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Analysis of Developmental Relevance of Cdk1 Inhibitory Phosphorylation in *Drosophila*

by

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ABSTRACT

Cyclin dependent kinase 1, Cdk1, inhibitory phosphorylation is at the core of conserved checkpoint mechanisms that prevent mitosis from interfering with DNA replication or repair. It is also crucial for coordinating cell cycle progression with morphogenetic processes during organismal development. During interphase, Wee1 and Myt1 function as Cdk1 inhibitory kinases. Both kinases phosphorylate Cdk1 on a conserved tyrosine, Y15, however the dual specificity Myt1 kinase also phosphorylates an adjacent threonine, T14. In spite of the functional redundancy with Wee1 for Y15 phosphorylation, Myt1 serves specialized developmental functions that may reflect its unique capabilities as a T14 or dual specificity Cdk1 inhibitor. To define distinct developmental requirements for T14 and Y15 as well as dual phosphorylation of Cdk1, new transgenic strains expressing Gal4-inducible VFP-tagged wild-type Cdk1 and three Cdk1 phospho-acceptor mutants: Cdk1(T14A)-VFP, Cdk1(Y15F)-VFP and Cdk1(T14AY15F)-VFP, were engineered. Genetic and biochemical evidence revealed that T14 and Y15 inhibitory phosphorylation are functionally distinct mechanisms for regulating Cdk1 activity. Y15 inhibitory phosphorylation was shown to be necessary and sufficient for developmentally regulated G2 phase arrest, while the T14 phosphorylation of Cdk1 may have evolved as a mechanism for accumulating dually inhibited Cdk1-Cyclin B complexes. The Myt1-mediated dual phosphorylation of Cdk1 was implicated as a genetic innovation evolved in metazoans for allowing cells to remain stably arrested in G2 phase for prolonged periods.

The new transgenic tools were also used to define how the temporal regulation of Cdk1 and the timing of G2-phase quiescence are linked with the developmentally regulated signal crucial for specifying neuronal cell fate during sensory organ development in *Drosophila*. Genetic evidence demonstrated that forced mitosis in G2 quiescent SOP cells conferred self-renewal potential on the cells that would normally terminally differentiate. Thus, linking the timing of G2 quiescence in SOP cells to the critically important developmental choice of self-renewal versus terminal differentiation. Further analysis of the transgenic Cdk1 fusion proteins in *cdc25*^{twe} mutant spermatocytes, lacking active endogenous Cdk1 proteins, suggested the existence of both Cdc25^{Twe}-dependent and –independent regulation of Cdk1 activity in male meiosis.

DEDICATION

Through the whirlwind of life, she navigated
Through the excrutiating pain of marriage, she endured
Through buffeting storm of uncertainties, she persisted

Sometimes a married woman, an oasis for six kids
Most times a single mum, a refreshing spring for her own four
Gave all; asked for little; dealh denied her everything

Although no longer here, she remains an inseparable part of my life. Her values and virtues; persistence, endurance and determination, are my invaluable inheritance. My source of strength and motivation in the thoughest moments of my live, most especially throughout my graduate studies!

In memory of my late mum, Princess Titilade Agnes Ayeni, I dedicate my PhD thesis to all mums across the globe who toil days and nights, labouring relentlessly to secure a better live for their kids;

"*Your pain is but momentary; it will*
give way to an unending joy"

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LIST OF ABBREVIATIONS

APC/C	Anaphase Promoting Complex/Cyclosome
A/P	Anterio-posterior
CAK	Cdk Activating Kinase
cdc2	mutant for "cell division cycle"
Cdk	Cyclin Dependent Kinase
DNA	Deoxyribonucleic Acid
D/V	Dorso-ventral
CyO	Second chromosome balancer
CKIs	Cdk Kinase Inhibitors
G1-phase	First "gap" phase in the cell cycle
G2-phase	Second "gap" phase in the cell cycle
GFP	Green fluorescent protein
Gy	Gray
Hts	the gene product of hu li tai shao, an adducin-like protein
IR	Ionizing Radiation
min	Minutes
mg	milligram
ml	millilitre
MPF	Maturation Promoting Factor (M-phase Promoting Factor)
MPM2	Mitotic Phosphoproteins Monoclonal antibody 2, a marker for M-phase cells
PH3	Phospho-Histone H3
SDS-PAGE	Sodium dedocyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy

SOP	Sensory Organ Precursor
Thr 14 (T14)	Threonine 14 residue
Tyr 15 (Y15)	Tyrosine 15 residue
UAS	Upstream Activatory Sequence
VFP	Venus fluorescent protein
ZNC	Zone of Non-proliferating Cells

SYNOPSIS OF THE THESIS

Chapter 1 provides a summary of relevant literature on over 3 decades of scientific inquiries on the mechanisms of Cdk1 regulation in metazoans. This chapter acknowledged the enormous progress that has been made in understanding how Cdk1 activity coordinates cell proliferation with key development events. More importantly, the review reveals existing gaps on the mechanism(s) of Cdk1 regulation, providing justification for careful re-examination and the motivation for my PhD thesis.

Chapter 2 focuses on understanding how the specialized developmental functions of Wee1 and Myt1 relate to their biochemically-distinct Cdk1 regulatory mechanisms. Specifically, it examines whether the specialized developmental roles of dMyt1 kinase reflect unique properties conferred by Myt1-mediated T14 or dual specificity Cdk1 inhibitory phosphorylation. Transgenic Cdk1 strains expressing single and double phospho-acceptor Cdk1 mutant proteins were used to genetically define distinct requirements for Y15 and/or T14 phosphorylation in the context of imaginal disc and neuroblast development. This chapter provided new insights into a conserved cell cycle regulatory mechanism used for coordinating cell cycle progression with crucial developmental processes and described new sets of genetic tools suitable for dissecting, genetically and biochemically, the mechanisms of Cdk1 inhibition during pre-mitotic checkpoint.

In Chapter 3, I used the new genetic tools (i.e transgenic Cdk1 phospho-acceptor mutants) described in Chapter 2 to address a key developmental question

involving how Cdk1 activity is regulated in differentiating neural cells to ensure that an appropriate numbers of the correct types of cells are generated in the organism. Specifically, I addressed the question of how the timing of neural precursor re-entry from quiescence into a proliferative state is developmentally coordinated with the process of cell fate specification. I provided insight into the importance of the developmentally regulated G2 phase quiescence in the choice of neuronal differentiation versus self-renewal during sensory bristle development. In chapter 4, I examined the requirement for Twine-dependent activation of Cdk1 protein in male meiotic G2/MI versus MI/MII by ectopically expressing Cdk1 transgenes in *twine* mutant spermatocytes.

Appendix 1 focused on re-assessing the sequence of regulatory events involved in controlling Cdk1 activity via Y15/T14 inhibitory and T161 activatory phosphorylations during interphase. I ectopically expressed the Cdk1 fusion proteins in *Drosophila* salivary gland cells and examined Y15, T14 and T161 phosphorylation in the absence or presence of mitotic cyclin. The study also addressed the role of Y15 Inhibitory Phosphorylation as a novel localization mechanism and explored the potential mechanism by which the ectopic Cdk1 expression in salivary gland cells inhibits endocycling. Finally, I presented in Appendix 2 the results from a preliminary characterization of the phenotypic consequences of expressing Cdk1 fusion proteins during syncytial embryonic development.

1. INTRODUCTION

1.1 OVERVIEW OF THE CELL CYCLE

Since first proposed by Robert Hooke in 17th century and later espoused by Rudolf Virchow in his succinctly framed concept *"omnis cellula e cellula"* i.e cell begets cell (1855), our knowledge of a cell as the fundamental unit of life has grown quite dramatically. We now have a clear picture of what Rudolf Virchow intended when he said "*whole organism does not get sick; only certain cells or groups of cells (1855)*". The development and continuity of life across generations rely on the ability of a cell to produce other cells. This requires a cell to duplicate its DNA content and partition it faithfully into two daughter cells. Eukaryotic cells have evolved a universal control mechanism known as the cell cycle to ensure that these intricately interrelated events are executed with absolute precision to facilitate the survival of living organisms, while loss of the precision can lead to genomic instability, a hallmark of disease formation (Nurse, 2000). Cell cycle acts like a clock that precisely times how and when a cell reproduces itself, effectively and efficiently, while maintaining unidirectionality of the processes of copying and partitioning genetic materials. Two major events are common to all cell cycles in proliferating eukaryotic cells: synthesis phase (S-phase), when the chromosomes are replicated and mitotic phase (M-phase), when the replicated chromosomes are segregated into the two daughter cells (Figure 1-1A).

Early embryonic cell divisions in *Drosophila* and *Xenopus* rely on rapid S/M cell cycles, but as development progresses gap phases, G1 and/or G2, are introduced between S and M phases. Other cell cycle variations exist in metazoans, however. The canonical G1-S-G2-M cell cycle is characterized by

alternation of a DNA replication phase (S-phase) and mitosis (M-phase) separated by two Gap-phases G1 and G2 phases between M/S and S/M respectively (Figure 1-1B). Another cell cycle variation involves S-G2-M, where a G2-phase is introduced between S-phase and M-phase but skips the gap phase G1 (Figure 1-1C). It is worth mentioning that other cell cycle variations exist in eukaryotes that allow a cell to fulfil the developmental imperatives of growth by repeatedly duplicating their genome without dividing. A good example of such variation is endocycle, which is composed of alternating DNA synthesis (S) phases and gap (G) phases without chromosome segregation during a M-phase (Figure 1-1D).

There are no visible signs of cellular activity during the G1, S and G2 phases; as such they are collectively referred to as the cell cycle interphase. However, M-phase is the unique phase of a cell cycle that is associated with dramatic cellular choreographies; replicated chromosomes condense and become visible under the microscope, nuclear envelope breaks down, bipolar spindles are organized, condensed chromosomes are aligned on the metaphase plate and the replicated chromosomes are segregated and pulled to the opposite poles. Mammalian cell fusion studies reveal that the S and M phases are the dominant states of a cell cycle (Rao and Johnson, 1970). Interphase cells were forced to condense their chromosomes when fused with M-phase cell, while a G1 cell but not G2 cells replicated their DNA when fused with an S-phase cell. This elegant cell fusion study pioneered the idea that there are diffusible components important for regulating cell cycle (Rao and Johnson, 1970).

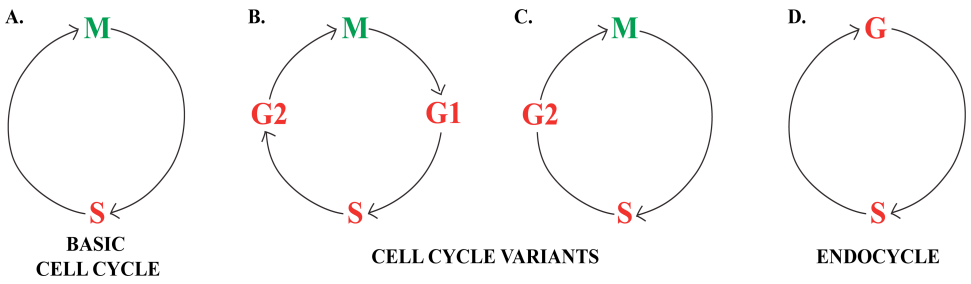


Figure 1-1: Basic cell cycle and its variations in metazoans

The basic cell cycle in metazoans involves repeated cycle of chromosome duplication (S-phase) and segregation (M-phase) without intervening gap phases (A), which is typical of cell cycle in early embryonic development. The introduction of the gap phases (G1 and G2) results in different variations of the basic S/M cell cycle later during development in metazoans (B and C). Endocycle is another unique modification of the basic cell cycle, which involves repeated rounds of chromosome duplication (S-phase) separated by a brief gap phase without M-phase.

1.2 CYCLIN DEPENDENT KINASES (CDKS): …BEHIND THE WHEEL BUT UNDER THE RADAR!

The temporality of cell cycle events is driven by the activity of a family of serine/threonine protein kinases known as the cyclin-dependent kinases (Cdks). Cdks are small proteins with molecular weight of between 34 - 40 kDa (Amon et al., 1992; Morgan, 2007). Cdks act by phosphorylating a large number of cell cycle substrates on the [S/T*]PX[K/R] consensus sequence, the serine or threonine residue which is phosphorylated next to a Proline, often followed by a basic amino acid, lysine or arginine, two positions away from the target residue (Amon et al., 1992; Morgan, 2007). In addition to this phosphorylation site, the presence of a hydrophobic patch on the mitotic cyclin enhances Cdk substrate recognition by binding to substrates that contain an RXL sequence motif (Adams et al., 1996; Chen et al., 1996; Adams et al., 1999).

Cdk kinase subunits have a bi-partite structure consisting of a small N-terminal and a large C-terminal lobe with a cleft for ATP binding and substrate recognition in between the two lobes (Knighton et al., 1991), see Figure 1-2. In its monomeric form, the conserved T-loop or activation loop, which arises from the C-terminal lobe of Cdks obscures the catalytic cleft and prevents substrate binding. Also in this configuration, the side chains in the ATP binding sites are oriented so that the ATP phosphates are poorly positioned and prevent efficient phospho-transfer (Morgan, 1995; John et al., 2001). The conserved PSTAIRE and L12 alpha helices on Cdks play important role in their activation. The N-terminal located PSTAIRE motif is important for cyclin binding (Ducommun et al., 1991),

while the L12 helix facilitates structural re-configuration of the active site/T-loop (Amon et al., 1992; Morgan, 2007).

To date, 11 Cdks have been identified in metazoans but only one member of the family is present in fission yeast (Cdc2) or budding yeast (Cdc28). Not all Cdks are involved in cell cycle regulation. For example Cdk7 and Cdk8 are involved in control of basal gene transcription by regulation of RNA polymerase II (Long et al., 1998; Akoulitchev et al., 2000). Here, I will focus specifically on Cdk1, one of four metazoan classes of Cdks that are directly involved in cell cycle regulation (Cdk1, 2, 4 & 6), which is also representative of the first Cdk to be uncovered, in yeasts (Hartwell, 1974; Nurse, 1975; Hunt, 1989). In its simplest sense, the prevailing model for how Cdks drive cell cycle events is that Cdk4 and/or Cdk6 interacts with G1 phase specific cyclins to drive the events of G1 phase. Cdk2 complexes with S phase cyclin to promote S phase activity and Cdk1 in complexes with mitotic cyclins regulates G2/M events (Amon et al., 1992; Vermeulen et al., 2003). An emerging paradigm suggests that Cdks regulation of the cell cycle phases may be more complicated than the above simplistic model. This alternative view will be explored later in this introduction.

1.3 THE CYCLIN DEPENDENT KINASE 1 (CDK1) AND CELL CYCLE REGULATION

The identity of the diffusible molecules regulating the cell cycle was first unveiled by studies of frog oocytes, showing that G2 arrested meiotic-incompetent oocytes could be induced to enter M phase by microinjection of cytoplasm from hormonally stimulated meiotic-competent oocytes (Masui and

Markert, 1971; Smith and Ecker, 1971). The cytoplasmic factor present in the hormone-treated oocytes was referred to as the maturation-promoting factor (MPF) but was appropriately renamed the M-phase promoting factor concomitant with the discovery that MPF activation is not restricted to oocyte maturation but is a general phenomenon that also promotes entry into M phase in G2-arrested somatic cells (Lohka et al., 1988).

The components of MPF were uncovered in elegant genetic screens for cell division cycle (cdc) mutants in yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and later validated by biochemical studies in sea urchin and clam embryos (Hartwell, 1974; Nurse, 1975; Hunt, 1989). MPF is a heterodimer consisting of regulatory subunits known as cyclins and kinase subunits called cell division cycle 2 (cdc2) (Swenson et al., 1986; Draetta et al., 1989; Pines and Hunter, 1989; Gautier et al., 1990). Epistatic interactions between *cdc25* and *wee1* demonstrated that the two genes are involved in regulating MPF activity, where Cdc25 acts antagonistically to Wee1 in regulating cdc2 functions (Fantes, 1981). Subsequently, *cdc2* homologues were discovered in several eukaryotes such as *Drosophila*, frogs, clams, starfish, mammals and plants (Arion et al., 1988; Lee and Nurse, 1988; Krek and Nigg, 1989; Lehner and O'Farrell, 1990a; Lehner and O'Farrell, 1990b; Hirt et al., 1993). The discovery that the mammalian Cdc2 homologs bind to cyclins (Draetta et al., 1987; Pines and Hunter, 1990) and the observations that other closely related Cdc2-like proteins that bound to cyclins exist during the cell cycle led to a new convention for naming kinases that are associated with cyclins, which was established by

consensus at the Cold Spring Harbor Symposium on the Cell Cycle in 1991. The first cell division cycle gene i.e. *Cdc2* therefore became *Cdk1* (Dorée and Hunt, 2002). It is important to note that the yeast homologs are still commonly referred their original nomenclature; cdc2 in fission yeast and Cdc28 in budding yeast.

1.3.1 Regulation of Cdk1 Activity

M-phase entry involves elaborate sub-cellular re-organization such as chromatin condensation, nuclear envelope breakdown, fragmentation of the endoplasmic reticulum and Golgi apparatus, and reorganization of microtubules to form the mitotic spindle. Execution of this intricate cellular remodelling depends on strict regulation of the conserved Cyclin-dependent kinase 1 (Cdk1) activity, which in turn phosphorylates many proteins involved in these processes. Cdk1 regulation is central to the pre-mitotic checkpoint mechanism for maintaining genomic integrity in response to chromosomal insults and developmental signals. It ensures that mitosis does not interfere with DNA damage repair or DNA replication (Rhind et al., 1997; Russell, 1998; Fletcher et al., 2002). Failure to fine-tune Cdk1 regulation with these crucial molecular processes could be devastating to the cell or lead to disease biogenesis (Malumbres and Barbacid, 2009).

Indeed, the abnormal activation of Cdk1 is implicated in the massive cell loss associated with HIV-1 infection and neurodegenerative diseases, and in various forms of human cancers (Castedo et al., 2002). Therefore, Cdk1 activity must be strictly regulated spatially and temporally for proper coordination of the cellular events characterizing mitotic entry. Multiple conserved biochemical

mechanisms, acting cooperatively or independently, modulate Cdk1 activity, its substrate recognition and its subcellular location. These mechanisms include (but are probably not limited to) cyclin binding, activating phosphorylation, inhibitory phosphorylation, de-phosphorylation, ubiquitin-mediated proteolytic degradation and interaction with Cdk inhibitors called CKIs (Morgan, 1995; Morgan, 1997).

1.3.1.1 Cdk1 Regulation via Cyclin Binding

Upon binding to the PSTAIRE motif of Cdk1, mitotic cyclins induce structural changes that re-orient the ATP binding sites and displacement of the T loop away from the catalytic cleft, thereby relieving structural restraints on Cdk1 activity (Jeffrey et al., 1995). In addition to these structural rearrangements that promote kinase catalytic potential, cyclin binding also facilitates Cdk1 substrate recognition (Morgan, 1995; John et al., 2001). Unlike in *S. pombe* where there is only one mitotic cyclin (Cdc13), four mitotic cyclins have been described in *S. cerevisiae* (Clb 1, 2, 3, & 4) and all of them interact with a single Cdk1 homolog (Richardson et al., 1992). Multiple mitotic cycles are also found in multicellular eukaryotes. Three mitotic cyclins (A, B and B3) are reported in *Drosophila* (Jacobs et al., 1998), two B type mitotic cyclin (B1 and B2) exit in Xenopus (MINSHULL et al., 1989) and three B-type mitotic cyclins (B1, B2 and B3) are also reported in mammals (Brandeis et al., 1998).

1.3.1.2 Cdk1 Activating Phosphorylation (T161p and CAK)

In addition to cyclin binding, Cdk1 activation requires phosphorylation on its conserved threonine-161 residue, which is located in the T-loop/activation loop of the kinase (Ducommun et al., 1991; Gould et al., 1991; Desai et al., 1992; Krek and Nigg, 1992; Solomon et al., 1992). The T161, (T167 in *S. pombe*), residue is

phosphorylated by a protein complex known as Cdk1 activating kinase (CAK) (Gould et al., 1991; Solomon et al., 1992). In vertebrates and *Drosophila*, CAK is a trimeric protein complex that is made up of a Cdk7 kinase subunit with cyclin H and Mat1 interacting partners (Larochelle et al., 1998; Kaldis, 1999). While the Cdk1 activating phosphorylation on T161 residue depends on cyclin binding (Desai et al., 1992; Solomon et al., 1992), stable cyclin B interaction with Cdk1 also requires T161 phosphorylation (Ducommun et al., 1991; Gould et al., 1991). Whether T161 phosphorylation occurs prior to or after Cdk1 inhibitory phosphorylation during interphase remains a subject of intense scientific inquiry. This issue is further clouded by a recent report, suggesting dependency of the Cdk1 T161 activating phosphorylation on T14 inhibitory phosphorylation (Coulonval et al., 2011). These observations reveal that our understanding of Cdk1 regulation, at least via T161 phosphorylation, is incomplete.

1.3.1.3 Proteolytic degradation of cyclin

Exiting M-phase requires that the activity of Cdk1 be extinguished (Murray et al., 1989; Glotzer et al., 1991). Eukaryotic cells rely on the destruction of mitotic cyclins as a reliable mechanism for controlling Cdk1 activity. The role of cyclin degradation in mitotic exit was first demonstrated via a biochemical study showing that a proteolysis-resistant mutant of cyclin prevents MPF inactivation and exit from mitosis, both *in vivo* and *in vitro* (Murray et al., 1989). A conserved RxxLxxxxN motif known as the destruction box exists in the N-terminal region of cyclins (both A and B types) that serves as the signal targeting different mitotic cyclin proteins for ubiquitin-dependent degradation, although at

different times, during mitosis (Glotzer et al., 1991). Cyclin degradation is catalyzed by multimeric ubiquitin ligase termed of the anaphase promoting complex (APC) or cyclosome, which interacts with Cdc20 for its activity and the interaction is facilitated by Cdk1/cyclin activity.

