^{R6})	60	45	27	28	160	62.5	20

Frequency of bristle defect was estimated based on the appearance of the shaft (*Jin et al, 2008*). This data was generated based on the eight major macrochaetae bristles located on the scutum. Normal bristles have a single long shaft. Bristle defects (showed in gray columns) are classified in three categories: short shafts are noticed with distinctively reduced bristle length, no shaft indicates the absence of bristle and 2x shaft denotes the bristle duplication. Equal number of male and female flies was included in this data collection. Transgenes of Myt1(WT), Myt1 (N229A) and *neuGal4* are genetically combined with *myt1^{R6}* mutant allele to enable the expression of these transgenes in *myt1* mutant background.

Myt1(WT) control embryos showed a complete absence of syncytial nuclear divisions. In contrast, *nos>VFP-Myt1(N229A)* embryos showed no apparent defects in early embryo development and the progeny were able to continue into the larval stage, however the post-larval development was not examined in my experiments. Early embryos are therefore sensitive to wild-type levels of Myt1 activity but not to the activity of a catalytically compromised variant, indicating that this phenotype is dosage-sensitive.

3.3.4 Expression of Myt1 (N229A) does not rescue *myt1* mutant fusomes but does restore centriole engagement in immature spermatocytes.

Having demonstrated that Myt1(N229A) was both hypomorphic for catalytic activity and compromised for function in early embryos, I examined this allele for rescue of *myt1* mutant defects in pre-meiotic spermatocytes. VFP-tagged wild-type Myt1 and Myt1(N229A) were expressed in *myt1* mutant early spermatocytes using a *bam-Gal4* driver and the fusomes were labeled with Hts antibodies in fixed testes. Expression of EGFP-Myt1(WT) completely restored the fusome in *myt1* mutant spermatocytes, whereas VFP-Myt1(N229A) did not (Fig. 3-2A). To examine rescue of the centriole engagement defect in *myt1* mutants, I labeled fixed spermatocytes with antibodies against Aur A-T288p (which detect an M phase-specific phospho-isoform) and β-Tubulin antibodies. Unlike the fusome results, VFP-Myt1 (N229A) expressed either at early or late stages of spermatocyte development both completely rescued the *myt1* mutants centriole dis-engagement defect similar to EGFP-Myt1 (WT) as shown in Fig. 2-3A. The *myt1* mutants expressing *bam>VFP-Myt1 (N229A)* were also weakly fertile (Fig. 3-2B), implying that chromosome mis-segregation and other meiotic defects that we hypothesize to be the reason for sterility were also suppressed. Thus, partially restoring Myt1

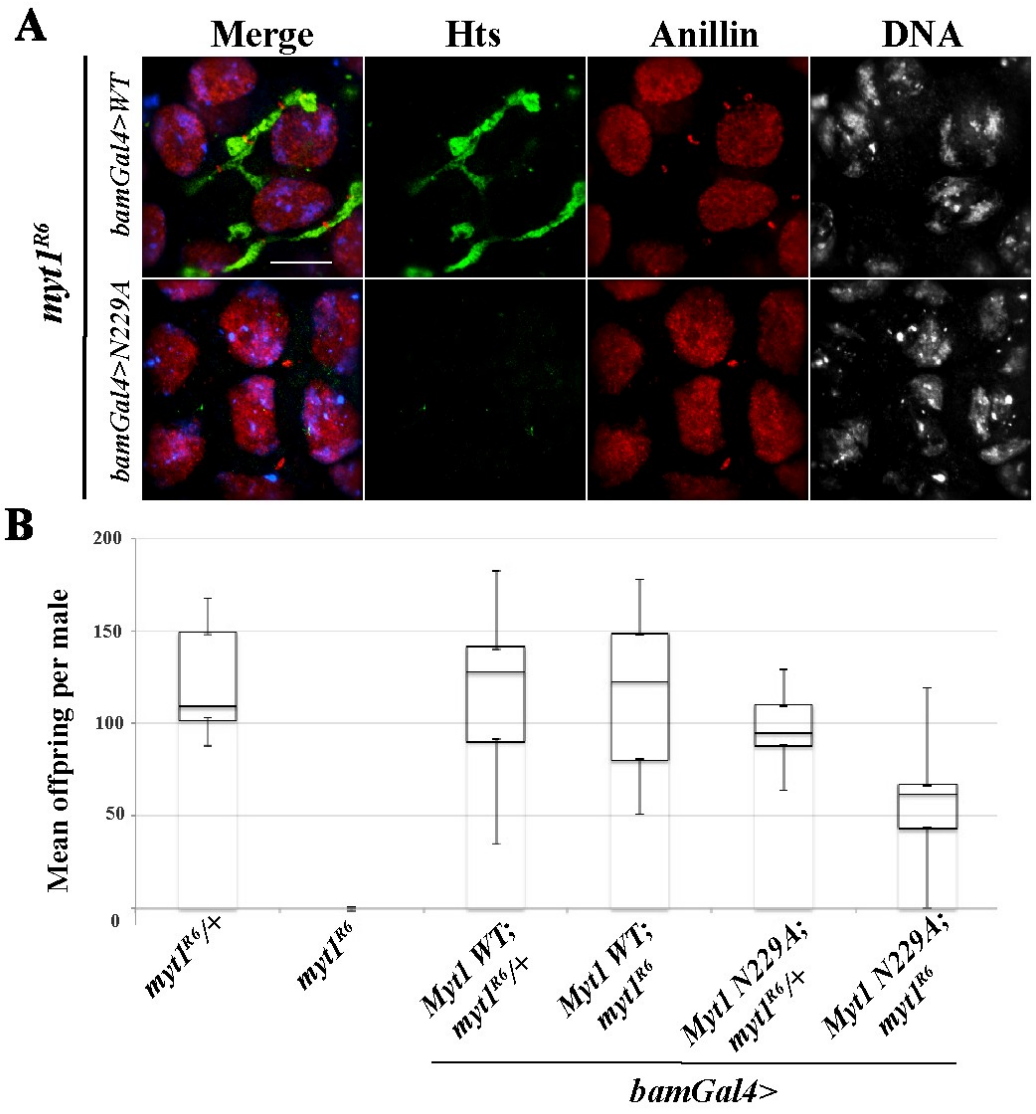

Fig. 3-2: Ectopic expression of Myt1 (N229A) does not rescue *myt1* mutant fusomes, but partially rescue sterility

(A) The germ line specific inter-cellular bridges are labeled with fusome (Hts, Green) and ring canal (Anillin, red) specific markers. *myt1* mutant testes expressing *bamGal4 >Myt1(WT)* reveals complete restoration of fusome branches in 16 cell (S3-4) spermatocytes, whereas the *Myt1(N229)* shows no rescue the these structures. The DNA labels represent the S3-S4 stage of *myt1* spermatocytes. Scale bar -10 microns. (B) Individual males of indicated genotypes were crossed to 3 *yw* females and the number of progeny obtained from each of this cross was counted. For each genotype 30 males were tested, except *Myt1(N229A); myt1,* in which only 10 were used. The graphs shows, an average of 150±20 progeny per male in *myt1/+*control and no progeny in *myt1* mutants. Expression of *bamGal4* driven EGFP-Myt1(WT) in *myt1* mutants spermatocytes produced an average of 150±30 progeny per male, whereas VFP-Myt1(N229A) produced approximately 70±65.

activity with a hypomorphic allele was not sufficient for regulating Cdk1-Cyclin A to satisfy the fusome checkpoint of *myt1* mutants, however, it appeared to be adequate to rescue the centriole engagement phenotype. Based on these observations I speculate that Myt1-mediated meiotic organelle checkpoint mechanisms require different levels of Cdk1 phospho-inhibition to protect different cytoplasmic structures. Importantly, these results show that centriole disengagement rather than fusome defects were the primary cause of *myt1* mutant sterility.

3.3.5 Wee1-VFP can also rescue centriole dis-engagement and male sterility without suppressing *myt1* mutant fusome defects

Drosophila Wee1 and Myt1 function redundantly in most developmental contexts, however the phenotypes I have described in *myt1* mutant spermatocytes indicate that endogenous Wee1 does not normally substitute for Myt1 in regulation of pre-meiotic organelle checkpoints (Jin *et al.*, 2008). To examine how ectopic Wee1 expression might affect Myt1 organelle checkpoint functions described in Chapter 2, I examined *myt1* mutant phenotypes in spermatocytes expressing *Wee1-VFP* under *bam-Gal4* control. First, I examined sub-cellular localization of Wee1-VFP. Previous studies have shown that in most developmental contexts, endogenous Wee1 detected by immuno-labeling is a primarily nuclear protein (E. Homola, pers. comm.), as is Wee1-VFP expressed using the UAS/Gal4 system (S. Campbell, pers. comm). In *myt1*/+ early (polar) S1-S2 spermatocytes, Wee1-VFP expressed under *bam-Gal4* control was detected primarily in the cytoplasm, where it appeared to be enriched at spectrosome/fusome structures stretching between cells in each cyst (Fig 3-3A). Fixed *bam>Wee1-VFP* spermatocytes labeled with Hts antibodies also showed Wee1-VFP co-

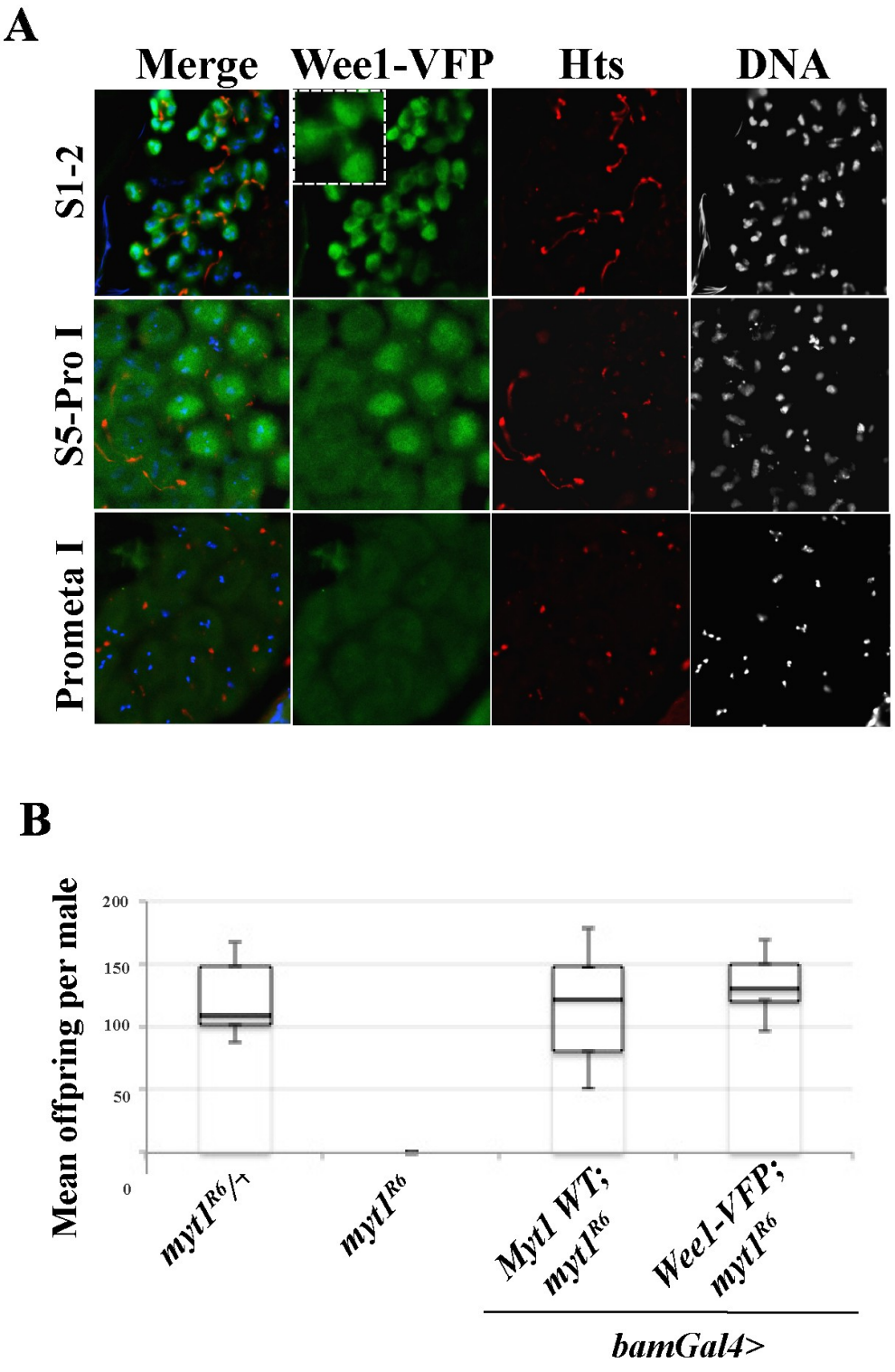

Fig. 3-3: Transgenic Wee1-VFP completely rescues *myt1* mutant sterility

(A) Fixed 16 cell spermatocytes expressing Wee1-VFP (Green) are co-labeled with Hts fusome marker (red). Wee1-VFP signals in polar spermatocyte (S1-2) are co-localized with Hts labels (arrowheads). Wee1-VFP appears both in cytoplasm and nucleus of S4-5 spermatocytes and in the prophaseI stage it is detected primarily in nucleus. No or less Wee1-VFP was detected in the late prometaphaseI spermatocytes. Prophase I and prometaphase I stage spermatocytes (encircled) are staged based on their DNA pattern and fragmented hts labeled fusomes. (B) The fertility graphs shows, an average of 150±20 progeny per male in *myt1/+*control and no progeny in *myt1* mutants. Expression of *bamGal4* driven EGFP-Myt1(WT) in *myt1* mutants spermatocytes produced an average of 150±30 progeny per male, and Wee1-VFP produced 150±10 per male. For each genotype approximately 30 individual males were tested.

localization with the fusome (Fig 3-3A). In mature S5 and prophase spermatocytes however, Wee1-VFP was found either primarily in the nucleus or in the cytoplasm, suggesting there was a developmental transition where the Wee1 protein moves into the nucleus at G2/MI, similar to the translocation of Cyclin A and Cdk1 into the nucleus at this time. At prometaphase I when the chromosomes condense, Wee1-VFP was primarily detected in the nucleus, but by early metaphase I the protein had largely disappeared. This evidence therefore suggests that Wee1 is degraded during MI of male meiosis, reminiscent of descriptions of Wee1 degradation in HeLa cells (Watanabe *et al.*, 1995; Smith *et al.*, 2007) and in *Xenopus* oocyte maturation (Tang *et al.*, 1993; Mueller *et al.*, 1995).

To assay how ectopic expression of Wee1-VFP in spermatocytes using *bamGal4* would affect *myt1* mutant spermatogenesis, I performed fertility assays, mating individual males (n=30) with 3 females each. I could confirm that the males were in fact *myt1* mutants because they exhibited bristle defects (Jin *et al.*, 2008). Surprisingly, *bam>Wee1-VFP* expression completely rescued *myt1* mutant male sterility (Fig.3-3B), indicating that Wee1 can functionally substitute for loss of Myt1 activity during *Drosophila* male meiosis when expressed at high levels in early spermatocytes. Although we have not characterized endogenous Wee1 expression in the testes ourselves, information posted for this gene on FlyBase indicates that the endogenous gene is expressed at relatively high levels in this tissue (no spatial information, however). These results therefore indicate that presence of normal endogenous Wee1 expression is perhaps inadequate for loss of Myt1 activity.

I also examined whether ectopic expression of Wee1-VFP could rescue *myt1* mutant fusome defects that are detectable in early spermatocytes. Immuno-labeling with Hts showed normal fusomes in *myt1*/+ spermatocyte controls and also in *myt1* mutant *bam>Wee1-VFP*

spermatogonia, however *myt1* mutant *bam>Wee1-VFP* spermatocytes showed little or no Hts fusome labeling (Fig.3-4A). These results show that Wee1-VFP does not substitute for Myt1 with respect to the spermatocyte fusome checkpoint, in spite of functional complementation of the sterility defect.

To study how ectopic Wee1 expression affected centriole engagement I examined *bam>Wee1-VFP* expression in *myt1* mutant spermatocytes that were co-labeled with Aur-A T228p and β-tubulin antibodies. Centriole dis-engagement was completely restored in these spermatocytes (Fig. 3-4B), showing that Wee1 could substitute for Myt1 in this meiotic role and re-inforcing the conclusion that rescue of *myt1* mutant sterility depends on the centriole engagement checkpoint. Since Wee1-VFP was clearly present and abundant in the cytoplasm of early spermatocytes, failure to rescue the fusome defect of *myt1* mutants could not easily be explained by a lack of expression or an inability to interact with cytoplasmic Cdk1. Instead, these paradoxical findings suggest that differences in the ability of Wee1 to inhibit Cyclin A-Cdk1 (present throughout spermatocyte development) versus Cyclin B-Cdk1 (which is synthesized in S6 stage spermatocytes) might be the explanation for these discrepancies. Alternatively, the failure of endogenous or transgenic Wee1 to complement specialized functions of Myt1 that involve the spermatocyte fusomes may reflect an alternative mechanism for regulating Cyclin A-Cdk1 by Myt1 that is not shared with Wee1. In Chapter-1 I showed that Rux (CKI specific to CyclinA complex) ectopic expression suppressed *myt1* mutant fusome phenotype further indicating that Wee1 may contribute dispensable inhibitory regulations towards CyclinA-Cdk1 as compare to the CyclinB-Cdk1.

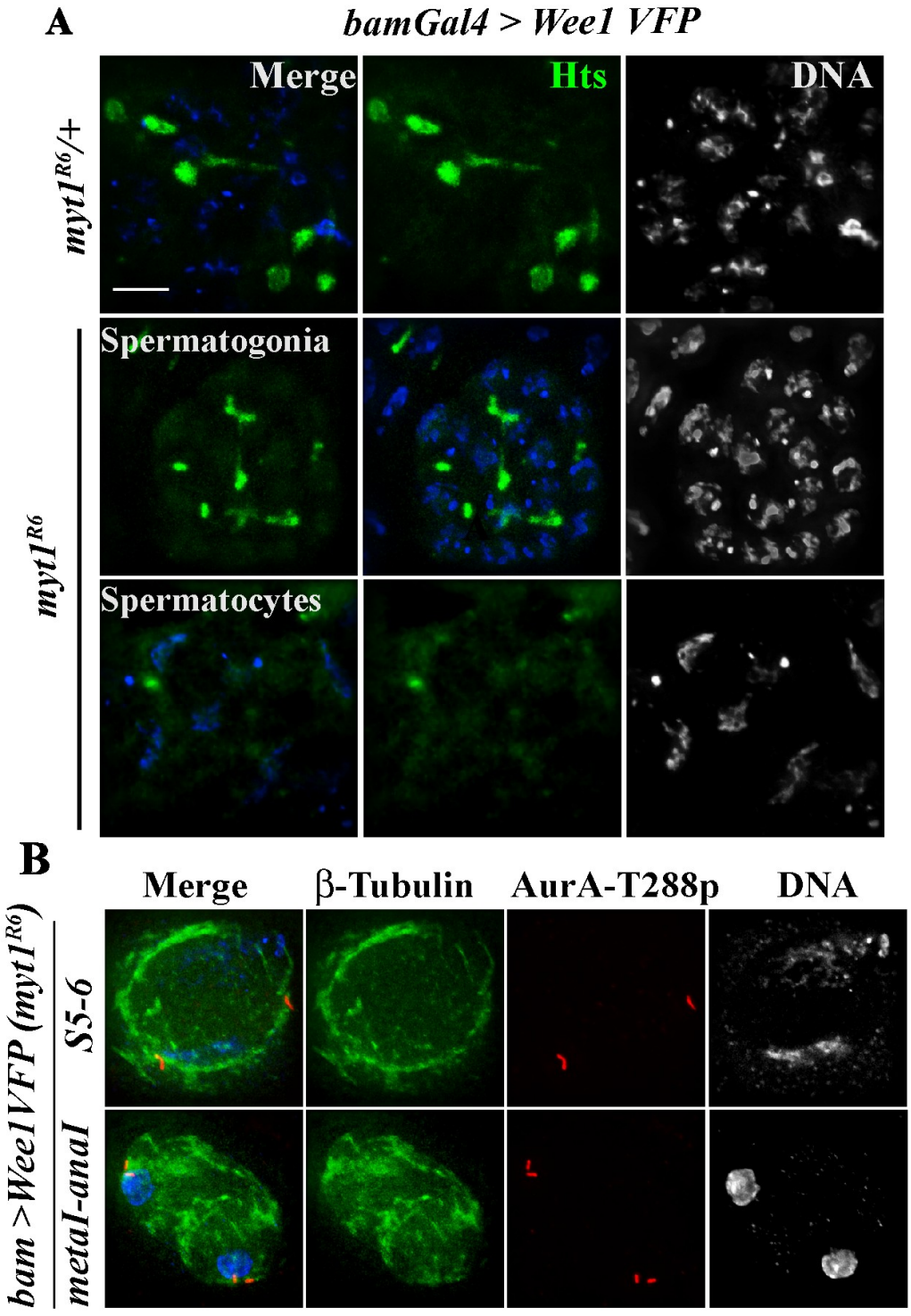

Fig. 3-4: Transgenic Wee1-VFP does not rescue *myt1* fusome defects, but completely suppresses meiotic centrosome abnormalities

(A) The Hts immuno-labeling in *myt* spermatogonia shows normal fusomes, whereas the spermatocytes expressing *bam>Wee1-VFP* show no fusome branches as compare to the *myt1/+*. (B) *myt1* spermatocytes expressing *bam>Wee1-VFP* were labeled with β–Tubulin (green) and AurA-T288p isoform (red) antibodies. The mature S5-6 spermatocytes show two normal centrosomes, each contained a pair of V shaped centrioles indicating the restoration of mutant centriole dis-engagement defects. Centriole dis-engagement is detected only during the metaI-anaI stage. Scalebar-10 microns**.**

3.3.6 Myt1 fusome checkpoint function is independent of Cdc25^{Twe} activity

The BrdU pulse chase timing experiment described in Chapter 2 provided no evidence for a precocious G2/MI transition during *myt1* mutant meiosis, indicating that the timing of C*dc25^{twe}* expression was not perturbed in these spermatocytes (Fig. 2-2). To assay the effects of Cdc25^{Twe} on Cdk1 activity in *myt1* spermatocytes I performed western blots of whole testes extracts probed with the MPM2 antibody (Fig. 3-5A), which detects mitotic and meiotic phospho-epitopes targeted by Cdk1 (Maro *et al.*, 1988; Westendorf *et al.*, 1994). In *myt1*/+ controls, MPM2 labeling detected multiple proteins between 25 kDa to 200 kDa. As expected, there was a major reduction in number and intensity of MPM2-labeled proteins in the *twe* mutant sample, as previously reported (White-Cooper *et al.*, 1993). These results indicate that most of the MPM2 phospho-epitopes observed in these testes extracts were in fact meiotic substrates of Cdk1. In contrast, loss of either *wee1* or *myt1* did not markedly affect the MPM2 signal compared to the heterozygous control. These results suggest that proteins detected by MPM2 antibodies were largely phosphorylated by Cyclin B-Cdk1 kinase, which was not expressed until late stages of G2 phase arrested spermatocytes, not Cyclin A/Cdk1 which was present throughout this stage of development (White-Cooper *et al.*, 1998; White-Cooper, 2010). This explanation presumably accounts for the fact that the timing of MI is not grossly perturbed in the *myt1* mutants, because Cyclin B and Cdc25^{Twe} required for active Cyclin B/Cdk1 are not synthesized until late in stage S6.

In chapter II, I showed that *myt1* mutant spermatocytes undergo normal meiotic progression in most part of the MI specific events including the timing in which the chromosome condensed or the lamin broke down. However, cytoplasm associated meiotic progression including fusome dis-assembly or centriole dis-engagement was temporally

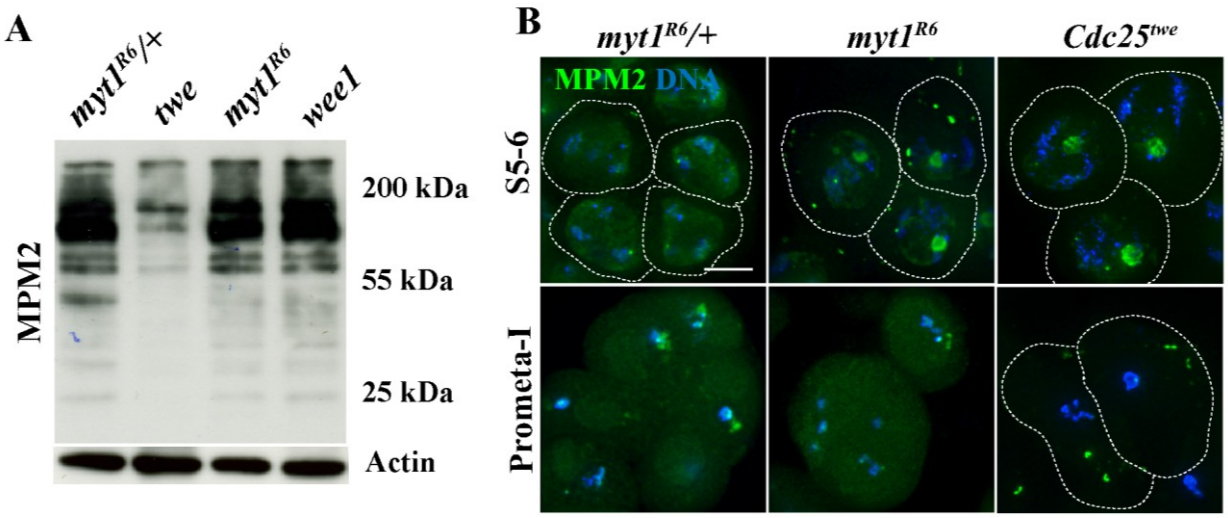

Fig. 3-5: *myt1* mutant pre-meiotic centriole defects are independent Cdc25^{Twe} activity

(A) Western blot data depicting the levels of meiotic Cdk1 targeted phospho-substrates detected from 10 testes extract. MPM2 antibodies recognize multiple Cdk1 targeted substrates that range between 200 to 25 kDa, and the 150 kDa size double band appear distinctive, however. *myt1/+*(lane 1) shows the normal levels of MPM2 signals, and *twe* reveals no or less signals, therefore used as a negative control for examining meiotic Cdk1 activity. Either *myt1* or *wee1* (lane 3,4) show no difference in the levels of MPM2 signals, as compare to the *myt1/+* control. (B) Fixed spermatocytes were immuno-labled with mMPM2 antibodies (green). The blue indicates the Hoechst DNA label. Appearance of the condensed chromosomes state indicates the prometaphaseI. Scale bar -10 microns.

uncoordinated in *myt1* mutants due to a premature Cdk1 activation. The above mentioned western blot detection of Cdk1 targeted phospho-substrates in total testes extract provided an estimate of meiotic Cdk1 activity, which appear unchanged in *myt1* mutant testes compared to the controls. This type of data did not provide a possible way of resolving the cytoplasmic or nuclear-specific meiotic activity. I therefore performed immunofluorescence-labeling experiments to examine fixed testes using MPM2 antibodies (Fig 3-5B). In *myt1/+* control spermatocytes, diffuse MPM2 signals were detected primarily in the nucleus during most of the G2 phase arrest (S3-S6) but appeared to diffuse into the cytoplasm during prometaphase-I, suggesting that Cdk1 activation was spreading from inside the nucleus to the cytoplasm during G2/MI. In *myt1* mutants, G2 phase arrested immature spermatocytes exhibited strong MPM2 labeling of a nuclear structure resembling the nucleolus, as well a small number of cytoplasmic foci (Fig 3-5B), however, prometaphase looked similar to the controls. Observations of nucleolar enrichment of MPM2 labels in *myt1* mutant are not consistent and also difficult to explain at this point. In the $Cdc25^{twe}$ mutants I also observed what appeared to be nucleolar labeling in immature spermatocytes, however the overall signal for MPM2 appeared reduced, as expected. Intriguingly, the MPM2 antibodies distinctively labeled structures resembling mature V-shaped centrosomes in $Cdc25^{twe}$ prometaphase-I spermatocytes (Riparbelli *et al.*, 2014). These data suggesting that Cdk1 phospho-substrates accumulate at centrosomes in $Cdc25^{twe}$ mutant could be evidence that $Cdc25^{Twe}$-independent Cdk1 activity has a role in centrosome-associated meiotic initiation during *Drosophila* male meiosis. In chapter-II, I showed that meiotic centriole behavior is primarily depend on the regulation of Cyclin A-Cdk1. Considering Chapter-II results I therefore presume that the centrosome associated Cyclin A-Cdk1 activity is independent of $Cdc25^{Twe}$.