1.3.1.4 Regulation of Cdk1 by Inhibitory Phosphorylation/Dephosphorylation

Cdk1 activity is temporally regulated during the cell cycle, being "OFF" during interphase and "ON" during M-phase, respectively. During interphase, Cdk1 is inactive due to inhibitory phosphorylation of threonine-14 (T14) and tyrosine-15 (Y15) residues (Gould and Nurse, 1989; Nurse, 1990; Krek and Nigg, 1991a; Krek and Nigg, 1991b; Lundgren et al., 1991; Norbury et al., 1991; Mueller et al., 1995a). Phosphorylation on Y15 and/or T14 residues occurs via Wee1-related inhibitory kinases (Parker and Piwnica-Worms, 1992; Parker et al., 1995; Booher et al., 1997; Liu et al., 1997).

There are two types of Wee1-like inhibitory kinases, Wee1 and Myt1, found in metazoans (Norbury et al., 1991; Mueller et al., 1995a; Parker et al., 1995; Booher et al., 1997; Liu et al., 1997; Jin et al., 2005). Wee1 is a nuclear kinase that specifically phosphorylates Cdk1 on the Y15 residue (Parker et al., 1991; McGowan and Russell, 1995). There are two isoforms of Wee1 kinase (Wee1A and Wee1B) present in *C. elegans*, *X. laevis*, *M. musculus* and *H. sapiens* (Honda et al., 1995; Parker et al., 1995; Nakanishi et al., 2000; Leise III and Mueller, 2002; Okamoto et al., 2002). In contrast, *Drosophila* has only one Wee1 kinase (Campbell et al., 1995).

The other type of Wee1-like kinase found in metazoans is called Myt1. Unlike nuclear Wee1 kinase, Myt1 is cytoplasmic, bound to membranous

organelles such as Golgi and endoplasmic reticulum (ER), and phosphorylates Cdk1 on both T14 and Y15 residues (Kornbluth et al., 1994; Mueller et al., 1995b; Fattaey and Booher, 1997; Liu et al., 1997; Jin et al., 2005). Two distinct mechanisms for Myt1-mediated Cdk1 inhibition have been proposed. One involves a kinase-dependent catalytic inhibition of Cdk1 via Y15 and/or T14 phosphorylation whereas the other acts through a kinase-independent cytoplasmic tethering mechanism involving Myt1 binding to cyclin B through an RXL motif (Liu et al., 1999; Wells et al., 1999). These mechanisms can both promote the cytoplasmic accumulation of inactive Cdk1/cyclin B complexes and influence the timing of the G2/M transition.

Dynamic control of spatial and temporal expression of the Cdc25-related phosphatases relieves Cdk1 of its inhibition via dephosphorylation. This cycle of phosphorylation/de-phosphorylation is an important mechanism for coordinating mitosis with important morphogenetic processes such as cell movements (Edgar and O'Farrell, 1989; Großhans and Wieschaus, 2000; Seher and Leptin, 2000). It also serves as a conserved regulatory mechanism for preventing the initiation of premature mitotic events during DNA replication and to allow time for DNA repair that can otherwise result in developmental defects or cell lethality (Krek and Nigg, 1991b; Norbury et al., 1991; Jin et al., 2005; Jin et al., 2008). Three Cdc25-like phosphatases are present in vertebrates (Cdc25A, Cdc25B and Cdc25C) and have overlapping functions, but *Drosophila* has two functionally similar Cdc25 phosphatases; String ($Cdc25^{stg}$), which is expressed exclusively in somatic cells and required for Cdk1 activation during mitotic division and Twine,

which is expressed in both mitotic and meiotic cells (Alphey et al., 1992; White-Cooper et al., 1993; Edgar and Datar, 1996), but specifically required for meiotic division (Edgar and O'Farrell, 1990; Alphey et al., 1992; Courtot et al., 1992).

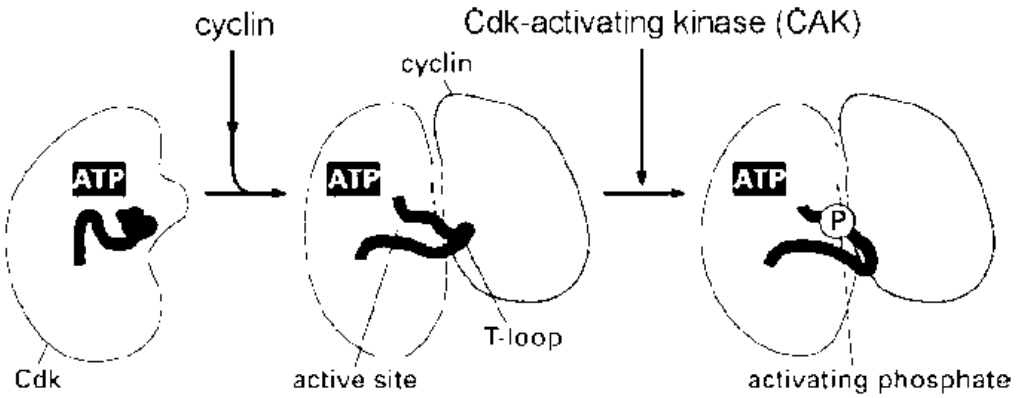


Figure 1-2:The structural basis for Cdk activation (Adapted from Alberts et al., 2002)

In their monomeric forms, Cdks are inactive due to T-loop or activation loop obscuring the catalytic cleft and prevents substrates binding, and mis-orientation of the ATP binding site. Activation of Cdk is a two-step process involving cyclin binding, which relieve the structural restraint. This is followed by phosphorylation on the T-loop, stabilizing the complex and properly re-orients the ATP binding site.

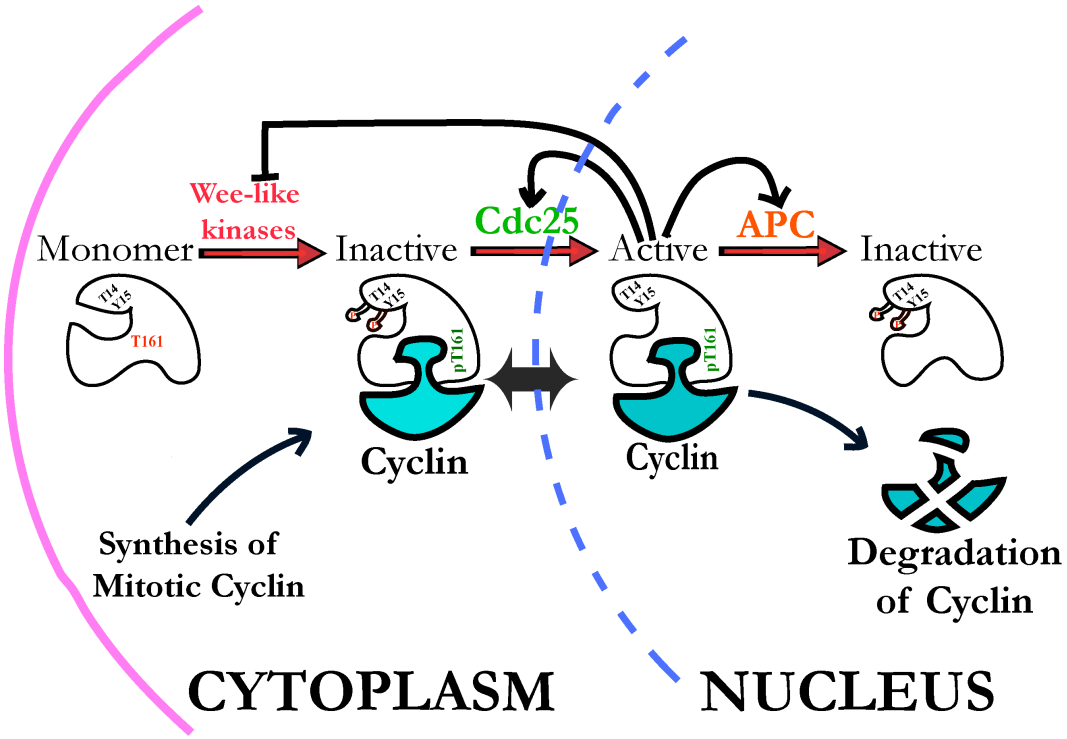


Figure 1-3: The yin and yang of Cdk1 regulation

Figure 1-3: THE YIN AND YANG OF CDK1 REGULATION

The standard model is that inactive Cdk1 monomer is activated by simultaneously binding to its mitotic cyclin partners and by activatory phosphorylation on threonine-161 (T161) residue by CAK complexes, but kept inactive via inhibitory phosphorylation on Y15 and T14 residues catalyze by Wee1-like inhibitory kinases (Myt1 and Wee1). During interphase, Cdk1/cyclin complexes shuttle between cytoplasm and nucleus (Heald et al., 1993; Hagting et al., 1998; Hagting et al., 1999; Gavet and Pines, 2010a; Gavet and Pines, 2010b). To enter mitosis, Cdk1/cyclin complexes activate Cdc25 phosphatases, which remove the inhibitory phosphates on Cdk1, and inactivate Wee1-like kinases, setting up a cascade of positive feedback loops that lead to a burst in Cdk1 activity. To exit mitosis, Cdk1/cyclin activates APC complexes, which subsequently extinguish Cdk1 activity via ubiquitin-mediated cyclin proteolysis. In summary, Cdk1 activity is regulated by two opposing forces, one inhibitory and the other stimulatory, and the activity of Cdk1/cyclin is also crucial to the regulatory forces.

1.3.2 Cdk1 Inhibitory Phosphorylation is conserved in Yeast

The yeast species, *Schizosaccharomyces pombe* (*S. pombe*), has two Y15-specific Cdk1 inhibitory kinases, Wee1 and Mik1 (Lundgren et al., 1991) and one Cdc25 phosphatase that removes the inhibitory phosphate (Russell and Nurse, 1986). *S. pombe wee1* mutants enter mitosis before sufficient growth has occurred during G2 phase, leading to abnormally small cells (Nurse, 1975), whereas *cdc25* mutants are unable to initiate mitosis and become abnormally large (Nurse, 1975; Russell and Nurse, 1986). Similarly**,** budding yeast, *Saccharomyces cerevisiae,* has one Cdk1 homologue known as Cdc28 with tyrosine-19 (Y19) residue, which is analogous to the conserved Y15 residue in other eukaryotic Cdk1s (Amon et al., 1992; Sorger and Murray, 1992; Booher et al., 1993). Cdc28 activity is regulated via inhibitory phosphorylation on tyrosine 19 by Swe1, a Wee1 ortholog, and dephosphorylated by Mih1, a Cdc25-like phosphatase (Russell et al., 1989; Booher et al., 1993). Although not essential, loss of *swe1* function also causes premature mitosis and a reduced cell size (Lim et al., 1996; Harvey and Kellogg, 2003; Rahal and Amon, 2008), whereas loss of Mih1 only causes slight delay in mitosis (Russell et al., 1989).

1.3.3 Single versus Dual Cdk1 Inhibitory Phosphorylation

Despite the broad conservation of Y15 and T14 residues, the regulation of Cdk1 inhibitory phosphorylation on a single residue Y15 (Y19 in *S. cerevisiae*) by Wee1-related kinases is nonetheless sufficient to prevent mitotic catastrophe in single-celled eukaryotes (Lundgren et al., 1991), to couple cell growth with cell division (Gould and Nurse, 1989; Rhind et al., 1997; Xiang et al., 1997) and to

facilitate repair of DNA damage by delaying mitosis (Morgan, 1995; Russell, 1998; Rhind and Russell, 2001). In metazoans, however, Cdk1 is regulated by a dual inhibitory phosphorylation mechanism that phosphorylates Y15 as well as the adjacent threonine residue, T14 (Nurse, 1990; Krek and Nigg, 1991a; Norbury et al., 1991; Jin et al., 1996); see Figure 1-4.

Cdk1 phospho-acceptor mutants have been engineered and used in both *in vivo* and *in vitro* experimental systems to study the functional significance of single and dual phosphorylated Cdk1 forms (Krek and Nigg, 1991a; Norbury et al., 1991; Heald et al., 1993; Jin et al., 1996; Blasina et al., 1997; Su et al., 1998; Fletcher et al., 2002). In cultured mammalian cells, completely non-inhibitable Cdk1(T14A,Y15F) in complexes with mitotic cyclins has high catalytic activity that shortens the length of G2-phase and forces the cells into a mitotic catastrophe (Krek and Nigg, 1991a; Heald et al., 1993; Jin et al., 1996; Blasina et al., 1997; Fletcher et al., 2002). Similar expression of completely non-inhibitable Cdk1(T14A,Y15F) mutants in *Drosophila* somatic and germline cells also produced deleterious developmental defects (Edgar et al., 1994; Su et al., 1998). On the contrary, *Xenopus* egg extract expressing the non-inhibitable Cdk1(T14A,Y15F) was associated with high catalytic activity but did not accelerate mitotic entry from G2-phase (Norbury et al., 1991). *In vitro* studies using single site mutant Cdk1 also yielded inconsistent results, with respect to Cdk1 catalytic activity and induction of precocious mitosis (Krek and Nigg, 1991a; Norbury et al., 1991; Blasina et al., 1997; Fletcher et al., 2002). These

discrepancies are intriguing and suggest that our understanding of Cdk1 inhibitory phosphorylation mechanisms remains incomplete.

These questions regarding the cellular function of single and dual phosphorylated Cdk1 isoforms may reflect inherent limitations of *in vitro* experiments and demonstrate that the issue of how inhibitory phosphorylation of these two residues affects Cdk1 regulation is far from being resolved, especially given the complexity of M-phase regulation in metazoans. Several possible explanations could be proposed to account for the evolution of a dual Cdk1 inhibitory phosphorylation mechanism in metazoans. The phosphorylation of Cdk1 on the T14 residue might occur early in the cytoplasm with Y15 phosphorylation having a later nuclear role in maintaining Cdk1 complexes in an inactive state (Fletcher et al., 2002). Susceptibility to Cdc25 de-phosphorylation may differ between singly and dual phosphorylated Cdk1 (Liu et al., 1997). Differential Cdk1 inhibitory phosphorylation may also restrict access to specific substrates, either by directly modulating physical interactions or indirectly, by creating distinct intracellular zones of Cdk1 activity (Jin et al., 1998a; Kao et al., 1999; Fletcher et al., 2002). Clear *in vivo* evidence for any of these possibilities is presently lacking, however.

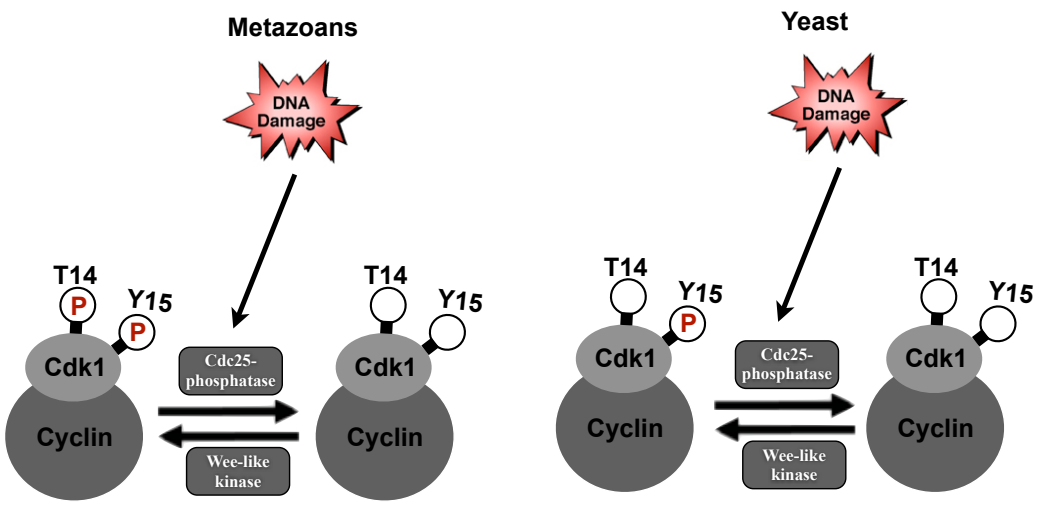


Figure 1-4: Single versus dual phosphorylation of Cdk1/cyclin complexes

One of the key dogmas in the field of Cdk1 regulation is that phosphorylation of Cdk1 on Y15 residue is sufficient in yeast to maintain G2 phase checkpoint following DNA damage, even though T14 is conserved. In contrast, in metazoans, in addition to Y15, T14 phosphorylation is required for a robust Cdk1 regulation following genomic insult. An important question from this dogma is: "What is the developmental relevance of dual versus single inhibitory phosphorylation of Cdk1"

1.4 CDK1 REGULATION DURING G2/M PRE-MITOTIC CHECKPOINT

Mitotic entry following DNA damage could result in mitotic catastrophy and apoptosis. To guard against such an unwanted fate, eukaryotic cells have evolved mechanism(s) to prevent cells with damaged DNA from initiating M phase and ensure that mitosis does not interfere with DNA replication or repair. This mechanism relies on Cdk1 inhibitory phosphorylation and it is broadly conserved among all eukaryotes (Gould and Nurse, 1989; Krek and Nigg, 1991b; Jin et al., 1996; Jin et al., 2008). In single-celled eukaryotes, phosphorylation of Cdk1 on a conserved tyrosine residue (Y15) is both necessary and sufficient for checkpoint arrest of cells in S or G2 phase until DNA replication and repair are completed and cells reach a critical size (Gould and Nurse, 1989; O'Connell et al., 1997; Russell, 1998). In metazoans, surveillance mechanisms that depend on Cdk1 phosphorylation on both Y15 and the adjacent T14 residues are used to coordinate the timing of entry into mitosis with dynamic developmental processes (Edgar and O'Farrell, 1989; Krek and Nigg, 1991a; Norbury et al., 1991; Krek and Nigg, 1992; Edgar et al., 1994; Stumpff et al., 2004; Jin et al., 2005).

Although there is broad consensus on how the pre-mitotic checkpoint is implemented via Cdk1 inhibitory phosphorylation, there are questions about the universality of Y15 and T14 inhibitory phosphorylation as the sole effector of G2-phase checkpoints. Expression of a constitutively active Cdk1(T14AY15F) in mammalian cells did not completely abolish DNA damage induced G2-phase arrest (Jin et al., 1996) and premature mitotic events were also infrequent in baby hamster kidney cells containing abundant level of the constitutively active

Cdk1(T14AY15F) (Heald et al., 1993). Other independent studies have also showed that the HeLa cells expressing either the Cdk1(Y15F) or Cdk1(T14AY15F) abolished DNA damaged induced G2-phase delay without increasing the number of mitotic cells (Fletcher et al., 2002). Indeed, cells expressing non-inhibitable Cdk1(T14A,Y15F) were characterized by condensed chromatin and disrupted lamin B, presumably reflecting a prophase-like arrest facilitated by yet unidentified mechanism(s) (Blasina et al., 1997; Fletcher et al., 2002). These observations suggest that Cdk1 inhibitory phosphorylation may not always be a universal effector of pre-mitotic checkpoints, supporting the notion that our understanding of pre-mitotic checkpoint via Cdk1 inhibitory phosphorylation is far from complete.

1.4.1 Cdk1 Regulates Developmental G2 Phase Quiescence

Eukaryotic cells can temporarily pause in G1, S or G2 phase of the cell cycle in response to environmental signals or as an intrinsic reaction to genomic insults (O'Farrell, 2011). The inactive state is generally termed “cell cycle quiescence”, and forms an integral part of metazoan development (Edgar and O'Farrell, 1989; Su et al., 1998; Shibutani et al., 2007). This transient cell cycle exit is characteristics of cells that are destined to undergo unique cellular and morphogenetic reorganizations (Schubiger and Palka, 1987; Edgar and O'Farrell, 1989; Milán et al., 1996; Neufeld et al., 1998; Shibutani et al., 2007). Of specific interest to my project, developmentally regulated G1 and G2 quiescence are common phenomena during *Drosophila* development. Ectodermal cells of embryonic cell cycle 17 are transiently arrested in G1 phase, such that forced mitosis in these cells alters cell morphogenesis and results in embryonic lethality

(Edgar and O'Farrell, 1989), and cells within the morphogenetic furrow are normally G1 quiescent. Failure to synchronize cells at this stage disrupts ommatidial patterning (Thomas et al., 1994). Similarly, G2-phase quiescence plays an important role in specialized *Drosophila* cells such as the abdominal histoblasts, embryonic pole cells, wing imaginal cells, spermatocytes (see Figure 1-5 for the process of spermatogenesis, highlighting G2 phase arrest) and sensory organ precursor (SOP) cells (Hayashi, 1996; Johnston and Edgar, 1998; Nègre et al., 2003; Fichelson and Gho, 2004; Jin et al., 2005; O'Farrell and Kylsten, 2008; Ninov et al., 2009).

Drosophila abdominal histoblasts remain in G2 throughout larval development but are poised to divide during larval/pre-pupae metamorphosis via ecdysone-dependent transcriptional activation of String, a Cdk1 activating Cdc25-related phosphatase (Hayashi, 1996; Ninov et al., 2009). Embryonic germ cells that give rise to adult gonads are also G2 quiescent throughout embryogenesis (Su et al., 1998), but can be driven into aberrant mitosis by expressing non-inhibitable Cdk1 mutants, indicating that inhibitory phosphorylation of Cdk1 is a key requirement for maintaining the G2 quiescent state. Another example are the G2-arrested cells composing the zone of non-proliferating cells at the dorsal/ventral boundary of the presumptive wing margin during late third instar larval development. These cells are maintained in a quiescent state via Wingless mediated transcriptional down-regulation of String, a mitotic homolog of the Cdk1 activating phosphatase (O'Brochta and Bryant, 1985; Johnston and Edgar, 1998). *Drosophila* sensory organ precursor cells are selected from this cluster of

quiescent G2 cells and stay arrested for a developmentally regulated time (Usui and Kimura, 1992; Nègre et al., 2003). Precocious activation of Cdk1 via String misexpression or loss of *myt1* function and inhibition of Cdk1 by overexpressing its inhibitors in SOP impairs thoracic sensory bristle formation (Fichelson and Gho, 2004; Jin et al., 2008; O'Farrell and Kylsten, 2008).

The role of Cdk1 during developmentally regulated G2 quiescence is also demonstrated by the fact that loss of *myt1,* encoding a Cdk1 inhibitory kinase, during gametogenesis disrupted important cytoplasmic structures in G2 arrested spermatocytes (Jin et al., 2005), even though the *myt1* mutant spermatocytes do not appear to undergo premature division. Collectively, these observations raise many questions about the physiologic relevance of differential Cdk1 inhibitory phosphorylation during developmentally regulated G2 arrest during metazoan development.

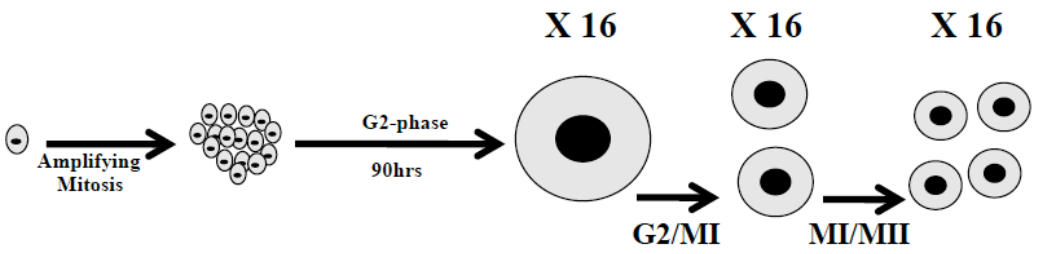


Figure 1-5: A cartoon highlighting the key events of spermatogenesis.

Spermatogenesis occurs in a tubular structure known as testis and involves significant changes in cell-cycle activity; a spermatogonia cell undergoes four rounds of trans-amplifying mitotic divisions to produce 16-cell spermatocytes. The spermatocytes undergo a prolonged G2 phase arrest for about 90 hours before undergoing two consecutive divisions in meiosis to produce 64 haploid spermatids. This is then followed by a post-replicative differentiation to produce sperms. (X 16 implies the number of cell shown multiply 16).

1.4.2 Nucleo-cytoplasmic Shuttling of CDK1/Cyclin Complexes

In addition to the temporal regulation via alternative phosphorylation and dephosphorylation, the biological functions of Cdk1/cyclin complexes are subjected to spatial regulation of sub-cellular localization during G2 phase and the G2/M transition. Cdk1/cyclin complexes localize mainly to the cytoplasm during interphase, but become predominantly nuclear during prophase just before the nuclear envelope breakdown (Li et al., 1997). The characteristic localization of Cdk1/cyclin complexes has been attributed to the sub-cellular re-distribution of mitotic cyclins (Heald et al., 1993; Li et al., 1997; Hagting et al., 1998; Jin et al., 1998b). The cytoplasmic accumulation of cyclin B depends on the balance between the rates of nuclear import and export (Hagting et al., 1998). While the nuclear import of cyclin B1 is facilitated initially by activation of Cdk1 that sets in motion a cascade of positive feedback loops culminating in auto-phosphorylation of cyclin B on its CRS region (Yang et al., 1998; Hagting et al., 1999), its nuclear export is mediated by a highly conserved atypical hydrophobic nuclear export signal (NES) in the CRS, and requires a functional exportin 1/CRM1 protein (Yang et al., 1998).

In starfish and *Xenopus* oocytes, cyclin B1 localizes predominantly in the cytoplasm during the S and G2 phases, but just before mitosis it accumulates in the nucleus (Pickham et al., 1992; Li et al., 1995). Similarly, mammalian cyclin B1 constantly shuttles between the nucleus and the cytoplasm during interphase (Hagting et al., 1998). Cyclin B1 in mouse two-cell embryos localizes in

cytoplasm until the late G2 phase, before suddenly accumulating in the nucleus just before mitotic initiation (Ohashi et al., 2001). Similar cytoplasmic localization of cyclin B1 was observed when Cdk1 activity was inhibited by butyroacetone I treatment in the mouse two-cell embryo (Ohashi et al., 2001), whereas Okadaic acid treatment (that activates Cdk1) promoted nuclear accumulation of cyclin B1 and allowed the two-cell arrested embryo to proceed into the M phase (Aoki et al., 1992). The universality of Cdk1-mediated nuclear import/export of cyclin B is called to question by a report in *Xenopus* oocytes demonstrating that cyclin B1 does not require Cdk1 for its nuclear import (Moore et al., 1999), and the observation that cyclin B1 in DNA-damaged HeLa cells localizes in the cytoplasm even when Cdk1 is active (Toyoshima et al., 1998).

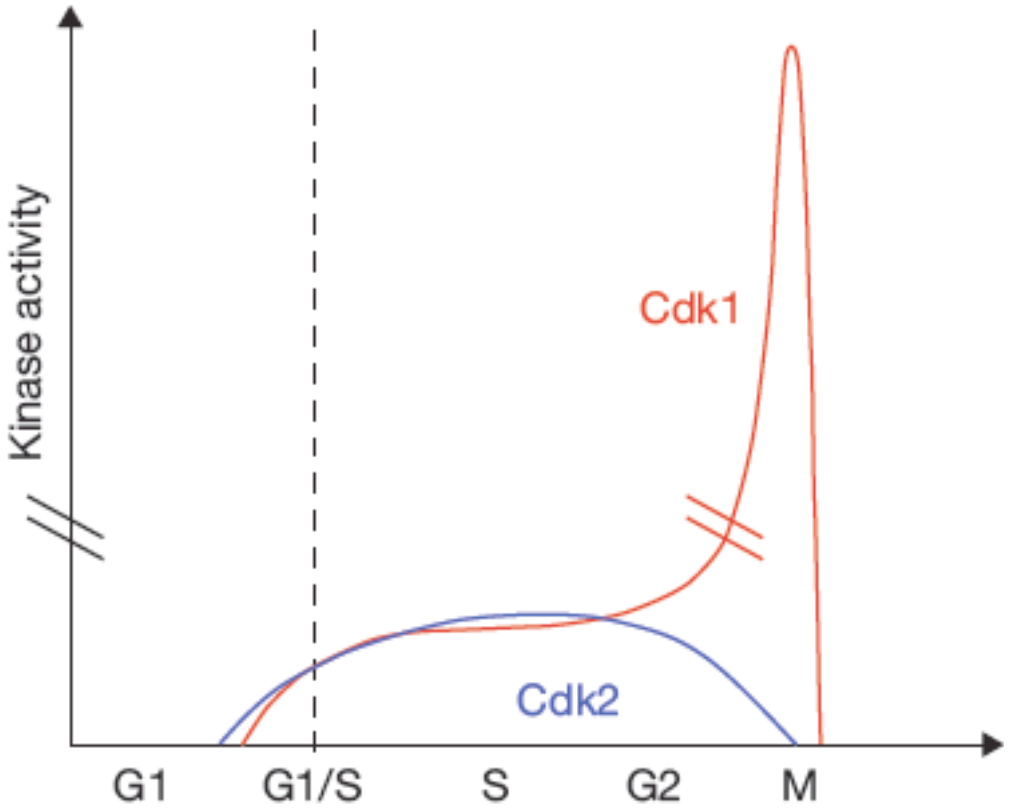
The extent to which the temporal regulation of Cdk1/cyclin B controls its spatial re-distribution or vice-versa remains a subject of intense research, however, an emerging paradigm suggests a dependency between the two regulatory mechanisms (Gavet and Pines, 2010a; Gavet and Pines, 2010b). The prevailing model suggests that activation of Cdk1/cyclin itself serves as the molecular pump that facilitates the nucleo-cytoplasmic shuttling of Cdk1/cyclin complexes during prophase to orchestrate the complex cellular reorganizations that accompany mitotic entry (Lindqvist, 2010). This idea is congruent with a model for Cdk1/cyclin nuclear entry involving changes in active export/import rates, rather than changes in the permeability of the nuclear envelope (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998; Hagting et al., 1999; Terasaki et al., 2003).

The biological relevance of this incredibly dynamic sub-cellular trafficking of Cdk1/cyclin complexes remains a subject of intensive research, however. It is not beyond the realm of possibility to speculate that such tightly regulated nucleo-cytoplasmic movements function to restrict untimely access to Cdk1 mitotic substrates. In fact, it has long been known that nuclear localization of Wee1 kinase prevents precocious accumulation of active Cdk1/cyclin B complex in the nucleus during interphase via Y15 inhibitory phosphorylation (Heald et al., 1993), and the accumulation of activated Cdk1/cyclin B in the nucleus in mouse oocytes promotes the export of Wee1 to the cytoplasm, preventing Wee1 access to nuclear Cdk1/Cyclin B substrates (Oh et al., 2010). The evidence that Myt1 kinase also serves a non-catalytic role in sequestering Cdk1/cyclin complexes in the cytoplasm during interphase is also consistent with a role in restricting access to Cdk1 substrates (Liu et al., 1999; Wells et al., 1999). As of yet, none of these ideas has been validated *in vivo*, in a physiologically relevant context.

1.4.3 Unusual role for Cdk1 in G1/S regulation

Cdk1 in complex with cyclin A or B is considered the G2/M specific kinase, while G1/S relies on Cdk2 kinase in complex with cyclin E. Recent studies in mouse have challenged this simple paradigm, however, by implicating Cdk1 in S phase regulation and as part of the regulatory circuitry used for controlling G1/S transitions (Berthet et al., 2003; Aleem et al., 2005; Pomerening et al., 2008; Potapova et al., 2009; Ma et al., 2012). Specifically, *Cdk2*$^{-/-}$ knockout mice were viable and this unexpected phenotypic outcome was due to the ability

of Cdk1 to substitute for Cdk2 in promoting G1/S phase transition (Berthet et al., 2003; Aleem et al., 2005). Cdk1 can also bind cyclin E to form an active complex; driving cells into S phase (Aleem et al., 2005). These observations suggest that cell cycle regulation in metazoans could be similar to the yeast cell cycle, where only one Cdk dynamically interacts with different cyclins to control passage through the cell cycle phases. Two possible explanations were offered for how Cdk1 activity at G1/S may have been overlooked despite decades of study by Tarig Bashir and Michele Pagano (2005). The fact that Cdk2 activity peaks at G1/S phase may obscure the presence of a basal level of Cdk1 activity. Alternatively, the basal level of Cdk1 activity, although sufficient to drive S phase events, may be negligible compared with the Cdk1 activity peak in early M phase, see Figure 1-6, (Bashir and Pagano, 2005). These proposed ideas are consistent with the findings that the levels of Cdk activity required for triggering S phase are significantly less than those required to activate mitosis (Coudreuse and Nurse, 2010). Indeed, the existence of a G1/S phase function for Cdk1 has also enjoyed further experimental support from studies in cultured mammalian cells showing that the expression of completely non-inhibitable Cdk1 mutant form produced phenotypes that are characteristic of perturbation of G1 and S events (Heald et al., 1993; Pomerening et al., 2008; Potapova et al., 2009; Ma et al., 2012).



(Adapted from; Bashir and Pagano, 2005)

Figure 1-6: The proposed cell cycle profile for Cdk1 and Cdk2 kinase activity (Adapted from; Bashir and Panago, 2005)

Even though Cdk1 activity may be sufficient to facilitate S phase events, the basal levels of Cdk1 activity at G1/S may be obscured by peaking of Cdk2 activity at G1/S phase or because the levels of Cdk1 activity during G1/S are negligible compared with the massive burst of Cdk1 activity peak in early M phase.

1.4.3.1 Cdk1 in mitotic exit regulation

Experiments using *Xenopus* egg extracts showed that the kinetics of Cdk1/cyclin inactivation occurred more rapidly when compared to the slow degradation of mitotic cyclin due to transient inhibitory phosphorylation of Cdk1 during M-phase exit (D'Angiolella et al., 2007). Concomitantly, M-phase egg extracts expressing non-inhibitable Cdk1 mutant failed to assemble functional APC/Cdc20 complexes, thereby blocking M phase exit (D'Angiolella et al., 2007). Mammalian cells also employ Cdk1 inhibitory phosphorylation as a compensating mechanism during M-phase exit, when APC/Cdc20 activity is insufficient to facilitate the process (Chow et al., 2011). This is consistent with the proposed role for Myt1-mediated inhibition of Cdk1 activity in Golgi and ER reassembly prior to mitotic exit (Nakajima et al., 2008).

1.5 DROSOPHILA CDK1 INHIBITORY KINASES: A TALE OF TWO SIMILAR, YET DIFFERENT ACTORS?

Genetic studies from our laboratory have demonstrated that *Drosophila* dWee1 and dMyt1 are partially redundant, but also serve specialized developmental functions. Their functional redundancies have made it difficult to ascertain specific molecular functions of the Wee1 and Myt1 kinases. Phosphorylation of Cdk1 on Y15 by maternally expressed Wee1 is required for an S/M checkpoint needed for completing the rapid nuclear cleavage divisions of early *Drosophila* embryogenesis (Price et al., 2000; Stumpff et al., 2004). Consistent with the requirement for Wee1 activity during early embryogenesis in mouse (Tominaga et al., 2006), *Drosophila* embryos lacking maternal Wee1 activity initiate normal development but fail to

coordinate transitions between S and M phase as interphase lengthens in the late syncytial cycles, resulting in lethal mitotic catastrophe (Price et al., 2000; Stumpff et al., 2004). Later during development, however, zygotic Wee1 activity is largely dispensable and levels of Y15-specific inhibitory phosphorylation of Cdk1 are unaffected in *wee1* mutants because of Myt1 activity (Jin et al., 2008).

The *Drosophila* dual specificity Cdk1 inhibitory kinase, dMyt1, is required during *Drosophila* spermatogenesis for male fertility and for proper specification of the cells that form *Drosophila* mechanosensory bristles on the head and thorax (Jin et al., 2005; Jin et al., 2008). *Drosophila* Myt1 is also crucial for G2-phase pre-mitotic checkpoint responses to ionizing radiation during imaginal wing development (Jin et al., 2008). Curiously, cellular defects caused by loss of dMyt1 activity include ectopic cell divisions of terminally differentiating germline-associated somatic cells, during both male and female gametogenesis (Jin et al., 2005). Biochemically, loss of dMyt1 activity results in complete loss of Cdk1-T14 phosphorylation as well as reduced Cdk1Y15p levels (Jin et al., 2008). However, it remains unclear whether these specialized developmental functions of Myt1 are due to phosphorylation of Cdk1 specifically on T14 or Y15 residues, or dual inhibitory phosphorylation. Mitotic cyclin-bounded Cdk1 proteins are detected in four distinct phosphorylation states during interphase due to Wee1 and Myt1-mediated inhibitory phosphorylation: T14-Y15, T14p-Y15, T14-Y15p and T14p-Y15p (Edgar et al., 1994; Mayya et al., 2006; Coulonval et al., 2011). The functional redundancy of Wee1 and Myt1 for Y15 inhibitory phosphorylation has made it

difficult to precisely assess, using *wee1* and *myt1*, how different Cdk1 phosphorylated isoforms impact developmental processes.

How can two kinases be functional redundant, yet have highly specialized developmental roles? Why is dual phosphorylation of T14 and Y15 by Myt1 kinase needed for Cdk1 regulation later in development, but not in early embryos? What factors could account for biochemically redundant kinases serving specialized developmental functions? One possible answer is that dual phosphorylation by Myt1 makes Cdk1 more refractory to de-phosphorylation by Cdc25 phosphatases than phosphorylation of T14 or Y15 alone, potentially as a developmental adaptation for enforcing a prolonged G2 phase arrest. Another possibility is that T14 and Y15 inhibitory phosphorylation occurs as an ordered reaction, with phosphorylation of one site depending on the other (Krek and Nigg, 1991c). Differential Cdk1 inhibitory phosphorylation could be important for regulating the subcellular localization of Cdk1 or its interactions with specific mitotic substrates (Holt et al., 2009; Gavet and Pines, 2010a; Gavet and Pines, 2010b; Kõivomägi et al., 2011). The differences in cytoplasmic dMyt1 versus nuclear dWee1 inhibitory kinases may also restrict access of different Cdk1 phosphoforms to specific mitotic substrates by directly modulating physical interactions or indirectly by creating distinct intracellular zones of Cdk1 activity (Jin et al., 1998a; Kao et al., 1999; Fletcher et al., 2002). An additional idea on differential roles of T14 and Y15 phosphorylation stems from the observation that the T14 inhibitory phosphorylation, but not Y15, is coupled to T161 activatory phosphorylation (Coulonval et al., 2011).

1.6 THESIS OBJECTIVE

The central goal of my thesis was to gain insight into specific effects of phosphorylating different inhibitory sites on the functional properties of Cdk1, its impacts on G2 phase checkpoints *in vivo* and the relevance of such differences in *Drosophila* developmental contexts, using site specific Cdk1 mutants. Four different Gal4 inducible phospho-acceptor variants of Cdk1 that were C-terminally tagged with venus fluorescent proteins (VFP) were developed; Cdk1(WT)-VFP, Cdk1(T14A)-VFP, Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F)-VFP (Figure 1-6). Tissue specific expression of these transgenes would allow us to understand the mechanics of a dual Cdk1 inhibitory phosphorylation mechanism that is used to coordinate cell proliferation with key developmental processes in all metazoans, using *Drosophila* as an experimental system.

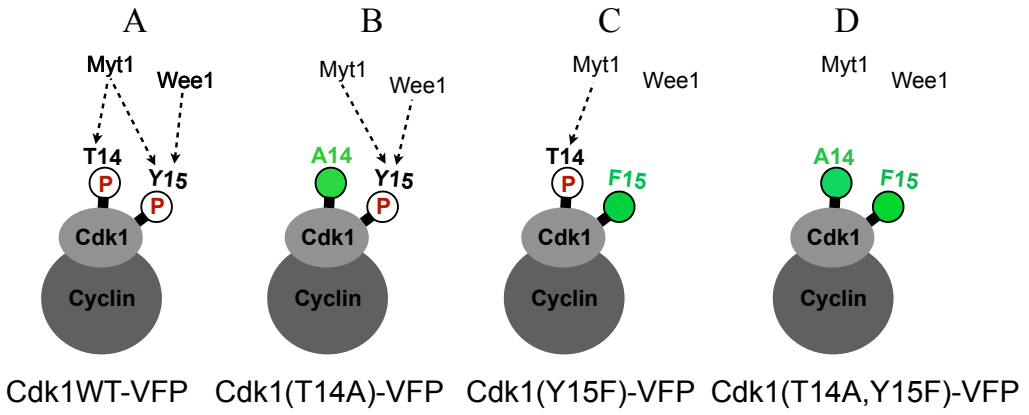


Figure 1-7: Cartoon showing the C-terminally tagged, Gal4 inducible Cdk1 transgenic proteins

(A) Shows the wild type Cdk1 fusion protein (Cdk1WT-VFP) containing inhibitable Y15 and T14 residues. (B) Shows Cdk1 mutant (Cdk1(T14A)-VFP) variants in which the threonine-14 residue is mutated to a non inhibitable alanine residue. (C) Shows Cdk1 mutant (Cdk1(Y15F)-VFP) variants in which the tyrosine-15 residue is mutated to a non inhibitable phenylalanine residue. (D) Represents constitutively active Cdk1 mutant (Cdk1(T14A,Y15F)-VFP) variants in which both tyrosine-15 and threonine-14 residue are mutated to non inhibitable alanine and phenylalanine residues, respectively.

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2. Functionally distinct mechanisms for regulating Cdk1 by T14 and Y15 inhibitory phosphorylation during Drosophila imaginal development[1]

[1] A version of this chapter has been published in the Journal of Genetics by Joseph O. Ayeni*, Ramya Varadarajan*, Oindrila Mukherjee*, David T. Stuart†, Frank Sprenger‡ and Martin Srayko*, Shelagh D. Campbell* ¶

2.1 INTRODUCTION

Cell cycle checkpoints delay entry into and exit from mitosis by inhibiting Cyclin-dependent Cdk1 (Cdk1) kinases, blocking cell-cycle progression when DNA replication or repair would threaten cell survival (Nurse, 1990). In *pombe*, phosphorylation of Cdk1 on a conserved tyrosine residue (Y15) is both necessary and sufficient for checkpoint arrest of cells in S or G2 phase until DNA replication and repair are completed and cells reach a critical size (Gould and Nurse, 1989; O'Connell et al., 1997; Rhind and Russell, 1998). Membrane-bound Myt1 kinases are Cdk1 inhibitors that co-evolved with Wee1 kinases, specifically in metazoans (Mueller et al., 1995; Booher et al., 1997; Liu et al., 1997). Myt1 kinases regulate Cdk1 by dual inhibitory phosphorylation of Y15 and the adjacent threonine residue, T14 (Gu et al., 1992; Blasina et al., 1997; Poon et al., 1997) and are also implicated in Cdk1/Cyclin B nucleo-cytoplasmic trafficking mechanisms responsible for coordinating the G2/M transition (Liu et al., 1999; Wells et al., 1999; Gavet and Pines, 2010). Gene duplications and functional redundancies have made it difficult to ascertain specific molecular functions of the Wee1 and Myt1 kinases (Okamoto et al., 2002; Burrows et al., 2006; Oh et al., 2010).