In Chapter 2, I also presented evidence that Myt1 inhibition of CyclinA-Cdk1 during the prolonged G2 phase arrest of spermatocyte development is required to prevent fusome defects and premature centriole disengagement that can first be detected in early (beginning in stage S1) and mid (beginning in S3) spermatocytes, respectively. The timing of these defects is therefore detected well before the meiotic Cdk1-activating phosphatase $Cdc25^{Twe}$ is normally expressed (Courtot *et al.*, 1992a; Sigrist *et al.*, 1995), implying that Cyclin A-Cdk1 activities normally regulated by Myt1 are not dependent on $Cdc25^{Twe}$. To test this hypothesis I compared control and *twe* spermatocytes labeled with Cyclin A antibodies to mark the fusomes as well as cytoplasmically localized endogenous protein. The Cyclin A-labeled fusomes appeared normal in *twe* mutant G2 phase arrested spermatocytes and they disassembled normally at prometaphase I, when the chromosomes condensed (Fig. 3-6A). Fusome disassembly in prophase I spermatocytes therefore does not require $Cdc25^{Twe}$ activity, consistent with published reports that early MI events including chromosome condensation and centrosome maturation are independent of $Cdc25^{Twe}$ mediated Cdk1 activation (Lin *et al.*, 1996). I also co-labeled control, *myt1* and *twe* mutant spermatocytes with Lamin and PH3 antibodies to examine requirements for $Cdc25^{Twe}$ for nuclear envelope breakdown (NEB) and histone H3(S10) phosphorylation, respectively, during MI (Fig. 3-6B). As discussed in Chapter 2, these meiotic events occur at late prometaphase I in *myt1/+* controls and loss of Myt1 did not appear to affect the temporality of these events. In *twe* mutants, the Lamin signal looked quite different and PH3 labeling was not detectable compared to similarly staged controls, indicating that nuclear envelope breakdown and histone H3-S10 phosphorylation both require $Cdc25^{Twe}$ activity. Moreover, although centrosome maturation and elongation appeared normal in *twe* mutant spermatocytes, loss of

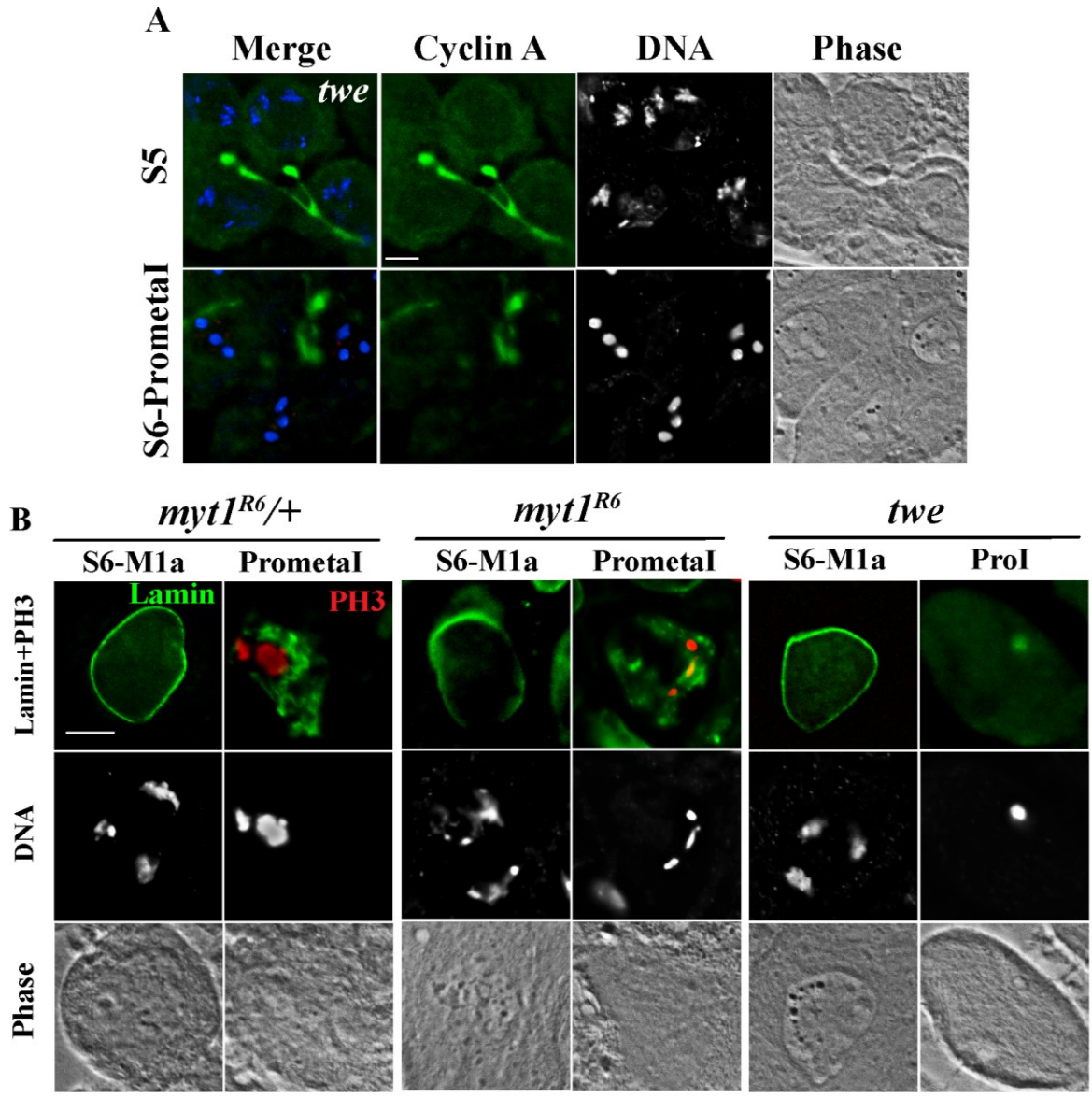

Fig. 3-6: *twe* mutants exhibit normal fusome disassembly.

(A) Fusomes of S5 stage *twe* mutant spermatocytes were shown with Cyclin A immuno-labels. The blue indicates the Hoechst DNA label. Fusome looks disrupted in the prometaphaseI *twe* spermatocytes. (B) The spermatocytes are co-labeled with lamin (green) and PH3 (red) antibodies. S6-ProphaseI spermatocytes of *myt1/+*,*myt1* and *twe* genotypes show an intact nuclear lamina. In both *myt1/+*and *myt1,* this structure appears disrupted during the prometaphase-I, the stage in which the PH3 labels are detected. In *twe* mutants no clear lamin disruption or PH3 labeling were detected. Scale bar -10 microns.

Cdc25^{Twe} blocked meiotic spindle assembly in metaphase-I and therefore all events that would normally occur after this stage (including centriole dis-engagement) could not be detected in *twe* mutants (not shown; this result was also reported in Dr. Ayeni's thesis). Collectively, all of these results confirm that meiotic progression consists of events that occur before prometaphase I which are independent of Cdc25^{Twe} as well as events after late prometaphase I that are dependent on Cdc25^{Twe}-mediated Cdk1 activation (White-Cooper *et al.*, 1993; Lin *et al.*, 1996).

3.3.7 Cdc25^{Stg}-dependent Cdk1 activation causes premature centriole disengagement and partial sterility

Having shown that *myt1* mutant meiotic organelle defects are independent of Cdc25^{Twe} mediated Cyclin A-Cdk1 activation, I examined if the mitotic homolog Cdc25^{Stg} has any role in triggering premature activation of Cyclin A-Cdk1 during early spermatocyte stage. I therefore ectopically expressed *Cdc2^{Stg}* transgene in early spermatocyte using *bam-Gal4* driver. Previous work from the Fuller lab had reported that ectopic expression of *bam-Gal4>Cdc25^{Stg}* resulted in spermatogonia undergoing 1 or 2 extra mitotic divisions to produce 32 and 64-cell pre-meiotic cysts (Insco *et al.*, 2009). I also observed similar defects with *bam*-driven Cdc25^{Stg} (Fig. 3-7A). Intriguingly, previously published work from our laboratory had shown that ~20% of *myt1* mutant germline cysts also undergo extra mitotic divisions (Jin *et al.*, 2005). Both observations indicate that limiting Cdc25^{Stg} activity after the fourth mitotic division is an essential step for restricting the number of spermatogonial divisions and making meiosis dependent on expression of Cdc25^{Twe} (Alphey *et al.*, 1992a).

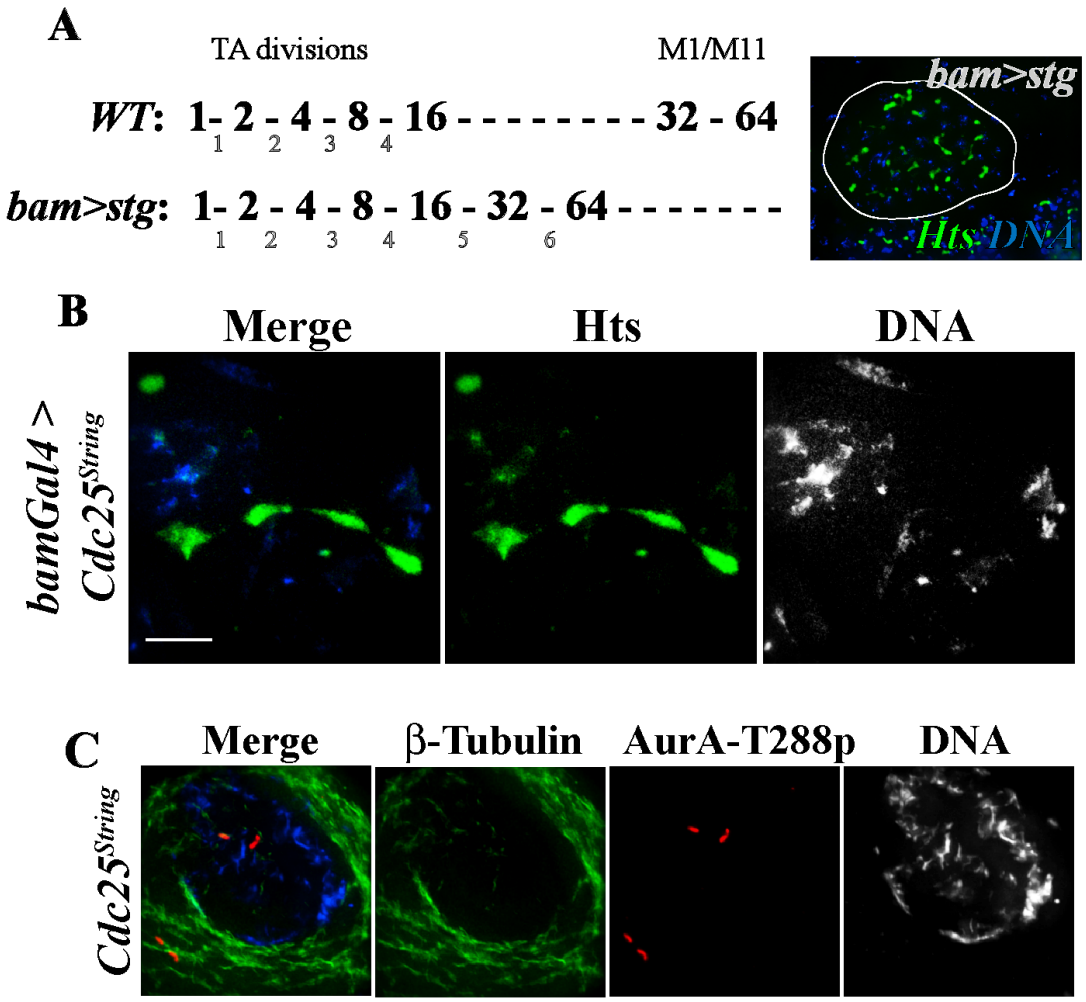

Fig. 3-7: Ectopic expression of *Cdc25^{Stg}* phenocopies *myt1* meiotic centriole defects

(A) Illustration of mitotic over proliferation phenotype observed in germ cells overexpressing Cdc25^{Stg} (bamGal4 driven). Normally, spermatogonia cells undergo only 4 round of TA divisions before differentiating into spermatocytes. *bam>Cdc25^{Stg}* germcells, however undergo 5 or 6 TA division resulting in 32 (right panel) or 64 spermatogonia cyst.(B) Hts immuno-labeled S3-4 *bam>Cdc25^{Stg}* spermatocytes exhibit normal fusomes (Green). (C) Late *bam>Cdc25^{Stg}* spermatocytes labeled with β–Tubulin (green) and AurA-T288p isoform (red) antibodies exhibit abnormal number of centrosomes/centrioles foci per cell that resemble *myt1* centriole dis-engagement phenotype.

To assess whether $Cdc25^{Stg}$ expression had any effect on the Myt1-dependent organelle checkpoints described in Chapter II, I examined fusome and centriole behavior in pre-meiotic *bam>Cdc25^{Stg}* spermatocytes. Fusomes labeled with Hts appeared normal (Fig. 3-7B), indicating that the Myt1-mediated fusome checkpoint mechanism was refractory to $Cdc25^{Stg}$ activity. When *bam>Cdc25^{Stg}* spermatocytes were labeled with AurA-T288p however the centrioles were prematurely disengaged (Fig. 3-7C) and 60% of *bam>Cdc25^{Stg}* spermatocytes exhibited multipolar spindles during MI, resulting in partial male sterility (Table 3-2). These results indicated that ectopic activation of Cyclin A-Cdk1 by *bam>Cdc25^{Stg}* only could bypass Myt1 mediated meiotic centriole engagement. I also noticed that most aspects of pre-meiotic arrest and the G2/MI transition appeared to be normal in *bam>Cdc25^{Stg}* spermatocytes, although *Cdc25^{Stg}* appear to trigger positive feedback response by its phosphatase activity targeting Cyclin A-Cdk1 (Fig. A-6, Appendix A). In most respects these results were similar to the phenotype of *myt1* mutants, indicating that Myt1-regulated fusome and centriole checkpoint mechanisms are different in their sensitivities to $Cdc25^{Stg}$ and $Cdc25^{Twe}$. The explanation for these differences may involve different activities of Cyclin A-Cdk1 and Cyclin B-Cdk1 complexes during early and late pre-meiotic G2 phase arrest, respectively.

3.3.8 Expression of phospho-acceptor Cdk1 mutants partially phenocopy *myt1* mutants

In Chapter II, I presented data showing that *bam-Gal4* driven expression of a Cdk1 phospho-acceptor mutant (Cdk1Y15F) in early spermatocytes could trigger premature fusome disassembly and disrupt centriole engagement, as indicated by Hts and AurA T288p immuno-labeling, respectively. Such effects were not observed upon expression of Cdk1(WT)

however, showing that Cdk1 activity rather than overall levels of Cdk1 protein were not rate-limiting for any of these effects. I also observed that Cdk1(Y15F) expression resulted in increased numbers of mitotic cells near the tip of the testes (Fig. 3-8A) labeled by antibodies against mitotic histone H3 (PH3). Mitotic over-proliferation in the *myt1* mutants can be attributed to two distinct factors: supernumerary transit-amplifying mitotic divisions (mentioned earlier) as well as ectopic divisions of the somatic cyst cells (Jin *et al.*, 2005). These results were therefore consistent with previous data indicating that inactivation of Cdk1 during exit from the 4th mitotic cycle is critical to properly co-ordinate the number of spermatogonial TA divisions.

In Fig. 3-8B, I show results from assaying the effects of different Cdk1 phospho-isoforms expressed in early spermatocytes by western blotting testes extracts and probing with anti-MPM2 (mitotic/meiotic phospho-epitopes), anti-Cdk1 T14p and anti-Cdk1 Y15p (phospho-inhibited isoforms), GFP (to detect the tagged transgenic proteins) and actin (as a loading control). As controls I used homozygous *myt1* mutants (lane 1) and *twe* mutants (lane 7) and expression of a germline-specific TRiP-Cdk1 (lane 2, equivalent to siRNA: *bam>TRiP-Cdk1*) to deplete endogenous Cdk1 as a negative control (Ni *et al.*, 2011). In testes extracts expressing transgenic Cdk1(WT), Cdk1(T14A), Cdk1(Y15F) or Cdk1(T14A,Y15F) the GFP antibodies detected a 61 kDa fusion protein (lanes 3 to 6), as expected for a VFP-tagged Cdk1 transgene. This was not detected in lanes 1, 2 or 7, also as expected. The MPM2 antibodies detected high levels of mitotic phospho-epitopes in the ~150 kDa range of extracts from the transgenic samples (lanes 3 to 6), whereas the *TRiP-Cdk1* (lane 2) and *twe* (lane 7) extracts from testes that cannot undergo meiosis showed reduced or no labeling. Curiously, MPM2 labeling appeared attenuated in the *myt1* mutant sample (lane

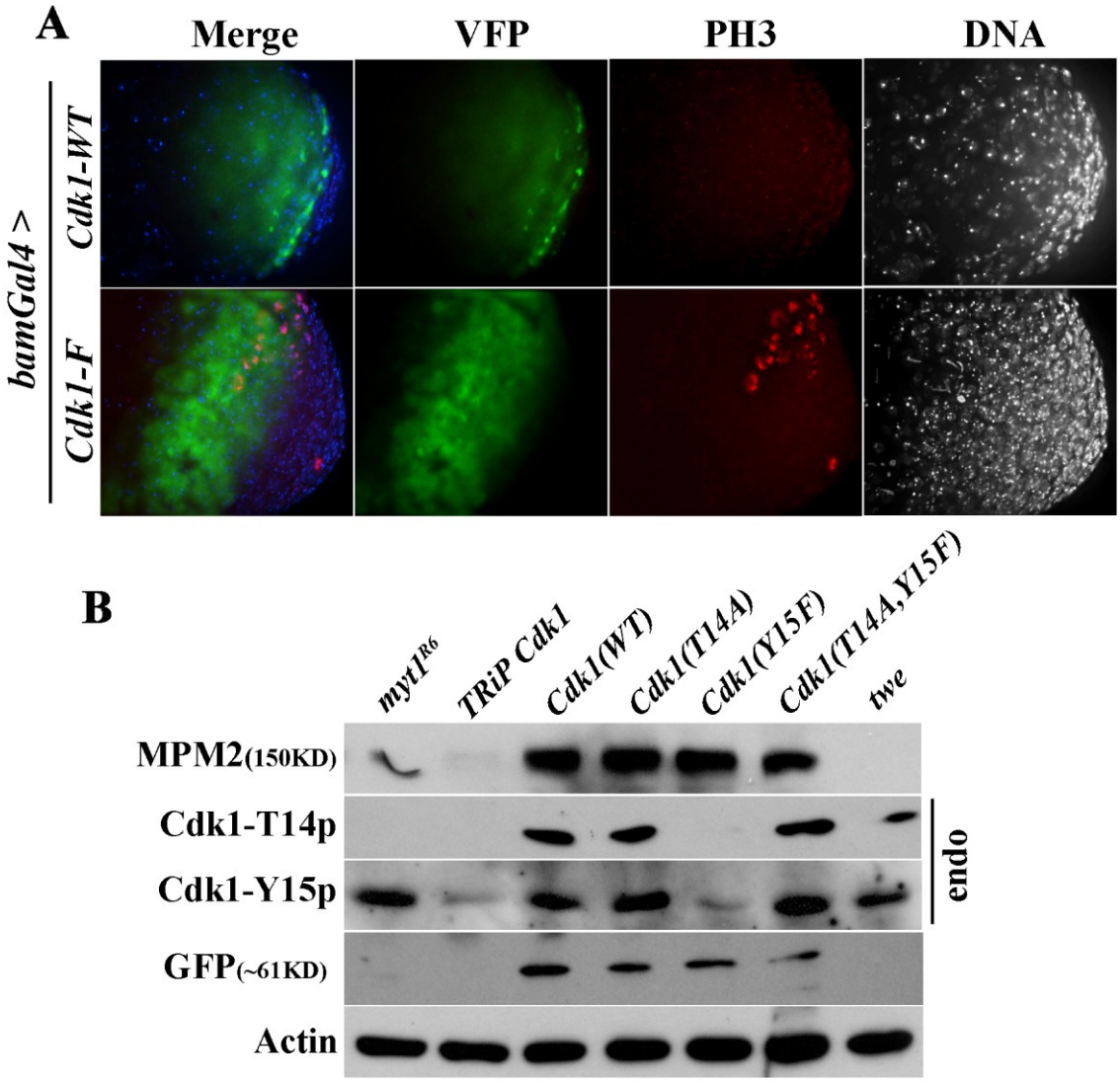

Fig. 3-8: *bam> Cdk1 (Y15F)* exhibits mitotic over proliferation and phenocopies *myt1*

(A) Testes expressing VFP tagged Cdk1(WT) or Cdk1(Y15F) are labeled with PH3 antibodies. VFP (green) panel indicates bamGal4 driven transgenic Cdk1 expression in late spermatogonia and early spermatocytes, and PH3 signal labels the proliferating cell. Germ cells expressing Cdk1(Y15F) reveal more PH3 positive cells compare to the control Cdk1(WT) cells. Scale bar-10 microns. (B) Western blot data characterizes the biochemical consequences of Cdk1 overexpression in spermatocytes. *bamGal4* >Trip Cdk1 expression knocked down the endogenous Cdk1 level, therefore this protein extract was used as a negative control for detecting endogenous Cdk1 phospho-isoforms (lane 2). *myt1*(lane 1) and *twe* (lane 7) testes extracts were used as negative controls for detecting MPM2 and Cdk1-T14p immuno-labels, respectively. The four Cdk1 phospho-isoforms including Cdk1 (WT),

Cdk1 (T14A), Cdk1 (Y15F) and Cdk1 (T14A,Y15F) were individually expressed in early spermatocytes using *bam-Gal4* driver and their testes extracts were loaded from lane 3 to 6. Single blot was stripped each time for re-probing with the listed antibodies. MPM2 antibodies detect multiple Cdk1 phospho-substrates, but detection of 150 kDa size band remained most consistent compare to other bands therefore only 150 kDa size bands are shown here. Levels of endogenous Cdk1 (34 kDa) phosphorylation at T14 and Y15 residues are detecting using their specific phopsho epitope tagged Cdk1 antibodies. GFP antibodies were used to detect the presence of the transgenic VFP tagged Cdk1 fusion protein (61kDa). Actin immuno-labels were used as loading controls.

1) compared to data shown in Fig. 3-5A. The ~150 kDa labeling appeared distorted in lane 1, however, suggesting that this was an experimental artefact.To determine how *bam*-driven expression of transgenic Cdk1 proteins affected Myt1 or Wee1 inhibitory phosphorylation activity, I also examined levels of endogenous Cdk1 (34 kDa) with phospho-specific Cdk1-T14p and Cdk1-Y15p antibodies (Fig. 3-8B). The *myt1* mutant sample (lane 1) had no Cdk1-T14p since this is a Myt1-specific modification, but normal levels of Cdk1-Y15p (due to Wee1). The levels of T14 phosphorylation in Cdk1(WT), (T14A) and Cdk1(T14A,Y15F) samples were all similar, however expression of Cdk1(Y15F) resulted in little or no T14 phosphorylation of endogenous Cdk1 as well as a strong reduction in the Cdk1-Y15p. This experiment therefore confirmed that MPM labeling is primarily a reflection of meiotic Cdk1 activity and reveals an unexpected relationship between Cdk1(Y15F) expression and inhibitory phosphorylation of endogenous Cdk1 that was not observed with the Cdk1(T14AY15F mutant) .

In Chapter 2 (Fig. 2-12), I described how expression of Cdk1(Y15F) in early spermatocytes perturbed the fusome, presumably by interfering with inhibitory phosphorylation of endogenous Cdk1. In Fig. 3-9A I show further data related to this point in prophase I spermatocytes immuno-labeled for β-tubulin (meiotic spindle), AurA-T288p (centrioles) and DNA. As noted before, expression of Cdk1(WT) had no effect on bi-polar spindle assembly or the timing of centriole disengagement. In contrast, expression of Cdk1(Y15F) resulted not only in premature centriole disengagement but also in amplification of the number of AurA-T288p labeled foci. When the completely non-inhibitable form of Cdk1 (Cdk1T14A,Y15F) was expressed using *bam-Gal4* however, there was premature centriole disengagement but no amplification. These abnormalities also partially affected the

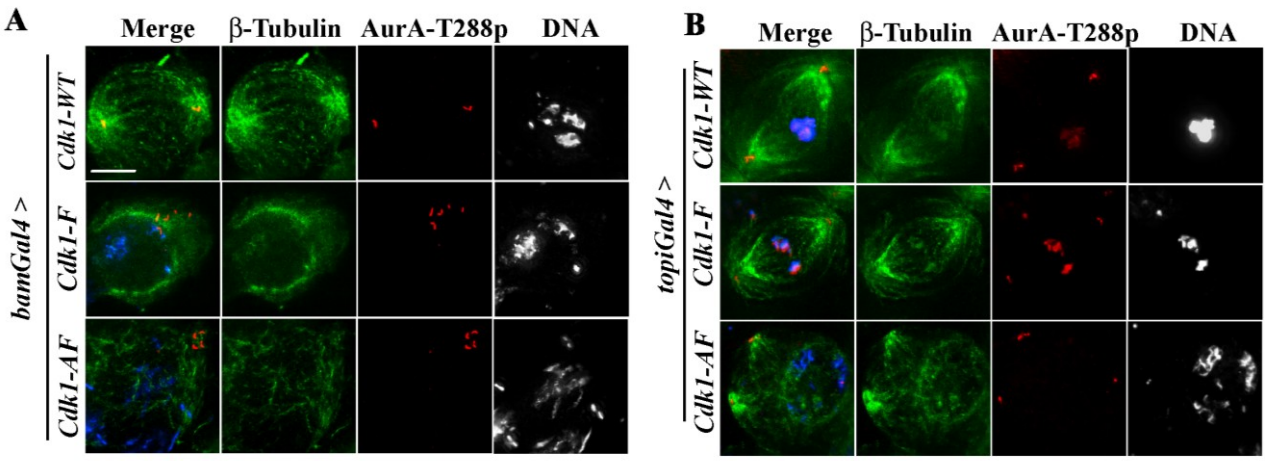

Fig. 3-9: *bam>Cdk1 (Y15F)* recapitulates *myt1* mutant meiotic centriole defects

(A,B) Mature spermatocytes are labeled with β–Tubulin (green) and AurA-T288p isoform (red) antibodies. Three major transgenic Cdk1 phospho-isoforms including Cdk1 (WT), Cdk1 (Y15F) and Cdk1 (T14A,Y15F) were individually expressed in either early (A using *bam-Gal4*) or late (B using *topi-Gal4*) spermatocytes. Scale bar-10 microns.

male fertility (Table. 3-2) When these Cdk1 variants were expressed later in spermatocyte development using *topi-Gal4* (stage S5-6), expression of neither Cdk1(WT), Cdk1(Y15F) nor Cdk1(Y15F) resulted in centriole engagement abnormalities or meiotic spindle formation defects (Fig. 3-9B) or the fertility (Table 3-3). These results suggest that there might be a defect in centriole duplication during pre-meiotic S phase, which did not occur in either *myt1* mutant or Cdk1 (T14AY15F) expressing spermatocytes . This intriguing observation provides a new starting point for experiments to address a potential role for Y15 phospho-regulation of Cyclin A-Cdk1 for S phase organelle checkpoints.