During interphase, Cdk1 bound to mitotic cyclins is detected in four distinct phosphorylation states with respect to Wee1 and Myt1-mediated dual inhibitory phosphorylation: T14-Y15, T14p-Y15, T14-Y15p and T14p-Y15p (Edgar et al., 1994; Mayya et al., 2006; Coulonval et al., 2011). Developmentally regulated expression of $Cdc25^{Stg}$ phosphatase activates Cdk1 in a dynamic

mechanism used for coordinating mitosis with cell movements during gastrulation in *Drosophila* (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). The expression of non-inhibitable Cdk1 (Cdk1AF) mutants cause cells to by-pass G2 phase arrest by triggering auto-amplification of feedback mechanisms used to activate endogenous Cdk1, in *Drosophila* and other experimental systems (Krek and Nigg, 1991; Norbury et al., 1991; Jin et al., 1996; Su et al., 1998). However, much less is known about the properties of singly phosphorylated Cdk1 isoforms *in vivo*. Studies of cultured mammalian cells suggested that Y15 phosphorylation is a more potent inhibitor of Cdk1 activity than T14 phosphorylation, but these experiments relied on conditions that may not reflect physiological constraints (Fletcher et al., 2002; Potapova et al., 2009). Moreover, a recent study reported that T14 phosphorylation of Cdk1/Cyclin B complexes promote T161 activating phosphorylation of Cdk1 by CAK (Cdk1 Activating Kinase) kinases whereas Cdk1/Cyclin B complexes phosphorylated only on Y15 were unstable during G2 phase (Coulonval et al., 2011), suggesting that T14 phosphorylation promotes accumulation of T161 phosphorylated but inhibited Cdk1 complexes. These intriguing observations raise questions regarding the physiological significance and functional properties of different Cdk1 isoforms produced by Wee1 and Myt1 phosphorylation during metazoan development.

We and others established that during the rapid syncytial cycles of early embryogenesis, maternal Wee1 regulates checkpoint responses that slow the cell cycle to accommodate late firing DNA replication origins and control chromosome condensation responses to DNA damage (Price et al., 2000; Stumpff

et al., 2004; Shermoen et al., 2010; Fasulo et al., 2012). Later in development Myt1 becomes the predominant biochemically detectable Cdk1 inhibitory kinase, and thus zygotic *myt1* mutants are male sterile with sensory bristle defects (Jin et al., 2005; Jin et al., 2008). Moreover, zygotic Wee1 and Myt1 are also functionally redundant for cell viability, implying that these two kinases also share essential house keeping functions. However, we do not yet know if these specialized developmental functions of Wee1 and Myt1 are due to differences in gene expression or biochemically distinct mechanisms for regulating Cdk1.

We addressed this issue by expressing fluorescently tagged Cdk1 wild-type and phospho-acceptor mutant proteins to examine how different inhibitory phosphorylation mechanisms affected *Drosophila* wing, eye and neuroblast development. The transgenic Cdk1 fusion proteins physically interacted with endogenous mitotic cyclins, were phosphorylated by endogenous inhibitory and activating kinases and functionally complemented conditional loss of function *cdc2* (*cdk1*) mutants. With these new genetic tools we were able to distinguish phenotypic effects of regulating Cdk1 by T14 and Y15 inhibitory phosphorylation for the first time *in vivo*, providing new insights into cell cycle regulatory mechanisms adapted for coordinating animal development.

2.2 MATERIALS AND METHODS

Construction of Gal-4 Inducible Cdk1-VFP Strains

Constructs were made from pSP64 plasmids carrying *Cdk1WT, Cdk1(T14A)*, *Cdk1(Y15F)* and *Cdk1(T14A,Y15F)* sequences that were amplified using high fidelity Platinum® *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA) with the following primers: LPE 192 (5'-CACCATGGAGGATTTTGAGAA-3') and RPE 192 (5'-ATTTCGAACTAAGCCCGATTG-3'). The amplified DNA was sub-cloned into a pENTR directional TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with One Shot® chemically competent *E.coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). Gateway recombination of the pENTR clones with the *Drosophila* destination vector pPWV (a UASp vector with 14 Gal4 UAS sites and a C-terminal Venus tag) was then used to generate Gal4-inducible, C-terminal VFP tagged expression constructs (Brand and Perrimon, 1993). Each construct was verified by sequencing on both strands before being used for P element transformation (Best Gene), with strains showing similar expression chosen for the experiments

Analysis of Wing Imaginal Disc Development and Checkpoint Responses to Radiation

Imaginal wing discs were dissected in phosphate-buffered saline (PBS) from larvae cultured at 22^{o} on standard *Drosophila* media supplemented with 0.05% bromophenol. Late third instar larvae were selected by disappearance of bromophenol blue from the gut and initiation of wandering behavior (Maroni and Stamey, 1983). The discs were fixed for 15 minutes in 4% paraformaldehyde at

room temperature, washed three times for 5 minutes each in PBT (1X PBS and 0.3% Triton X-100) and blocked for 30 minutes with 5% bovine serum albumin in 1X PBT before incubation with primary antibodies overnight at 4^{o}. Antibodies were used at the following dilutions: rabbit anti-phospho-Histone H3 (Upstate Biotechnology), 1:4000; rabbit anti-cleaved Caspase-3 (Cell Signaling Technology), 1:1000; mouse anti-Cyclin A, 1: 200; mouse anti-Cut, 1:200 and mouse anti-Wingless, 1:10 (these were all from Developmental Studies Hybridoma Bank). Secondary antibodies (Invitrogen) conjugated with Alexa Fluor-488 or Alexa Fluor-568 were used at a working dilution of 1:1000. Figures 4, 5 and 6 were composed from overlapping images so as to show the entire imaginal wing disc and were de-convolved by iterative restoration using Volocity software then compiled with Adobe Photoshop software using identical manipulations for each experimental set.

Radiation induced pre-mitotic checkpoint assays were performed using established protocols (Brodsky et al., 2000). Briefly, staged late third instar larvae were irradiated with 40 Gy using a Co^{60} gamma-ray source, then allowed to recover for 1 hour at room temperature before dissecting, fixing and labeling the wing discs with phospho-(S10)-Histone H3 antibodies to mark mitotic cells.

Scanning Electron Microscopy of Adult Drosophila Eyes

Pharate adults were dissected from their pupal cases and fixed for 2 hours with 2% gluteraldehyde in PBS (adding a drop of 0.2% Tween-20 to reduce surface tension). Following fixation, the samples were rinsed with distilled water and then dehydrated by passage through a graded ethanol series (once each for 30 minutes:

25%, 50%, and 75%, followed by twice with 100% ethanol) and mounted for imaging with a Philips/FEI LaB6 environmental scanning electron microscope (ESEM).

Western Blot Analysis of Protein Extracts

Wing imaginal discs were dissected from late third instar larvae and kept on ice. Ten wing discs per sample were homogenized in SDS-PAGE sample buffer and the protein extracts were separated by 10% SDS-PAGE and transferred to Hybond P membranes (Amersham). After blocking with 5% bovine serum albumin in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 1 hr the blots were incubated with primary antibodies over night at 4^{o}. Labelled proteins were then detected with anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Amersham), using a GE Healthcare ECL Plus chemiluminescence kit. The primary antibodies used for these experiments were rabbit anti-pT14-Cdk1 (1:500), pY15-Cdk1 (1:1000) and rabbit pT161-Cdk1 (1:1000), all from Cell Signaling Technology as well as mouse anti-GFP (1:5000; Clontech), and mouse anti-Actin (1:5000; Chemicon).

Immunoprecipitation of Transgenic Cdk1 Protein and Histone H1 Kinase Assays

Thirty to thirty-five wing discs from larvae expressing VFP-tagged Cdk1 transgenes under control of *Engrailed-Gal4* were dissected for each sample into ice-cold PBS and stored at -80^{o} until proceeding. After thawing, the tissue was homogenized in 200 μL of ice-cold lysis buffer containing phosphatase and protease inhibitors (20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 2 mM

EGTA, 0.1% NP-40, 1 mM Benzamidine, 2 mM Sodium orthovanadate, 80 mM Glycerophosphate, 2.5 mM β-Mercaptoethanol, 10 mM NaF, 2 mM PMSF, 10 mM Pepstatin A, 0.5 μg/mL Leupeptin, and 2.5 mg/mL Aprotinin). Lysates were kept at 4^{o} throughout the remainder of the experiment. Cellular debris was pelleted by centrifugation at 14000g for 15 minutes and the supernatant was removed and pre-cleared by incubation with 20 mL of a 50% slurry of lysis buffer and protein A beads for 1 hour, followed by centrifugation at 3000g for 5 minutes. The pre-cleared lysates were then incubated with 0.5 μL of rabbit anti-GFP for 1 hour at 4^{o}C and the GFP-fused proteins were immunoprecipitated by adding 20 μL of a 50% slurry of protein A beads and incubated at 4^{o} overnight. The precipitated proteins, the remaining supernatant (adsorbed) fractions and aliquots of the initial pre-cleared lysates were then analyzed on western blots by probing with mouse anti-Cyclin B and Cyclin A antibodies (1:1 dilution), from the Developmental Studies Hybridoma Bank (Lehner and O'Farrell, 1990) and then with horseradish peroxidase-coupled secondary antibodies (Sigma) and ECL chemiluminescence to detect the labeled proteins.

For Histone H1 kinase assays, immunoprecipitates were prepared as above except that 180 dissected third instar wing discs were used for each genotype. Precipitates were then washed three times with lysis buffer and twice with H1 kinase buffer (20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$), before incubation in a reaction mixture containing kinase buffer supplemented with 10 μM un-labeled ATP, 1 mg/mL histone H1, and 30 μCi of [γ-^{32}P]-labeled ATP for 15 min at 30°. The reactions were quenched by addition of one volume of 2× SDS loading buffer

and incubated for 5 min at 100°. Reaction products were then separated on 10% SDS-PAGE gels and the phosphorylated histone H1 was detected and quantified by scanning the dried gels with a Molecular Dynamics STORM Phosphoimager. To estimate the amount of transgenic protein in each sample, 20 μL aliquots of each precipitate were western blotted and probed with anti-Cdk1 (PSTAIR) antibodies (diluted 1: 5000), then quantified using IMAGEJ software. Catalytic activity was determined by normalizing the H1 kinase activity of each sample to the amount of protein, and shown relative to Cdk1(WT) activity set as 100%. Data from three independent experiments were compiled for Figure 1D.

Analysis of mitosis in larval neuroblasts

To study larval neuroblasts we dissected third-instar larvae in PBS to remove eye-antennal discs from the brains, which were incubated for 1.5 hours in 10^{-5} M colchicine at room temperature. This was followed by 10 minutes incubation in hypotonic solution (0.5% sodium citrate), then incubation for 2 minutes in 45% acetic acid and incubation for 20sec in 60% acetic acid before squashing hard between a poly-lysine-coated slide and siliconized coverslip. The slides were then frozen immediately in liquid nitrogen before removing the coverslip and dehydrated in absolute ethanol at room temperature then left to air-dry, before rehydration, staining with Hoechst 33258 and mounting in anti-fade glycerol-based medium. The slides were then examined for metaphase karyotypes to look for chromosome aberrations and to quantify the mitotic index by counting the number of cells with condensed, mitotic chromosomes per microscopic field (the area observed with a 40X Zeiss objective). To account for experimental variation,

10-20 fields were sampled from each of 4 brains per slide, with 3 slides per genotype examined.

Live analysis of mitosis was performed using published protocols to visualize both VFP expressed from each of the Cdk1 transgenes and microtubules (Buffin et al., 2005), using an α-*Tubulin-RFP* transgene (Goshima et al., 2007). Data for fluorescent time-lapse movies were acquired with an inverted microscope (IX81; Olympus; 60×, NA 1.42 oil objective) equipped with a spinning-disc confocal head (CSU10; Yokogawa). Image capture with a CCD camera (ORCA-R2; Hamamatsu Photonics) was controlled by MetaMorph software (Molecular Devices). Stacks of 5 planes at 0.5 micron intervals were collected every 30 seconds. Images showing maximum intensity projections were adjusted for brightness and contrast, with mitosis defined as the interval between the appearance of centrosomes at opposite poles of the dividing cell to the end of cytokinesis.

2.3 RESULTS

Functional Characterization of Cdk1-VFP Proteins Expressed in Wing Imaginal Discs

The unique developmental roles of Wee1 and Myt1 kinases may reflect specialized properties of Cdk1 proteins that are phosphorylated on T14 and/or Y15 residues. To test this idea, we made Gal4-inducible transgenes to compare the behaviour of different phospho-isoforms, *in vivo*. We made four constructs, each C-terminally tagged with Venus Fluorescent Protein (VFP): Cdk1(WT)-VFP, Cdk1(T14A)-VFP, Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F)-VFP, allowing us to visualize exactly where each protein was expressed. By genetic crosses to *engrailed-Gal4* (*en-Gal4*), we induced transgene expression in the posterior regions of late third instar wing imaginal discs. This allowed characterization of biochemical and functional properties of these Cdk1 fusion proteins in a tissue where Wee1 and Myt1 are both expressed (Jin et al., 2008). As expected, Western blots of protein extracts (from 30-35 wing discs, per sample) that were probed with anti-Cdk1 (PSTAIR) antibodies detected both endogenous Cdk1 (34 kDa) and the Cdk1-VFP (61 kDa) fusion proteins (Figure 2-1A**)**. Next, we used the same expression system to determine if the Cdk1-VFP fusion proteins interacted with endogenous mitotic cyclins. We first optimized conditions for quantitatively immuno-precipitating Cdk1(WT)-VFP from proteins extracted from wing discs (Figure 2-2A). Using this protocol, Western blots of immuno-precipitated proteins were then sequentially probed with mouse anti-*Drosophila* Cyclin B, Cyclin A and mouse anti-GFP antibodies (Figure 2-1B). The results

showed that Cyclin A and Cyclin B proteins co-precipitated with each of the transgenic Cdk1 fusion proteins, demonstrating that the tagged proteins physically interacted with endogenous mitotic cyclins.

When Cdk1 proteins are bound to mitotic cyclins they are subject to inhibitory phosphorylation by Wee1 and Myt1 (Meijer et al., 1991) as well as activating phosphorylation on residue T161 by the Cdk activating kinase, CAK (Solomon et al., 1992). To analyze the phosphorylation status of transgenic Cdk1 expressed in wing discs with *en-Gal4*, western blots of wing disc protein extracts were sequentially labeled with antibodies against phospho-T14-Cdk1 (pT14), phospho-Y15-Cdk1 (pY15) and phospho-T161-Cdk1 (pT161). These antibodies all recognized Cdk1WT-VFP, confirming presence of pT14, pY15 and pT161 Cdk1 phospho-isoforms (Figure 2-1C). However Cdk1(Y15F)-VFP was only labeled by pT14-Cdk1 antibodies whereas Cdk1(T14A)-VFP was only labeled by pY15-Cdk1 antibodies, showing that both T14 and Y15 residues of transgenic Cdk1 were independently phosphorylated by endogenous inhibitory kinases. Cdk1(T14A-Y15F)-VFP was not recognized by antibodies against either pT14 or pY15, also as expected. Anti-pT161 antibodies detected Cdk1(WT)-VFP, Cdk1(Y15F)-VFP and Cdk1(T14A-Y15F)-VFP fusion proteins. These antibodies did not detect Cdk1(T14A)-VFP and this observation was reproducible when the transgene was expressed in other tissues (data not shown). Although we cannot exclude alternative explanations for these intriguing results because we are working with an unsynchronized population of proliferating cells, they suggest that T161-activating phosphorylation may be compromised when only the Y15p

residue of Cdk1 is phosphorylated, consistent with the conclusions of Coulonval *et al*, 2011.

To assess Cdk1 catalytic activity, we immuno-precipitated VFP-tagged fusion proteins from wing disc extracts using GFP antibodies for histone H1 kinase assays, using 180 wing discs for each sample (Figure 2-1D and 2-2B). The incorporation of radioactive phosphate into histone H1 was quantified by densitometry after normalizing each sample for protein concentration, setting Cdk1(WT)-VFP activity as 100%. Mock immuno-precipitates from a non-transgenic *yw* strain were used as a negative control and showed negligible background levels of histone H1 kinase activity (Figure 2-2B). Pooled data compiled from three independent experiments were used to prepare Figure 2-1D, showing that the relative levels of Cdk1WT-VFP and Cdk1(T14A)-VFP activity were indistinguishable. In contrast, the Cdk1(Y15F)-VFP precipitates had roughly 2-fold higher activity than Cdk1WT-VFP whereas Cdk1(T14A,Y15F)-VFP precipitates had roughly 3-fold higher activity (Figure 2-1D) . Phosphorylation of Cdk1(Y15F)-VFP on the T14 residue therefore partially inhibited Cdk1 catalytic activity, relative to Cdk1(T14A,Y15F)-VFP. We cannot make a similar argument regarding the effect of Y15 phosphorylation alone, however, because the low activity of Cdk1(T14A)-VFP precipitates could be attributable both to low levels of activating T161 phosphorylation and to inhibitory (Y15) phosphorylation (as shown in Figure 2-1C).

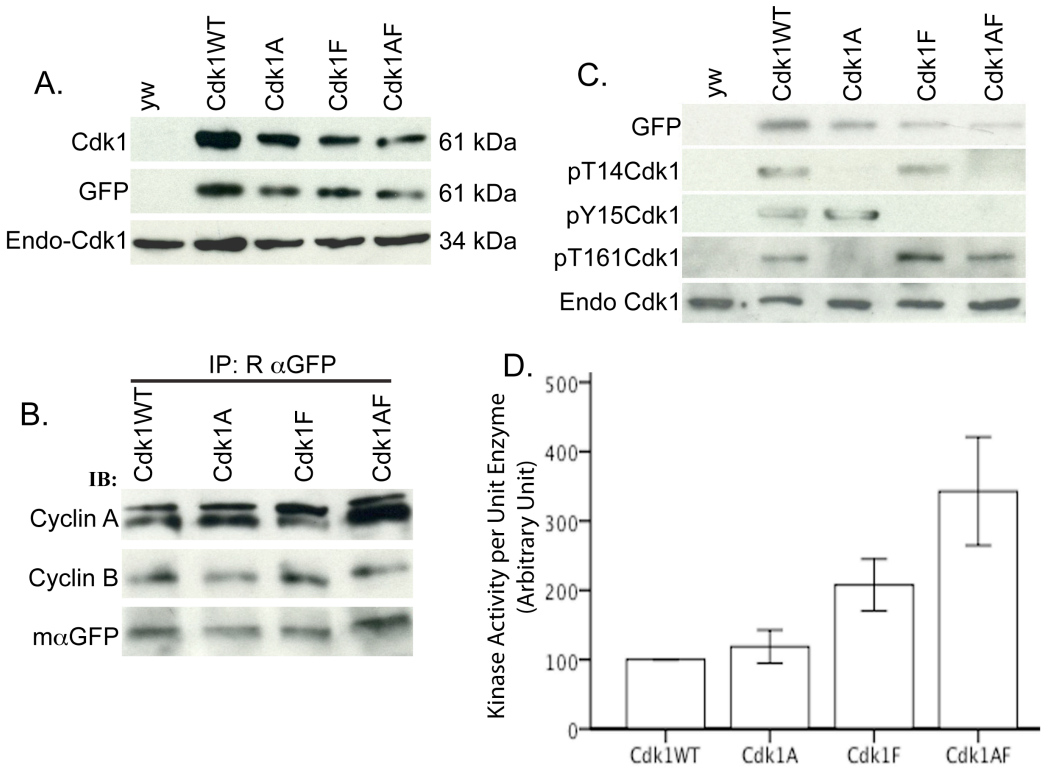

Figure 2-1: Biochemical analysis of transgenic Cdk1 fusion proteins expressed in wing disc with *en-Gal4*

In panels A and C, each sample was extracted from ten, late third instar wing discs. (A) Western blot of wing disc extracts probed with PSTAIRE antibodies (total Cdk1) detected both endogenous (34 kDa) and transgenic fusion (61 kDa) proteins, except in the non-transgenic *yw* control. (B) Western blot of transgenic Cdk1 strains that was immuno-precipitated from wing disc lysates (30-35 discs, each sample) using rabbit anti-GFP. The blot was sequentially probed with mouse anti-GFP, *Drosophila* Cyclin B and Cyclin A. Note that Cyclin A runs as a doublet (Lehner and O'Farrell, 1990). (C) Western blot of wing disc extracts, with the top section (proteins above 48kDa) probed sequentially with antibodies against pT14-Cdk1, GFP, pY15-Cdk1, pT161-Cdk1, stripping between each re-probing. The lower part of the blot containing 34kDa endogenous Cdk1 was probed with PSTAIRE antibodies, as a loading control. (D) Compilation of data from three independent experiments for each genotype that measured Histone H1 kinase activity of Cdk1 fusion proteins immuno-precipitated from wing disc extracts with anti-GFP antibodies (180 discs for each sample). We determined kinase activity for each Cdk1 variant by normalizing the H1 kinase activity per unit of total Cdk1 protein, estimated by probing western blots of aliquots of the

immuno-precipitates with anti-PSTAIRE, for each experiment. Relative percentage kinase activity was plotted with respect to Cdk1(WT) activity, which was set as 100%. Error bars show standard deviation calculated from data from three experimental replicates, for each genotype.