Table 3-2: Degree of male fertility affected by ectopic activation of Cdk1 during <u>early</u> pre-meiotic G2 arrest

Degree of Fertility	*myt1*^{R6}/+	*myt1*^{R6}	*bamGal4 >*				
			String	***Cdk1WT***	***Cdk1 A***	***Cdk1F***	***Cdk1AF***
Fertile (> 20)	30	0	18	23	19	18	7
Semi-Fertile (< 20)	0	0	7	0	0	6	2
Sterile (= 0)	0	30	2	2	1*	8	15
# of male tested(n)	30	30	27	25	20	32	24

Table 3-3: Degree of male fertility affected by ectopic activation of Cdk1 during <u>late</u> pre-meiotic G2 arrest

Degree of Fertility	*myt1*^{R6}/+	*myt1*^{R6}	*topiGal4 >*				
			String	***Cdk1WT***	***Cdk1 A***	***Cdk1F***	***Cdk1AF***
Fertile (> 20)	30	0	15	22	17	18	4
Semi-Fertile (< 20)	0	0	0	0	0	3	5
Sterile (= 0)	0	30	0	0	0	3	13
# of male tested (n)	30	30	15	22	17	24	22

Fertility Assay : Single test male was crossed with 3-5 *yw* virgin females in an individual vial at 25C incubator. The first brood progenies from this cross were counted. Fertility of the single test males was scored based on the following criteria; progenies count over 20 flies (fertile), less than 20(semi-fertile) and no progenies (sterile). In most case, fertile males produced over 100 progenies/cross.
* Indicates the exceptional case, where a single male does not fit with the most observed categories of the corresponding genotype.

3.4 Discussion

In this section I have presented data showing that catalytic function of Myt1 is essential for meiotic fusome checkpoint and this function is irreplaceable by the closest kinase family protein Wee1. Centriole engagement checkpoint is however, less sensitive to loss of Myt1 function. I have also shown that *myt1* meiotic organelle defects are independent of $Cdc25^{Twe}$ mediated CyclinA-Cdk1 regulation indicating the presence of an unknown mechanism of activating CyclinA-Cdk1 complex during the pre-meiotic arrest.

3.4.1 Fusome checkpoint controls Cyclin A-Cdk1 regulation during pre-meiotic G2 arrest

In chapter-II, I showed that Myt1 mediated down regulation of Cyclin A-Cdk1 is directly involved in meiotic fusome and centriole engagement checkpoints. In this Chapter I showed that each of these checkpoints requires differential levels of CyclinA-Cdk1 inhibitory regulation.Transgenic expression of Wee1-VFP or Myt1(N229A) fully or partially restored male fertility, respectively, without suppressing the *myt1* mutant fusome defect. However, both manipulations restore centriole engagement in *myt1* mutant spermatocytes revealed that centriole dis-engagement is the primary cause for *myt1* male sterility. Wee1-VFP expressed in *myt1* mutant spermatocytes can only catalyze Y15 inhibitory phosphorylation of endogenous Cdk1, not T14 phosphorylation, suggesting that T14-phosphorylation of Cyclin A-Cdk1 could serves as a signal for fusome localization. Consequently loss of Myt1 activity would result in active complexes being free to diffuse within the cytoplasmic compartment and triggering premature fusome disassembly and centriole disengagement. Previous studies in human tissue culture cells showed that Myt1- C terminal interaction with CyclinB serve as a mechanism of

sequestering CyclinB-Cdk1 complex in cytoplasm therefore prevent its subcellular trafficking (Liu *et al.*, 1999; Wells *et al.*, 1999). This tethering mechanism was also shown to influence Myt1 catalytic activity *in vitro*. I attempted to test this proposed mechanism in fusome checkpoint, by generating Myt1-Cyclin tethering mutant variant and the results are discussed in Appendix A. In this model, expression of a Myt1 (N229A) hypomorphic allele that only partially restored T14 phosphorylation in *myt1* mutants would compromise both inhibition of Cdk1 activity and tethering of Cyclin A-Cdk1 on fusomes. This could explain why loss of function of Myt1 and Wee1 result in distinct phenotypes, even though the two kinases both inhibit Cdk1. Collectively my results suggest that accumulation of Cyclin A-Cdk1 on fusomes and the mechanism regulating fusome checkpoint could serves as a key factor for sequestering inactive Cyclin A-Cdk1 away from the nucleus during the prolonged G2 phase pre-meiotic arrest (Fig. 3-10). This regulation could be essential for preventing spontaneous or partial activation of Cyclin A-Cdk1 until G2/MI when $Cdc25^{Twe}$ is expressed.

3.4.2 Regulation of CyclinA-Cdk1 is essential for centriole engagement but dispensable for pre-meiotic arrest.

Models derived from observing the behavior of a Cdk1 biosensor expressed in HeLa cells indicated that Cdk1 activation begins slowly during early G2 phase and progresses to a threshold in a classic bistable, switch-like mechanism that requires Cdc25 activity (Gavet and Pines, 2010a; Gavet and Pines, 2010b). The interplay between Cdk1 inhibitory kinases and phosphatases is therefore thought to establish a positive feedback loop so that when the threshold of active Cyclin B-Cdk1 exceeds the inactive pool, cells enter mitosis. Although

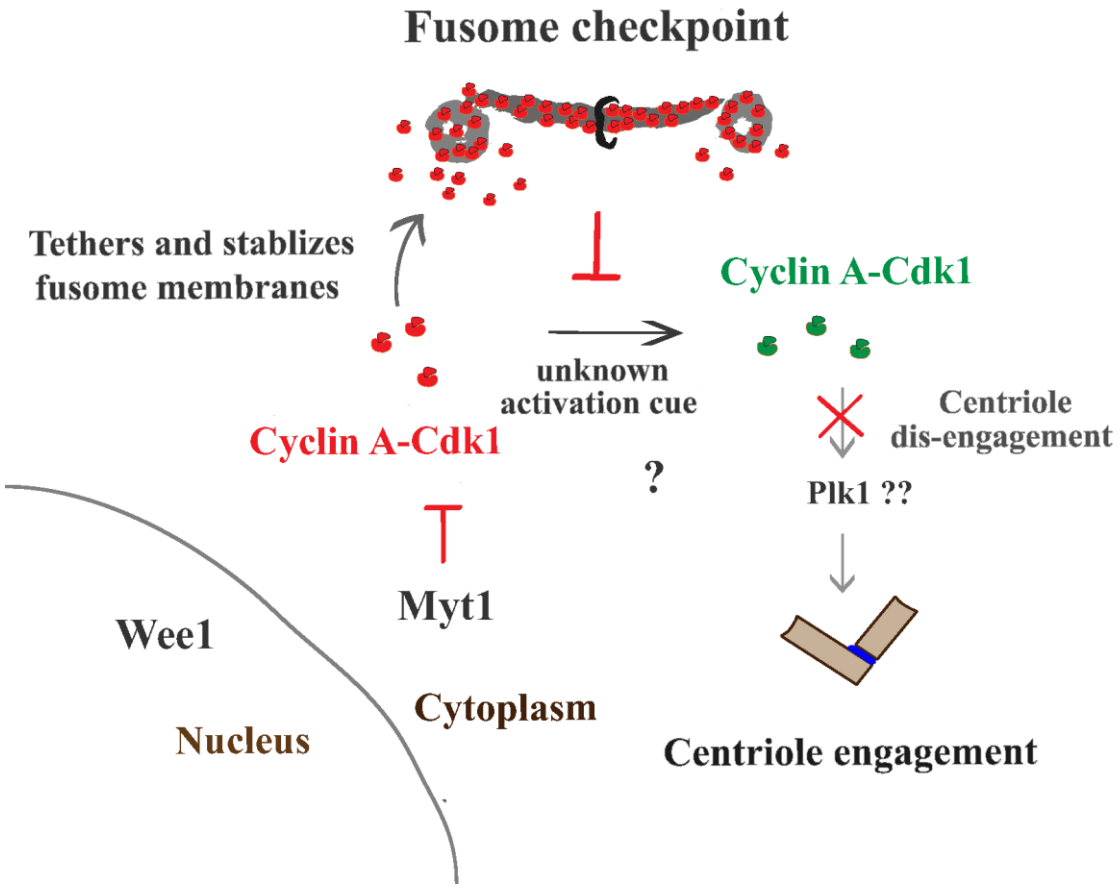

Fig. 3-10: Fusome checkpoint controls the meiotic centriole engagement.

This image depicts the observations drawn from chapter-III. Myt1-mediated inactivation of Cyclin A-Cdk1 is essential to establishing premeiotic fusome checkpoint. This checkpoint mechanism maintains fusome integrity and ensures continuous inhibition of Cyclin A-Cdk1 throughout the G2 phase arrest. Fusome checkpoint mediated Cyclin A-Cdk1 regulation is perhaps important for preventing Plk1 mediated premature centriole dis-engagement.

this theoretical model fits *in vivo* data for mitotic and meiotic progression in most animal models, my studies of *Drosophila* meiotic progression showing that spermatocytes prematurely undergo meiotic events during G2 phase when there is no Cdc25, which raise questions about its universality (White-Cooper, 2010). Ectopic expression of *bam-Gal4>Cdc25*^{Stg} phosphatase was observed to by pass Myt1 mediated centriole engagement checkpoint, however its regulation of CyclinA-Cdk1 did not abruptly triggered bistable feedback mechanism therefore no G2/MI transition is triggered. Detection of Cdk1 targeted substrates through MPM2 antibodies showed in this chapter also revealed no evidence for increased meiotic Cdk1 activity in either Myt1 or Wee1 deficient spermatocytes, further supported that premature activation of Cyclin A-Cdk1 does not elevate their meiotic activity. Instead, the consequences of premature Cyclin A-Cdk1activation in *myt1* mutant spermatocytes were detected at a sub-cellular level, resulting in fusome and centriole engagement defects, with multipolar meiotic spindles similar to those observed in *myt1* mutants. These observation indicated that CyclinA-Cdk1 may be initially activated through a specialized and Cdc25 independent mechanism to initiate meiotic maturation. Progression into prophase I or meiotic maturation causing chromosome condensation and nucleoli breakdown can proceed without Cdc25 activity in a *twe* mutant, arguing that these events may not have to be triggered by canonical bistable Cdk1 activation mechanisms. I therefore suggest that Cdc25^{Twe} mediated bistable feedback mechanisms are required only for the core MI progression that occur during/after metaphase-I. A model shown in Fig. 3-11 summarizes my ideas about how Cdk1 may be regulated during *Drosophila* male meiosis.

3.4.3 Why does ectopic expression of Cdk1(Y15F) phenocopy *myt1* mutant defects?

In both chapter-II and III, I showed evidences indicated that ectopic expression Cdk1(Y15F) is capable of bypassing Myt1 meiotic organelle checkpoint defects. Biochemical evidence presented in this chapter also revealed that ectopic expression of Cdk1(Y15F) depleted endogenous Cdk1-T14 phospho-isoforms, but retained Cdk1-Y15 resembling the *myt1* mutant phenotype. Ectopic expression of the commonly used dual phospho-mutant Cdk1(T14A,Y15F), which has been shown known to bypass mitotic G2 phase checkpoint (Ayeni and Campbell, 2014) did affect the endogenous Cdk1-T14 phospho-isoforms, unlike Cdk1(Y15F). My explanation for these intriguing results are the following: First, ectopic expression of Cdk1 (Y15F) may establish a positive feed-back mechanism similar to mitotic Cdk1 (T14A,Y15F) overexpression, which could result in activation of Cdc25 and inhibition of Myt1kinase that could attenuate endogenous Cdk1-T14 phosphorylation. However, the caveat associated with this interpretation could rise from the detection of endogenous Cdk1-T14p in (lane 6, Fig. 3-8B) spermatocyte expressing Cdk1(T14A,Y15F), which argues against the possibility of Myt1 inactivation. Alternatively, I interpret that transgenic Cdk1(Y15F) may compete with endogenous Cdk1 for Myt1 catalytic activity, as Cdk1(Y15F) has an available T14 phosphorylatable site unlike Cdk1(T14A) or Cdk1(T14A,Y15F). Cdk1(Y15F) may also exhibit some form of stoichiometric physical interaction with Myt1 that could reflect its inhibitory regulation.

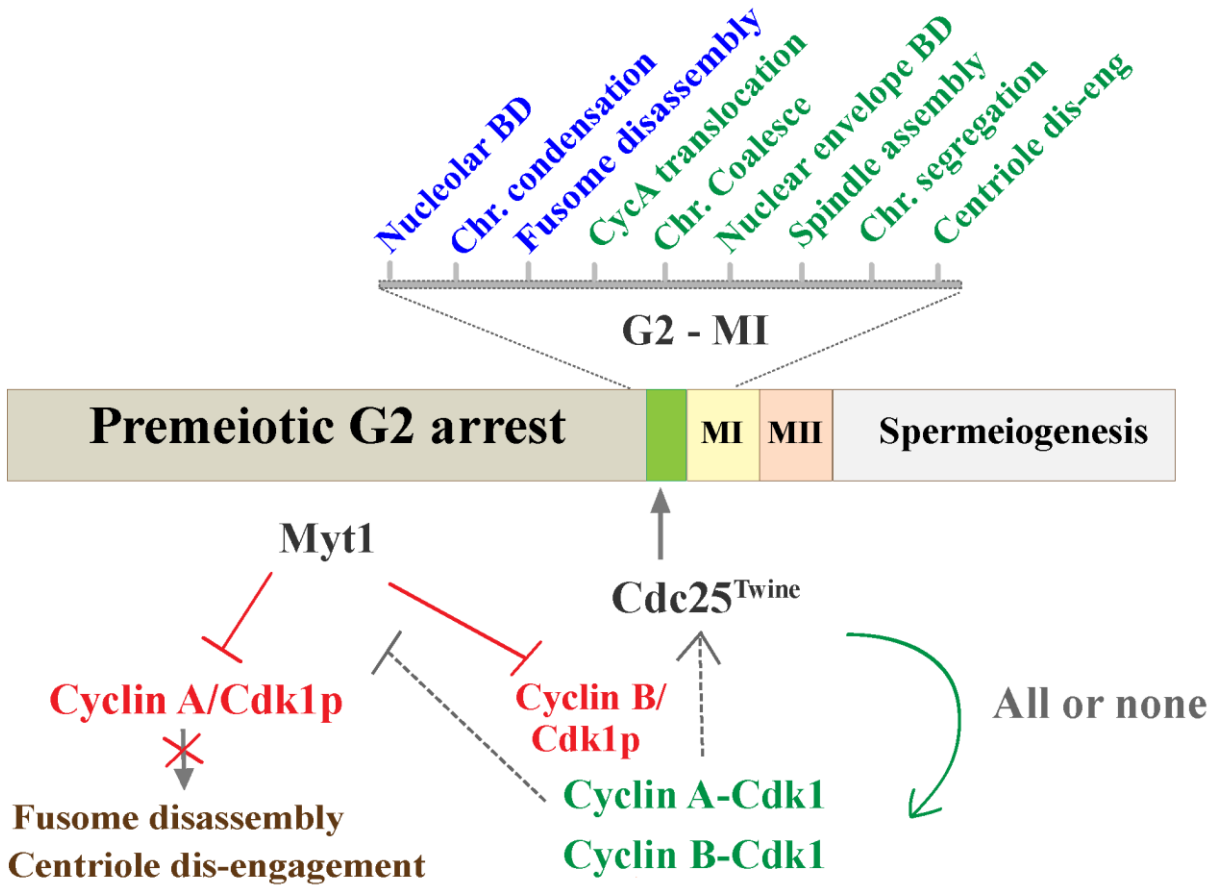

Fig. 3-11: Co-ordination of Cdc25^{Twe} independent and dependent meiotic Cdk1 regulation.

Our model illustrates the differential regulation of Cdk1 during spermatocyte development. During the premeiotic G2 arrest, Myt1 kinase is primarily involved in inactivating CyclinA-Cdk1 therefore premature fusome disassembly and the centriole dis-engagement are prevented. Pro-activation of CyclinA-Cdk1 and/or CyclinB-Cdk1 occur independent of Cdc25^{Twe} mediated phosphatase function, and this pro-regulation is sufficient to trigger the early meiotic initiation/events (highlighted in blue). Accumulation of Cdc25^{Twe} during Prophase I, however trigger the major meiotic progression through establishing bistable 'switch' like or 'all or none' Cdk1 activation.

3.5 References

1. Alphey, L, J Jimenez, H White-Cooper, I Dawson, P Nurse, and DM Glover. 1992a. "twine, a cdc25 homolog that functions in the male and female germline of Drosophila." *Cell* 69(6): 977–88.
2. Alphey, L, J Jimenez, H White-Cooper, I Dawson, P Nurse, and DM Glover. 1992b. "twine, a cdc25 homolog that functions in the male and female germline of Drosophila." *Cell* 69(6): 977–88.
3. Ayeni, JO, and SD Campbell. 2014. ""Ready, set, go": checkpoint regulation by Cdk1 inhibitory phosphorylation." *Fly (Austin)* 8(3): 140–47.
4. Bergman, ZJ, JD Mclaurin, AS Eritano, BM Johnson, AQ Sims, and B Riggs. 2015. "Spatial reorganization of the endoplasmic reticulum during mitosis relies on mitotic kinase cyclin A in the early Drosophila embryo." *PLoS One* 10(2): e0117859.
5. Chen, D, and DM McKearin. 2003. "A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell." *Development* 130(6): 1159–70.
6. Courtot, C, C Fankhauser, V Simanis, and CF Lehner. 1992. "The Drosophila cdc25 homolog twine is required for meiosis." *Development* 116(2): 405–16.
7. De Boer, L, V Oakes, H Beamish, N Giles, F Stevens, M Somodevilla-Torres, C Desouza, and B Gabrielli. 2008. "Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events." *Oncogene* 27(31): 4261–68.
8. Dienemann, A, and F Sprenger. 2004. "Requirements of cyclin a for mitosis are independent of its subcellular localization." *Curr Biol* 14(12): 1117–23.
9. Fichelson, P, A Audibert, F Simon, and M Gho. 2005. "Cell cycle and cell-fate

determination in Drosophila neural cell lineages." *Trends Genet* 21(7): 413–20.

10. Fung, TK, HT Ma, and RY Poon. 2007. "Specialized roles of the two mitotic cyclins in somatic cells: cyclin A as an activator of M phase-promoting factor." *Mol Biol Cell* 18(5): 1861–73.
11. Furuno, N, N den Elzen, and J Pines. 1999. "Human cyclin A is required for mitosis until mid prophase." *J Cell Biol* 147(2): 295–306.
12. Gavet, O, and J Pines. 2010a. "Activation of cyclin B1-Cdk1 synchronizes events in the nucleus and the cytoplasm at mitosis." *J Cell Biol* 189(2): 247–59.
13. Gavet, O, and J Pines. 2010b. "Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis." *Dev Cell* 18(4): 533–43.
14. Gonczy, P, BJ Thomas, and S DiNardo. 1994. "roughex is a dose-dependent regulator of the second meiotic division during Drosophila spermatogenesis." *Cell* 77(7): 1015–25.
15. Insco, ML, A Leon, CH Tam, DM McKearin, and MT Fuller. 2009. "Accumulation of a differentiation regulator specifies transit amplifying division number in an adult stem cell lineage." *Proc Natl Acad Sci U S A* 106(52): 22311–16.
16. Jin, Z, E Homola, S Tiong, and SD Campbell. 2008. "Drosophila myt1 is the major cdk1 inhibitory kinase for wing imaginal disc development." *Genetics* 180(4): 2123–33.
17. Jin, Z, EM Homola, P Goldbach, Y Choi, JA Brill, and SD Campbell. 2005. "Drosophila Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis." *Development* 132(18): 4075–85.
18. Knoblich, JA, and CF Lehner. 1993. "Synergistic action of Drosophila cyclins A and B during the G2-M transition." *EMBO J* 12(1): 65–74.
19. Lehner, CF, and PH O'Farrell. 1989a. "Expression and function of Drosophila cyclin A

during embryonic cell cycle progression." *Cell* 56(6): 957–68.

20. Lehner, CF, and PH O'Farrell. 1989b. "Expression and function of Drosophila cyclin A during embryonic cell cycle progression." *Cell* 56(6): 957–68.
21. Lehner, CF, and PH O'Farrell. 1990. "The roles of Drosophila cyclins A and B in mitotic control." *Cell* 61(3): 535–47.
22. Lin, TY, S Viswanathan, C Wood, PG Wilson, N Wolf, and MT Fuller. 1996. "Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in Drosophila males." *Development* 122(4): 1331–41.
23. Liu, D, MM Matzuk, WK Sung, Q Guo, P Wang, and DJ Wolgemuth. 1998. "Cyclin A1 is required for meiosis in the male mouse." *Nat Genet* 20(4): 377–80.
24. Liu, F, C Rothblum-Oviatt, CE Ryan, and H Piwnica-Worms. 1999. "Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes." *Mol Cell Biol* 19(7): 5113–23.
25. Liu, F, JJ Stanton, Z Wu, and H Piwnica-Worms. 1997. "The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex." *Mol Cell Biol* 17(2): 571–83.
26. Maro, B, E Houliston, and M Paintrand. 1988. "Purification of meiotic spindles and cytoplasmic asters from mouse oocytes." *Dev Biol* 129(2): 275–82.
27. McCleland, ML, JA Farrell, and PH O'Farrell. 2009. "Influence of cyclin type and dose on mitotic entry and progression in the early Drosophila embryo." *J Cell Biol* 184(5): 639–46.
28. McKearin, DM, and AC Spradling. 1990. "bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis." *Genes Dev* 4(12B): 2242–51.

29. Mueller, PR, TR Coleman, and WG Dunphy. 1995. "Cell cycle regulation of a Xenopus Wee1-like kinase." *Mol Biol Cell* 6(1): 119–34.

30. Ni, JQ, R Zhou, B Czech, LP Liu, L Holderbaum, D Yang-Zhou, HS Shim, R Tao, D Handler, P Karpowicz, R Binari, M Booker, J Brennecke, LA Perkins, GJ Hannon, and N Perrimon. 2011. "A genome-scale shRNA resource for transgenic RNAi in Drosophila." *Nat Methods* 8(5): 405–07.

31. Raychaudhuri, N, R Dubruille, GA Orsi, HC Bagheri, B Loppin, and CF Lehner. 2012. "Transgenerational propagation and quantitative maintenance of paternal centromeres depends on Cid/Cenp-A presence in Drosophila sperm." *PLoS Biol* 10(12): e1001434.

32. Riparbelli, MG, M Gottardo, DM Glover, and G Callaini. 2014. "Inhibition of Polo kinase by BI2536 affects centriole separation during Drosophila male meiosis." *Cell Cycle* 13(13): 2064–72.

33. Roy, LM, KI Swenson, DH Walker, BG Gabrielli, RS Li, H Piwnica-Worms, and JL Maller. 1991. "Activation of p34cdc2 kinase by cyclin A." *J Cell Biol* 113(3): 507–14.

34. Sigrist, S, G Ried, and CF Lehner. 1995. "Dmcdc2 kinase is required for both meiotic divisions during Drosophila spermatogenesis and is activated by the Twine/cdc25 phosphatase." *Mech Dev* 53(2): 247–60.

35. Sigrist, SJ, and CF Lehner. 1997. "Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles." *Cell* 90(4): 671–81.

36. Smith, A, S Simanski, M Fallahi, and NG Ayad. 2007. "Redundant ubiquitin ligase activities regulate wee1 degradation and mitotic entry." *Cell Cycle* 6(22): 2795–99.

37. Sprenger, F, N Yakubovich, and PH O'Farrell. 1997. "S-phase function of Drosophila

cyclin A and its downregulation in G1 phase." *Curr Biol* 7(7): 488–99.

38. Strausfeld, UP, M Howell, P Descombes, S Chevalier, RE Rempel, J Adamczewski, JL Maller, T Hunt, and JJ Blow. 1996. "Both cyclin A and cyclin E have S-phase promoting (SPF) activity in Xenopus egg extracts." *J Cell Sci* 109 (Pt 6): 1555–63.
39. Swenson, KI, KM Farrell, and JV Ruderman. 1986. "The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in Xenopus oocytes." *Cell* 47(6): 861–70.
40. Tang, Z, TR Coleman, and WG Dunphy. 1993. "Two distinct mechanisms for negative regulation of the Wee1 protein kinase." *EMBO J* 12(9): 3427–36.
41. van der Meer, T, WY Chan, LS Palazon, C Nieduszynski, M Murphy, J Sobczak-Thepot, M Carrington, and WH Colledge. 2004. "Cyclin A1 protein shows haplo-insufficiency for normal fertility in male mice." *Reproduction* 127(4): 503–11.
42. Voronina, E, WF Marzluff, and GM Wessel. 2003. "Cyclin B synthesis is required for sea urchin oocyte maturation." *Dev Biol* 256(2): 258–75.
43. Wang, C, and R Lehmann. 1991. "Nanos is the localized posterior determinant in Drosophila." *Cell* 66(4): 637–47.
44. Watanabe, N, M Broome, and T Hunter. 1995. "Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle." *EMBO J* 14(9): 1878–91.
45. Wells, NJ, N Watanabe, T Tokusumi, W Jiang, MA Verdecia, and T Hunter. 1999. "The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression." *J Cell Sci* 112 (Pt 19): 3361–71.
46. Westendorf, JM, PN Rao, and L Gerace. 1994. "Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of

the phosphorylated epitope." *Proc Natl Acad Sci U S A* 91(2): 714–18.

47. White-Cooper, H. 2010. "Molecular mechanisms of gene regulation during Drosophila spermatogenesis." *Reproduction* 139(1): 11–21.

48. White-Cooper, H, L Alphey, and DM Glover. 1993. "The cdc25 homologue twine is required for only some aspects of the entry into meiosis in Drosophila." *J Cell Sci* 106 (Pt 4): 1035–44.

49. White-Cooper, H, MA Schafer, LS Alphey, and MT Fuller. 1998. "Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid differentiation with meiosis I in Drosophila." *Development* 125(1): 125–34.

50. Wong, R, I Hadjiyanni, HC Wei, G Polevoy, R McBride, KP Sem, and JA Brill. 2005. "PIP2 hydrolysis and calcium release are required for cytokinesis in Drosophila spermatocytes." *Curr Biol* 15(15): 1401–06.

51. Yang, R, C Muller, V Huynh, YK Fung, AS Yee, and HP Koeffler. 1999. "Functions of cyclin A1 in the cell cycle and its interactions with transcription factor E2F-1 and the Rb family of proteins." *Mol Cell Biol* 19(3): 2400–07.

52. Yeh, E, L Zhou, N Rudzik, and GL Boulianne. 2000. "Neuralized functions cell autonomously to regulate Drosophila sense organ development." *EMBO J* 19(17): 4827–37.

Chapter IV

Discussion and Conclusion

In the field of cell cycle, Cdk1-Cyclin B (MPF) has been implicated as a major regulator of G2/M since it was discovered in *Xenopus* oocytes. Mechanisms controlling the Cdk1 either by Wee1/Myt1 mediated inhibition or Cdc25 driven activation are well established for the Cyclin B associated Cdk1 complex. Although Cyclin A and Cdk1 were shown to be the most essential cell cycle regulators in many developmental systems, their regulatory mechanism still remains elusive. This thesis provides direct evidence for an essential role of Myt1 in regulating Cyclin A-Cdk1 in establishing novel pre-meiotic organelle checkpoints in *Drosophila* spermatocytes.

4.1 Pre-meiotic translational regulations control spermatocyte arrest and G2/MI

Conclusions drawn from Chapter 2 show that phosphorylation-dependent inhibition of meiotic Cdk1 is dispensable for maintaining spermatocyte G2 phase arrest. Our BrdU-pulse chase experiments revealed that loss of either Myt1 or Wee1 activity did not cause any premature entry into MI. This observation is unexpected compare to what has been reported in other systems including *Xenopus* and *C. elegans* oocytes (Nebreda and Ferby, 2000; Han *et al.*, 2005; Burrows *et al.*, 2006; Kishimoto, 2011). The G2 like prophase I meiotic arrest was shown to be primarily dependent on inhibitory regulation of Cdk1. Either depleting Myt1 or ectopically injecting Cdc25 in *Xenopus* oocytes caused abrupt entry into meiosis by initiating nuclear envelop breakdown/GVBD followed by other early meiotic events (Duckworth *et al.*, 2002; Gaffre *et al.*, 2011). Meiotic resumption is normally regulated based on the

physiological changes caused by Ca^{2+}/cCAMP levels upon external hormonal stimuli or entry of sperm (Nebreda and Ferby, 2000). Downstream signaling through MAPK/Mos/ERK activity induces meiotic translational program to promote formation and activation of Cyclin B- Cdk1.

In *Drosophila,* neither the oocyte nor spermatocytes have been demonstrated to resume meiosis upon hormonal stimuli (Von Stetina and Orr-Weaver, 2011); however, the requirement of Cyclin B- Cdk1activation for G2/MI progression remains conserved (Alphey *et al.*, 1992). In spermatocytes, this progression is primarily triggered by growth associated translational mechanism that regulates testes specific protein expression through eIF4G (translation initiation complex) (Baker and Fuller, 2007). Spermatocytes have four major classes of 'meiotic arrest' genes including *always early (aly), cannonball (can), spermatocyte arrest (sa) and meiosis I arrest (mia)* (Lin *et al.*, 1996). Always early (*aly*) regulates the transcription of $Cdc25^{Twe}$, *boule* and *cyclin B* and *can* mediate the post-transcriptional modification of $Cdc25^{Twe}$. Translation of Cyclin B during the early prophase-I allows the formation of CyclinB-Cdk1 and accumulation of $Cdc25^{Twe}$ induces activation of these complexes, therefore the mature spermatocytes resume meiosis.

We noticed that preventing Cyclin B synthesis using siRNA mediated knockdown did not prevent G2/MI transition and neither affected the meiotic divisions, indicating that $Cdc25^{Twe}$ dependent activation of previously accumulated Cyclin A-Cdk1 alone is sufficient to trigger completion of meiosis. Unlike Cyclin B, Cyclin A is available during the entire pre-meiotic G2 phase. Curiously, ectopic activation of Cyclin A-Cdk1 through $bam>Cdc25^{Stg}$ overexpression did not appear to abruptly trigger G2/MI transition. Although $Cdc25^{Stg}$ has been shown to partially complement loss of $Cdc25^{Twe}$ activity in *twe* mutant late

spermatocytes(Sigrist *et al.*, 1995), its ectopic activity during early G2 phase arrest does not accelerate meiotic entry. These observations revealed that CyclinA-Cdk1 is not capable of inducing the G2/MI in the absence of meiotic $Cdc25^{Twe}$ indicating that synthesis of $Cdc25^{Twe}$ is a key factor determining G2/MI in spermatocytes. Meiotic translational regulations, which control the synthesis of $Cdc25^{Twe}$ therefore temporally coordinating the 90 hour long spermatocyte arrest and G2/MI transition. Apart from its requirement for meiotic progression, regulation of pre-meiotic translation also controls spermatid differentiation (Lin *et al.*, 1996). Experimental evidences form previous studies and my results have demonstrated that spermatid differentiation is independent of meiotic progression. Depletion of $Cdc25^{Twe}$ mediated Cdk1 activation either by temperature sensitive $Cdk1^{ts}$ or *twe* or siRNA-mediated Cyclin A knockdown (our data) prevented meiotic entry, however spermatid like differentiation was noticed to occur normally (Alphey *et al.*, 1992). These observations therefore imply that spermatocytes are equipped with intrinsic robust translational regulation that not just determine the timing of G2/MI and spermatid differentiation, but also ensure an intact 90 hour long pre-meiotic arrest.

4.2 How could Myt1 involve with spermatocyte translational control?

Drosophila spermatocyte arrest is regulated by distinct mechanisms that can partly be explained by pre-meiotic translational control that facilitate their G2 phase growth. Insulin signaling has been implicated in spermatocyte growth and cell cycle progression through downstream activation of Akt (Ueishi *et al.*, 2009). The signaling pathway mediated by MAPK/MEK/Mos/ prsk90 has also been demonstrated to promote meiotic resumption in vertebrate oocytes (Nebreda and Ferby, 2000), however their requirement is not known in

Drosophila spermatocytes. Mos was not required for most of the *Drosophila* development including oocyte maturation (Ivanovska *et al.*, 2004). However, S6K ribosome kinase (p90(RSK) homolog) has been demonstrated to facilitate growth and translational regulation during larval development (Volarevic and Thomas, 2001). Interestingly, earlier studies have also shown p90(RSK) as a inhibitor of Myt1 (Frodin and Gammeltoft, 1999; Palmer and Nebreda, 2000), therefore examining the requirement for S6K during spermatocyte arrest might provide a possibility to further understand the Cdk1 dependent or independent pre-meiotic regulation.

4.3 Regulation of Cyclin A-Cdk1 is essential for stabilizing meiotic centriole engagement

Myt1 has been implicated in the regulation of Cyclin B-Cdk1 in preventing mitotic or meiotic entry (Pomerening *et al.*, 2003; Slepchenko and Terasaki, 2003; Ruiz *et al.*, 2010; Tuck *et al.*, 2013). In this thesis, I have demonstrated that Myt1 regulation of CyclinA-Cdk1 is essential for the pre-meiotic fusome and centriole behavior during spermatocyte development. However, manipulation in the regulation of Cyclin A-Cdk1 either by activating or inhibiting its function caused different responses in fusomes and centrosomes. Establishing a reduced catalytic activity of Myt1, using N229A mutation that can only partially inhibit Cyclin A-Cdk1 and this partial regulation was sufficient to suppress *myt1* mutant centriole dis-engagement, whereas it is not adequate for restoring normal fusomes. Overexpression of Wee1-VFP or Rux in *myt1* mutant spermatocytes also revealed similar responses from these organelles that further suggested a differential requirement for CyclinA-Cdk1 regulation in maintaining these two sub-cellular structures. Restoring the regulation of CyclinA-Cdk1 in

myt1 spermatocytes not only rescued abnormal centriole behavior, but also suppressed the *myt1* mutant sterility. These results therefore indicated that fusome defects associated with loss of Myt1 *per se* are not responsible for male infertility.

4.4 Fusomes checkpoint could be the prerequisite for meiotic centriole engagement

The function of fusomes has been connected with centriole/centrosome behavior during oogenesis (Megraw and Kaufman, 2000). In cystocytes, centrosomes associate with spectrosomes to specify polarity for mitotic division and oocyte positioning. Centrosome and fusome connections facilitate centriole migration from nurse cells to the posterior pole of oocytes through inter-cellular bridges. In oocytes, the meiotic divisions are acentriolar, as they lose the maternal centrosomes. In zygotes, only the paternal sperm centrioles ensure the first embryonic division. In male germline stem cells, one out of two centrosomes have been shown to remain proximal to the spectrosome that indicate the direction of gonioblast division (Yamashita, 2009; Salzmann *et al.*, 2014). A direct link between centrosome and fusomes was demonstrated in *hts* mutant (Wilson, 2005). Perturbations in the fusome structures were shown to result in abnormal pre-meiotic centrosome behavior in the *hts* spermatocytes. *hts* spermatocytes often showed more than two or four centrosome labeled foci per cell resulting in multipolar meiotic spindles. The question of how does fusome influence meiotic centrosome behavior however remained elusive.

myt1 mutant provides the second most convincing evidence indicating the link between fusome and centrosome in spermatogenesis. Fusome abnormalities were the earliest defects detected in *myt1* mutant spermatocytes, whereas the centriole dis-engagements were

noticed a stage (approximately 2 days) after. Down-regulation of Cyclin A-Cdk1 has been shown to suppress *myt1* mutant centriole dis-engagement phenotype, which was shown to occur independent of fusome restoration. This raises a possibility that fusome could therefore directly be involved in down-regulating CyclinA-Cdk1 activity to facilitate normal pre-meiotic centriole behavior. Enrichment of Cyclin A and Cdk1 on fusomes could also support this possibility, therefore, I propose a two-step model that depicts the potential link between fusome and centriole engagement.

First, the newly formed CyclinA-Cdk1 complexes are targeted by Myt1 mediated phospho-regulation. Second, inhibited form for these complexes may be tethered to fusome structures mostly on the membranes. Fusomes could be serving as a reservoir to accumulate most of the inhibited CyclinA-Cdk1 complexes and therefore could prevent their sub-cellular trafficking and spontaneous activation until $Cdc25^{Twe}$ is synthesized. Fusome mediated CyclinA-Cdk1 regulation may provide a meiotic checkpoint of restricting Cdk1 activity throughout the G2 phase arrest. This fusome checkpoint therefore provides a new insight into the regulation of germline cell meiotic divisions.

4.5 Would *hts* meiotic centriole defects be suppressed by down-regulation of Cyclin A-Cdk1?

Hts is an essential membrane component that stabilizes fusomes throughout germ-cell development (Wilson, 2005; Petrella *et al.*, 2007). Loss of Hts perturbed fusome integrity and also resulted in abnormal centrosome behavior in *hts* mutant spermatocytes (Wilson, 2005). Both *myt1* and *hts* mutant spermatocytes have therefore revealed a strong connection between fusomes and pre-meiotic centrosomes. The entire germ-cell developments including MI, MII

and spermiogenesis appeared to occur normally in these two mutants, except for obvious perturbations in the meiotic fusomes and centrosomes. Based on my experimental evidences, my current model depicted that the association between these two organelles is perhaps through regulation of Cyclin A-Cdk1. Therefore I hypothesize that the abnormalities reported in *hts* mutant spermatocytes could be due to the absence of fusome checkpoint function involving the Cyclin A-Cdk1 inhibition. This idea can be further tested with the Cyclin A knock down in *hts* spermatocytes. Similar to the results shown in *myt1* mutants, down-regulation of Cyclin A-Cdk1 in *hts* mutants, may not suppress the fusome defects, but I expect that might rescue centriole abnormalities. The experimental outcomes could therefore validate our current fusome-centrosome model and will also further clarify the understanding of novel fusome mediated meiotic checkpoint functions.

4.6 Myt1 mediated Cdk1 inhibition may regulate meiotic cytokinesis?

In *Drosophila,* most of the molecular players regulating cytokinesis appeared to be conserved between somatic and germ-line cells, however, meiotic cells requires a specialized mechanisms to facilitate the incomplete cytokinesis (Giansanti *et al.*, 2004; Cabernard, 2012). Fusomes are defined as an intercellular bridge, which are formed as a result of incomplete cytokinesis (IC) and they resemble the midbody structures normally seen in abscission phase of the process (Wilson, 1999). Although fusomes are equivalent to the midbody structures, the essential membrane-remodeling components including Hts and Spectrin poise these structures to remain stable through out the germ-cell development (de Cuevas *et al.*, 1996; Wilson, 2005). Anillin, which is a ring canal component, was also thought to stabilize the cleavage furrow by linking the actin rich actomyosin ring to Septin filaments on the fusome

membrane(Giansanti *et al.*, 1999). Klp3A motor protein and CLASP ortholog Orbit were also shown to assemble central spindle microtubules in the fusomes (Williams *et al.*, 1995; Miyauchi *et al.*, 2013). Regulation of IC therefore can be explained in two steps; Germ cells exit ana phase and undergo normal cytokinesis until abscission step, however the midbody remodeling could result in a cytokinesis arrest.

Our experimental evidences revealed an obvious requirement of Myt1 mediated Cdk1 inhibitory regulation for stabilizing meiotic fusome structures. Rescue experimental results discussed in chapter-II indicated that expressing Myt1 during the late spermatogonia or early 16 cell cysts is critical to rescue *myt1* mutant fusomes. These observations therefore suggested that Myt1 might play a key role in regulating incomplete cytokinesis during mitotic to meiotic transition. The fourth mitotic exit is distinctive from the three previous exits, since the resulting 16 cell cysts differentiate into spermatocytes and undergo a prolonged pre-meiotic interphase rather than entering the 5^{th} transit amplification. Regulation of midbody remodeling during the 4^{th} IC might require stringent mechanisms to withstand the enormous stretching that occur during spermatocyte growth. Therefore, I predicted that Myt1 might contribute an additional role in stabilizing this structure through its catalytic activity.

Exit from anaphase or entry into cytokinesis requires a brief inactivation of Cdk1, however completion of cytokinesis requires reestablishment of Cdk1 activity to mediate Plk1 and AuroraB associated regulations (Glover, 2005; Takaki *et al.*, 2008; Archambault and Carmena, 2012). In *Drosophila* germline cells, the requirements of Cdk1/Plk1 regulation to arrest the meiotic cytokinesis have not been demonstrated. Since our results reveal a striking clue that relate the IC and Myt1 mediated Cdk1 regulation, further studies can be addressed in

the direction of identifying IC substrates of Cdk1, which might provide novel understanding about meiotic cytokinesis.

4.7 Does Myt1 have any role in spermatocyte DNA damage response?

siRNA mediated Myt1 knockdown in *Drosophila* and *in vitro* somatic cells revealed a dispensable role of Myt1 in regulating normal DNA damage checkpoint functions (Glover, 2005; Takaki *et al.*, 2008; Archambault and Carmena, 2012). However, ionization radiation experiments revealed a critical requirement of Myt1, when cells encounter an increased DNA damage that overwhelms the normal p53 mediated DNA repair machinery (Jin *et al.*, 2008; Chow and Poon, 2013). A previous graduate student Dr. Jin has reported a genetic interaction between Myt1 and Chk1/2 in the context of over all viability. Dr. Ayeni in our lab also demonstrated the critical requirement of Myt1 mediated Cdk1 T14 phosphorylation for preventing genomic catastrophe/instability in somatic neuroblast cells (Ayeni *et al.*, 2014). I have also noticed an uncharacterized chromosomal sensitivity of *myt1* mutant's spermatocytes to the acid treatment indicating a possible genomic instability due to loss of Myt1.

Furthermore, In chapter II, I have shown that over expression of Wee1 can completely rescue the *myt1* mutant male sterility through restoring centriole engagement, whereas Rux could only partially rescue the sterility, although it completely complemented the mutant centriole defects. These observations reveal a compelling need for phosphorylation dependent Cdk1 inhibitory regulation for restoring *myt1* mutant defects, beyond rescuing abnormal centrosome behavior. Therefore, I hypothesize that *myt1* mutant spermatocytes may have an additional defects other than fusomes and centriole phenotypes, which can be complemented by Wee1 mediated regulations. One possibility that propose here could exist at the level of

genomic instability, which might be due to loss of Myt1 regulation perturbing pre-meiotic DNA replication and repair.

4.8 Significance of this thesis

The foremost significance of this thesis research is involved with the identification of two novel meiotic organelle checkpoints: namely the fusome checkpoint and the centriole engagement checkpoint. Experimental evidence presented in this thesis revealed a requirement of Myt1 kinase in regulating CyclinA-Cdk1 that serves as a direct molecular mechanism of these new organelle checkpoints. The requirements of fusome or the human equivalent "intercellular bridges" is not widely connected with the human reproductive disorders. Extrapolating these research outcomes towards the human reproductive system may provide major implications in understanding the abnormalities associated with human infertility. Defects in centriole/ centrosome behavior are already well co-related with cancer development, and in fact is used as a marker in clinical diagnosis. The mechanistic connections demonstrated between centrosome and Myt1 may provide new perspectives in approaching the cancer diagnosis and therapy.

4.9 References

1. Alphey, L, J Jimenez, H White-Cooper, I Dawson, P Nurse, and DM Glover. 1992. "twine, a cdc25 homolog that functions in the male and female germline of Drosophila." *Cell* 69(6): 977–88.
2. Archambault, V, and M Carmena. 2012. "Polo-like kinase-activating kinases: Aurora A, Aurora B and what else?" *Cell Cycle* 11(8): 1490–95.
3. Ayeni, JO, R Varadarajan, O Mukherjee, DT Stuart, F Sprenger, M Srayko, and SD Campbell. 2014. "Dual phosphorylation of cdk1 coordinates cell proliferation with key developmental processes in Drosophila." *Genetics* 196(1): 197–210.
4. Baker, CC, and MT Fuller. 2007. "Translational control of meiotic cell cycle progression and spermatid differentiation in male germ cells by a novel eIF4G homolog." *Development* 134(15): 2863–69.
5. Burrows, AE, BK Sceurman, ME Kosinski, CT Richie, PL Sadler, JM Schumacher, and A Golden. 2006. "The C. elegans Myt1 ortholog is required for the proper timing of oocyte maturation." *Development* 133(4): 697–709.
6. Cabernard, C. 2012. "Cytokinesis in Drosophila melanogaster." *Cytoskeleton (Hoboken)* 69(10): 791–809.
7. Chow, JP, and RY Poon. 2013. "The CDK1 inhibitory kinase MYT1 in DNA damage checkpoint recovery." *Oncogene* 32(40): 4778–88.
8. de Cuevas, M, JK Lee, and AC Spradling. 1996. "alpha-spectrin is required for germline cell division and differentiation in the Drosophila ovary." *Development* 122(12): 3959–68.
9. Duckworth, BC, JS Weaver, and JV Ruderman. 2002. "G2 arrest in Xenopus oocytes

depends on phosphorylation of cdc25 by protein kinase A." *Proc Natl Acad Sci U S A* 99(26): 16794–99.

10. Frodin, M, and S Gammeltoft. 1999. "Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction." *Mol Cell Endocrinol* 151(1-2): 65–77.
11. Gaffre, M, A Martoriati, N Belhachemi, JP Chambon, E Houliston, C Jessus, and A Karaiskou. 2011. "A critical balance between Cyclin B synthesis and Myt1 activity controls meiosis entry in Xenopus oocytes." *Development* 138(17): 3735–44.
12. Giansanti, MG, S Bonaccorsi, and M Gatti. 1999. "The role of anillin in meiotic cytokinesis of Drosophila males." *J Cell Sci* 112 (Pt 14): 2323–34.
13. Giansanti, MG, RM Farkas, S Bonaccorsi, DL Lindsley, BT Wakimoto, MT Fuller, and M Gatti. 2004. "Genetic dissection of meiotic cytokinesis in Drosophila males." *Mol Biol Cell* 15(5): 2509–22.
14. Glover, DM. 2005. "Polo kinase and progression through M phase in Drosophila: a perspective from the spindle poles." *Oncogene* 24(2): 230–37.
15. Han, SJ, R Chen, MP Paronetto, and M Conti. 2005. "Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse." *Curr Biol* 15(18): 1670–76.
16. Ivanovska, I, E Lee, KM Kwan, DD Fenger, and TL Orr-Weaver. 2004. "The Drosophila MOS ortholog is not essential for meiosis." *Curr Biol* 14(1): 75–80.
17. Jin, Z, E Homola, S Tiong, and SD Campbell. 2008. "Drosophila myt1 is the major cdk1 inhibitory kinase for wing imaginal disc development." *Genetics* 180(4): 2123–33.
18. Kishimoto, T. 2011. "A primer on meiotic resumption in starfish oocytes: the proposed signaling pathway triggered by maturation-inducing hormone." *Mol Reprod Dev* 78(10-

11): 704–07.

19. Lin, TY, S Viswanathan, C Wood, PG Wilson, N Wolf, and MT Fuller. 1996. "Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in Drosophila males." *Development* 122(4): 1331–41.

20. Megraw, TL, and TC Kaufman. 2000. "The centrosome in Drosophila oocyte development." *Curr Top Dev Biol* 49: 385–407.

21. Miyauchi, C, D Kitazawa, I Ando, D Hayashi, and YH Inoue. 2013. "Orbit/CLASP is required for germline cyst formation through its developmental control of fusomes and ring canals in Drosophila males." *PLoS One* 8(3): e58220.

22. Nebreda, AR, and I Ferby. 2000. "Regulation of the meiotic cell cycle in oocytes." *Curr Opin Cell Biol* 12(6): 666–75.

23. Palmer, A, and AR Nebreda. 2000. "The activation of MAP kinase and p34cdc2/cyclin B during the meiotic maturation of Xenopus oocytes." *Prog Cell Cycle Res* 4: 131–43.

24. Petrella, LN, T Smith-Leiker, and L Cooley. 2007. "The Ovhts polyprotein is cleaved to produce fusome and ring canal proteins required for Drosophila oogenesis." *Development* 134(4): 703–12.

25. Pomerening, JR, ED Sontag, and JE Jr Ferrell. 2003. "Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2." *Nat Cell Biol* 5(4): 346–51.

26. Ruiz, EJ, M Vilar, and AR Nebreda. 2010. "A two-step inactivation mechanism of Myt1 ensures CDK1/cyclin B activation and meiosis I entry." *Curr Biol* 20(8): 717–23.

27. Salzmann, V, C Chen, CY Chiang, A Tiyaboonchai, M Mayer, and YM Yamashita. 2014. "Centrosome-dependent asymmetric inheritance of the midbody ring in Drosophila germline stem cell division." *Mol Biol Cell* 25(2): 267–75.

28. Sigrist, S, G Ried, and CF Lehner. 1995. “Dmcdc2 kinase is required for both meiotic divisions during Drosophila spermatogenesis and is activated by the Twine/cdc25 phosphatase.” *Mech Dev* 53(2): 247–60.

29. Slepchenko, BM, and M Terasaki. 2003. “Cyclin aggregation and robustness of bio-switching.” *Mol Biol Cell* 14(11): 4695–706.

30. Takaki, T, K Trenz, V Costanzo, and M Petronczki. 2008. “Polo-like kinase 1 reaches beyond mitosis--cytokinesis, DNA damage response, and development.” *Curr Opin Cell Biol* 20(6): 650–60.

31. Tuck, C, T Zhang, T Potapova, M Malumbres, and B Novak. 2013. “Robust mitotic entry is ensured by a latching switch.” *Biol Open* 2(9): 924–31.

32. Ueishi, S, H Shimizu, and Y H Inoue. 2009. “Male germline stem cell division and spermatocyte growth require insulin signaling in Drosophila.” *Cell Struct Funct* 34(1): 61–69.

33. Volarevic, S, and G Thomas. 2001. “Role of S6 phosphorylation and S6 kinase in cell growth.” *Prog Nucleic Acid Res Mol Biol* 65: 101–27.

34. Von Stetina, JR, and TL Orr-Weaver. 2011. “Developmental control of oocyte maturation and egg activation in metazoan models.” *Cold Spring Harb Perspect Biol* 3(10): a005553.

35. Williams, BC, MF Riedy, EV Williams, M Gatti, and ML Goldberg. 1995. “The Drosophila kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis.” *J Cell Biol* 129(3): 709–23.

36. Wilson, PG. 1999. “BimC motor protein KLP61F cycles between mitotic spindles and fusomes in Drosophila germ cells.” *Curr Biol* 9(16): 923–26.

37. Wilson, PG. 2005. “Centrosome inheritance in the male germ line of Drosophila requires hu-li tai-shao function.” *Cell Biol Int* 29(5): 360–69.

38. Yamashita, YM. 2009. “The centrosome and asymmetric cell division.” *Prion* 3(2): 84

Bibliography

Abraham, RT. 2001. “Cell cycle checkpoint signaling through the ATM and ATR kinases.” *Genes Dev* 15(17): 2177–96.

Adhikari, D, and K Liu. 2014. “The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes.” *Mol Cell Endocrinol* 382(1): 480–87.

Ajduk, A, A Malagocki, and M Maleszewski. 2008. “Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca2+ oscillations.” *Reprod Biol* 8(1): 3–22.

Allen, AK, JE Nesmith, and A Golden. 2014. “An RNAi-based suppressor screen identifies interactors of the Myt1 ortholog of Caenorhabditis elegans.” *G3 (Bethesda)* 4(12): 2329–43.

Alphey, L, J Jimenez, H White-Cooper, I Dawson, P Nurse, and DM Glover. 1992. “twine, a cdc25 homolog that functions in the male and female germline of Drosophila.” *Cell* 69(6): 977–88.

Archambault, V, X Zhao, H White-Cooper, AT Carpenter, and DM Glover. 2007. “Mutations in Drosophila Greatwall/Scant reveal its roles in mitosis and meiosis and interdependence with Polo kinase.” *PLoS Genet* 3(11): e200.

Avazeri, N, AM Courtot, A Pesty, and B Lefevre. 2003. “Meiosis resumption, calcium-sensitive period, and PLC-beta1 relocation into the nucleus in the mouse oocyte.” *Cell Signal* 15(11): 1003–10.

Ayeni, JO, and SD Campbell. 2014. “”Ready, set, go”: checkpoint regulation by Cdk1 inhibitory phosphorylation.” *Fly (Austin)* 8(3): 140–47.

Ayeni, JO, R Varadarajan, O Mukherjee, DT Stuart, F Sprenger, M Srayko, and SD Campbell. 2014. “Dual phosphorylation of cdk1 coordinates cell proliferation with key developmental processes in Drosophila.” *Genetics* 196(1): 197–210.