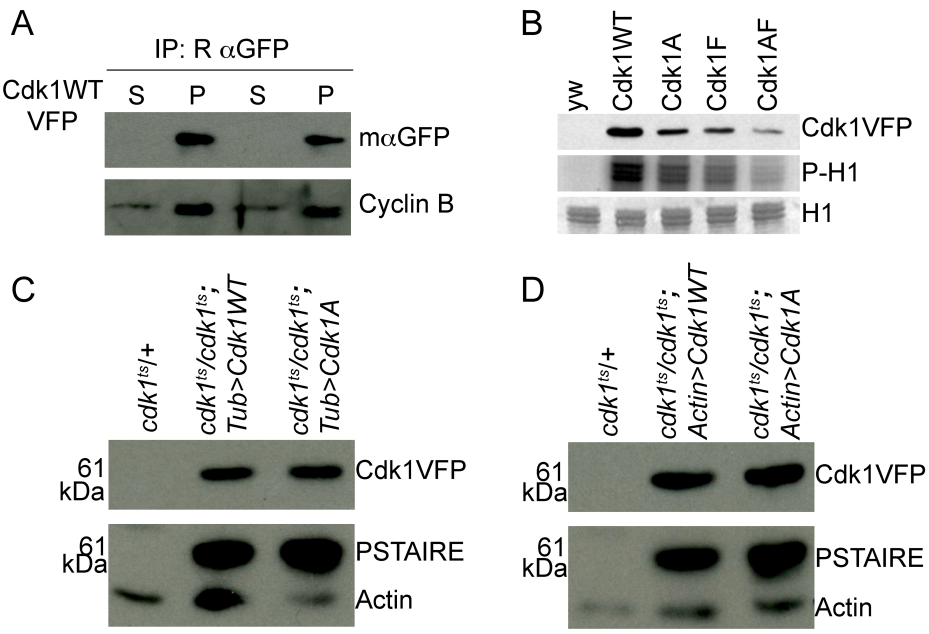

Figure 2-2: Data showing protocol for efficiently precipitating transgenic Cdk1(WT) proteins from wing disc extracts with rabbit anti-GFP

(A) Precipitate (P) and supernatant (S) fractions were probed with antibodies against Cyclin B which detect a single band of 63 kD , then stripped and re-probed with mouse anti-GFP antibodies, showing that Cdk1WT-VFP was efficiently precipitated (and depleted from the supernatant). The blot shows two experimental replicates. Panel B shows aliquots from one experiment where Cdk1 fusion proteins were immunoprecipitated from larval wing discs with rabbit anti-GFP antibodies (*yw* as a negative control) and tested for kinase activity determination using histone H1 as a substrate. Anti-histone H1 and mouse anti-Cdk1 antibodies were used for labeling the blot as positive controls. Each sample represents immunoprecipitated protein from 180 wing discs per genotype. Relative kinase activity of each of the Cdk1 variants was determined by normalizing kinase activity per unit of total immunoprecipitated protein. Panels C and D show western blots of protein extracts from $cdc2^{ts}$ mutants rescued with Cdk1(WT)-VFP expressed using *Tub-Gal4* and *Actin-Gal4,* respectively. Control extracts ($cdk1^{ts}$/+) probed with anti-GFP, anti-Cdk(PSTAIR) or anti-Actin antibodies showed no transgenic protein, whereas extracts from $cdc2^{B47}/cdc2^{E1-E24}$*;UAS-Cdk1WT-VFP/Tubulin-Gal4* or $cdc2^{B47}/cdc2^{E1-E24}$*;UAS-Cdk1WT-VFP/Actin-Gal4* showed that the transgenic protein was well expressed.

Table 2-1: Data showing the results of the rescue assay used to assess the functionality of the transgenic proteins.

Class of Progeny	Predicted Genotypes	Cdk1WT	Cdk1A	CdK1F	Cdk1AF
A	$\frac{cdc2^{B47}}{cdc2^{E1\text{-}E24}}$; $\frac{TRANSGENE}{Tubulin\text{-}Gal4}$	49	24	0	0
B	$\frac{cdc2^{B47}}{cdc2^{E1\text{-}E24}}$; $\frac{Tubulin\text{-}Gal4}{TM6,Tb}$ $\frac{cdc2^{B47}}{cdc2^{E1\text{-}E24}}$; $\frac{TRANSGENE}{TM6,Tb}$	0	0	0	0
C	$\frac{cdc2^{B47}}{CyO}$; $\frac{TRANSGENE}{Tubulin\text{-}Gal4}$ $\frac{cdc2^{E1\text{-}E24}}{CyO}$; $\frac{TRANSGENE}{Tubulin\text{-}Gal4}$	94	53	0	0
D	$\frac{cdc2^{B47}}{CyO}$; $\frac{TRANSGENE}{TM6,Tb}$ $\frac{cdc2^{E1\text{-}E24}}{CyO}$; $\frac{TRANSGENE}{TM6,Tb}$ $\frac{cdc2^{B47}}{CyO}$; $\frac{Tubulin\text{-}Gal4}{TM6,Tb}$ $\frac{cdc2^{E1\text{-}E24}}{CyO}$; $\frac{Tubulin\text{-}Gal4}{TM6,Tb}$	177	110	247	320
p-Value (expected ratio of classes A:C:D was 1:2:4)		0.78	0.80	0.0001*	0.0001*

Table 2-1 legends:

Compilation of progeny data resulting from genetic crosses used to test whether the Cdk1-VFP transgenes ubiquitously expressed with either *Tubulin-Gal4* or *Actin-Gal4* could rescue pupal lethality in a *cdk1* temperature-sensitive mutant background (*w; cdc2*^{B47}*/cdc2*$^{E1\text{-}E24}$*; Cdk1-VFP/Tubulin-Gal4)*. Flies were cultured at 25 ^{o}C throughout development. Expression of Cdk1(WT)-VFP completely rescued *cdk1* lethality (as indicated by progeny in Class A). Expression of Cdk1(T14A)-VFP also completely rescued the temperature sensitive *cdk1* lethality (see Class A). In contrast, neither expression of Cdk1(Y15F)-VFP nor Cdk1(T14A,Y15F)-VFP were capable of rescuing the lethality of *cdk1* mutants (no progeny in Class A).

To assess the functionality of the Cdk1 fusion proteins *in vivo* we tested for genetic complementation of $cdc2^{ts}$ (*cdk1)* mutant lethality. *Tubulin-Gal4* and *Actin-Gal4* strains were used to express each variant ubiquitously in $cdc2^{E1\text{-}24}/cdc2^{B47}$ mutant background that was temperature-sensitive for Cdk1 activity (Stern et al., 1993). Progeny from the genetic crosses used to create these combinations were cultured at the restrictive temperature for the $cdc2^{E1\text{-}24}$ allele (25º) until adulthood, when phenotypic markers carried on the balancer chromosomes were analyzed to score the genotype of each survivor (Figure 2-3 and Figure 2-2 C-E). Expression of Cdk1(WT)-VFP completely rescued *cdc2* pupal lethality (Figure 2-3 B, B' and D, Class A in Figure 2-2E). The rescued adults were morphologically indistinguishable from sibling heterozygous controls (Figure 2-2A, A' and D, Class D in Figure 2-2E) and western blots (Figure 2-2C and D) confirmed that the fusion proteins were expressed in $cdc2^{B47}/cdc2^{E1\text{-}E24}$*;UAS-Cdk1WT-VFP/Tubulin-Gal4* and $cdc2^{B47}/cdc2^{E1\text{-}E24}$*;UAS-Cdk1WT-VFP/Actin-Gal4* flies. Surprisingly, expression of transgenic Cdk1(T14A)-VFP also rescued *cdc2* mutant lethality (Figure 2-3 C, C' and D, Class A in Figure 2-2E), showing that Cdk1 activity produced by this transgene was sufficient for complementation despite the defects in T161 phosphorylation noted earlier (Figure 2-1 C). In both, the percentage of rescued progeny was not significantly different from the maximum expected for genetic complementation (Figure 2-3D). In contrast, expression of Cdk1(Y15F)-VFP or Cdk1(T14A,Y15F)-VFP did not rescue *cdc2* pupal lethality (Class A, Figure 2-2E). In fact, ubiquitous expression of either transgene was lethal in a heterozygous $cdc2^{ts}/+$ background

(Class C in Figure 2-2E). While dominant lethality arising from expression of non-inhibitable Cdk1 was not unexpected, seeing the same effect with both of the Cdk1 transgenes that were not phosphorylated on Y15 provided our first clue that regulation of this particular residue was critical for normal *Drosophila* development.

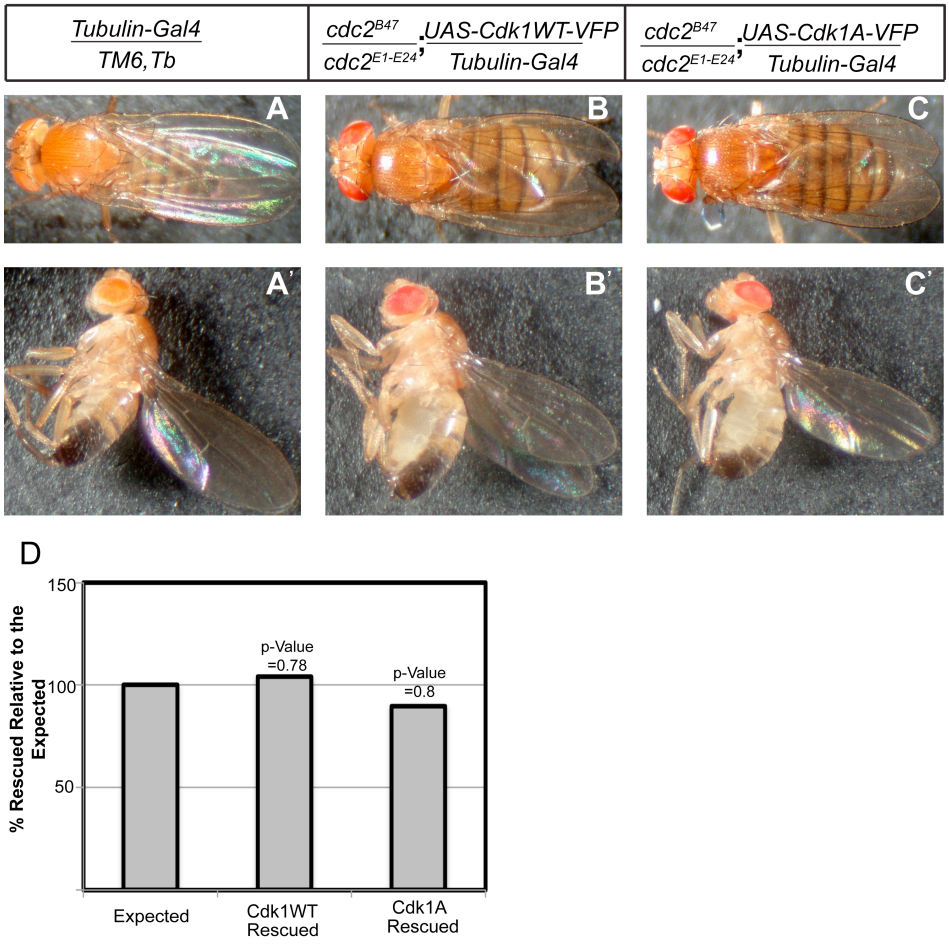

Figure 2-3: Conditional *cdk1* (*cdc2*) mutants are rescued only by Cdk1 transgene that can be phosphorylated on Y15

Panels A and A' show the dorsal and side views of wild-type control flies, respectively. Panels B and B' show flies ubiquitously expressing the *Cdk1WT-VFP* transgene with *Tubulin-Gal4* in temperature sensitive *cdc2* mutant background (*cdc2*^{B47}/*cdc2*$^{E1-E24}$;*UAS-Cdk1WT-VFP/Gal4*). Panels C and C' show adult flies ubiquitously expressing the *Cdk1(T14A)-VFP* transgene with *Tubulin-Gal4* (*cdc2*^{B47}/*cdc2*$^{E1-E24}$;*UAS-Cdk1(T14A)-VFP/Gal4*). Panel D summarizes progeny data tabulated in Figure S1, Panel E, showing the percentage of rescued flies relative to the expected maximum percentage rescue.

Regulation of Cdk1 by T14 and Y15 Phosphorylation During Adult Eye and Wing Development

Expression of non-inhibitable Cdk1 in *Drosophila* induces G2-arrested cells to enter premature mitosis and is incompatible with normal development of germline and somatic tissue (Sprenger et al., 1997; Su et al., 1998; Jin et al., 2005). To determine if Cdk1 proteins phosphorylated only on T14 or Y15 residues caused visible developmental defects we expressed the transgenes throughout wing disc development in an otherwise wild-type genetic background (Campbell et al., 1992). *Sd-Gal4*-driven expression of Cdk1(WT)-VFP, Cdk1(T14A)-VFP or Cdk1(Y15F)-VFP did not noticeably affect wing development (Figure 2-4A-C), however Cdk1(T14A,Y15F)-VFP caused varying degrees of wing margin notching (Figure 2-4D). We also induced the transgenes during eye-antennal disc development using *ey-Gal4* (Hazelett et al., 1998). Expression of Cdk1(WT)-VFP, Cdk1(T14A)-VFP or Cdk1(Y15F)-VFP produced adults with normal eyes (Figure 2-4E-G), however Cdk1(T14A,Y15F)-VFP produced eyeless, pharate adult flies that were unable to eclose (Figure 2-4H). These results showed for the first time *in vivo* that singly phosphorylated Cdk1 isoforms caused phenotypic effects that were qualitatively distinct from non-inhibitable Cdk1 mutants.

Previous studies have shown that the dominant phenotypic defects and mitotic catastrophe caused by expression of non-inhibitable Cdk1 mutants are dependent on the levels of endogenous mitotic cyclins (Heald et al., 1993; Jin et al., 1998; Su et al., 1998). To assess how this variable affected our results we co-expressed transgenic *Drosophila* Cyclin B with each Cdk1 variant, again using

sd-Gal4. Examples shown in Figure 2-4 are representative of at least 100 adult flies examined for each genotype. Expression of Cyclin B alone (Figure 2-4I) had no detectable effect on the adult wing, nor did Cyclin B co-expressed with Cdk1WT-VFP (Figure 3J) or with Cdk1(T14A)-VFP (Figure 2-4K). However when Cyclin B was co-expressed with Cdk1(Y15F)-VFP, we did observe wing margin defects (compare Figure 2-4C with 2-4L). Co-expression of Cyclin B with Cdk1(T14A,Y15F)-VFP also caused more severe phenotypic defects, resulting in complete loss of the adult wing (compare Figure 2-4D with 2-4M). These results showed that expression of non-inhibitable Cdk1(T14A,Y15F)-VFP consistently caused more severe dominant wing and eye defects than Cdk1(Y15F)-VFP, even when Cyclin levels were not limiting. We concluded from these results that phosphorylation of Cdk1(Y15F)-VFP on T14 had an inhibitory effect of Cdk1 activity *in vivo*, consistent with the *in vitro* H1 kinase measurements shown in Figure 2-1D. We also examined *en-Gal4* and *neur-Gal4*-driven Cdk1(Y15F)-VFP expression in a *myt1* mutant background (Jin et al., 2005). Expression of Cdk1(Y15F)-VFP was lethal in *myt1* mutants but not in *myt1/+* heterozygote controls (data not shown), showing that Myt1 phosphorylation of the T14 residue was what prevented the phenotypic defects associated with expression of non-inhibitable Cdk1 .

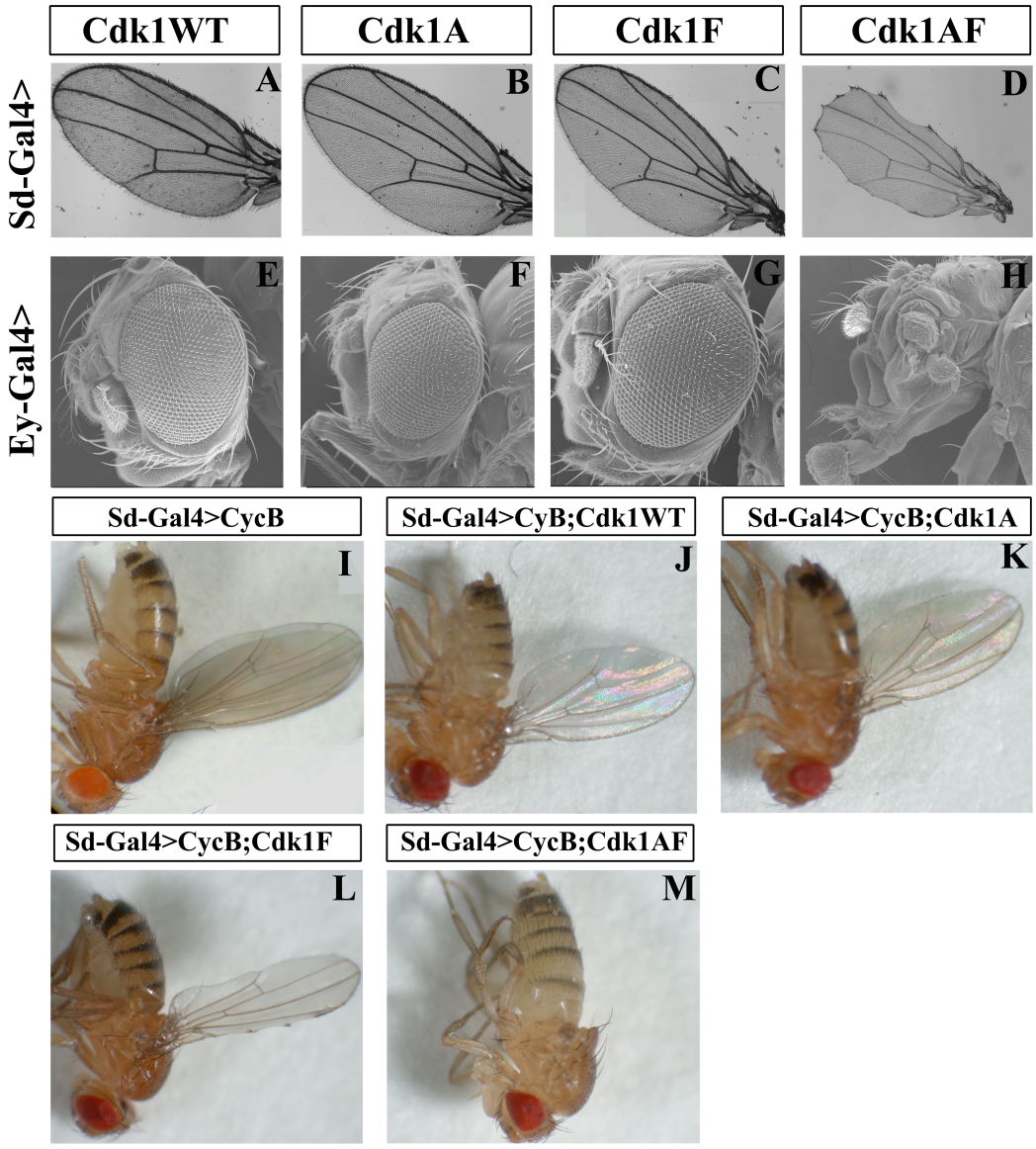

Figure 2-4: Phenotypic effects of expression of Cdk1 transgenes during adult wing and eye development are enhanced by co-expression of cyclin B

Figure 2-4 Legend

Cdk1-VFP transgenes were expressed using *sd-Gal4* and *ey-Gal4* respectively, with ~500 adults examined for each genotype unless otherwise noted. Panels A to C show progeny expressing Cdk1(WT), Cdk1(T14A) or Cdk1(Y15F) in an otherwise wild-type background that cause no defect in adult wing morphology. Panel D show that Cdk1(T14A,Y15F) expression caused wing margin defects. Panels E to G, show scanning electron micrographs of adult eyes from progeny expressing Cdk1(WT), Cdk1(T14A) or Cdk1(Y15F) that did not affect adult eye development. Panel H shows that Cdk1(T14A,Y15F) expression severely affected adult eye and head structures, causing pharate adult lethality (N=200). Panels I to K show that expression of Cyclin B alone, co-expression of Cyclin B with Cdk1(WT), or co-expression of Cyclin B with Cdk1(T14A) had no detectable effect on adult wings. Panel L shows that co-expression of Cdk1(Y15F) with Cyclin B caused extensive loss of adult wing margin. Panel M shows that co-expression of Cdk1(T14A,Y15F) with Cyclin B also caused enhanced resulting in complete loss of the adult wing. Panels L to M show the entire adult fly to emphasize the complete loss of wing tissue observed with co-expression of Cdk1(T14A,Y15F) and Cyclin B.