Baker, CC, and MT Fuller. 2007. “Translational control of meiotic cell cycle progression and spermatid differentiation in male germ cells by a novel eIF4G homolog.” *Development* 134(15): 2863–69.

Bate, Michael. 1994. *The Development of Drosophila Melanogaster*. Cold Spring Harbor

Laboratory Pr.

Becker, EB, and A Bonni. 2004. “Cell cycle regulation of neuronal apoptosis in development and disease.” *Prog Neurobiol* 72(1): 1–25.

Belloni, G, S Sechi, MG Riparbelli, MT Fuller, G Callaini, and MG Giansanti. 2012. “Mutations in Cog7 affect Golgi structure, meiotic cytokinesis and sperm development during Drosophila spermatogenesis.” *J Cell Sci* 125(Pt 22): 5441–52.

Bergman, ZJ, JD Mclaurin, AS Eritano, BM Johnson, AQ Sims, and B Riggs. 2015. “Spatial Reorganization of the Endoplasmic Reticulum during Mitosis Relies on Mitotic Kinase Cyclin A in the Early Drosophila Embryo.” *PLoS One* 10(2): e0117859.

Bettencourt-Dias, M, A Rodrigues-Martins, L Carpenter, M Riparbelli, L Lehmann, MK Gatt, N Carmo, F Balloux, G Callaini, and DM Glover. 2005. “SAK/PLK4 is required for centriole duplication and flagella development.” *Curr Biol* 15(24): 2199–207.

Blachon, S, X Cai, KA Roberts, K Yang, A Polyanovsky, A Church, and T Avidor-Reiss. 2009. “A proximal centriole-like structure is present in Drosophila spermatids and can serve as a model to study centriole duplication.” *Genetics* 182(1): 133–44.

Bolanos-Garcia, VM. 2014. “Formation of multiprotein assemblies in the nucleus: the spindle assembly checkpoint.” *Int Rev Cell Mol Biol* 307: 151–74.

Bolivar, J, JR Huynh, H Lopez-Schier, C Gonzalez, D St Johnston, and A Gonzalez-Reyes. 2001. “Centrosome migration into the Drosophila oocyte is independent of BicD and egl, and of the organisation of the microtubule cytoskeleton.” *Development* 128(10): 1889–97.

Booher, RN, PS Holman, and A Fattaey. 1997. “Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity.” *J Biol Chem* 272(35): 22300–06.

Brand, AH, and N Perrimon. 1993. “Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.” *Development* 118(2): 401–15.

Brink, NG. 1968. “Protein synthesis during spermatogenesis in Drosophila melanogaster.” *Mutat Res* 5(1): 192–94.

Brodsky, MH, JJ Sekelsky, G Tsang, RS Hawley, and GM Rubin. 2000. “mus304 encodes a novel DNA damage checkpoint protein required during Drosophila development.” *Genes Dev* 14(6): 666–78.

Burrows, AE, BK Sceurman, ME Kosinski, CT Richie, PL Sadler, JM Schumacher, and A

Golden. 2006. “The C. elegans Myt1 ortholog is required for the proper timing of oocyte maturation.” *Development* 133(4): 697–709.

Cabernard, C. 2012. “Cytokinesis in Drosophila melanogaster.” *Cytoskeleton (Hoboken)* 69(10): 791–809.

Cabral, G, SS Sans, CR Cowan, and A Dammermann. 2013. “Multiple mechanisms contribute to centriole separation in C. elegans.” *Curr Biol* 23(14): 1380–87.

Campbell, SD, F Sprenger, BA Edgar, and PH O’Farrell. 1995. “Drosophila Wee1 kinase rescues fission yeast from mitotic catastrophe and phosphorylates Drosophila Cdc2 in vitro.” *Mol Biol Cell* 6(10): 1333–47.

Cantu, JM, F Rivas, P Hernandez-Jauregui, M Diaz, V Cortes-Gallegos, G Vaca, A Velazquez, and B Ibarra. 1981. “Meiotic arrest at first spermatocyte level: a new inherited infertility disorder.” *Hum Genet* 59(4): 380–85.

Castedo, M, JL Perfettini, T Roumier, and G Kroemer. 2002. “Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe.” *Cell Death Differ* 9(12): 1287–93.

Cenci, G, S Bonaccorsi, C Pisano, F Verni, and M Gatti. 1994. “Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of Drosophila melanogaster spermatogenesis.” *J Cell Sci* 107 (Pt 12): 3521–34.

Chan, PC, RY Hsu, CW Liu, CC Lai, and HC Chen. 2014. “Adducin-1 is essential for mitotic spindle assembly through its interaction with myosin-X.” *J Cell Biol* 204(1): 19–28.

Channing, CP, T Hillensjo, and FW Schaerf. 1978. “Hormonal control of oocyte meiosis, ovulation and luteinization in mammals.” *Clin Endocrinol Metab* 7(3): 601–24.

Chen, D, and DM McKearin. 2003. “A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell.” *Development* 130(6): 1159–70.

Chen, JV, and TL Megraw. 2014. “Mother centrioles do a cartwheel to produce just one daughter.” *Dev Cell* 30(2): 111–12.

Chow, JP, and RY Poon. 2013. “The CDK1 inhibitory kinase MYT1 in DNA damage checkpoint recovery.” *Oncogene* 32(40): 4778–88.

Chow, JP, RY Poon, and HT Ma. 2011. “Inhibitory phosphorylation of cyclin-dependent kinase 1 as a compensatory mechanism for mitosis exit.” *Mol Cell Biol* 31(7): 1478–91.

Chu, T, G Henrion, V Haegeli, and S Strickland. 2001. “Cortex, a Drosophila gene required to complete oocyte meiosis, is a member of the Cdc20/fizzy protein family.” *Genesis* 29(3): 141–52.

Clute, P, and J Pines. 1999. “Temporal and spatial control of cyclin B1 destruction in metaphase.” *Nat Cell Biol* 1(2): 82–87.

Cornwell, WD, PJ Kaminski, and JR Jackson. 2002. “Identification of Drosophila Myt1 kinase and its role in Golgi during mitosis.” *Cell Signal* 14(5): 467–76.

Coulonval, K, H Kooken, and PP Roger. 2011. “Coupling of T161 and T14 phosphorylations protects cyclin B-CDK1 from premature activation.” *Mol Biol Cell* 22(21): 3971–85.

Courtot, C, C Fankhauser, V Simanis, and CF Lehner. 1992. “The Drosophila cdc25 homolog twine is required for meiosis.” *Development* 116(2): 405–16.

Cox, DN, B Lu, TQ Sun, LT Williams, and YN Jan. 2001a. “Drosophila par-1 is required for oocyte differentiation and microtubule organization.” *Curr Biol* 11(2): 75–87.

Cox, DN, SA Seyfried, LY Jan, and YN Jan. 2001b. “Bazooka and atypical protein kinase C are required to regulate oocyte differentiation in the Drosophila ovary.” *Proc Natl Acad Sci U S A* 98(25): 14475–80.

Cunha-Ferreira, I, A Rodrigues-Martins, I Bento, M Riparbelli, W Zhang, E Laue, G Callaini, DM Glover, and M Bettencourt-Dias. 2009. “The SCF/Slimb ubiquitin ligase limits centrosome amplification through degradation of SAK/PLK4.” *Curr Biol* 19(1): 43–49.

De Boer, L, V Oakes, H Beamish, N Giles, F Stevens, M Somodevilla-Torres, C Desouza, and B Gabrielli. 2008. “Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events.” *Oncogene* 27(31): 4261–68.

de Cuevas, M, JK Lee, and AC Spradling. 1996. “alpha-spectrin is required for germline cell division and differentiation in the Drosophila ovary.” *Development* 122(12): 3959–68.

de Cuevas, M, MA Lilly, and AC Spradling. 1997. “Germline cyst formation in Drosophila.” *Annu Rev Genet* 31: 405–28.

de Cuevas, M, and AC Spradling. 1998. “Morphogenesis of the Drosophila fusome and its implications for oocyte specification.” *Development* 125(15): 2781–89.

de Vries, HI, L Uyetake, W Lemstra, JF Brunsting, TT Su, HH Kampinga, and OC Sibon. 2005. “Grp/DChk1 is required for G2-M checkpoint activation in Drosophila S2 cells, whereas Dmnk/DChk2 is dispensable.” *J Cell Sci* 118(Pt 9): 1833–42.

Deng, W, and H Lin. 2001. “Asymmetric germ cell division and oocyte determination during Drosophila oogenesis.” *Int Rev Cytol* 203: 93–138.

Dienemann, A, and F Sprenger. 2004. “Requirements of cyclin a for mitosis are independent of its subcellular localization.” *Curr Biol* 14(12): 1117–23.

Doniach, T. 1986. “Activity of the sex-determining gene tra-2 is modulated to allow spermatogenesis in the C. elegans hermaphrodite.” *Genetics* 114(1): 53–76.

Duckworth, BC, JS Weaver, and JV Ruderman. 2002. “G2 arrest in Xenopus oocytes depends on phosphorylation of cdc25 by protein kinase A.” *Proc Natl Acad Sci U S A* 99(26): 16794–99.

Duronio, RJ. 2012. “Developing S-phase control.” *Genes Dev* 26(8): 746–50.

Duronio, RJ, and Y Xiong. 2013. “Signaling pathways that control cell proliferation.” *Cold Spring Harb Perspect Biol* 5(3): a008904.

Duronio, Robert J, and Patrick H O’Farrell. 1995. “Developmental control of the G1 to S transition in Drosophila: cyclin Eis a limiting downstream target of E2F.” *Genes & Development* 9(12): 1456–68.

Dzhindzhev, NS, G Tzolovsky, Z Lipinszki, S Schneider, R Lattao, J Fu, J Debski, M Dadlez, and DM Glover. 2014. “Plk4 phosphorylates Ana2 to trigger Sas6 recruitment and procentriole formation.” *Curr Biol* 24(21): 2526–32.

Edgar, BA, F Sprenger, RJ Duronio, P Leopold, and PH O’Farrell. 1994. “Distinct molecular mechanism regulate cell cycle timing at successive stages of Drosophila embryogenesis.” *Genes Dev* 8(4): 440–52.

Eikenes, AH, A Brech, H Stenmark, and K Haglund. 2013. “Spatiotemporal control of Cindr at ring canals during incomplete cytokinesis in the Drosophila male germline.” *Dev Biol* 377(1): 9–20.

Enserink, JM, and RD Kolodner. 2010. “An overview of Cdk1-controlled targets and processes.” *Cell Div* 5: 11.

Fagarasanu, A, FD Mast, B Knoblach, and RA Rachubinski. 2010. “Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast.” *Nat Rev Mol Cell Biol* 11(9): 644–54.

Fattaey, A, and RN Booher. 1997. “Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14.” *Prog Cell Cycle Res* 3: 233–40.

Fichelson, P, A Audibert, F Simon, and M Gho. 2005. “Cell cycle and cell-fate determination in Drosophila neural cell lineages.” *Trends Genet* 21(7): 413–20.

Fichelson, P, and JR Huynh. 2007. “Asymmetric divisions of germline cells.” *Prog Mol Subcell Biol* 45: 97–120.

Field, CM, and BM Alberts. 1995. “Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex.” *J Cell Biol* 131(1): 165–78.

Firat-Karalar, EN, and T Stearns. 2014. “The centriole duplication cycle.” *Philos Trans R Soc Lond B Biol Sci* 369(1650):

Fogarty, P, RF Kalpin, and W Sullivan. 1994. “The Drosophila maternal-effect mutation grapes causes a metaphase arrest at nuclear cycle 13.” *Development* 120(8): 2131–42.

Foley, E, PH O’Farrell, and F Sprenger. 1999. “Rux is a cyclin-dependent kinase inhibitor (CKI) specific for mitotic cyclin-Cdk complexes.” *Curr Biol* 9(23): 1392–402.

Fong, CS, M Kim, TT Yang, JC Liao, and MF Tsou. 2014. “SAS-6 assembly templated by the lumen of cartwheel-less centrioles precedes centriole duplication.” *Dev Cell* 30(2): 238–45.

Frodin, M, and S Gammeltoft. 1999. “Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction.” *Mol Cell Endocrinol* 151(1-2): 65–77.

Fry, AM. 2015. “Solving the centriole disengagement puzzle.” *Nat Cell Biol* 17(1): 3–5.

Fu, J, IM Hagan, and DM Glover. 2015. “The centrosome and its duplication cycle.” *Cold Spring Harb Perspect Biol* 7(2): a015800.

Fuller, MT. 1998. “Genetic control of cell proliferation and differentiation in Drosophila spermatogenesis.” *Semin Cell Dev Biol* 9(4): 433–44.

Fung, TK, HT Ma, and RY Poon. 2007. “Specialized roles of the two mitotic cyclins in somatic cells: cyclin A as an activator of M phase-promoting factor.” *Mol Biol Cell* 18(5): 1861–73.

Furman, DP, and TA Bukharina. 2012. “Morphogenesis of Drosophila melanogaster macrochaetes: cell fate determination for bristle organ.” *J Stem Cells* 7(1): 19–41.

Furuno, N, N den Elzen, and J Pines. 1999. “Human cyclin A is required for mitosis until mid prophase.” *J Cell Biol* 147(2): 295–306.

Furuno, N, A Kawasaki, and N Sagata. 2003. “Expression of cell-cycle regulators during Xenopus oogenesis.” *Gene Expr Patterns* 3(2): 165–68.

Gaffre, M, A Martoriati, N Belhachemi, JP Chambon, E Houliston, C Jessus, and A Karaiskou. 2011. “A critical balance between Cyclin B synthesis and Myt1 activity controls meiosis entry in Xenopus oocytes.” *Development* 138(17): 3735–44.

Gavet, O, and J Pines. 2010a. “Activation of cyclin B1-Cdk1 synchronizes events in the nucleus and the cytoplasm at mitosis.” *J Cell Biol* 189(2): 247–59.

Gavet, O, and J Pines. 2010. “Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis.” *Dev Cell* 18(4): 533–43.

Giansanti, MG, S Bonaccorsi, and M Gatti. 1999. “The role of anillin in meiotic cytokinesis of Drosophila males.” *J Cell Sci* 112 (Pt 14): 2323–34.

Giansanti, MG, RM Farkas, S Bonaccorsi, DL Lindsley, BT Wakimoto, MT Fuller, and M Gatti. 2004. “Genetic dissection of meiotic cytokinesis in Drosophila males.” *Mol Biol Cell* 15(5): 2509–22.

Glover, DM. 2005. “Polo kinase and progression through M phase in Drosophila: a perspective from the spindle poles.” *Oncogene* 24(2): 230–37.

Gonczy, P, BJ Thomas, and S DiNardo. 1994. “roughex is a dose-dependent regulator of the second meiotic division during Drosophila spermatogenesis.” *Cell* 77(7): 1015–25.

Gonczy, P, E Matunis, and S DiNardo. 1997. “bag-of-marbles and benign gonial cell neoplasm act in the germline to restrict proliferation during Drosophila spermatogenesis.” *Development* 124(21): 4361–71.

Gorbsky, GJ. 2015. “The spindle checkpoint and chromosome segregation in meiosis.” *FEBS J* 282(13): 2458–74.

Gorr, IH, D Boos, and O Stemmann. 2005. “Mutual inhibition of separase and Cdk1 by two-step complex formation.” *Mol Cell* 19(1): 135–41.

Gottardo, M, G Callaini, and MG Riparbelli. 2013. “The cilium-like region of the Drosophila spermatocyte: an emerging flagellum?” *J Cell Sci* 126(Pt 23): 5441–52.

Gottardo, M, G Callaini, and MG Riparbelli. 2014. “Procentriole assembly without centriole disengagement - a paradox of male gametogenesis.” *J Cell Sci* 127(Pt 16): 3434–39.

Gould, KL, and P Nurse. 1989. “Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis.” *Nature* 342(6245): 39–45.

Habedanck, R, YD Stierhof, CJ Wilkinson, and EA Nigg. 2005. “The Polo kinase Plk4 functions in centriole duplication.” *Nat Cell Biol* 7(11): 1140–46.

Hackstein, JH. 1991. “Spermatogenesis in Drosophila. A genetic approach to cellular and subcellular differentiation.” *Eur J Cell Biol* 56(2): 151–69.

Han, SJ, R Chen, MP Paronetto, and M Conti. 2005. “Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse.” *Curr Biol* 15(18): 1670–76.

Harper, JW, and SJ Elledge. 1998. “The role of Cdk7 in CAK function, a retro-retrospective.” *Genes Dev* 12(3): 285–89.

Hartwell, LH, and TA Weinert. 1989. “Checkpoints: controls that ensure the order of cell cycle events.” *Science* 246(4930): 629–34.

Hatano, T, and G Sluder. 2012. “The interrelationship between APC/C and Plk1 activities in centriole disengagement.” *Biol Open* 1(11): 1153–60.

Hime, GR, JA Brill, and MT Fuller. 1996. “Assembly of ring canals in the male germ line from structural components of the contractile ring.” *J Cell Sci* 109 (Pt 12): 2779–88.

Holt, JE, SI Lane, and KT Jones. 2013. “The control of meiotic maturation in mammalian oocytes.” *Curr Top Dev Biol* 102: 207–26.

Hormanseder, E, T Tischer, and TU Mayer. 2013. “Modulation of cell cycle control during oocyte-to-embryo transitions.” *EMBO J* 32(16): 2191–203.

Hoyt, MA, L Totis, and BT Roberts. 1991. “S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function.” *Cell* 66(3): 507–17.

Huynh, JR, JM Shulman, R Benton, and D St Johnston. 2001. “PAR-1 is required for the maintenance of oocyte fate in Drosophila.” *Development* 128(7): 1201–09.

Huynh, JR, and D St Johnston. 2004. “The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte.” *Curr Biol* 14(11): R438–49.

Ikeda, MA, L Jakoi, and JR Nevins. 1996. “A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation.” *Proc Natl Acad Sci U S A* 93(8): 3215–20.

Inanc, B, H Dodson, and CG Morrison. 2010. “A centrosome-autonomous signal that involves centriole disengagement permits centrosome duplication in G2 phase after DNA damage.” *Mol Biol Cell* 21(22): 3866–77.

Inoue, D, and N Sagata. 2005. “The Polo-like kinase Plx1 interacts with and inhibits Myt1 after fertilization of Xenopus eggs.” *EMBO J* 24(5): 1057–67.

Insco, ML, A Leon, CH Tam, DM McKearin, and MT Fuller. 2009. “Accumulation of a

differentiation regulator specifies transit amplifying division number in an adult stem cell lineage." *Proc Natl Acad Sci U S A* 106(52): 22311–16.

Irniger, S, S Piatti, C Michaelis, and K Nasmyth. 1995. "Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast." *Cell* 81(2): 269–78.

Ivanovska, I, E Lee, KM Kwan, DD Fenger, and TL Orr-Weaver. 2004. "The Drosophila MOS ortholog is not essential for meiosis." *Curr Biol* 14(1): 75–80.

Jin, Z, E Homola, S Tiong, and SD Campbell. 2008. "Drosophila myt1 is the major cdk1 inhibitory kinase for wing imaginal disc development." *Genetics* 180(4): 2123–33.

Jin, Z, EM Homola, P Goldbach, Y Choi, JA Brill, and SD Campbell. 2005. "Drosophila Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis." *Development* 132(18): 4075–85.

Kannan, R, I Kuzina, S Wincovitch, SH Nowotarski, and E Giniger. 2014. "The Abl/enabled signaling pathway regulates Golgi architecture in Drosophila photoreceptor neurons." *Mol Biol Cell* 25(19): 2993–3005.

Karaiskou, A, AC Lepretre, G Pahlavan, D Du Pasquier, R Ozon, and C Jessus. 2004. "Polo-like kinase confers MPF autoamplification competence to growing Xenopus oocytes." *Development* 131(7): 1543–52.

Kierszenbaum, AL. 2002. "Sperm axoneme: a tale of tubulin posttranslation diversity." *Mol Reprod Dev* 62(1): 1–3.

Kiger, AA, DL Jones, C Schulz, MB Rogers, and MT Fuller. 2001. "Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue." *Science* 294(5551): 2542–45.

Kim, M, CS Fong, and MF Tsou. 2014. "Centriole duplication: when PLK4 meets Ana2/STIL." *Curr Biol* 24(21): R1046–48.

Kim, S, C Spike, and D Greenstein. 2013. "Control of oocyte growth and meiotic maturation in Caenorhabditis elegans." *Adv Exp Med Biol* 757: 277–320.

Kimata, Y, JE Baxter, AM Fry, and H Yamano. 2008. "A role for the Fizzy/Cdc20 family of proteins in activation of the APC/C distinct from substrate recruitment." *Mol Cell* 32(4): 576–83.

Kishimoto, T. 2003. "Cell-cycle control during meiotic maturation." *Curr Opin Cell Biol*

15(6): 654–63.

Kishimoto, T. 2011. “A primer on meiotic resumption in starfish oocytes: the proposed signaling pathway triggered by maturation-inducing hormone.” *Mol Reprod Dev* 78(10-11): 704–07.

Kleylein-Sohn, J, J Westendorf, M Le Clech, R Habedanck, YD Stierhof, and EA Nigg. 2007. “Plk4-induced centriole biogenesis in human cells.” *Dev Cell* 13(2): 190–202.

Klumperman, J. 2011. “Architecture of the mammalian Golgi.” *Cold Spring Harb Perspect Biol* 3(7)

Knoblich, JA, and CF Lehner. 1993. “Synergistic action of Drosophila cyclins A and B during the G2-M transition.” *EMBO J* 12(1): 65–74.

Kondylis, V, and C Rabouille. 2009. “The Golgi apparatus: lessons from Drosophila.” *FEBS Lett* 583(23): 3827–38.

Kondylis, V, HE van Nispen tot Pannerden, B Herpers, F Friggi-Grelin, and C Rabouille. 2007. “The golgi comprises a paired stack that is separated at G2 by modulation of the actin cytoskeleton through Abi and Scar/WAVE.” *Dev Cell* 12(6): 901–15.

Kornbluth, S, B Sebastian, T Hunter, and J Newport. 1994. “Membrane localization of the kinase which phosphorylates p34cdc2 on threonine 14.” *Mol Biol Cell* 5(3): 273–82.

Krek, W, J Marks, N Schmitz, EA Nigg, and V Simanis. 1992. “Vertebrate p34cdc2 phosphorylation site mutants: effects upon cell cycle progression in the fission yeast Schizosaccharomyces pombe.” *J Cell Sci* 102 (Pt 1): 43–53.

Krek, W, and EA Nigg. 1991. “Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates.” *EMBO J* 10(11): 3331–41.

Kristjansdottir, K, A Safi, C Shah, and J Rudolph. 2006. “Autophosphorylation of Ser66 on Xenopus Myt1 is a prerequisite for meiotic inactivation of Myt1.” *Cell Cycle* 5(4): 421–27.

Kronja, I, ZJ Whitfield, B Yuan, K Dzeyk, J Kirkpatrick, J Krijgsveld, and TL Orr-Weaver. 2014. “Quantitative proteomics reveals the dynamics of protein changes during Drosophila oocyte maturation and the oocyte-to-embryo transition.” *Proc Natl Acad Sci U S A* 111(45): 16023–28.

Kulawiec, M, V Ayyasamy, and KK Singh. 2009. “p53 regulates mtDNA copy number and

mitocheckpoint pathway." *J Carcinog* 8: 8.

Lamitina, ST, and SW L'Hernault. 2002. "Dominant mutations in the Caenorhabditis elegans Myt1 ortholog wee-1.3 reveal a novel domain that controls M-phase entry during spermatogenesis." *Development* 129(21): 5009–18.

Lee, K, and K Rhee. 2012. "Separase-dependent cleavage of pericentrin B is necessary and sufficient for centriole disengagement during mitosis." *Cell Cycle* 11(13): 2476–85.

Lehner, CF, and PH O'Farrell. 1989. "Expression and function of Drosophila cyclin A during embryonic cell cycle progression." *Cell* 56(6): 957–68.

Lehner, CF, and PH O'Farrell. 1990. "The roles of Drosophila cyclins A and B in mitotic control." *Cell* 61(3): 535–47.

Levine, MS, and AJ Holland. 2014. "Polo-like kinase 4 shapes up." *Structure* 22(8): 1071–73.

Lighthouse, DV, M Buszczak, and AC Spradling. 2008. "New components of the Drosophila fusome suggest it plays novel roles in signaling and transport." *Dev Biol* 317(1): 59–71.

Lilly, MA, M de Cuevas, and AC Spradling. 2000. "Cyclin A associates with the fusome during germline cyst formation in the Drosophila ovary." *Dev Biol* 218(1): 53–63.

Lin, H, and AC Spradling. 1995. "Fusome asymmetry and oocyte determination in Drosophila." *Dev Genet* 16(1): 6–12.

Lin, H, L Yue, and AC Spradling. 1994. "The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation." *Development* 120(4): 947–56.

Lin, TY, S Viswanathan, C Wood, PG Wilson, N Wolf, and MT Fuller. 1996. "Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in Drosophila males." *Development* 122(4): 1331–41.

Liu, D, MM Matzuk, WK Sung, Q Guo, P Wang, and DJ Wolgemuth. 1998. "Cyclin A1 is required for meiosis in the male mouse." *Nat Genet* 20(4): 377–80.

Liu, F, C Rothblum-Oviatt, CE Ryan, and H Piwnica-Worms. 1999a. "Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes." *Mol Cell Biol* 19(7): 5113–23.

Liu, F, JJ Stanton, Z Wu, and H Piwnica-Worms. 1997. "The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex." *Mol Cell Biol* 17(2): 571–83.

Liu, Z, T Xie, and R Steward. 1999b. "Lis1, the Drosophila homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation." *Development* 126(20): 4477–88.

Lowe, M, and FA Barr. 2007. "Inheritance and biogenesis of organelles in the secretory pathway." *Nat Rev Mol Cell Biol* 8(6): 429–39.

Luders, J. 2012. "The amorphous pericentriolar cloud takes shape." *Nat Cell Biol* 14(11): 1126–28.

Lundgren, K, N Walworth, R Booher, M Dembski, M Kirschner, and D Beach. 1991. "mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2." *Cell* 64(6): 1111–22.

Mardin, BR, and E Schiebel. 2012. "Breaking the ties that bind: new advances in centrosome biology." *J Cell Biol* 197(1): 11–18.

Maro, B, E Houliston, and M Paintrand. 1988. "Purification of meiotic spindles and cytoplasmic asters from mouse oocytes." *Dev Biol* 129(2): 275–82.

Mathe, E, YH Inoue, W Palframan, G Brown, and DM Glover. 2003. "Orbit/Mast, the CLASP orthologue of Drosophila, is required for asymmetric stem cell and cystocyte divisions and development of the polarised microtubule network that interconnects oocyte and nurse cells during oogenesis." *Development* 130(5): 901–15.

Matsuura, I, and JH Wang. 1996. "Demonstration of cyclin-dependent kinase inhibitory serine/threonine kinase in bovine thymus." *J Biol Chem* 271(10): 5443–50.

McCleland, ML, JA Farrell, and PH O'Farrell. 2009. "Influence of cyclin type and dose on mitotic entry and progression in the early Drosophila embryo." *J Cell Biol* 184(5): 639–46.

McCullough, S, and J Lucocq. 2005. "Endoplasmic reticulum positioning and partitioning in mitotic HeLa cells." *J Anat* 206(5): 415–25.

McKearin, D. 1997. "The Drosophila fusome, organelle biogenesis and germ cell differentiation: if you build it." *Bioessays* 19(2): 147–52.

McKearin, DM, and AC Spradling. 1990. "bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis." *Genes Dev* 4(12B): 2242–51.

Megraw, TL, and TC Kaufman. 2000. "The centrosome in Drosophila oocyte development." *Curr Top Dev Biol* 49: 385–407.

Miller, MA, VQ Nguyen, MH Lee, M Kosinski, T Schedl, RM Caprioli, and D Greenstein. 2001. "A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation." *Science* 291(5511): 2144–47.

Minocherhomji, S, TO Tollefsbol, and KK Singh. 2012. "Mitochondrial regulation of epigenetics and its role in human diseases." *Epigenetics* 7(4): 326–34.

Miyauchi, C, D Kitazawa, I Ando, D Hayashi, and YH Inoue. 2013. "Orbit/CLASP is required for germline cyst formation through its developmental control of fusomes and ring canals in Drosophila males." *PLoS One* 8(3): e58220.

Morris, LX, and AC Spradling. 2012. "Steroid signaling within Drosophila ovarian epithelial cells sex-specifically modulates early germ cell development and meiotic entry." *PLoS One* 7(10): e46109.

Mottier-Pavie, V, and TL Megraw. 2009. "Drosophila bld10 is a centriolar protein that regulates centriole, basal body, and motile cilium assembly." *Mol Biol Cell* 20(10): 2605–14.

Moyer, TC, KM Clutario, BG Lambrus, V Daggubati, and AJ Holland. 2015. "Binding of STIL to Plk4 activates kinase activity to promote centriole assembly." *J Cell Biol* 209(6): 863–78.

Mueller, PR, TR Coleman, A Kumagai, and WG Dunphy. 1995. "Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15." *Science* 270(5233): 86–90.

Mueller, PR, TR Coleman, and WG Dunphy. 1995. "Cell cycle regulation of a Xenopus Wee1-like kinase." *Mol Biol Cell* 6(1): 119–34.

Murakami, MS, and GF Vande Woude. 1998. "Analysis of the early embryonic cell cycles of Xenopus; regulation of cell cycle length by Xe-wee1 and Mos." *Development* 125(2): 237–48.

Nakajima, H, S Yonemura, M Murata, N Nakamura, H Piwnica-Worms, and E Nishida. 2008. "Myt1 protein kinase is essential for Golgi and ER assembly during mitotic exit." *J Cell Biol* 181(1): 89–103.

Nakajo, N, S Yoshitome, J Iwashita, M Iida, K Uto, S Ueno, K Okamoto, and N Sagata. 2000. "Absence of Wee1 ensures the meiotic cell cycle in Xenopus oocytes." *Genes Dev* 14(3): 328–38.

Nash, B, and B Bowerman. 2004. “Uncoiling centriole duplication.” *Nat Cell Biol* 6(7): 573–75.

Nebreda, AR, and I Ferby. 2000. “Regulation of the meiotic cell cycle in oocytes.” *Curr Opin Cell Biol* 12(6): 666–75.

Ni, JQ, R Zhou, B Czech, LP Liu, L Holderbaum, D Yang-Zhou, HS Shim, R Tao, D Handler, P Karpowicz, R Binari, M Booker, J Brennecke, LA Perkins, GJ Hannon, and N Perrimon. 2011. “A genome-scale shRNA resource for transgenic RNAi in Drosophila.” *Nat Methods* 8(5): 405–07.

Nigg, EA. 2006. “Cell biology: a licence for duplication.” *Nature* 442(7105): 874–75.

Norbury, C, J Blow, and P Nurse. 1991. “Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates.” *EMBO J* 10(11): 3321–29.

Nurse, P, and P Thuriaux. 1980. “Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe.” *Genetics* 96(3): 627–37.

Oegema, K, MS Savoian, TJ Mitchison, and CM Field. 2000. “Functional analysis of a human homologue of the Drosophila actin binding protein anillin suggests a role in cytokinesis.” *J Cell Biol* 150(3): 539–52.

O’Farrell, PH. 2001. “Triggering the all-or-nothing switch into mitosis.” *Trends Cell Biol* 11(12): 512–19.

Oh, JS, SJ Han, and M Conti. 2010. “Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption.” *J Cell Biol* 188(2): 199–207.

Ohi, R, and KL Gould. 1999. “Regulating the onset of mitosis.” *Curr Opin Cell Biol* 11(2): 267–73.

Ohlmeyer, JT, and T Schupbach. 2003. “Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during Drosophila oogenesis.” *Development* 130(25): 6339–49.

Ohsumi, K, W Sawada, and T Kishimoto. 1994. “Meiosis-specific cell cycle regulation in maturing Xenopus oocytes.” *J Cell Sci* 107 (Pt 11): 3005–13.

Okano-Uchida, T, E Okumura, M Iwashita, H Yoshida, K Tachibana, and T Kishimoto. 2003. “Distinct regulators for Plk1 activation in starfish meiotic and early embryonic cycles.” *EMBO J* 22(20): 5633–42.

Okumura, E, T Fukuhara, H Yoshida, S Hanada Si, R Kozutsumi, M Mori, K Tachibana, and

T Kishimoto. 2002. “Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition.” *Nat Cell Biol* 4(2): 111–16.

Oliveira, RA, and K Nasmyth. 2013. “Cohesin cleavage is insufficient for centriole disengagement in Drosophila.” *Curr Biol* 23(14): R601–03.

Orr-Weaver, TL. 1995. “Meiosis in Drosophila: seeing is believing.” *Proc Natl Acad Sci U S A* 92(23): 10443–49.

Ou, Y, M Zhang, and JB Rattner. 2004. “The centrosome: The centriole-PCM coalition.” *Cell Motil Cytoskeleton* 57(1): 1–7.

Palmer, A, AC Gavin, and AR Nebreda. 1998. “A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1.” *EMBO J* 17(17): 5037–47.

Palmer, A, and AR Nebreda. 2000. “The activation of MAP kinase and p34cdc2/cyclin B during the meiotic maturation of Xenopus oocytes.” *Prog Cell Cycle Res* 4: 131–43.

Parker, LL, S Atherton-Fessler, and H Piwnica-Worms. 1992. “p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15.” *Proc Natl Acad Sci U S A* 89(7): 2917–21.

Perezgasga, L, J Jiang, B Jr Bolival, M Hiller, E Benson, MT Fuller, and H White-Cooper. 2004. “Regulation of transcription of meiotic cell cycle and terminal differentiation genes by the testis-specific Zn-finger protein matotopetli.” *Development* 131(8): 1691–702.

Perry, AC, and MH Verlhac. 2008. “Second meiotic arrest and exit in frogs and mice.” *EMBO Rep* 9(3): 246–51.

Persico, A, RI Cervigni, ML Barretta, and A Colanzi. 2009. “Mitotic inheritance of the Golgi complex.” *FEBS Lett* 583(23): 3857–62.

Peter, M, JC Labbe, M Doree, and E Mandart. 2002. “A new role for Mos in Xenopus oocyte maturation: targeting Myt1 independently of MAPK.” *Development* 129(9): 2129–39.

Petrella, LN, T Smith-Leiker, and L Cooley. 2007. “The Ovhts polyprotein is cleaved to produce fusome and ring canal proteins required for Drosophila oogenesis.” *Development* 134(4): 703–12.

Pirrotta, V. 1988. “Vectors for P-mediated transformation in Drosophila.” *Biotechnology* 10: 437–56.

Pokrywka, NJ, A Payne-Tobin, KM Raley-Susman, and S Swartzman. 2009. "Microtubules, the ER and Exu: new associations revealed by analysis of mini spindles mutations." *Mech Dev* 126(5-6): 289–300.

Pomerening, JR, ED Sontag, and JE Jr Ferrell. 2003. "Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2." *Nat Cell Biol* 5(4): 346–51.

Potapova, TA, JR Daum, KS Byrd, and GJ Gorbsky. 2009. "Fine tuning the cell cycle: activation of the Cdk1 inhibitory phosphorylation pathway during mitotic exit." *Mol Biol Cell* 20(6): 1737–48.

Price, D, S Rabinovitch, PH O'Farrell, and SD Campbell. 2000. "Drosophila wee1 has an essential role in the nuclear divisions of early embryogenesis." *Genetics* 155(1): 159–66.

Price, DM, Z Jin, S Rabinovitch, and SD Campbell. 2002. "Ectopic expression of the Drosophila Cdk1 inhibitory kinases, Wee1 and Myt1, interferes with the second mitotic wave and disrupts pattern formation during eye development." *Genetics* 161(2): 721–31.

Priyadarshini, A, D Basu, AK Navneet, A Bhattacharya, S Bhattacharya, S Maitra, and S Bhattacharya. 2009. "Activation of both Mos and Cdc25 is required for G2-M transition in perch oocyte." *Mol Reprod Dev* 76(3): 289–300.

Raychaudhuri, N, R Dubruille, GA Orsi, HC Bagheri, B Loppin, and CF Lehner. 2012. "Transgenerational propagation and quantitative maintenance of paternal centromeres depends on Cid/Cenp-A presence in Drosophila sperm." *PLoS Biol* 10(12): e1001434.

Rhind, N, and P Russell. 2001. "Roles of the mitotic inhibitors Wee1 and Mik1 in the G(2) DNA damage and replication checkpoints." *Mol Cell Biol* 21(5): 1499–508.

Riparbelli, MG, OA Cabrera, G Callaini, and TL Megraw. 2013. "Unique properties of Drosophila spermatocyte primary cilia." *Biol Open* 2(11): 1137–47.

Riparbelli, MG, and G Callaini. 2011. "Male gametogenesis without centrioles." *Dev Biol* 349(2): 427–39.

Riparbelli, MG, G Callaini, and TL Megraw. 2012. "Assembly and persistence of primary cilia in dividing Drosophila spermatocytes." *Dev Cell* 23(2): 425–32.

Riparbelli, MG, R Dallai, D Mercati, Y Bu, and G Callaini. 2009. "Centriole symmetry: a big tale from small organisms." *Cell Motil Cytoskeleton* 66(12): 1100–05.

Riparbelli, MG, M Gottardo, DM Glover, and G Callaini. 2014. "Inhibition of Polo kinase by BI2536 affects centriole separation during Drosophila male meiosis." *Cell Cycle* 13(13):

2064–72.

Rodrigues-Martins, A, M Riparbelli, G Callaini, DM Glover, and M Bettencourt-Dias. 2008. "From centriole biogenesis to cellular function: centrioles are essential for cell division at critical developmental stages." *Cell Cycle* 7(1): 11–16.

Roegiers, F, S Younger-Shepherd, LY Jan, and YN Jan. 2001. "Two types of asymmetric divisions in the Drosophila sensory organ precursor cell lineage." *Nat Cell Biol* 3(1): 58–67.

Roper, K, and NH Brown. 2004. "A spectraplakin is enriched on the fusome and organizes microtubules during oocyte specification in Drosophila." *Curr Biol* 14(2): 99–110.

Roy, LM, KI Swenson, DH Walker, BG Gabrielli, RS Li, H Piwnica-Worms, and JL Maller. 1991. "Activation of p34cdc2 kinase by cyclin A." *J Cell Biol* 113(3): 507–14.

Ruiz, EJ, T Hunt, and AR Nebreda. 2008. "Meiotic inactivation of Xenopus Myt1 by CDK/XRINGO, but not CDK/cyclin, via site-specific phosphorylation." *Mol Cell* 32(2): 210–20.

Ruiz, EJ, M Vilar, and AR Nebreda. 2010. "A two-step inactivation mechanism of Myt1 ensures CDK1/cyclin B activation and meiosis I entry." *Curr Biol* 20(8): 717–23.

Russell, P, and P Nurse. 1987. "The mitotic inducer nim1+ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis." *Cell* 49(4): 569–76.

Salzmann, V, C Chen, CY Chiang, A Tiyaboonchai, M Mayer, and YM Yamashita. 2014. "Centrosome-dependent asymmetric inheritance of the midbody ring in Drosophila germline stem cell division." *Mol Biol Cell* 25(2): 267–75.

Santamaria, D, C Barriere, A Cerqueira, S Hunt, C Tardy, K Newton, JF Caceres, P Dubus, M Malumbres, and M Barbacid. 2007. "Cdk1 is sufficient to drive the mammalian cell cycle." *Nature* 448(7155): 811–15.

Schafer, M, K Nayernia, W Engel, and U Schafer. 1995. "Translational control in spermatogenesis." *Dev Biol* 172(2): 344–52.

Scheer, U. 2014. "Historical roots of centrosome research: discovery of Boveri's microscope slides in Wurzburg." *Philos Trans R Soc Lond B Biol Sci* 369(1650)

Schmitt, A, and AR Nebreda. 2002. "Signalling pathways in oocyte meiotic maturation." *J Cell Sci* 115(Pt 12): 2457–59.

Schockel, L, M Mockel, B Mayer, D Boos, and O Stemmann. 2011. "Cleavage of cohesin

rings coordinates the separation of centrioles and chromatids." *Nat Cell Biol* 13(8): 966–72.

Sharp, DJ, KL McDonald, HM Brown, HJ Matthies, C Walczak, RD Vale, TJ Mitchison, and JM Scholey. 1999. "The bipolar kinesin, KLP61F, cross-links microtubules within interpolar microtubule bundles of Drosophila embryonic mitotic spindles." *J Cell Biol* 144(1): 125–38.

Sigrist, S, G Ried, and CF Lehner. 1995. "Dmcdc2 kinase is required for both meiotic divisions during Drosophila spermatogenesis and is activated by the Twine/cdc25 phosphatase." *Mech Dev* 53(2): 247–60.

Sigrist, SJ, and CF Lehner. 1997. "Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles." *Cell* 90(4): 671–81.

Singh, KK. 2006. "Mitochondria damage checkpoint, aging, and cancer." *Ann N Y Acad Sci* 1067: 182–90.

Sisson, JC, C Field, R Ventura, A Royou, and W Sullivan. 2000. "Lava lamp, a novel peripheral golgi protein, is required for Drosophila melanogaster cellularization." *J Cell Biol* 151(4): 905–18.

Slepchenko, BM, and M Terasaki. 2003. "Cyclin aggregation and robustness of bio-switching." *Mol Biol Cell* 14(11): 4695–706.

Sluder, G. 2013. "Centriole engagement: it's not just cohesin any more." *Curr Biol* 23(15): R659–60.

Smith, A, S Simanski, M Fallahi, and NG Ayad. 2007. "Redundant ubiquitin ligase activities regulate wee1 degradation and mitotic entry." *Cell Cycle* 6(22): 2795–99.

Snapp, EL, T Iida, D Frescas, J Lippincott-Schwartz, and MA Lilly. 2004. "The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts." *Mol Biol Cell* 15(10): 4512–21.

Solc, P, RM Schultz, and J Motlik. 2010. "Prophase I arrest and progression to metaphase I in mouse oocytes: comparison of resumption of meiosis and recovery from G2-arrest in somatic cells." *Mol Hum Reprod* 16(9): 654–64.

Spradling, AC. 1993. "Germline cysts: communes that work." *Cell* 72(5): 649–51.

Sprenger, F, N Yakubovich, and PH O'Farrell. 1997. "S-phase function of Drosophila cyclin

A and its downregulation in G1 phase." *Curr Biol* 7(7): 488–99.

Stevens, NR, H Roque, and JW Raff. 2010. "DSas-6 and Ana2 coassemble into tubules to promote centriole duplication and engagement." *Dev Cell* 19(6): 913–19.

Stevens, NR, H Roque, and JW Raff. 2010. "DSas-6 and Ana2 coassemble into tubules to promote centriole duplication and engagement." *Dev Cell* 19(6): 913–19.

Strausfeld, UP, M Howell, P Descombes, S Chevalier, RE Rempel, J Adamczewski, JL Maller, T Hunt, and JJ Blow. 1996. "Both cyclin A and cyclin E have S-phase promoting (SPF) activity in Xenopus egg extracts." *J Cell Sci* 109 (Pt 6): 1555–63.

Strnad, P, S Leidel, T Vinogradova, U Euteneuer, A Khodjakov, and P Gonczy. 2007. "Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle." *Dev Cell* 13(2): 203–13.

Stumpff, J, T Duncan, E Homola, SD Campbell, and TT Su. 2004. "Drosophila Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis." *Curr Biol* 14(23): 2143–48.

Su, TT, SD Campbell, and PH O'Farrell. 1998. "The cell cycle program in germ cells of the Drosophila embryo." *Dev Biol* 196(2): 160–70.

Sutterlin, C, P Hsu, A Mallabiabarrena, and V Malhotra. 2002. "Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells." *Cell* 109(3): 359–69.

Swenson, KI, KM Farrell, and JV Ruderman. 1986. "The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in Xenopus oocytes." *Cell* 47(6): 861–70.

Takaki, T, K Trenz, V Costanzo, and M Petronczki. 2008. "Polo-like kinase 1 reaches beyond mitosis--cytokinesis, DNA damage response, and development." *Curr Opin Cell Biol* 20(6): 650–60.

Tang, Z, TR Coleman, and WG Dunphy. 1993. "Two distinct mechanisms for negative regulation of the Wee1 protein kinase." *EMBO J* 12(9): 3427–36.

Tang, N, and WF Marshall. 2012. "Centrosome positioning in vertebrate development." *J Cell Sci* 125(Pt 21): 4951–61.

Taylor, WR, and GR Stark. 2001. "Regulation of the G2/M transition by p53." *Oncogene* 20(15): 1803–15.

Thomas, BJ, DA Gunning, J Cho, and L Zipursky. 1994. "Cell cycle progression in the

developing Drosophila eye: roughex encodes a novel protein required for the establishment of G1." *Cell* 77(7): 1003–14.

Tokuyasu, KT, WJ Peacock, and RW Hardy. 1972. "Dynamics of spermiogenesis in Drosophila melanogaster. I. Individualization process." *Z Zellforsch Mikrosk Anat* 124(4): 479–506.

Tokuyasu, KT. 1975. "Dynamics of spermiogenesis in Drosophila melanogaster. VI. Significance of "onion" nebenkern formation." *J Ultrastruct Res* 53(1): 93–112.

Tootle, TL, D Williams, A Hubb, R Frederick, and A Spradling. 2011. "Drosophila eggshell production: identification of new genes and coordination by Pxt." *PLoS One* 6(5): e19943.

Tsou, MF, and T Stearns. 2006. "Controlling centrosome number: licenses and blocks." *Curr Opin Cell Biol* 18(1): 74–78.

Tuck, C, T Zhang, T Potapova, M Malumbres, and B Novak. 2013. "Robust mitotic entry is ensured by a latching switch." *Biol Open* 2(9): 924–31.

Ueishi, S, H Shimizu, and Y H Inoue. 2009. "Male germline stem cell division and spermatocyte growth require insulin signaling in Drosophila." *Cell Struct Funct* 34(1): 61–69.

Vaccari, T, and A Ephrussi. 2002. "The fusome and microtubules enrich Par-1 in the oocyte, where it effects polarization in conjunction with Par-3, BicD, Egl, and dynein." *Curr Biol* 12(17): 1524–28.

van der Meer, T, WY Chan, LS Palazon, C Nieduszynski, M Murphy, J Sobczak-Thepot, M Carrington, and WH Colledge. 2004. "Cyclin A1 protein shows haplo-insufficiency for normal fertility in male mice." *Reproduction* 127(4): 503–11.

van Vugt, MA, and MB Yaffe. 2010. "Cell cycle re-entry mechanisms after DNA damage checkpoints: giving it some gas to shut off the breaks!" *Cell Cycle* 9(11): 2097–101.

van Zon, W, J Ogink, B ter Riet, RH Medema, H te Riele, and RM Wolthuis. 2010. "The APC/C recruits cyclin B1-Cdk1-Cks in prometaphase before D box recognition to control mitotic exit." *J Cell Biol* 190(4): 587–602.

Villeneuve, J, M Scarpa, M Ortega-Bellido, and V Malhotra. 2013. "MEK1 inactivates Myt1 to regulate Golgi membrane fragmentation and mitotic entry in mammalian cells." *EMBO J* 32(1): 72–85.

Vincent, I, G Jicha, M Rosado, and DW Dickson. 1997. "Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain." *J Neurosci* 17(10): 3588–98.

Volarevic, S, and G Thomas. 2001. "Role of S6 phosphorylation and S6 kinase in cell growth." *Prog Nucleic Acid Res Mol Biol* 65: 101–27.

Von Stetina, JR, and TL Orr-Weaver. 2011. "Developmental control of oocyte maturation and egg activation in metazoan models." *Cold Spring Harb Perspect Biol* 3(10): a005553.

Von Stetina, JR, S Tranguch, SK Dey, LA Lee, B Cha, and D Drummond-Barbosa. 2008. "alpha-Endosulfine is a conserved protein required for oocyte meiotic maturation in Drosophila." *Development* 135(22): 3697–706.

Voronina, E, WF Marzluff, and GM Wessel. 2003. "Cyclin B synthesis is required for sea urchin oocyte maturation." *Dev Biol* 256(2): 258–75.

Walter, SA, SN Guadagno, and JE Jr Ferrell. 2000. "Activation of Wee1 by p42 MAPK in vitro and in cycling xenopus egg extracts." *Mol Biol Cell* 11(3): 887–96.

Wang, C, and R Lehmann. 1991. "Nanos is the localized posterior determinant in Drosophila." *Cell* 66(4): 637–47.

Warren, G, and V Malhotra. 1998. "The organisation of the Golgi apparatus." *Curr Opin Cell Biol* 10(4): 493–98.

Watanabe, N, M Broome, and T Hunter. 1995. "Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle." *EMBO J* 14(9): 1878–91.

Wehr, K, A Swan, and T Schupbach. 2006. "Deadlock, a novel protein of Drosophila, is required for germline maintenance, fusome morphogenesis and axial patterning in oogenesis and associates with centrosomes in the early embryo." *Dev Biol* 294(2): 406–17.

Wei, JH, and J Seemann. 2010. "Unraveling the Golgi ribbon." *Traffic* 11(11): 1391–400.

Wells, NJ, N Watanabe, T Tokusumi, W Jiang, MA Verdecia, and T Hunter. 1999. "The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression." *J Cell Sci* 112 (Pt 19): 3361–71.

Westendorf, JM, PN Rao, and L Gerace. 1994. "Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope." *Proc Natl Acad Sci U S A* 91(2): 714–18.

Whitaker, M. 1996. “Control of meiotic arrest.” *Rev Reprod* 1(2): 127–35.

White-Cooper, H. 2010. “Molecular mechanisms of gene regulation during Drosophila spermatogenesis.” *Reproduction* 139(1): 11–21.

White-Cooper, H, L Alphey, and DM Glover. 1993. “The cdc25 homologue twine is required for only some aspects of the entry into meiosis in Drosophila.” *J Cell Sci* 106 (Pt 4): 1035–44.

White-Cooper, H, MA Schafer, LS Alphey, and MT Fuller. 1998. “Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid differentiation with meiosis I in Drosophila.” *Development* 125(1): 125–34.

Williams, BC, MF Riedy, EV Williams, M Gatti, and ML Goldberg. 1995. “The Drosophila kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis.” *J Cell Biol* 129(3): 709–23.

Wilson, PG. 1999. “BimC motor protein KLP61F cycles between mitotic spindles and fusomes in Drosophila germ cells.” *Curr Biol* 9(16): 923–26.

Wilson, PG. 2005. “Centrosome inheritance in the male germ line of Drosophila requires hu-li tai-shao function.” *Cell Biol Int* 29(5): 360–69.

Wong, R, I Hadjiyanni, HC Wei, G Polevoy, R McBride, KP Sem, and JA Brill. 2005. “PIP2 hydrolysis and calcium release are required for cytokinesis in Drosophila spermatocytes.” *Curr Biol* 15(15): 1401–06.

Woodruff, JB, O Wueseke, and AA Hyman. 2014. “Pericentriolar material structure and dynamics.” *Philos Trans R Soc Lond B Biol Sci* 369(1650)

Yamashita, YM. 2009. “The centrosome and asymmetric cell division.” *Prion* 3(2): 84

Yang, R, C Muller, V Huynh, YK Fung, AS Yee, and HP Koeffler. 1999. “Functions of cyclin A1 in the cell cycle and its interactions with transcription factor E2F-1 and the Rb family of proteins.” *Mol Cell Biol* 19(3): 2400–07.

Yano, H, M Yamamoto-Hino, M Abe, R Kuwahara, S Haraguchi, I Kusaka, W Awano, A Kinoshita-Toyoda, H Toyoda, and S Goto. 2005. “Distinct functional units of the Golgi complex in Drosophila cells.” *Proc Natl Acad Sci U S A* 102(38): 13467–72.

Yasuno, Y, J Kawano, YH Inoue, and MT Yamamoto. 2013. “Distribution and morphological changes of the Golgi apparatus during Drosophila spermatogenesis.” *Dev Growth Differ*

55(6): 635–47.

Yeh, E, L Zhou, N Rudzik, and GL Boulianne. 2000. “Neuralized functions cell autonomously to regulate Drosophila sense organ development.” *EMBO J* 19(17): 4827–37.

Yue, L, and AC Spradling. 1992. “hu-li tai shao, a gene required for ring canal formation during Drosophila oogenesis, encodes a homolog of adducin.” *Genes Dev* 6(12B): 2443–54.

Zaccai, M, and HD Lipshitz. 1996. “Role of Adducin-like (hu-li tai shao) mRNA and protein localization in regulating cytoskeletal structure and function during Drosophila Oogenesis and early embryogenesis.” *Dev Genet* 19(3): 249–57.

Zhang, M, and G Xia. 2012. “Hormonal control of mammalian oocyte meiosis at diplotene stage.” *Cell Mol Life Sci* 69(8): 1279–88.

Zitouni, S, C Nabais, SC Jana, A Guerrero, and M Bettencourt-Dias. 2014. “Polo-like kinases: structural variations lead to multiple functions.” *Nat Rev Mol Cell Biol* 15(7): 433–52.

Appendix A

A.1. Functional characterization of Myt1 tethering to Cyclin B

Myt1 protein includes three major functional domains: a conserved kinase domain, trans-membrane hydrophobic domain and CyclinB interaction domain (Fig. A-1). Studies in human cultured cells revealed a small C-terminal region of Myt1 that can bind to Cyclin B through a Cyclin-interacting RXL motif (Liu *et al.*, 1997; Liu *et al.*, 1999; Wells *et al.*, 1999: Jin *et al.*, 2005). This transient interaction was proposed to physically tether the Cyclin B-Cdk1 complex in the cytoplasm and influence nuclear-cytoplasmic shuttling, in addition to Myt1 catalytic activity restricting premature mitotic initiation by inhibitory phosphorylation. I therefore hypothesized that ER-localized-Myt1 transiently tethers and phospho-inhibits Cyclin A-Cdk1 during early spermatocyte development to promote stable association with fusomes until $Cdc25^{Twe}$-mediated Cdk1 activation is established. To test this possibility I generated a new Myt1 variant bearing mutation at the Cyclin-tethering motif and examined its functional consequences in *Drosophila* development.