Loss of Y15 Inhibitory Phosphorylation Caused Cell Proliferation Defects and Apoptosis

Tissue homeostasis is maintained by compensatory mechanisms that control the balance of cell proliferation and apoptosis in *Drosophila* wing discs (Reis and Edgar, 2004; Davidson and Duronio, 2012). Our results showing that adult wing development was exquisitely sensitive to Cdk1(T14A,Y15F)-VFP expression but not to expression of singly phosphorylated isoforms suggested that T14 or Y15 phosphorylation were functionally equivalent mechanisms for regulating Cdk1 during wing and eye development, at least with endogenous mitotic cyclin levels. Accordingly, there was no reason to expect that expression of either Cdk1(T14A)-VFP or Cdk1(Y15F)-VFP would cause problems with cell proliferation or apoptosis, since these proteins were phosphorylated on Y15 and T14, respectively (Figure 2-1C). To test this idea we used *en-Gal4* to express the transgenes in the posterior compartment of wing discs. The discs were fixed and stained using antibodies against activated cleaved-Caspase3 to label apoptotic cells (Jackman et al., 2002) and antibodies against phosphorylated Histone-H3 on S10 (PH3) to mark mitotic cells (Hendzel et al., 1997; Brodsky et al., 2000). Few apoptotic cells were observed in discs expressing Cdk1WT-VFP or Cdk1(T14A)-VFP (Figure 2-5A and 2-5E, B and F, N = 10). In contrast, many apoptotic cells were observed specifically in the posterior regions of discs expressing Cdk1(Y15F)-VFP (Figure 2-5C and 2-5G, N = 10) or Cdk1(T14A,Y15F)-VFP (Figure 2-5D and 2-5H, N = 10). The discs expressing Cdk1(T14A,Y15F)-VFP always appeared smaller than normal and abnormally shaped, as shown. These

results showed that inability to phosphorylate Cdk1 on Y15 resulted in increase apoptosis in some cells, even though this did not cause morphological defects in adult wing development (Figure 2-4C).

Wing discs expressing Cdk1WT-VFP (Figure 2-5I and 2-5M, N= 15) or Cdk1(T14A)-VFP (Figure 2-5J and 2-5N, N= 15) had similar numbers of PH3-positive mitotic cells in each compartment. Discs expressing Cdk1(Y15F)-VFP (Figure 2-5K and 2-5O, N = 15) or Cdk1(T14A,Y15F)-VFP (Figure 2-5L and 2-5P, N= 9) had markedly more PH3-labeled cells in the posterior compartment, however. We quantified this phenotype by using de-convolved images to count PH3-labeled cells in the posterior (VFP-positive) and anterior (PH3-negative) compartments of these wing discs (N = 7, per genotype) to obtain a (P/A) mitotic index ratio. The data is shown as a box plot in Figure 2-6, indicating significant differences between the different genotypes. Thus, expression of both Cdk1 transgenes that could not be phosphorylated on Y15 resulted in a higher than normal mitotic index and ectopic apoptosis, even though their impact on adult morphological development was very different.

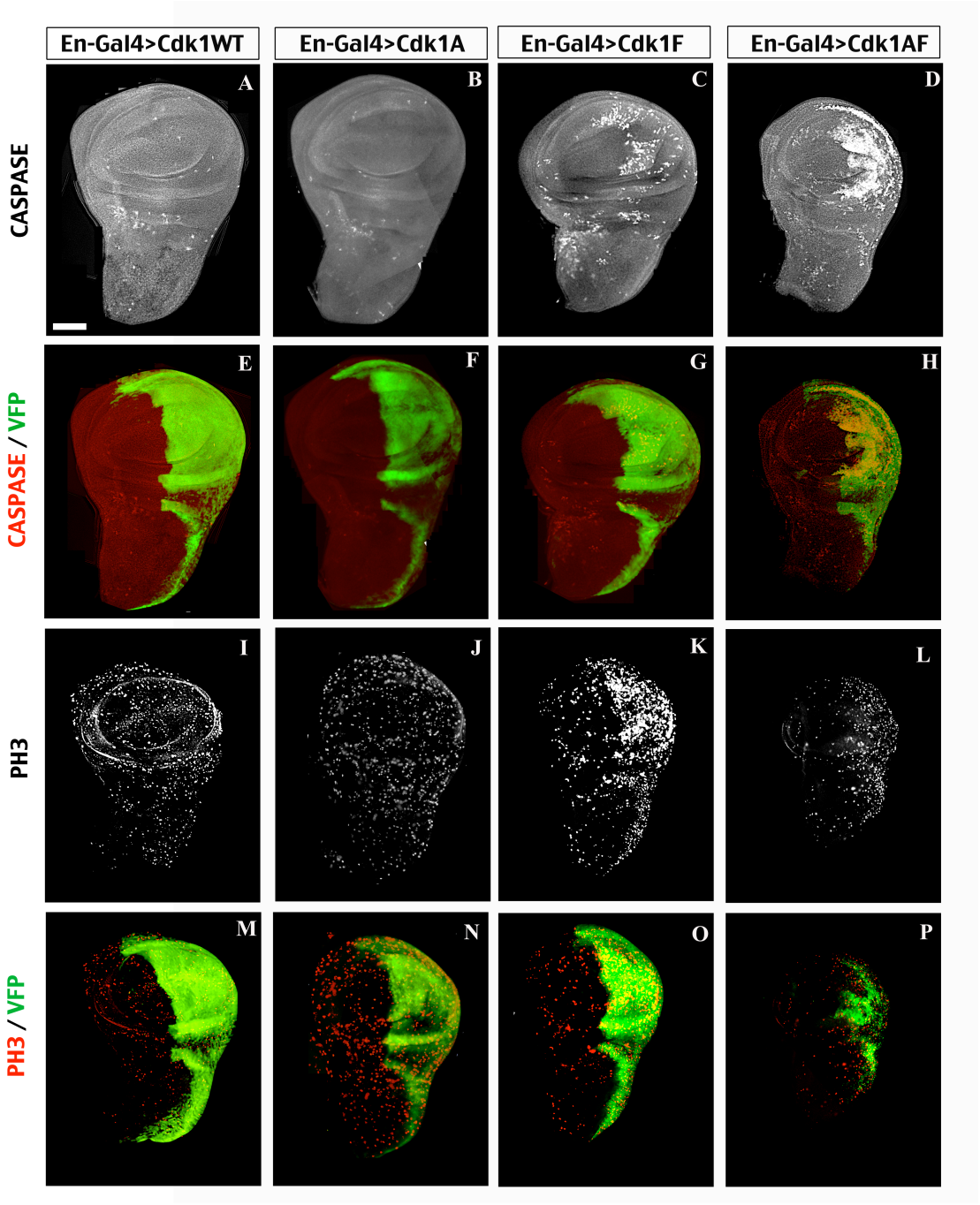

Figure 2-5: Expression of Cdk1(Y15F) and Cdk1(T14A,Y15F) caused increased apoptosis and mitotic index in late third instar larval wing discs

Figure 2-5 Legend:

VFP-tagged Cdk1 variants (Green label, in E-H and M-P) were expressed with *En-Gal4* in the posterior compartment and the discs were labeled with antibodies against activated Caspase-3 (Red in E, F, G and H) as an apoptosis marker or anti-phosphohistone H3(S10) (PH3; Red in M, N, O and P) as a mitotic marker. The VFP-negative anterior compartment serves as internal control. Panels A, B, E and F show that few apoptotic cells were observed in expressing Cdk1(WT) or Cdk1(T14A) discs (N =10, each genotype). Panels C, D, G and H show that expression of Cdk1(Y15F) or Cdk1(T14A,Y15F) resulted in elevated numbers of apoptotic cells in the posterior region of wing discs (N= 10). Panels I, J, M and N showed no noticeable differences in PH3-labeled cells from expression of Cdk1(WT) or Cdk1(T14A) in wing discs (N= 15, each genotype). Panels K, L, O and P show that more PH3-labeled cells were observed in posterior regions of Cdk1(Y15F) and Cdk1(T14A,Y15F)-expressing discs (N= 9, each genotype). Wing discs expressing Cdk1(T14A,Y15F) were always smaller and morphologically abnormal relative to the other genotypes, as shown. The scale bar in panel A represents 50 μm.

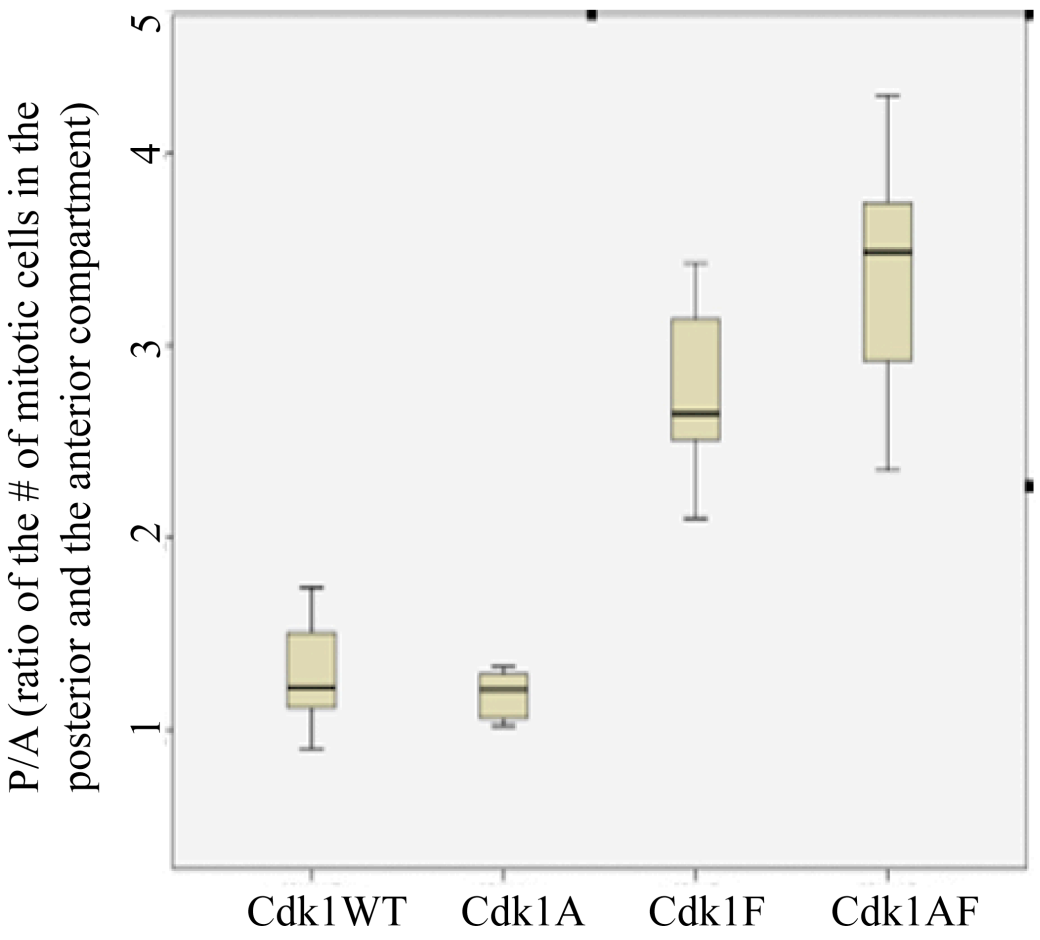

Figure 2-6: Quantitation of mitotic index in third instar wing discs expressing Cdk1-VFP transgenes in the posterior compartment under en-Gal4 control

PH3-labeled cells were counted in the posterior and anterior compartments of discs (N=7, per genotype) using deconvolved images to obtain a (P/A) mitotic index ratio, shown as box plots representative of the wing discs shown in Fig. 4 (panels I-L). Compared to Cdk1WT-VFP or Cdk1(T14A)-VFP, wing discs expressing either Cdk1(Y15F)-VFP or Cdk1(T14A,Y15F)-VFP had a significantly higher P/A ratio.

Developmental G2 phase arrest at the wing margin requires Y15 inhibitory phosphorylation

Cdk1 is activated by $Cdc25^{stg}$-catalyzed removal of dual inhibitory phosphorylation, a mechanism that is essential for coordinating mitosis with dynamic processes of *Drosophila* development (Edgar and O'Farrell, 1990; Fichelson and Gho, 2004). To assess how Y15 inhibitory phosphorylation of Cdk1 affected this developmental checkpoint mechanism, we examined cells of the presumptive dorsal-ventral wing margin in late third larval wing discs (O'Brochta and Bryant, 1985). This zone of non-proliferating cells (ZNC) consists of a central row of G1-arrested cells flanked by G2-arrested cells (Johnston and Edgar, 1998). We first examined whether the wing-margin patterning genes Cut and Wingless (Wg) were affected by *sd-Gal4*-driven Cdk1 variants (Jack et al., 1991; Williams et al., 1994). Cdk1WT-VFP-expressing discs showed normal Cut expression at the presumptive wing margin (Figure 2-7A and 2-7A', N = 10), as did discs expressing either Cdk1(T14A)-VFP (Figure 2-7B and 2-7B', N=10) or Cdk1(Y15F)-VFP (Figure 2-7C and 2-7C', N= 10). In contrast, discs expressing Cdk1(T14A,Y15F)-VFP showed reduced Cut labeling (Figure 2-7D and 2-7D', N= 10) and the discs were consistently smaller and abnormally shaped, as noted earlier with *en-Gal4* (Figure 2-5). Similar results were obtained from analyzing Wg expression, which was only disrupted by Cdk1(T14A,Y15F)-VFP expression (Figure 2-8). Although expression of Cdk1(Y15F)-VFP caused ectopic apoptosis and cell proliferation defects it did not disrupt adult wing development (unless cyclin B levels were raised), unlike Cdk1(T14A,Y15F)-VFP. These results,

therefore, provided further evidence that phosphorylation on the T14 residue partially inhibited Cdk1 activity, *in vivo*.

To specifically examine the effect of Cdk1(Y15F) on G2 phase arrest, we analyzed sensory organ precursor (SOP) cells of the ZNC using *neur*^{p72}, a P transposon Gal4 insertion allele to express the transgenes (Fichelson and Gho, 2004). VFP-positive SOP cells expressing either Cdk1(WT)-VFP (Figure 2-7E and 2-7E', N = 8) or Cdk1(T14A)-VFP (Figure 2-7F and 2-7F', N = 8) were not labeled by PH3 antibodies used to detect mitotic cells, consistent with expectations for G2 phase-arrested cells. In contrast, some of the SOP cells expressing Cdk1(Y15F)-VFP (Figure 2-7G and 2-7G', N = 8) or Cdk1(T14A,Y15F)-VFP (Figure 2-7H and 2-7H', N = 7) were PH3-positive, indicating that these cells had entered mitosis prematurely. We concluded from these results that Y15 phosphorylation of Cdk1 was specifically required for the developmental G2 phase arrest mechanism of wing margin SOP cells. In spite of Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F)-VFP appearing equally effective at bypassing the G2/M checkpoint, only Cdk1(T14A,Y15F)-VFP caused adult wing defects, implying that non-inhibitable Cdk1 expression was causing additional defects accounting for this discrepancy.

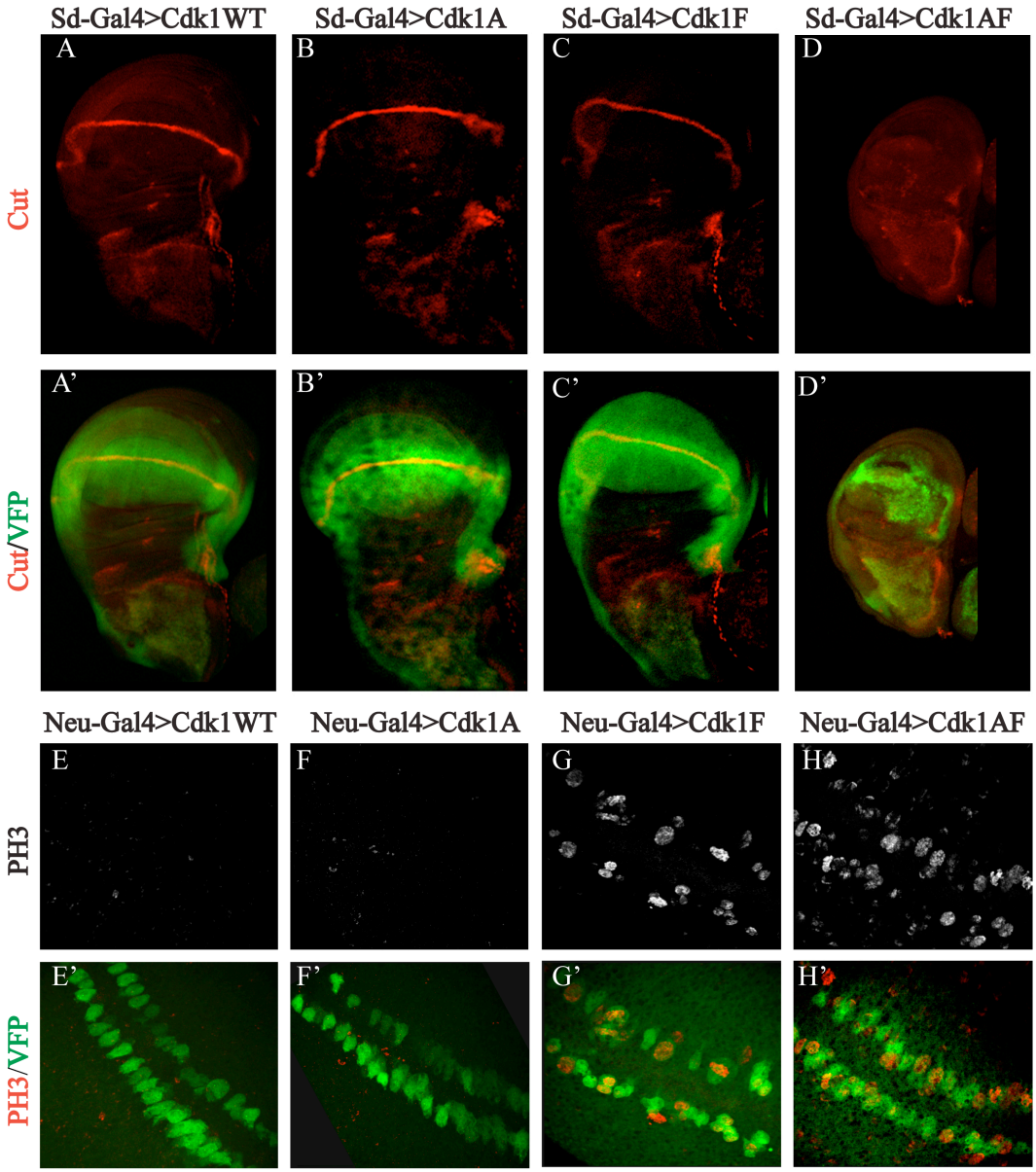

Figure 2-7: Wing margin specification and developmental regulation of G2 phase arrest are differentially affected by expression of Cdk1 transgenes during wing development with *sd-Gal4*.

Figure 2-7 Legend

At least ten labeled discs were examined for each genotype. Panels A, B and C shows wing discs from third instar larvae labeled with anti-Cut antibodies (Red). Cut expression at the presumptive wing margin was unaffected by expression of Cdk1(WT), Cdk1(T14A) or Cdk1(Y15F), respectively. Panel D shows that expression of Cdk1(T14A,Y15F) disrupted Cut expression. Panels A' to D' show that VFP-tagged transgenes were expressed throughout the wing pouch of each disc. Panels E to H show shows wing discs from third instar larvae expressing transgenes expressed in SOP cells (a subset of G2 phase-arrested ZNC cells) with *neur*^{p72}*-Gal4* that were labeled with anti-PH3(S10) antibodies (Red). Panels E and F show that SOP cells expressing Cdk1(WT)-VFP and Cdk1(T14A) were PH3-negative. Panels G and H show that SOP cells expressing Cdk1(Y15F)-VFP and Cdk1(T14A,Y15F) were a mixture of smaller, mitotic (PH3-positive) and non-mitotic cells. Panels E' to H' showing that the VFP-tagged transgenes were expressed in SOP cells.

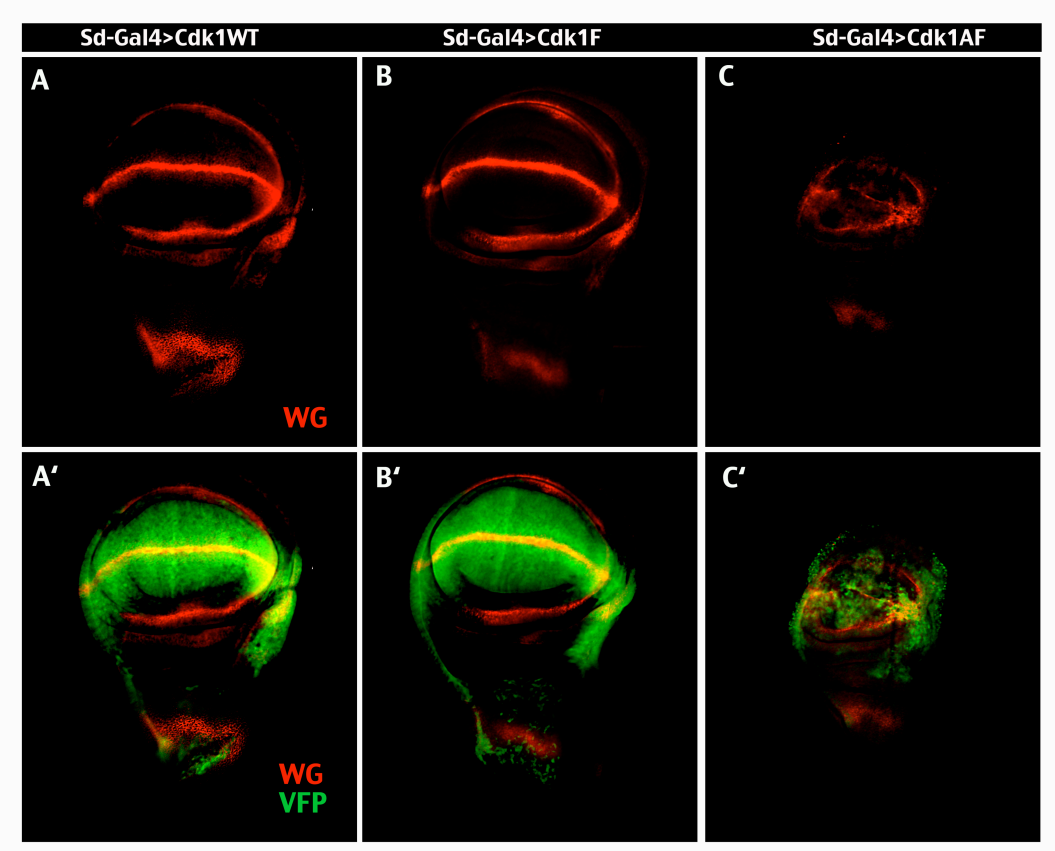

Figure 2-8: Expression of Cdk1-VFP in the wing pouch using *sd-Gal4* and effects on Wingless (Wg) expression at the presumptive wing margin

Wing discs from third instar larvae were immuno-labeled with Wingless antibodies (Red). At least 10 discs were examined for each genotype. Panels A, A' and B, B' show that Wg expression pattern was unaffected in wing discs expressing Cdk1WT and Cdk1(Y15F). Panel C, C' shows that Wg expression was disrupted in wing discs expressing Cdk1(T14A,Y15F).

The DNA Damage Checkpoint Response Depends on Y15 Phosphorylation of Cdk1

Exposure to ionizing radiation causes DNA damage, eliciting a Cdk1 inhibitory phosphorylation checkpoint response that provides interphase cells with time for DNA repair before mitosis begins. In *S. pombe*, the DNA damage checkpoint causes phosphorylation of Cdk1 on Y15 (O'Connell et al., 1997; Rhind et al., 1997). Both T14 and Y15 residues of Cdk1 are phosphorylated in response to DNA damage in metazoans, however (Blasina et al., 1997). Myt1 is required for normal DNA damage checkpoint responses in *Drosophila* wing discs, however it is not known if dual phosphorylation or specifically T14 or Y15 inhibitory phosphorylation of Cdk1 that is necessary (Jin et al., 2008). We addressed this question by expressing transgenic Cdk1 in the posterior compartment of wing discs using *en-Gal4* to examine DNA damage checkpoint responses caused by exposure to 40Gy of ionizing radiation (Brodsky et al., 2000). One hour after irradiation, the wing discs were dissected, fixed and immuno-labeled with PH3 antibodies. Mitotic (PH3-positive) cells were not observed in either the anterior or posterior (VFP-positive) compartments of irradiated discs expressing Cdk1WT-VFP (Figure 2-9A and 2-9A', N = 10) or Cdk1(T14A)-VFP (Figure 2-9B and 2-9B', N = 10), demonstrating a functional checkpoint response. In contrast, there were many PH3-positive cells in the posterior compartment of discs from irradiated Cdk1(Y15F)-VFP (Figure 2-9C and 2-9C', N = 10) or Cdk1(T14A,Y15F)-VFP larvae (Figure 2-9D and 2-9D', N

= 10). Thus, phosphorylation of Cdk1 on Y15 is also necessary for pre-mitotic checkpoint responses to DNA damage in late third instar wing discs.

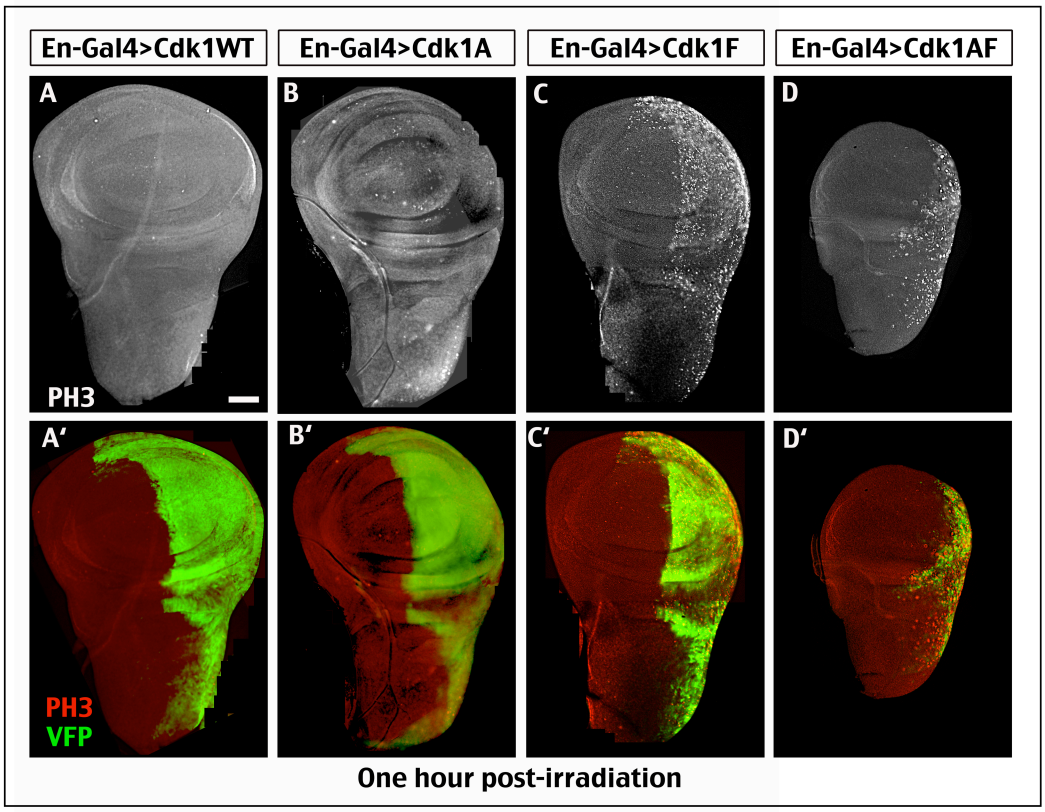

Figure 2-9: Assay for DNA damage-induced G2/M checkpoint responses in wing discs expressing Cdk1 transgenes.

VFP-tagged Cdk1 transgene variants (Green) were expressed with *En-Gal4* in the posterior compartment of each wing discs. Wing discs were dissected from late third instar larvae 60 min after exposure to 40 Gy of ionizing radiation and labeled for PH3 (White in A-D, Red in A'-D') to mark mitotic cells. At least 10 labeled discs were examined for each genotype. Panels A, A' and B, B' show no PH3-positive cells in either compartment of wing discs expressing Cdk1(WT) or Cdk1(T14A), indicating a functional pre-mitotic checkpoint response. Panels C, C' and D, D' show wing discs expressing Cdk1(Y15F) or Cdk1(T14A,Y15F), where PH3 labeling in the posterior compartment indicates a G2/M checkpoint defect. The scale bar in panel A represents 50 μm.

Inhibitory Phosphorylation of Cdk1 on T14 Prevents Chromosome Defects and Mitotic Delays

We wondered if the more severe developmental outcomes caused by expression of Cdk1 (T14AY15F) could be caused by defects in mitotic progression, as reported for HeLa cells (Santos et al., 2012). To address this possibility we used *Prospero-Gal4* to express each transgene in type I larval brain neuroblasts, which are large neural stem cells with well-characterized metaphase checkpoint mechanisms (Basto et al., 2000; Donaldson et al., 2001; Royou et al., 2005). First, squashed neuroblast preparations were treated with colchicine, a microtubule poison used to arrest cells in mitosis, and stained for DNA to quantify the mitotic index (Figure 2-10A). In neuroblasts expressing Cdk1WT-VFP or Cdk1(T14A)-VFP we observed 10-20 mitotic cells per field. Cdk1(Y15F)-VFP expression resulted in a much higher mitotic index, ranging from 50-80 mitotic cells per field. In contrast, neuroblasts expressing Cdk1(T14A,Y15F)-VFP had a much lower mitotic index, ranging from 5-10 mitotic cells per field. Thus, expression of different Cdk1 phospho-isoforms in larval neuroblasts had distinct effects on the mitotic index that could be caused by differences in rates of cell proliferation or mitotic progression.

A conserved metaphase checkpoint mechanism that monitors chromosome integrity and spindle attachment prior to anaphase can be assayed by treating neuroblasts with colchicine (Hardwick et al., 1996). In normal cells this results in a metaphase-like arrest with paired sister chromatids (Gonzalez et al., 1988; Gatti and Baker, 1989). We also observed that neuroblasts expressing Cdk1(WT)-VFP

or Cdk1(T14A)-VFP (Figure 2-10B and 2-10C) had paired sister chromatids in 99.5 % of the karyotypes, demonstrating a functional metaphase checkpoint response. The majority of neuroblasts expressing Cdk1(Y15F)-VFP showed similar results (89.5% with paired sister chromatids; Figure 2-10D), however in a small percentage of these cells (9.5%, see Figure 2-10E), sister chromatids appeared to have lost cohesion.

In contrast, we frequently observed gross chromosomal aberrations in colchicine-treated neuroblasts expressing Cdk1(T14A,Y15F)-VFP. The defects included polyploidy (4%, Figure 2-10F), abnormally thin, entangled chromosomes (5%, Figure 2-10G) and chromosome breaks (35%, Figure 2-10H). Such chromosomal aberrations are associated with genome instability and apoptosis, possibly explaining the developmental defects associated with expression of non-inhibitable Cdk1.

The elevated mitotic index associated with Cdk1(Y15F)-VFP (Figure 2-10A) could reflect delays in mitotic progression that resulted in an accumulation of mitotic cells. To test this idea we used *Prospero-Gal4* to co-express each of the Cdk1 variants and *Tubulin-RFP* to visualize mitosis in live neuroblasts, defined as the interval between the appearance of centrosomes at opposite poles and the completion of cytokinesis. In Cdk1(WT)-VFP-expressing neuroblasts, mitosis took approximately 12.6 min ± 1.2 min (N = 5, Figure 2-11A and 2-11E and Movie 2-1). Neuroblasts expressing Cdk1(T14A)-VFP- or Cdk1(Y15F)-VFP showed similar results, with mitosis lasting 13.5 min ± 1.3 min (N = 5, Figure 2-11B and 2-11E; Movie 2-2) and 13.0 min ± 0.7 min, respectively (N = 5, Figure

2-11C and 2-11E; Movie 2-3). These data therefore excluded the possibility that Cdk1(Y15F)-VFP expression caused delays in M-phase. Instead, the elevated mitotic index associated with Cdk1(Y15F)-VFP expression more likely reflects a faster cell cycle caused by dominant bypass of developmentally regulated G2 phase, as we saw with the SOP cells of the presumptive wing margin (Figure 2-7).

In contrast, mitosis took significantly longer than normal in Cdk1(T14A,Y15F)-VFP-expressing neuroblasts (28.5min ± 2.3 min, N= 3; Figure 2-11D and 2-11E), and delays in bipolar spindle assembly were observable (Movie 2-4). Collectively, these data showing mitotic defects and chromosome aberrations only when Cdk1(T14A,Y15F)-VFP was expressed provided further evidence that inhibitory phosphorylation of T14 significantly affected Cdk1 activity *in vivo*, even though this regulatory mechanism is not essential for the G2/M checkpoint functions that we assayed. These results imply that Myt1 regulation of Cdk1 by T14 phosphorylation also functions outside of the known role of Myt1 in G2 phase. This is the first report we are aware of describing such a function for Myt1 kinases, underscoring the importance of investigating molecular mechanisms of cell cycle control in a relevant physiological context.

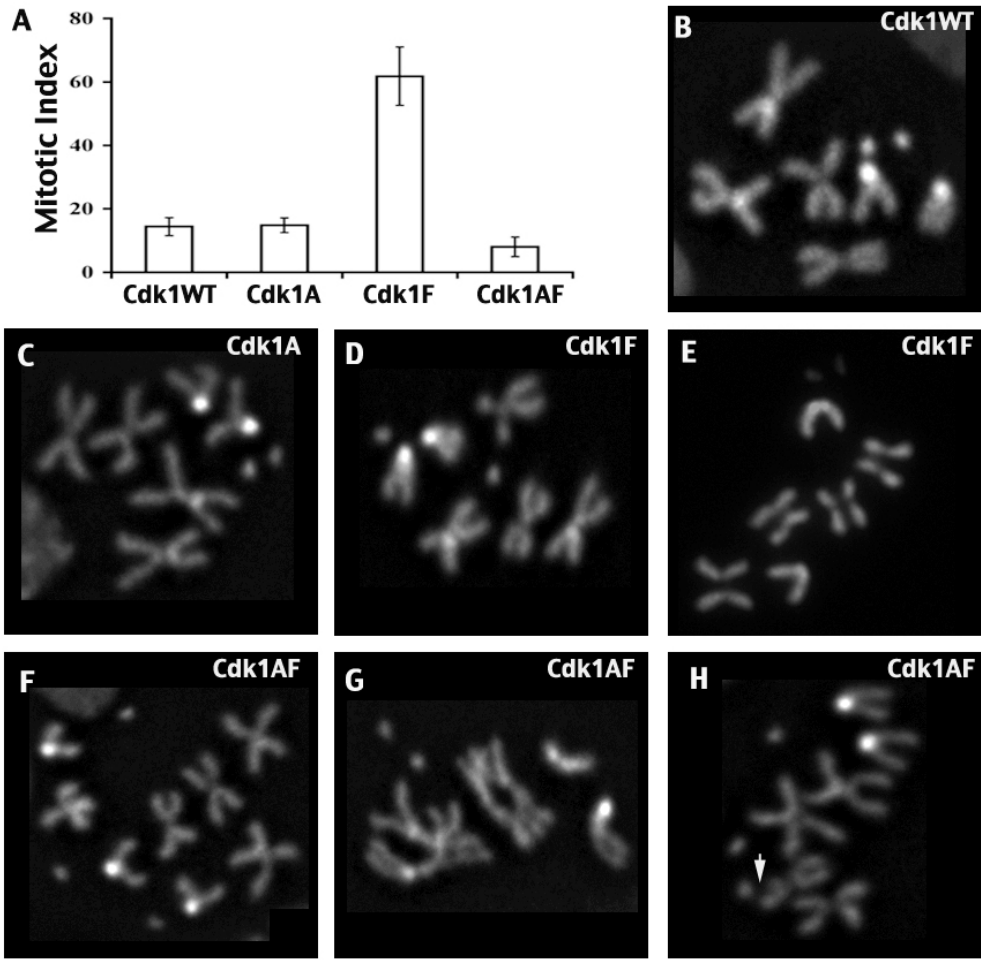

Figure 2-10: Mitotic index and karyotype defects in Type 1 larval neuroblasts expressing Cdk1 transgenes under control of *prospero-Gal4*.

Metaphase karyotypes of colchicine-treated brain squashes were labeled with Hoechst 33258 to identify mitotic chromosomes. At least 800 interpretable karyotypes were examined for each genotype. (A) Bar chart showing mitotic index associated with each Cdk1 transgene. Panels B and C of neuroblasts expressing Cdk1(WT) or Cdk1(T14A) show that chromosomes arrest in metaphase with cohered sister chromatids. (D) Approximately 90% of Cdk1(Y15F)-expressing mitotic neuroblasts also arrest in metaphase with cohered sister chromatids, however defects in sister chromatid cohesion were evident in 9.5% of the karyotypes (E, N=900). Approximately 45% of Cdk1(T14A,Y15F)-expressing neuroblasts exhibited chromosomal aberrations (N=850), including polyploidy (G), thin, poorly condensed chromosomes (H) or chromosome breaks, arrow (I).

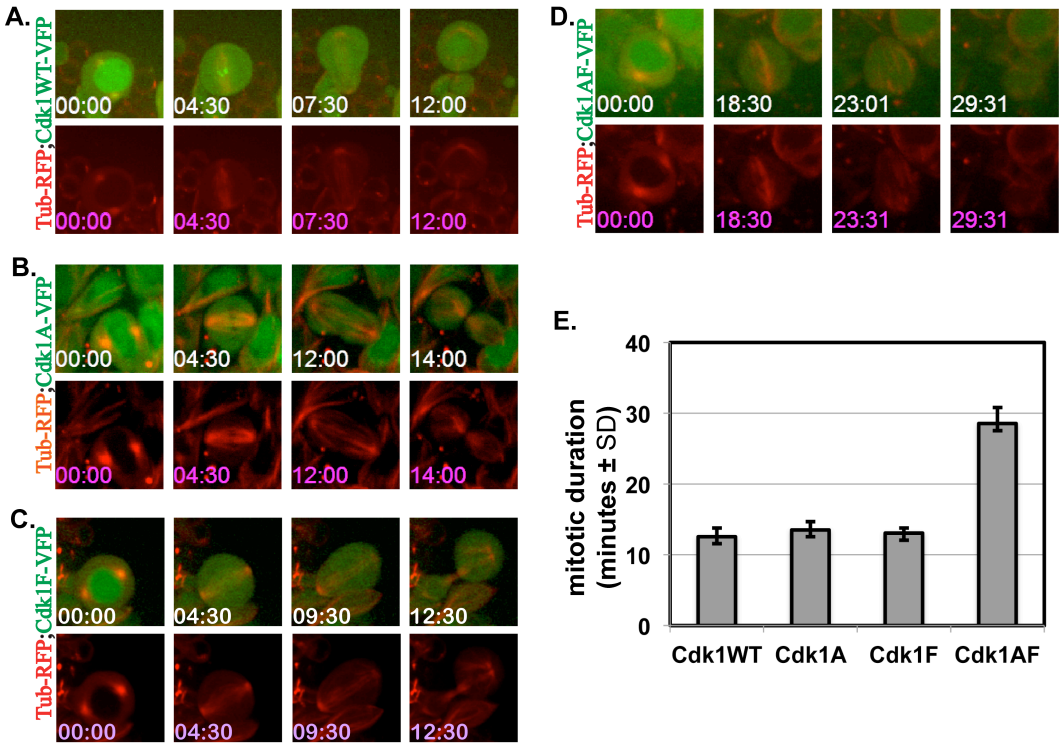

Figure 2-11: Live analysis of mitosis in larval neuroblasts expressing Cdk1 transgenes under control of prospero-Gal4.

Still images representing four different points from each of the movies in Movies S1 to S4 are shown. Top panels show merged images of the respective Cdk1-VFP (Green) and Tubulin (Red) fluorescent reporters, bottom panels show only Tubulin (Red). In panels A to D the zero time point marks the appearance of centrosomes at opposite poles, the second panel shows timing of bipolar spindle formation, while the third and fourth panels represent the beginning and end of cytokinesis, respectively. (A) Neuroblast expressing Cdk1WT-VFP, where mitosis took 12.56 min ± 1.2 min (Movie S1). (B) Neuroblast expressing Cdk1(T14A), where mitosis took 13.52 min ± 1.26 min (Movie S2). (C) Neuroblast expressing Cdk1(Y15F), where mitosis took 13.0 min ± 0.7 min (Movie S3). (D) Neuroblasts expressing Cdk1(T14A,Y15F), where mitosis lasted 28.54 min ± 2.25 min, (Movie S4). (E) Quantification of the duration of mitosis in neuroblasts expressing Cdk1WT-VFP (N= 5), Cdk1(T14A)-VFP (N= 5), Cdk1(Y15F)-VFP (N= 5) and Cdk1(T14A,Y15F)-VFP (N= 3). The bar graph depicts the mean duration of mitosis ± SD for each genotype.

Movie 2-1 Live analysis of mitotic timing in neuroblasts expressing *Cdk1(WT)-VFP* and *Tubulin-RFP*. The neuroblast spends about 12.6 min ± 1.2 min in mitosis. (Refer to the attached DVD)

Movie 2-2: Live analysis of mitotic timing in neuroblasts expressing *Cdk1(T14A)-VFP* and *Tubulin-RFP*. The movie shows similar mitotic timing with neuroblast expressing Cdk1WT-VFP. (Refer to the attached DVD)

Movie 2-3: Live analysis of mitotic timing in neuroblasts expressing *Cdk1(Y15F)-VFP* and *Tubulin-RFP*. The movie shows similar mitotic timing with neuroblast expressing Cdk1WT-VFP. (Refer to the attached DVD)

Movie 2-4: Live analysis of mitotic timing in neuroblasts expressing *Cdk1(T14A,Y15F)-VFP* and *Tubulin-RFP*. The neuroblast shows delayed spindle assembly and extended mitotic timing. (Refer to the attached DVD)

2.4 DISCUSSION

In this study we examined how Cdk1 dual inhibitory phosphorylation on T14 and Y15 residues affected *Drosophila* imaginal wing disc cells and larval neuroblasts, seeking to understand how biochemical differences in this regulatory mechanism relate to specialized developmental functions of the Wee1 and Myt1 kinases. Phenotypic analysis of Cdk1(Y15F)-VFP and non-inhibitable Cdk1(T14AY15F)-VFP expression showed that phosphorylation of Cdk1 on Y15 was necessary for both developmental and radiation-induced G2/M checkpoint arrest. The elevated mitotic index and apoptosis caused by Cdk1(Y15F)-VFP expression closely resembled mutant phenotypes of *myt1* mutant wing discs, confirming biochemical evidence that Myt1 is the predominant Y15-directed kinase at this stage of development (Jin et al., 2008). Our conclusion that Y15 phosphorylation of Cdk1 is essential for G2/M checkpoint arrest is also consistent with Wee1 and Myt1 kinases being functionally redundant for essential cell functions during post-embryonic development (Jin et al., 2008).