A.1.1. Generation of a putative CyclinB-tethering RXL mutant Myt1

Drosophila Myt1 has three RXL motifs; two of which are located within the kinase domain while a third is in the C terminal region (Fig. A-1). Based on the location of this site I chose to mutate the motif in the C terminal region of Myt, as the other two were located close to the trans-membrane domain, and therefore was expected to be less likely to interact with the cytosolic Cyclins. The 321RYL residue was mutated to triple alanine (AAA) using primers carrying the mutation. Primers used are the following: Forward (5'GTGCAATTGGAAAA CAGCCGCCTTTGCCGCCGCTCTATACTTCCTGGAGGTCCTGCATC 3') and reverse

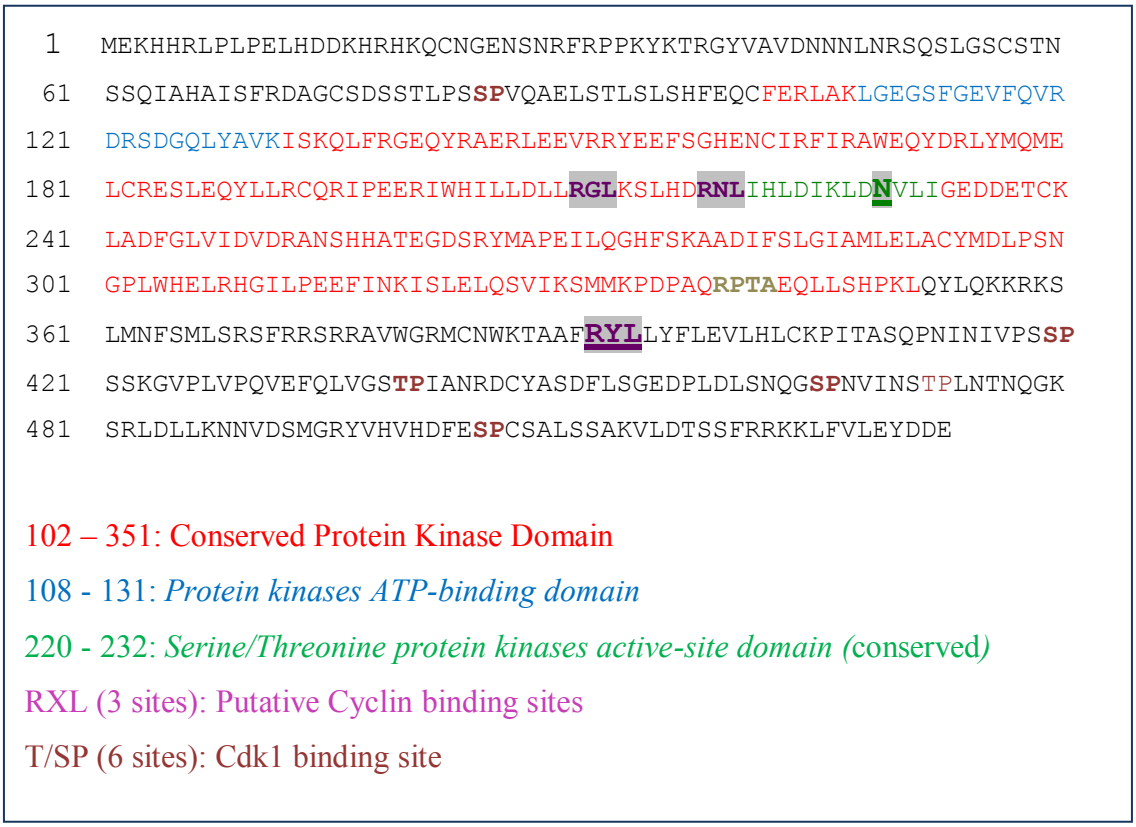

Fig. A-1: *Drosophila* Myt1 sequence and its functional motifs.

dMyt1 protein has 534 amino acids. Region indicated in red represent the conserved protein kinase domain and blue indicate the ATP binding site. Sequences labeled in green are the conserved active-site domain of Myt1. Asparagine (N229), (underlined, bold) is the critical residue that corresponds to the catalytic activity of Myt1. The three putative Cyclin binding RXL domains are shown in purple. The RYL392 motif (underlined) that is proximal to the C terminal region of Myt1 was targeted and mutated to generate RYL392AAA mutant variant. Myt1 includes 6 S/TP sites (brown) that are specific to Cdk1 binding. Five of those are located at the C-terminal region.

(5'GATGCAGGACCTCCAGGAAGTATAGAGCGGCGGCAAAGGCGGCTGTTTTCCA ATTGCAC 3') to introduce the appropriate sequence change. PCR amplification was performed using Stratagene QuikChange II site-directed mutagenesis protocol (Agilent). Two out of 8 clones obtained from site-specific mutagenesis condition were sequenced to confirm the presence of expected mutations. In Appendix B, I have showed the change in 321-323 triple codon that corresponded to RYL391AAA mutation. In order to express Myt1(321RYLAAA) in *Drosophila* somatic and germline cells, I cloned this variant from a pENTR vector into N-terminal GFP/VFP with UASp-VFP or *tv3* promoter (Wong *et al.*, 2005) destination vectors designed for Gal4-inducible or spermatocyte-specific expression, respectively. After sequencing to confirm their identity, these Myt1(321RYLAAA) constructs were microinjected into *Drosophila* embryos to generate transgenic lines (BestGene).

A.1.2. Analysis of Myt1-Cyclin B interaction

Mutation in Myt1 putative RXL domain (321RYLAAA) was expected to lack Cyclin B interaction. Therefore I analyzed Myt1 and CyclinB interaction by performing immuno-precipitation experiment. Testes expressing *tv3::GFP-Myt*1 or *tv3::GFP-Myt1*(321RYLAAA) were used for this analysis. 40 testes were dissected from one-day-old males and homogenized in freshly prepared lysis buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 1mM EDTA, 0.1%NP-40, 1X protease inhibitors, 1X phosphatase inhibitors). The lysate was then incubated on a nutator with 10 microlitre of 1:100 mouse anti-GFP (full length, JL-8: BD Biosciences) antibodies for 2 hour at 4 C. 50% protein A beads were added to the lysate plus anti-GFP mixture and incubated overnight at 4 C. The slurry was then centrifuged at 5000 rpm for 5 minutes to immuno-precipitate the GFP-tagged fusion proteins and the supernatant

was removed and saved for analysis. After washing the pellet three times with IP buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 0.5% TritonX-100, 1X protease inhibitors, 1X phosphatase inhibitors), the GFP pulldown (GFP + Protein A bead + GFP) was added with 6x sample buffer and boiled for 7-10 min. The supernatant and the immuno-precipitated proteins were then loaded onto SDS-mini gels for western blot detection with rabbit anti-CyclinB antibodies.

Fig. A-2A shows the western blot results obtained with this immuno-precipitation experiment. Supernatant and pellet fractions from heterozygous (*myt1/+*) negative control extracts in lane 1 and 2 respectively showed no GFP band in supernatant or pellet, confirming the absence of transgenic proteins. Cyclin B was detected in both the samples, however, suggesting that the pellets had not been washed under sufficiently stringent conditions. Curiously, the Cyclin B-labeled band exhibited different electrophoretic mobility in these samples (compare lane 1 and lane 2), possibly due to protein modifications differently affected by how the supernatant and immuno-precipitate were handled during the course of the experimental procedure. Supernatant and pellet fractions extracted from the *GFP-Myt1(WT)*-expressing testes revealed a 95kDa (GFP-Myt1) band only in the pellet (lane 4), not in the supernatant (lane 3), confirming that the conditions were sufficiently optimized to precipitate the membrane bound GFP-Myt1 protein. The *GFP Myt1(RYL392AAAI)* samples showed similar results (lanes 5 and 6). I can not make any conclusions about the ability of this transgenic protein to associate with Cyclin B however, because of issues discussed above. This experiment will need to be repeated under more stringent conditions to compare how the Myt1(WT) and Myt1(RYL392AAA) I variants physically interact with endogenous Cyclin B.

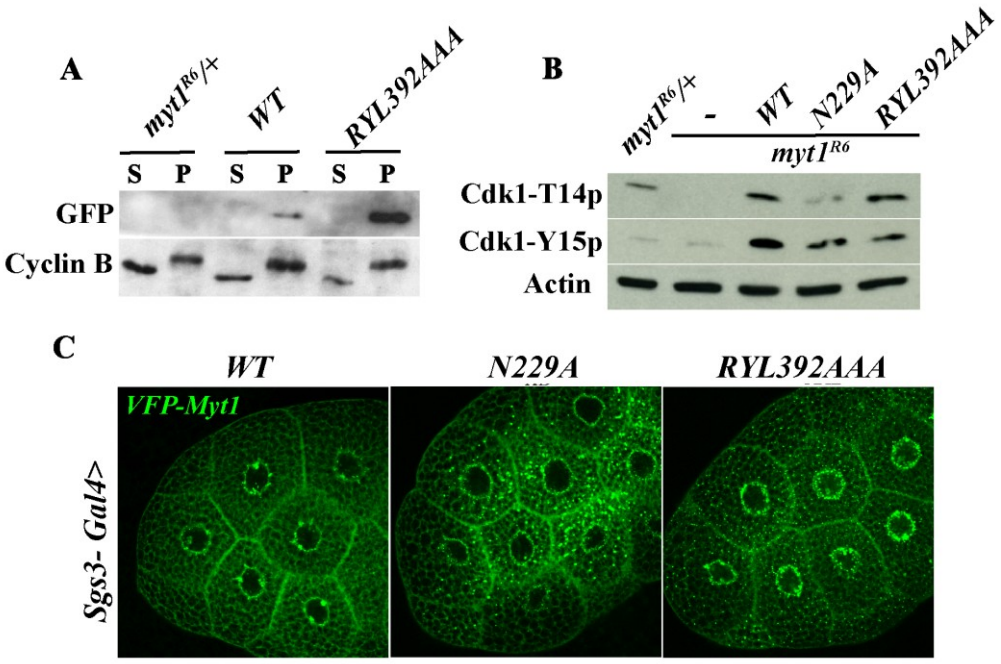

Fig. A-2: Characterization of Myt1(RYL392AAA)

(A) GFP tagged Myt1 expressed in *Drosophila* spermatocytes was immuno-precipitation with GFP antibodies and the pull downs (P) and supernatants (S) were examined for the presence of CyclinB by western blotting. Pulldown from *myt1/+* control (lane 2) shows no GFP, as expected and CyclinB was detected in both supernatant and precipitated samples (lane 1 and 2). The size of the Cyclin B band appears different in supernatant and pulldown samples due to the differences in experimental treatment (see Materials and Methods). Pulldown from testes expressing either *tv3::GFP-Myt1(WT)* (lane 4) or *tv3::GFP-Myt1(RYL392AAA)* (lane 6) shows GFP signals indicating the presence of Myt1 transgenic protein in the immuno-precipitates. Presence of Cyclin B was detected in both these pulldowns (lane 4 and6) indicating its association with *GFP-Myt1(WT)* as well as *GFP-Myt1(RYL392AAA)* mutants. (B) Western blot analysis of Myt1 catalytic activity shows normal levels of Cdk1 T14 and Y15 phosphorylation in *myt1/+* control testes sample (lane 1). Cdk1-T14 phosphorylation indicates Myt1 mediated Cdk1 regulation that is absent in *myt1* mutant protein sample (lane 2). Cdk1-Y15 phospho-isoforms are detected in both *myt1/+* and *myt1* (alone) samples, and the levels look equally reduced due to the stripping involved during the reprobing of blot. Transgenic expression of tv3 driven Myt1(WT) in *myt1* mutant spermatocytes restored Cdk1-T14 phosphorylation (lane 3). Myt1 having N229A mutation shows reduced T14p levels (lane), as compare to its WT control, whereas the levels in Cyclin-tethering mutant sample (lane 5) appear not different from WT. (C) UASp-Gal4 inducible expression of the VFP tagged variants including wildtype(WT), kinase inactive or hypomorph (N229A) and CyclinB tethering mutant (RYL392AAA) are shown in Salivary gland cells using Sgs3 Gal4 driver.

A.1.3. Developmental consequences of ectopic Myt1(RYL392AAA) expression resemble Myt1(WT)

In chapter3 (Fig. 3-1B) I showed that ectopic expression of GFP-Myt1(WT) was able to rescue *myt1* mutant biochemical defect, whereas GFP-Myt1(N229A) could only partially restore it. Here in Fig. A-2B, I have included the observation made with GFP-Myt1(RYL392AAA) expression. Western-blot experiments conditions used to assay the catalytic activity towards endogenous Cdk1 were mentioned in chapter-2. I used phospho-specific antibodies recognizing Cdk1-T14p and Cdk1-Y15p isoforms. Both Wee1 and Myt1 kinases can phosphorylate Cdk1 on residue Y15 (Y15p), however Myt1 alone phosphorylates residue T14 (T14p). In lane 1 of Fig A-2A, a *myt1*/+testes extract serving as a positive control showed the presence of both Cdk1 T14 and Y15 phospho-isoforms. The homozygous *myt1* null mutants shown in lane 2 had no detectable Cdk1-T14p isoform however, confirming the loss of Myt1 kinase activity. Expression of transgenic Myt1(WT) in *myt1* mutant spermatocytes (lane 3) completely rescued this biochemical defect, whereas expression of the N229A catalytic mutant Myt1 variant in a *myt1* mutant background showed lower but detectable Cdk1-T14p and Y15p isoforms (lane 4) indicating its hypomorphic activity. Expression of a putative Cyclin-tethering motif mutant (RYL392AAA) in *myt1* mutant spermatocytes completely rescued the T14 phosphorylation defect however (lane 5), showing that alteration of the RYL392 motif does not perturb Mty1 catalytic function.

I examined the sub-cellular localization of VFP-Myt1(RYL392AAA) in the fixed third instar larval salivary gland endo-replicating cells using *Sgs3-Gal4.* Expression of GFP-Myt1(WT) exhibited its sub-cellular localization at the reticular cytoplasmic network and appeared enriched at perinuclear foci (Fig. A-2C). In cells expressing VFP-Myt1(N229A)

however, cytoplasmic foci rather than perinuclear foci appeared to predominate. Expression of VFP-Myt1(RYL392AAA) appeared similar to the localization of GFP-Myt1(WT) indicating that RYL392AAA mutation does not majorly affect Myt1 localization.

To examine the developmental function of CyclinB tethering mutant (RYL392AAA), I examined Gal4-inducible *UASp*-VFP-Myt1(RYL392AAA) transgenes in *myt1* mutant sensory organ cells using the *neuralized Gal-4* driver. Similar to the observation made with EGFP-Myt1(WT), expression of VFP-Myt1(RYL392AAA) fully rescued the *myt1* mutant bristles (Table 3.1). I also examined the consequences of RYL392AAA in *myt1* meiotic organelle checkpoint defect. Fig. A-3 shows Hts immuno-labeling of fusomes observed in *myt1/+* and *myt1* mutant spermatocytes. *myt1/+* spermatocytes exhibited well branched fusome, whereas *myt1* lacked these structures as described in Chapter2. In Fig. A-3, I have shown that transgenic expression of *tv3::GFP-Myt1(WT)* in late *myt1* spermatocytes partially rescued the fusome, as expected, as Myt1 is required early during spermatocyte development for mediating fusome checkpoint. Expression of *tv3::GFP-Myt1(N229A)* did not rescue *myt1* fusomes, whereas *tv3::GFP-Myt1(RYL392AAA)* partially restored these structures similar to Myt1(WT). In most part of the *myt1* mutant developmental defects including meiotic centriole dis-engagement, transgenic expression of RYL392AAA appeared to pheno-copy Myt1(WT) rescue. Collectively these observations indicating that RYL392 motif may not serve a Cyclin-interaction function in *Drosophila* Myt1, or such functions may be specified by other interaction motifs, such two RXL domains located within the kinase domain of dMyt1.

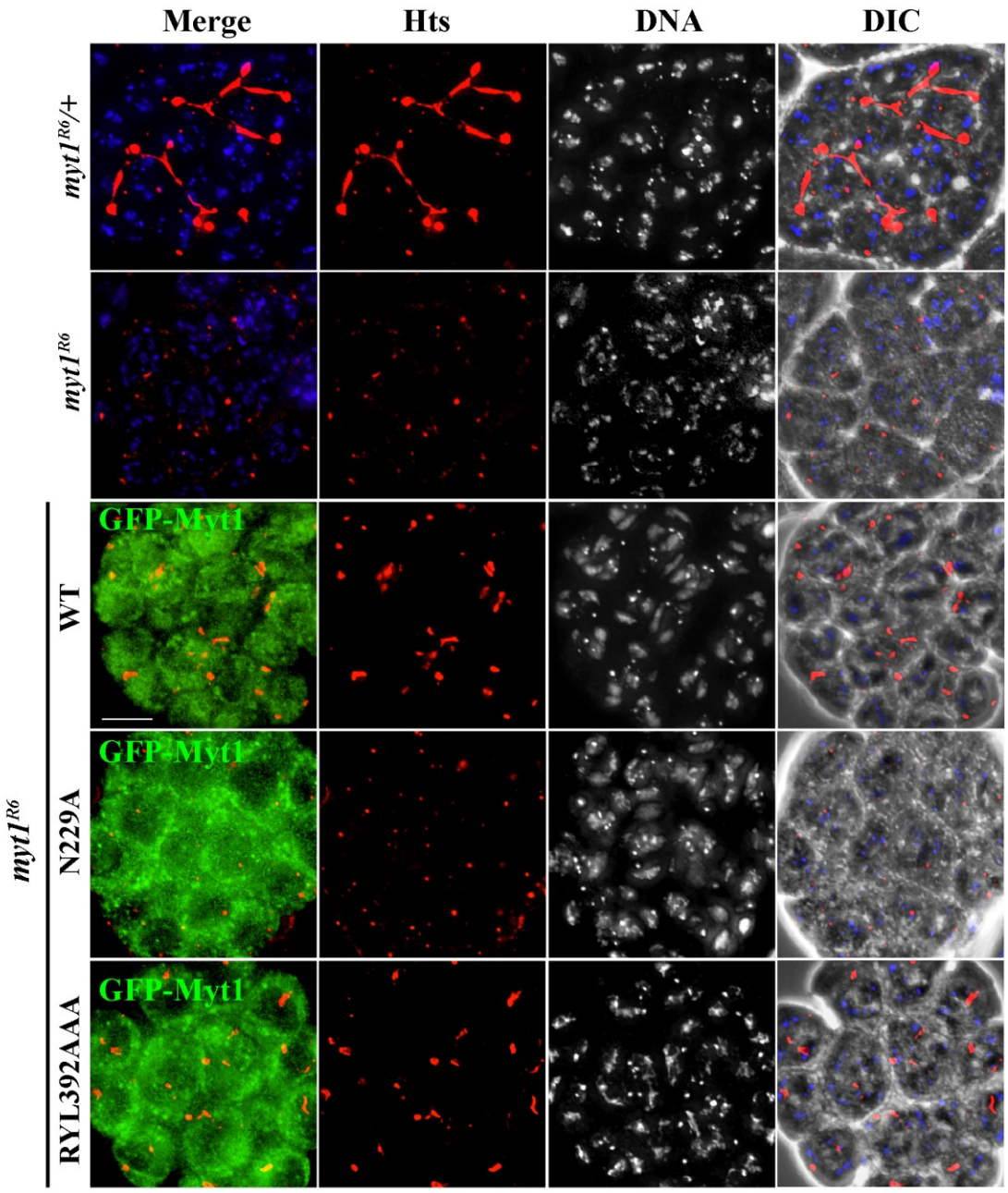

Fig. A-3: Expression of *Tv3::Myt1(RYL392AAA)* i partially rescues *myt1* fusome

The Hts immuno-labeling in *myt1* spermatocytes expressing *tv3:: GFP-Myt1(WT)* reveals partial restoration of fusome branches, whereas the *tv3:: GFP-Myt1(N229A)* shows no rescue of these structures. Expression of *tv3:: GFP-Myt1(RYL392AAA)* partial restorated *myt1* fusome branches that resembles *tv3:: GFP-Myt1(WT)*. DNA labels represent S3-4 stage of *myt1* spermatocytes. Scale bar -16 microns.

A.2. Other results

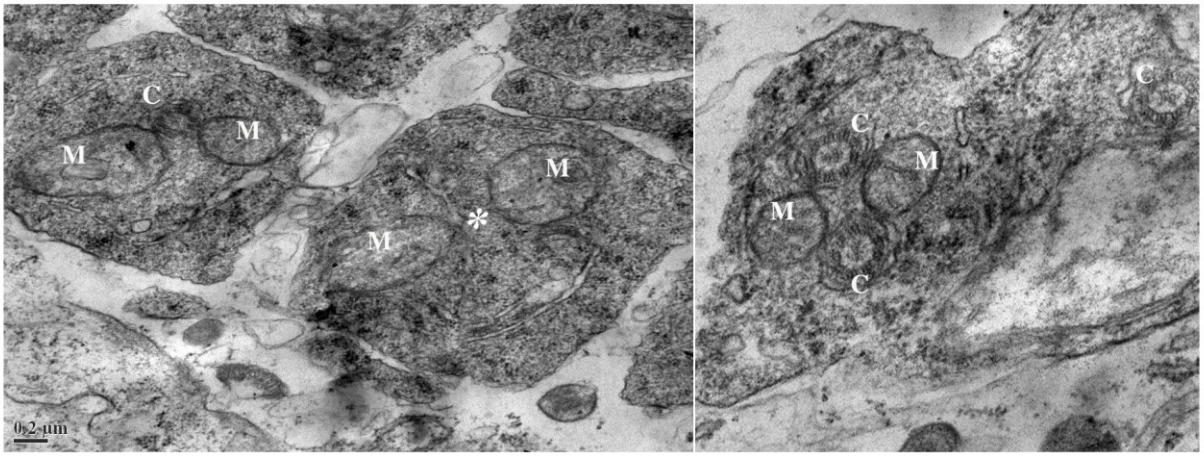

Fig. A-4: *myt1* mutant spermatids contain abnormal number of centrioles

Electron microscopic analysis on *myt1* early spermatids are shown here. M indicates the mitochondrial derivatives and C represents the cross section of centriole which forms the axoneme. *myt1/+* control exhibited a single centriole per spermatid indicating normal centrioles segregation in the secondary meiocytes. *myt1* mutants, however, were noticed with single(left most side of the image) as well as more than one centrioles per spermatids(right most side of the image). Fewer spermatids were found to have no centriole (middle, indicated with *). Scale bar - 200 nm.

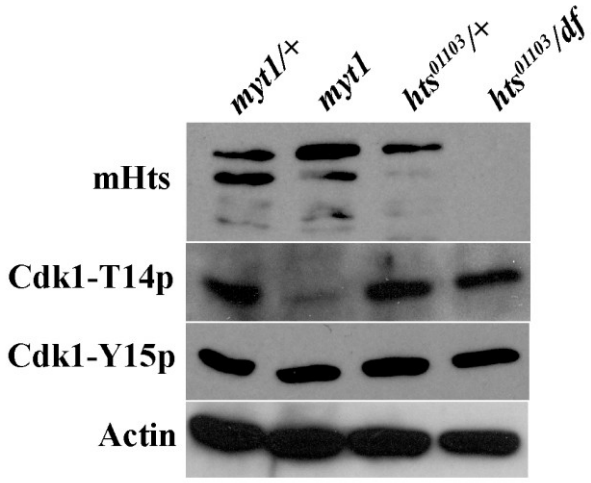

Fig. A-5: Endogenous Hts levels are relatively normal in *myt1* testes

Immuno-blot of testes extracts probed sequentially with mouse anti-hts (1:20) and phospho-specific antibodies against the endogenous Cdk1-T14p and Cdk1-Y15p. The blot was stripped between each reprobing. In *myt1*/+ control, mouse anti-Hts antibodies(1B1) detected a doublet that corresponded to 90 kDa and 80 kDa size fragments indicating the two modified forms of endogenous Hts. *myt1* testes extracts appeared to have both these band, although the fusome localized Hts immuno-fluorescence signals were undetectable in the mutant spermatocytes. *myt1* mutants lack Cdk1-T14p isoforms as expected. Heterozygous control of hts^{01103} allele showed only the 90 kDa size band and the mutant hts^{01103}/df had no band as expected. Molecular weight of the endogenous Cdk1 isoforms is around 34 kDa and the Actin is 47 kDa.

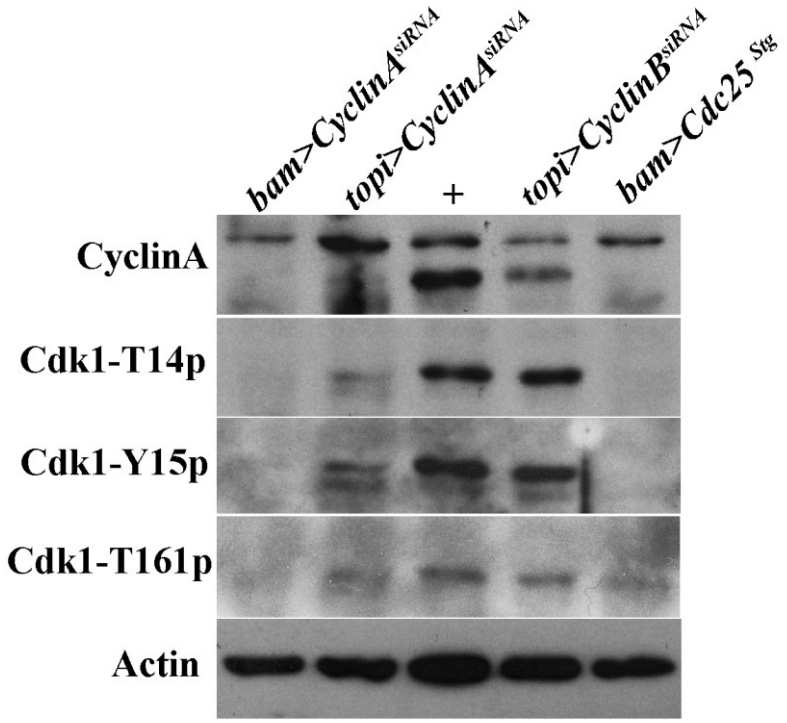

Fig. A-6: Endogenous levels of Cdk1 phospho-isoforms upon Cyclin A/B depletion from spermatocytes

Immuno-blot of testes extracts probed sequentially with mouse anti-Cyclin A and phospho-specific antibodies against the endogenous Cdk1-T14p, Cdk1-Y15p and Cdk1-T161p. The blot was stripped between each reprobing. The normal endogenous level of the probed proteins are shown in lane 3. Endogensous Cyclin A and Cyclin B were depleted using transgenic expressions of their corresponding siRNA using early(*bam-Gal4*) and late (*topi-Gal4*) stage spermatocyte drivers. *bam>Cyclin A*^{siRNA} (lane 1) completely lacked the lower band of Cyclin A (doublet), and exbihited more reduced levels of top band compare to the *topi>Cyclin A*^{siRNA} (lane 2). The three phospho-isoforms of endogenous Cdk1(Cdk1-T14p, Cdk1-Y15p and Cdk1-T161p) were also absent in *bam>Cyclin A*^{siRNA} testes extracts as compared to the controls in lane 3. The *topi>Cyclin A*^{siRNA} (lane 2) showed reduced levels of all three Cdk1 phospho-isoforms. Depletion of Cyclin B by *topi>Cyclin A*^{siRNA} (late, lane 4) spermatocyte stage showed relatively normal levels of Cdk1 phospho-isoforms compare to the lane 1. Ectopic expression of *Cdc25*^{Stg}, a mitotic Cdk1 phosphatase, in early spermatocytes (lane 5) exhibited reduced or no detection of two inhibitory Cdk1 phospho-isoforms (Cdk1-T14p and Cdk1-Y15p), while the activating phospho-isoforms(Cdk1-T161p) are still present. This could possibily indicate that *bam>Cdc25*^{Stg} removed the T14p and Y15p of Cdk1 that is in complex with Cyclin A at early spermatocyte stage. Significant reduction in Cyclin A levels upon *bam>Cdc25*^{Stg} expression is puzzling, however. Molecular weight of the endogenous Cdk1 isoforms is around 34 kDa and the Actin is 47 kDa.

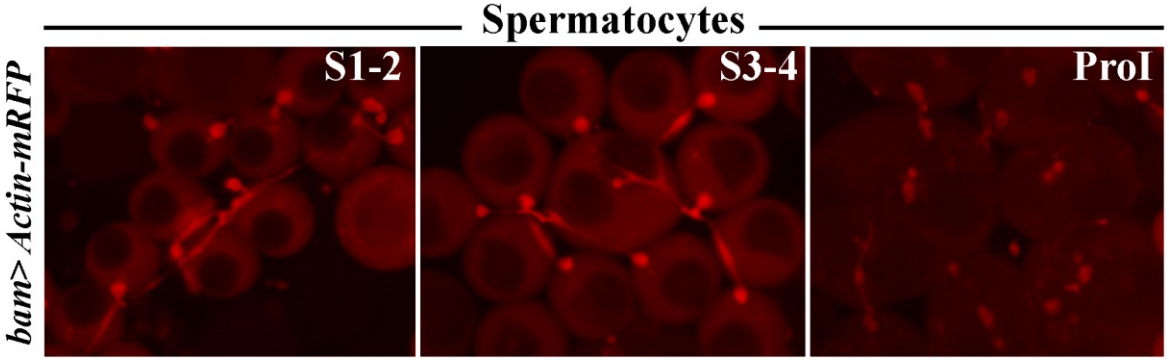

Fig. A-7: Actin-mRFP labels fusomes

Actin-mRFP trangene was expressed in spermatocytes using *bam-Gal4* driver. Actin-mRFP was detected both in cytoplasm and fusome structures of spermatocytes throughout the pre-meiotic G2 arrest(S1-6). Actin-mRFP labeled fusomes were found fragmented in prophase-I spermatocytes showing fusome disassembly during MI onset.

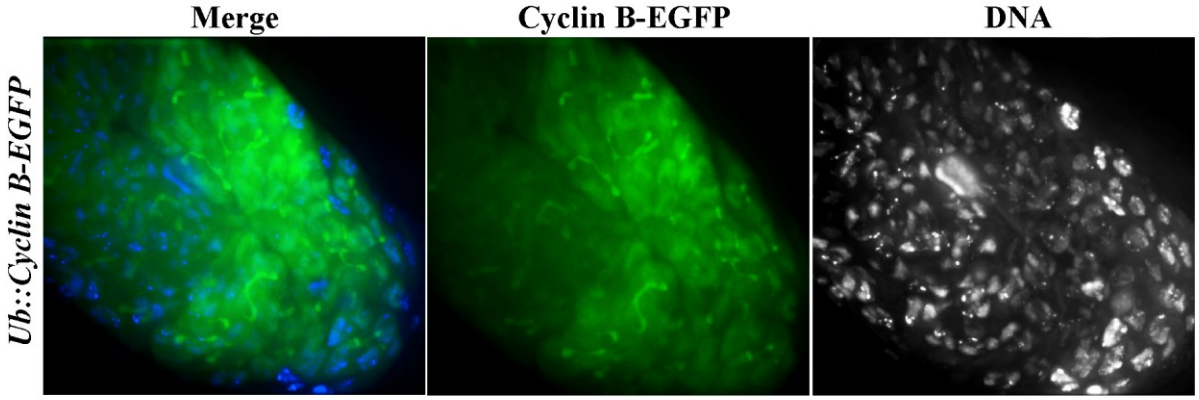

Fig. A-6: Cyclin B-EGFP labels fusomes

Whole-mount testis expressing *Ub::Cyclin B-EGFP* trangene is shown here. Ectopically expressed Cyclin B-EGFP transgenic proteins (green) were detected to label the fusome structures. Blue indicates the DNA label.

Appendix B

B.1. Molecular cloning

Information related to the molecular cloning of Myt1 or Cdk1 transgenes used in this thesis are mentioned in this section. Myt1 or Cdk1 cDNA was first cloned into the TOPO-pENTR vector using topo cloning protocol. Myt1(WT)-TOPO-pENTR or Cdk1(WT)-TOPO-pENTR was used as a template to perform the PCR based site directed mutagenesis. Myt1 or Cdk1 primers are listed in Table. B1 and 2. Change in nucleotides causing corresponding mutation was analyzed using DNA sequencing (done in MBSU). After confirming the mutational change, new Myt1 or Cdk1 variants were moved into destination vectors (TV3, UASp or AGW) using LR clonase reaction. One or two successful destination vector+Myt1/Cdk1 clones were handpicked and their sequences were re-analyzed before making the transgenic lines. Microinjecting these clones into *Drosophila* embryo was done by BestGene to generate transgenic flies.

B.1.1. PCR amplification of Myt1 cDNA

pCasper Myt1	-	1 (300 ng)
Forward primer (10 μM)	-	2
Reverse primer (10 μM)	□□	2
Pfu 10x buffer	-	3
10mM dNTP mix	-	3
Pfu DNA polymerase enzyme	-	1
Double distilled H_2O	-	made upto 30 μl

PCR Cycle - 94C, 3min; 25x (94C, 30sec; 51C, 30sec; 68C, 90sec); 68C, 10min

B.1.2. Topo-cloning reaction

pENTR specific Myt1 or Cdk1 cDNA	-	1 (25 ng/µl)
Topo vector	-	1
Salt solution		
Double distilled H_2O	-	made upto 6 µl

Reaction mix was stored at room temperature for 30 min and transformed into the competent cells using the following Heat shock transformation protocol: Topo-cloning reaction mix was combined with 50-100µl of thawed cold competent cells. The tube containing the mix was stored in ice for 15 min before heat shocking at 45C for 90 sec. The tube was then transferred to ice. The mix was combined with 0.5 ml liquid nutrient broth and incubated for 30 min at 37C. Transformation mix was centrifuged at 2000 rpm and excess supernatant was removed. Pellet was suspended in the remaining ~200 µl of supernatant and plated on solidified agar plates and incubated overnight at 37C. Multiple colonies growing on the plate were individually cultured in liquid media and the plasmids were isolated using miniprep (Qiagen). Plasmids clones were screened by restriction digestion using Sac II enzyme. Size of Topo-pENTR is around 3.9 kilobase(kb) and dMyt1 is 1.602 kb. Sac II cuts Topo-pENTR(alone) once producing single linear DNA fragment, whereas Myt1(WT) insertion in Topo-pENTR resulted in two different size fragments due to another Sac II located in Myt1(WT).

B.1.3. Site-directed mutagenesis PCR amplification

Myt1(WT)-Topo-pENTR Myt1	-	0.5 (200 ng)
Forward primer (125ng/**µl**)	-	1
Reverse primer (125ng/**µl**)		1
Pfu 10x buffer	-	5
10mM dNTP mix	-	2
Pfu ultra HF DNA polymerase enzyme	-	1
Double distilled H_2O	-	50 µl reaction

PCR Cycle - 94C, 3min; 16x (94 C, 30 sec; 55 C, 1 min; 68 C, 4 min); 68 C, 5min
Mutagenesis PCR mix was transformed into competent cells using heat-shocking conditions mentioned earlier. Specific mutation in Myt1(WT) was analyzed using the following DNA sequencing reaction.

B.1.4. DNA sequencing PCR reaction (BigDye - MBSU protocol)

Myt1(putative mutation)Topo-pENTR	-	1 (1 μg)
BD mix	-	4
BD 5x buffer	-	8
Sequencing primer (10 μM)	-	2
Double distilled H_2O	-	made upto 20 μl

PCR Cycle - 95C, 1 min; 25x (95 C, 30 sec; 50 C, 15 sec; 60 C, 2 min); 60C, 2min

This PCR product was cleaned up using the following protocol: Final PCR product was mixed wih 2 μl of 250mM EDTA+1.5m NaOAc and added with 80 μl of 95% ethanol to precipitate the DNA. The mix was centrifuged at maximum rpm for 15 min. The pellet was washed in 70% ethanol and air-dried. DNA sequencing was performed based on Sanger's method and the sequences were analyzed using FinchTV software. Nucleotide alignment and mutation change are shown in this section below. Successive Myt1-Topo-pENTR/ Cdk1-Topo-pENTR clones containing the expected mutations were used for LR clonase reaction to clone the new Myt1/Cdk1 variants in to the destination vectors.

B.1.5. LR clonase II recombinant reaction (10 μl)

Myt1-Topo-pENTR or Cdk1-Topo-pENTR	-	1 (300 ng)
Destination vectors TV3/UASp/AGW	-	1 (300 ng)
Topoisomerase	-	1
TE buffer	-	5
LR clonase II enzyme	-	2

The reaction mix was incubated in room temperature for 3 hours. Recombination reaction was terminated by adding 2 μl proteinase K followed by incubation at 37C for 10 min. Recombinant reaction mix was transformed in to competent cell and individual clones were sequence analyzed.

Table. B-1: List of clones generated

Clones	Mutation	TOPO-pENTR	TV3	UASp	AGW/HGW
Myt1(WT)	-	new	N'GFP tag	N'EGFP tag (ZJ)	N'GFP tag
Myt1 (N229A)	N229A	new	N'GFP tag	N'VFP tag	N'GFP tag
Myt1(RYL392 AAA)	RYL392AAA	new	N'GFP tag	N'VFP tag	N'GFP tag
Cdk1(WT)	-	OM	C'GFP tag	C'VFP tag (OM)	-
Cdk1(T14A)	T14A	new	C'GFP tag	C'VFP tag	-
Cdk1(Y15F)	Y15F	new	C'GFP tag	C'VFP tag	-
Cdk1(T14A, Y15F)	T14A,Y15F	OM	C'GFP tag	C'VFP tag (OM)	-

N' and C' indicate the N terminal and C terminal tagging of fluorescent (EGFP/GFP/VFP) cDNA, respectively. Clones generated by Ondrilla Mukherjee (OM) and Dr.Zhigang Jin (ZJ) are mentioned here.

Table. B-2: List of primers used for DNA sequencing

Primers	Sequence (5'-3')
GFP F	GGAGTACAACTACAACAGCC
Myt F1	CACCATGGAAAAGCATCATCG
Myt F2	GATCTCCAAGCAACTGTTCC
Myt F3	ATGGCTCCAGAGATCCTG
Myt F4	GGTCCTGCATCTATGCAAG
SV R	CATTCCACCACTGCTC
Myt R1	GTGTACATGAACGTACCTGC
Myt R2	AACTACGCGACAGCATG
Myt R3	CTCGCCGATCAGAACGTT
Myt R4	CACTGCTCGAAGTGTGAC
GW5 F	ATCGAGGCCTGTCTAGAGAAG
Cdk1 F1	GCCGCAGAACTTACTAATCGAC
Cdk1 F2	GATCCAGTTCATCGCATTTCC
GFP R	GACTTGAAGAAGTCGTGCTG
Cdk1 R1	ACCATCGCATCGAGATTC
Cdk1 R2	GCTACGGACCAATTCACTCT

Table. B-3: List of primers used in site-directed mutagenesis

Clone	Primer sequence (5'-3')
pENTR-Myt1 Fwd	CACCATGGAAAAGCATCATCG
pENTR-Myt1 Rev	TCACTCGTCGTCATATTCCAGGACG
Myt1(N229A) Fwd	GGACATTAAACTGGACGCCGTTCTGATCGGCGAG
Myt1(N229A)Rev	CTCGCCGATCAGAACGGCGTCCAGTTTAATGTCC
Myt1(RYL392AAA) Fwd	GTGCAATTGGAAAACAGCCGCCTTTGCCGCCGCTCTATACTT CCTGGAGGTCCTGCATC
Myt1(RYL392AAA) Rev	GATGCAGGACCTCCAGGAAGTATAGAGCGGCGGCAAAGGCG GCTGTTTTCCAATTGCAC
pENTR-Cdk1 Fwd	CACCATGGAGGATTTTGAGAAAATTGAGAA
pENTR-Cdk1 Rev	ATTTCGAACTAAGCCCGATTG

B.2. DNA sequence and protein alignment

Myt1(WT)-TOPO pENTR sequence

>pdMyt1 Entry clone, 2580 bp+1602 bp

CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAA
CGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCG
ATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATACGCGTACCGC
TAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCGTCAGGATGGCCTTCTGCTTAGTTTGATGCCTGGCAGTTTA
TGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCA
GGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTATTTGATGCCTG
GCAGTTCCCTACTCTCGCGTTAACGCTAGCATGGATGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTCTTAAGC
TCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATA
ATGCCAACTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTT**CACCATGGAAAAGCATCATCGCCTGCCCCTCCCGGA**
ATTGCACGACGACAAACACAGACACAAACAGTGCAATGGGGAGAACAGCAATCGCTTCCGGCCGCCCAAGTACAAGACGC
GTGGCTACGTCGCCGTGGACAACAACAATCTGAACCGAAGCCAATCGCTGGGCTCCTGTAGCACCAACAGTTCCCAGATC
GCGCACGCGATCTCCTTCCGGGACGCCGGATGTTCGGATTCCAGTACGCTGCCCTCGTCACCAGTCCAGGCCGAGCTGAG
CACCCTCTCCCTGTCACACTTCGAGCAGTGCTTCGAAAGGCTGGCCAAACTGGGCGAAGGATCCTTCGGCGAGGTATTCC
AGGTGCGCGATCGCTCCGACGGCCAGTTATATGCCGTCAAGATCTCCAAGCAACTGTTCCGCGGCGAACAGTACCGCGCA
GAGCGGCTGGAAGAGGTGCGGCGCTACGAGGAGTTCTCCGGCCATGAGAACTGCATCCGGTTCATCCGCGCCTGGGAGCA
GTACGACCGACTGTACATGCAAATGGAGCTGTGCCGCGAAAGTCTGGAGCAGTACTTGCTGCGCTGCCAAAGGATACCGG
AGGAGCGCATCTGGCACATCCTGCTGGATCTGCTGCGGGGTCTCAAGTCGCTGCACGACCGGAATCTCATCCATCTGGAC
ATTAAACTGGACAACGTTCTGATCGGCGAGGACGACGAGACGTGCAAGCTGGCAGACTTTGGACTGGTCATCGATGTGGA
CAGGGCCAACAGCCATCACGCCACGGAGGGAGATTCGAGGTATATGGCTCCAGAGATCCTGCAGGGTCACTTCTCCAAGG
CTGCGGACATCTTCAGTCTGGGCATTGCCATGCTAGAGCTGGCATGCTACATGGATCTTCCTTCCAACGGCCCACTGTGG
CACGAACTGAGGCACGGCATTCTGCCCGAGGAGTTCATAAACAAAATATCACTGGAGCTGCAGTCGGTAATTAAGTCCAT
GATGAAGCCCGATCCTGCGCAGAGGCCGACGGCCGAGCAGCTACTCTCACATCCCAAGCTGCAGTACCTGCAAAAGAAGC
GCAAGTCGCTGATGAACTTCAGCATGCTGTCGCGTAGTTTTAGGCGATCTCGCCGCGCCGTTTGGGGAAGAATGTGCAAT
TGGAAAACAGCCGCCTTTCGTTACCTTCTATACTTCCTGGAGGTCCTGCATCTATGCAAGCCCATAACGGCCTCACAGCC
CAATATTAACATAGTGCCCTCCTCGCCTTCGTCAAAGGGAGTGCCTCTGGTGCCTCAGGTGGAGTTCCAGCTGGTAGGAT
CCACACCCATTGCCAATCGTGACTGCTATGCCTCCGACTTCCTTTCCGGCGAGGACCCGCTGGACCTCTCCAATCAGGGT
AGCCCCAACGTAATAAATTCCACGCCATTGAACACAAACCAAGGCAAATCCCGTCTGGATTTGCTAAAGAATAATGTTGA
TTCAATGGGCAGGTACGTTCATGTACACGATTTCGAGAGTCCGTGTTCCGCCCTATCTTCCGCCAAGGTCCTGGACACCT
CATCGTTCCGACGCAAAAAGCTCTTCGTCCTGGAATATGACGACGAGTGAAAGGGTGGGCGCGCCGACCCAGCTTTCTTG
TACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCAT
TATTTGCCATCCAGCTGATATCCCCTATAGTGAGTCGTATTACATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCT
CAAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAA
TACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATG
GGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGTATGGGAAGCCCGATGCGCCAGAG
TTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATT
TATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGAAAAA
CAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTG
CATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAA
CGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAAC
TTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTA
ATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGA
GTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATT
TGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGAC
GGCGCAAGCTCATGACCAAAATCCCTTAACGTGAGTTACGCGTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAA
AGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTT
GTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTT
CTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACC
AGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGT
CGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAG
CATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCG
CACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGAT
TTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGC
TGGCCTTTTGCTCACATGTT

Gray shaded sequence - attL1 and attL2 flanking sites; Bold sequence - dMyt1 sequence

Sequence alignment of Myt1(WT) vs Myt1(N229A)

>**NA**

```
wt              MEKHHRLPLPELHDDKHRHKQCNGENSNRFRPPKYKTRGYVAVDNNNLNRSQSLGSCSTN 60
NA              MEKHHRLPLPELHDDKHRHKQCNGENSNRFRPPKYKTRGYVAVDNNNLNRSQSLGSCSTN 60
                ************************************************************

wt              SSQIAHAISFRDAGCSDSSTLPSSPVQAELSTLSLSHFEQCFERLAKLGEGSFGEVFQVR 120
NA              SSQIAHAISFRDAGCSDSSTLPSSPVQAELSTLSLSHFEQCFERLAKLGEGSFGEVFQVR 120
                ************************************************************

wt              DRSDGQLYAVKISKQLFRGEQYRAERLEEVRRYEEFSGHENCIRFIRAWEQYDRLYMQME 180
NA              DRSDGQLYAVKISKQLFRGEQYRAERLEEVRRYEEFSGHENCIRFIRAWEQYDRLYMQME 180
                ************************************************************

wt              LCRESLEQYLLRCQRIPEERIWHILLDLLRGLKSLHDRNLIHLDIKLDNVLIGEDDETCK 240
NA              LCRESLEQYLLRCQRIPEERIWHILLDLLRGLKSLHDRNLIHLDIKLDAVLIGEDDETCK 240
                ********************************************** ***********

wt              LADFGLVIDVDRANSHHATEGDSRYMAPEILQGHFSKAADIFSLGIAMLELACYMDLPSN 300
NA              LADFGLVIDVDRANSHHATEGDSRYMAPEILQGHFSKAADIFSLGIAMLELACYMDLPSN 300
                ************************************************************

wt              GPLWHELRHGILPEEFINKISLELQSVIKSMMKPDPAQRPTAEQLLSHPKLQYLQKKRKS 360
NA             GPLWHELRHGILPEEFINKISLELQSVIKSMMKPDPAQRPTAEQLLSHPKLQYLQKKRKS 360
                ************************************************************

wt              LMNFSMLSRSFRRSRRAVWGRMCNWKTAAFRYLLYFLEVLHLCKPITASQPNINIVPSSP 420
NA              LMNFSMLSRSFRRSRRAVWGRMCNWKTAAFAAALYFLEVLHLCKPITASQPNINIVPSSP 420
                ************************************************************

wt              SSKGVPLVPQVEFQLVGSTPIANRDCYASDFLSGEDPLDLSNQGSPNVINSTPLNTNQGK 480
NA              SSKGVPLVPQVEFQLVGSTPIANRDCYASDFLSGEDPLDLSNQGSPNVINSTPLNTNQGK 480
                ************************************************************

wt              SRLDLLKNNVDSMGRYVHVHDFESPCSALSSAKVLDTSSFRRKKLFVLEYDDE- 533
NA              SRLDLLKNNVDSMGRYVHVHDFESPCSALSSAKVLDTSSFRRKKLFVLEYDDE- 533
                ***************************************************
```

Sequence alignment of Myt1(WT) vs Myt1(RYL392AAA)

>**Rxl** Protein sequence

```
wt              MEKHHRLPLPELHDDKHRHKQCNGENSNRFRPPKYKTRGYVAVDNNNLNRSQSLGSCSTN 60
rxl             MEKHHRLPLPELHDDKHRHKQCNGENSNRFRPPKYKTRGYVAVDNNNLNRSQSLGSCSTN 60
                ************************************************************

wt              SSQIAHAISFRDAGCSDSSTLPSSPVQAELSTLSLSHFEQCFERLAKLGEGSFGEVFQVR 120
rxl             SSQIAHAISFRDAGCSDSSTLPSSPVQAELSTLSLSHFEQCFERLAKLGEGSFGEVFQVR 120
                ************************************************************

wt              DRSDGQLYAVKISKQLFRGEQYRAERLEEVRRYEEFSGHENCIRFIRAWEQYDRLYMQME 180
rxl             DRSDGQLYAVKISKQLFRGEQYRAERLEEVRRYEEFSGHENCIRFIRAWEQYDRLYMQME 180
                ************************************************************

wt              LCRESLEQYLLRCQRIPEERIWHILLDLLRGLKSLHDRNLIHLDIKLDNVLIGEDDETCK 240
rxl             LCRESLEQYLLRCQRIPEERIWHILLDLLRGLKSLHDRNLIHLDIKLDNVLIGEDDETCK 240
                ************************************************************

wt              LADFGLVIDVDRANSHHATEGDSRYMAPEILQGHFSKAADIFSLGIAMLELACYMDLPSN 300
rxl             LADFGLVIDVDRANSHHATEGDSRYMAPEILQGHFSKAADIFSLGIAMLELACYMDLPSN 300
                ************************************************************

wt              GPLWHELRHGILPEEFINKISLELQSVIKSMMKPDPAQRPTAEQLLSHPKLQYLQKKRKS 360
rxl             GPLWHELRHGILPEEFINKISLELQSVIKSMMKPDPAQRPTAEQLLSHPKLQYLQKKRKS 360
                ************************************************************

wt              LMNFSMLSRSFRRSRRAVWGRMCNWKTAAFRYLLYFLEVLHLCKPITASQPNINIVPSSP 420
rxl             LMNFSMLSRSFRRSRRAVWGRMCNWKTAAFAAALYFLEVLHLCKPITASQPNINIVPSSP 420
                ******************************   ************************

wt              SSKGVPLVPQVEFQLVGSTPIANRDCYASDFLSGEDPLDLSNQGSPNVINSTPLNTNQGK 480
rxl             SSKGVPLVPQVEFQLVGSTPIANRDCYASDFLSGEDPLDLSNQGSPNVINSTPLNTNQGK 480
                ************************************************************

wt              SRLDLLKNNVDSMGRYVHVHDFESPCSALSSAKVLDTSSFRRKKLFVLEYDDE- 533
rxl             SRLDLLKNNVDSMGRYVHVHDFESPCSALSSAKVLDTSSFRRKKLFVLEYDDE- 533
                ***************************************************
```

Sequence alignment of Cdk1(WT) vs Cdk1(T14A)

```
Cdk1-wt         MEDFEKIEKIGEGTYGVVYKGRNRLTGQIVAMKKIRLESDDEGVPSTAIREISLLKELKH 60
Cdk1-A          MEDFEKIEKIGEGAYGVVYKGRNRLTGQIAAMKKIRLESDDEGVPSTAIREISLLKELKH 60
                ************:***************.****************************

Cdk1-wt         ENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLYQITSAILFCH 120
Cdk1-A          ENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLYQITSAILFCH 120
                ************************************************************

Cdk1-wt         RRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGSPR 180
Cdk1-A          RRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGSPR 180
                ************************************************************

Cdk1-wt         YSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRILKTPTEDIWPGVTSLPDYKNT 240
Cdk1-A          YSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRILKTPTEDIWPGVTSLPDYKNT 240
                ************************************************************

Cdk1-wt         FPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHRISAKDILEHPYFNGFQSGLVRNX 298
Cdk1-A          FPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHRISAKDILEHPYFNGFQSGLVRN- 297
                ***********************************************************
```

Sequence alignment of Cdk1(WT) vs Cdk1(Y15F)

```
Cdk1-wt         MEDFEKIEKIGEGTYGVVYKGRNRLTGQIVAMKKIRLESDDEGVPSTAIREISLLKELKH 60
Cdk1-F          MEDFEKIEKIGEGTFGVVYKGRNRLTGQIVAMKKIRLESDDEGVPSTAIREISLLKELKH 60
                **************:*********************************************


Cdk1-wt         ENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLYQITSAILFCH 120
Cdk1-F          ENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLYQITSAILFCH 120
                ************************************************************


Cdk1-wt         RRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGSPR 180
Cdk1-F          RRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGSPR 180
                ************************************************************


Cdk1-wt         YSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRILKTPTEDIWPGVTSLPDYKNT 240
Cdk1-F          YSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRILKTPTEDIWPGVTSLPDYKNT 240
                ************************************************************


Cdk1-wt         FPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHRISAKDILEHPYFNGFQSGLVRNX 298
Cdk1-F          FPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHRISAKDILEHPYFNGFQSGLVRN- 297
                ***********************************************************
```

Sequence alignment of Cdk1(WT) vs Cdk1(T14A,Y15F)

```
Cdk1-wt         MEDFEKIEKIGEGTYGVVYKGRNRLTGQIVAMKKIRLESDDEGVPSTAIREISLLKELKH 60
Cdk1-AF         MEDFEKIEKIGEGAFGVVYKGRNRLTGQIVAMKKIRLESDDEGVPSTAIREISLLKELKH 60
                ************::************************************************

Cdk1-wt         ENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLYQITSAILFCH 120
Cdk1-AF         ENIVCLEDVLMEENRIYLIFEFLSMDLKKYMDSLPVDKHMESELVRSYLYQITSAILFCH 120
                ************************************************************

Cdk1-wt         RRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGSPR 180
Cdk1-AF         RRRVLHRDLKPQNLLIDKSGLIKVADFGLGRSFGIPVRIYTHEIVTLWYRAPEVLLGSPR 180
                ************************************************************

Cdk1-wt         YSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRILKTPTEDIWPGVTSLPDYKNT 240
Cdk1-AF         YSCPVDIWSIGCIFAEMATRKPLFQGDSEIDQLFRMFRILKTPTEDIWPGVTSLPDYKNT 240
                ************************************************************

Cdk1-wt         FPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHRISAKDILEHPYFNGFQSGLVRNX 298
Cdk1-AF         FPCWSTNQLTNQLKNLDANGIDLIQKMLIYDPVHRISAKDILEHPYFNGFQSGLVRN- 297
                ***********************************************************
```