Having established the importance of phosphorylating Cdk1 on Y15, do we have evidence that this regulatory mechanism is also sufficient for G2 phase arrest? Ubiquitous, high level expression of Cdk1(T14A)-VFP rescued $cdc2^{ts}$ mutant lethality, indicating that regulation of Cdk1 by phosphorylation of Y15 alone was compatible with development, at least under these circumstances. However, the intriguing observation that T161 activating phosphorylation of Cdk1(T14A)-VFP was compromised by Y15 phosphorylation alone (Figure 2-1) suggests this mechanism may not necessarily be compatible with stable G2 phase

arrest. Indeed, Myt1 activity is required in developmentally arrested G2 phase cells in *Drosophila* and other experimental systems (Karaiskou et al., 2004; Jin et al., 2005; Burrows et al., 2006), suggesting this dual phosphorylation mechanism is important for accumulating inhibited Cdk1/CyclinB complexes during G2 phase that can be rapidly activated once the G2/M transition begins (Coulonval et al., 2011).

Our study also provides insight into another role for T14 inhibitory phosphorylation of Cdk1 by Myt1 kinases. Although Cdk1(Y15F) and non-phosphorylatable Cdk1(T14AY15F) both overcome G2/M checkpoint arrest we only observed profound developmental and mitotic defects with Cdk1(T14AY15F) expression. This observation suggests that Myt1 regulation of Cdk1 activity by T14 phosphorylation can also function at another stage of the cell cycle to promote cell survival. What molecular mechanism could explain this proposed role for Myt1? Recent studies of cultured mammalian cells identified mis-regulation of APC/C causing defects in G1 phase (Ma et al., 2012) or defects in mitotic exit (Chow et al., 2011) as major problems associated with expression of non-inhibitable Cdk1(T14AY15F). Moreover, Myt1 regulation of Cdk1 is important for mitotic checkpoint mechanisms that regulate Golgi structural dynamics (Nakajima et al., 2008; Villeneuve et al., 2013). Although we cannot exclude any of these mechanisms as possible contributing factors, a more likely explanation for our results is that inappropriate Cdk1(T14AY15F) activity during S phase causes DNA replication defects that lead to genome instability and mitotic catastrophe (Heald et al., 1993). This idea that partial inhibition of Cdk1

activity by T14 phosphorylation contributes to an S/M checkpoint mechanism is also consistent with evidence that the levels of Cdk activity required for triggering S phase are less than those required to activate mitosis (Coudreuse and Nurse, 2010; Thomson et al., 2010). Further studies are needed to distinguish these alternative explanations and to define exactly how Myt1 regulation of Cdk1 via T14 phosphorylation prevents chromosome instability and mitotic defects.

In summary, we have presented genetic and biochemical evidence that T14 and Y15 inhibitory phosphorylation are functionally distinct mechanisms for regulating Cdk1 activity in the context of *Drosophila* wing, eye and neuroblast development. Phosphorylation of Cdk1 on Y15 (primarily by Myt1 at these stages of development) was essential for G2 phase checkpoint arrest, whereas T14 phosphorylation of Cdk1 was sufficient for maintaining chromosome stability when Y15 phosphorylation was compromised. We therefore propose that Myt1 functions in an S phase checkpoint mechanism that prevents premature mitosis from interfering with DNA replication in wing imaginal discs, explaining the cellular defects observed in *myt1* mutants at this stage of development (Jin *et al*., 2008). Intriguingly, we also found evidence consistent with the proposal that phosphorylation on Y15 alone can de-stabilize Cdk1-Cyclin B complexes and compromise Cdk1 activation by T161 phosphorylation (Coulonval et al., 2011). In this light, Myt1-mediated dual phosphorylation of Cdk1 may have evolved in metazoans to allow cells to arrest in G2 phase for prolonged periods, while remaining competent to enter mitosis without delay when triggered by Cdc25 de-phosphorylation.

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3. Mis-regulation of Cdk1 Inhibitory Phosphorylation Disrupts Neuronal Cell Fate Specification during Sensory Organ Development

3.1 INTRODUCTION

Neural precursor cells undergo transient mitotic inactivity during neural development but are poised to re-enter the cell division cycle to generate the specialized cells that constitute the adult nervous system (Liu et al., 2007; Liu et al., 2009; Furutachi et al., 2013). Maintaining neural precursor cells in this developmentally regulated quiescent state is fundamental to neurogenesis in metazoans, yet it is a less mechanistically explored process in the field of cell cycle regulation, where studies have focused mostly on experimental systems exhibiting unbridled proliferation (Humbert et al., 2003; Bilder, 2004; O'Farrell, 2011). Cellular quiescence is a common phenomenon in invertebrate and mammalian nervous system development (Tsuji et al., 2008), and quiescent cells are capable of adopting specialized fate and can differ dramatically (O'Farrell, 2011). Disruptions in the quiescence state have serious implications for neurodegenerative diseases and cancer formation (Egger et al., 2008; O'Farrell, 2011).

A well-studied example of a neural precursor cell that undergoes cell cycle quiescence is the *Drosophila* thoracic sensory organ precursors (SOPs) (Hartenstein and Posakony, 1989; Hartenstein and Posakony, 1990b; Gho et al., 1999; Furman and Bukharina, 2008) . These cells are selected from the clusters of proneural cells that are arrested in G2-phase of the cell cycle (Usui and Kimura, 1992; Kimura et al., 1997). After a period of quiescence, SOPs divide asymmetrically to produce two distinct and highly specialized daughter cells, pIIa and pIIb cells; the pIIb adopts a neuronal fate, while the pIIa maintains a

structural fate (Bodmer et al., 1989; Hartenstein and Posakony, 1989; Gho et al., 1999; Reddy and Rodrigues, 1999a; Fichelson and Gho, 2003). How the timing of mitotic entry in SOPs is developmentally synchronized with the adoption of a specialized cell fate remains unclear.

The molecular mechanism linking SOP cell division timing with cell fate specification is known to involve the master mitotic regulatory kinase, cyclin dependent kinase 1 (Cdk1) (Tio et al., 2001; Fichelson and Gho, 2004; O'Farrell and Kylsten, 2008). In particular, lowering Cdk1 activity by overexpressing Wee1, Myt1 or Tribbles during SOP division uncoupled cell division and cell fate determination by allowing undivided SOPs to adopt the fate of its pIIb daughter, thereby producing only the internal neuron and sheath cells (Fichelson and Gho, 2004). Conversely, ectopic activation of Cdk1 via dephosphorylation by misexpression of String (Stg) resulted mostly in the duplication and sometimes loss of external sensory (ES) organs (O'Farrell and Kylsten, 2008). Congruently, loss of function of myt1 resulted in extensive sensory bristle defects that ranged from missing shaft and duplicated socket to complete loss of ES organs (Jin et al., 2008). These results demonstrate that even though cell division and cell fate specification could be uncoupled, the strict temporal regulation of Cdk1 activity is intricately intertwined with the process of cell fate specification.

Although, the sensory bristle phenotypes caused by loss of *myt1* function or $Cdc25^{Stg}$ misexpression suggest that proper regulation of G2 phase quiescence via Cdk1 inhibitory phosphorylation could be important in maintaining the fidelity of the process of *Drosophila* sensory organ (SO) development (Negre et

al., 2003; Jin et al., 2008; O'Farrell and Kylsten, 2008), it does not rule out the fact that Myt1 and String could regulate neural development via mechanism unrelated to Cdk1 activation. Indeed, misexpression of String was shown to interfere with the process of lateral inhibition during SOP selection via downregulation of proneural gene expression (Negre et al., 2003). Hence, how short-circuiting G2-phase arrest via precocious activation of Cdk1 activity encroaches upon the process of sensory organ development remains an open question.

Ectopic activation of Cdk1 could precociously relieve SOP of its G2 quiescence state not allowing sufficient time in G2 phase to build-up or segregate cell-fate determinants, thereby disrupting cellular asymmetry (O'Farrell and Kylsten, 2008). Consistent with this idea, shortening of G2 phase timing could encroach on the molecular timer regulating how many times a precursor cell divides before terminally differentiating. This could force each SOP cell and/or its daughter cells to undergo multiple cell divisions. Alternatively, the ectopic Cdk1 activity may impinge on the temporality of the neural cell division thereby changing the developmental cues required for cell fate specification. The duplication of bristle and socket cells associated with ectopic activity of String will also be consistent with an hypothesis that ectopic Cdk1 activity promotes cell fate transformation of pIIb to pIIa (O'Farrell and Kylsten, 2008; O'Farrell, 2008). Additionally, the ectopic Cdk1 activity could reprogram the SOP such that precursor cells acquire a stem cell-like self-renewal potential rather than their

default differentiative ability. Of yet, there is no direct genetic or molecular evidence to support any of the stated hypotheses.

To understand the importance of Cdk1 inhibitory phosphorylation during the quiescence/proliferation transition in thoracic SOPs and how perturbation of the G2-phase quiescence impacts *Drosophila* sensory organ development, we expressed fluorescently tagged Cdk1 mutant, Cdk1(Y15F)-VFP, whose activity is sufficient to promote premature mitosis without causing genomic instability. We present evidence that short-circuiting G2-phase quiescence via ectopic Cdk1 activity induced self-renewal asymmetric division in sensory organ precursors (SOPs) rather than the default differentiative asymmetric cell division. This study provides a new insight into how temporal regulation of Cdk1 and the timing of G2-phase quiescence are linked with the developmentally regulated signal crucial for specifying neuronal cell fate during neurogenesis.

3.2 MATERIALS AND METHODS

Fly strains

The *UAS-Cdk1(WT)-VFP* and *UAS-Cdk1(Y15F)-VFP* transgenes were specifically expressed in sensory bristle lineage using *neur^{P72}Gal4* (Bloomington Stock Centre) or *w;neur^{P72}-Gal4,P*(UAS>mRFP1-Pon[LD])*[1.2]/TM3,Sb* (a kind gift from Knoblich lab) driver. To control the temporal expression of the transgenes, a *neur^{P72}Gal4* strain bearing Gal80^{ts} was used and fly culture were kept at 18°C for embryonic and larval development, and pupae were transferred at 18 h APF to 25°C to allow the expression of GAL4. Other sensory cell specific Gal4 lines used to express the transgenes are *elav-Gal4* (Bloomington Stock Centre) and *pros-Gal4* (a gift from Chris Doe).

Immunohistology

Dissected larvae or pupae were fixed in 4% paraformaldehyde (PFA) for 25 minutes and washed with 1X PBT (0.3% Triton X-100). The following primary antibodies were used: mouse anti-Cut (DSHB, 1:250); rat anti-ELAV (DSHB, 1:100); mouse anti-ELAV (DSHB, 1:100); mouse anti-Pros (DSHB, 1:5); rabbit anti-Su(H) (gift from Sarah Hughes, 1:200); rabbit anti-phospho-Histone H3 (Upstate, 1:4000); rabbit anti-Pon (a gift from Y.N. Jan (Lu et al., 1998b), 1:1000). Alexa 568- and 633-conjugated secondary antibodies were purchased from Molecular Probe and used at 1:1000 dilution.

Imaging live pupae.

Live imaging of bristle lineage in (neur-Gal4,UAS-Pon::RFP)*/+;UAS-Cdk1(WT)-VFP/+* and (neur-Gal4,UAS-Pon::RFP)*/+;UAS-Cdk1(Y15F)-VFP/+* pupa was performed as previously described (Zitserman and Roegiers, 2011). White pupae were collected and aged for 15 h at 25 °C before the pupae were prepared for mounting. Pupae were placed dorsal side up on a double-sided tape attached to a slide, then carefully dissected out of the pupa case. A frame of Whatman paper moistened with distilled water "dH_2O" was placed around the pupae and a ring of Silicon vacuum grease was used to support the cover slip and seal the chamber. A small drop of dH_2O was placed on the underside of the cover slip in the region that contacted the notum. Images were acquired every 5 minutes on a spinning disc confocal microscope (40× objective) driven by Metamorph. Temperature of the imaging chamber was maintained at 25°C.

3.3 RESULTS

Ectopic Cdk1 Activity Perturbs Sensory Bristle Formation in *Drosophila*

Drosophila sensory organ development involves a sequence of precisely timed asymmetric cell divisions that produce four clonally related terminally differentiated cells (shaft, socket, neuron, sheath) derived from a single sensory organ precursor SOP. Ectopic expression of $Cdc25^{String}$ interferes with the process of lateral inhibition during SOP selection (Negre et al., 2003), resulting in abnormal sensory organ formation. We sought to assess if the sensory organ phenotype was due to the ectopic Cdk1 activity, by expressing a mutant form of Cdk1 (Cdk1(Y15F)-VFP), whose activity is sufficient to promote premature mitosis without causing genomic instability, in *Drosophila* SOP cells.

We expressed Cdk1(WT)-VFP and Cdk1(Y15F)-VFP in the sensory organ precursor (SOP) cell using *neu-Gal4* to examine adult bristle phenotypes. Flies expressing Cdk1(WT)-VFP had macrochaetae and microchaetae with a single socket and a shaft in 100% of the flies examined (Figure 3-1A, Table 3-1 & 3-2). However, flies expressing Cdk1(Y15F)-VFP had frequent defects affecting both microchaetae and macrochaetae (Figure 3-1B). Sensory organs with more than one socket were observed in 63% of macrochaetae and 26.2%±4.2% of microchaetae examined and other types of defects were also frequently observed (Table 3-1 & 3-2). Macrochaetae defects were more pronounced than microchaetae defects, with total percentage of defective adult external sensory bristles being 86% and 45%, respectively. Next, we examined cells of the SO lineage using anti-Cut antibodies that label all sensory cells (Blochlinger et al.,

1990). At 24 hours after puparium formation (APF) there were 4 cells in each sensory cell cluster expressing Cdk1(WT)-VFP (Figure 3-1A' & A''). In contrast, sensory cell clusters expressing Cdk1(Y15F)-VFP at 24 hours APF had more than 4 cells (Figure 3-1B' & B''). These results show that expression of Cdk1(Y15F)-VFP in SOPs generated supernumerary SO cells, linking ectopic Cdk1 activity to sensory bristle defects in *Drosophila*.

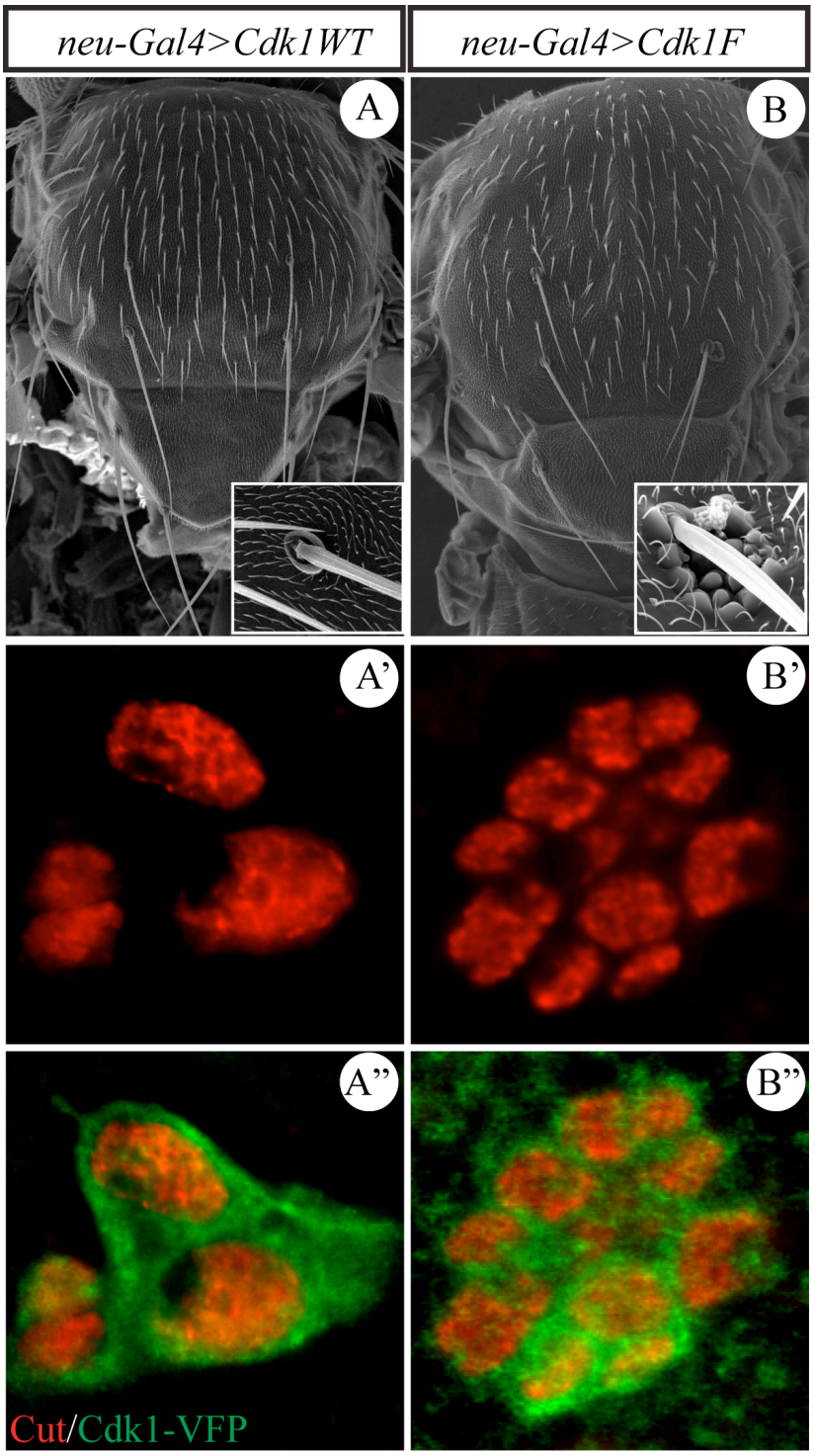

Figure 3-1: Effect of expressing non-inhibitable Cdk1 mutant on *Drosophila* sensory bristles development.

Figure 3-1 Legend:

Cdk1(WT)-VFP and Cdk1(Y15F)-VFP were each expressed in SOPs using *neur-Gal4* driver and examined adult flies as well as sensory cells for defects. The red label shows anti-Cut staining and the green represents transgenic protein expression. (A) Scanning electron micrograph showing normal bristles on adult thorax expressing Cdk1(WT)-VFP. (A' & A'') Indicate a Cut-labeled 4-cell cluster in SO lineage expressing Cdk1(WT)-VFP at 24 hours APF. (B) Defective sensory bristles on the adult thorax expressing Cdk1(Y15F)-VFP. (B' & B'') Show a Cut-labeled supernumerary-cell cluster in SO lineage expressing Cdk1(Y15F)-VFP at 24 hours APF.

Table 3-1: Percentage of macrochaetae defects in adults expressing neur-Gal4>Cdk1WT-VFP or neur-Gal4>Cdk1(Y15F)-GFP

	yw (*n*=80)	*Neu-Gal4/+; UAS-Cdk1WT/+* (*n*=80)	*Neu-Gal4/+; UAS-Cdk1F/+* (*n*=96)
1 socket/ 1 shaft	100%	100%	14%
>1 socket/ 1 shaft	0%	0%	63%
1 socket/ >1 shaft	0%	0%	6%
>1 socket/ >1 shaft	0%	0%	11%
>1 socket/ 0 shaft	0%	0%	6%

Table 3-2: Percentage of microchaetae defects in adults expressing neur-Gal4>Cdk1WT-VFP or neur-Gal4>Cdk1(Y15F)-GFP

	yw (*n*=7)	*Neu-Gal4/+; UAS-Cdk1WT/+* (*n*=9)	*Neu-Gal4/+; UAS-Cdk1F/+* (*n*=12)
# of short bristle/adult notum	229±19	224±11	198±9
1 socket/ 1 shaft	100%	100%	55±6.8%
>1 socket/ 1 shaft	0%	0%	26.2±4.2%
1 socket/ >1 shaft	0%	0%	10.8±4.7%
>1 socket/ >1 shaft	0%	0%	4.8±1.4%
>1 socket/ 0 shaft	0%	0%	2.6±1.6%

Supernumerary sensory cells are not produced at the expense of pIIb daughter cells.

The supernumerary socket and shaft cells in mutant SO lineages expressing Cdk1(Y15F)-VFP is similar to fate transformation of pIIb-to-pIIa associated with *lgl* mutants and *numb* loss-of-function as well as *Notch* gain-of-function phenotypes (Uemura et al., 1989; Rebay et al., 1993; Frise et al., 1996; Guo et al., 1996; Justice et al., 2003). To investigate whether the abnormal sensory bristles seen in Cdk1(Y15F)-VFP-expressing flies were due to a change in cell fate, we analyzed clusters with more than 1 socket cell by doubly-labeling sensory cells expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP with Cut antibodies (marking all of the cells) and Su(H) antibodies that specifically label socket cells (Gho et al., 1996). In presumptive microchaetae expressing Cdk1(WT)-VFP, we observed 4 Cut-positive cells, one of which was 1 Su(H)-positive cell at 24 hours APF (100% of the clusters, Figure 3-2A – 3-2A''). With Cdk1(Y15F)-VFP expression however, we noted normal clusters (45% of the clusters counted, Figure 3-2B – 3-2B'') as well as clusters with 5 or 6 Cut-positive cells and 2 Su(H)-positive socket cells (31% of the clusters counted, Figure 3-2C – 3-2C'') and clusters of 6 Cut-positive cells with 3 were Su(H)-positive socket cells (24% of the clusters counted, Figure 3-2D – 3-2D''). Examples of presumptive macrochaetae expressing Cdk1(Y15F)-VFP with even more Cut- and Su(H)-positive cells were also observed (Figure 3-2E – 3-2E'').

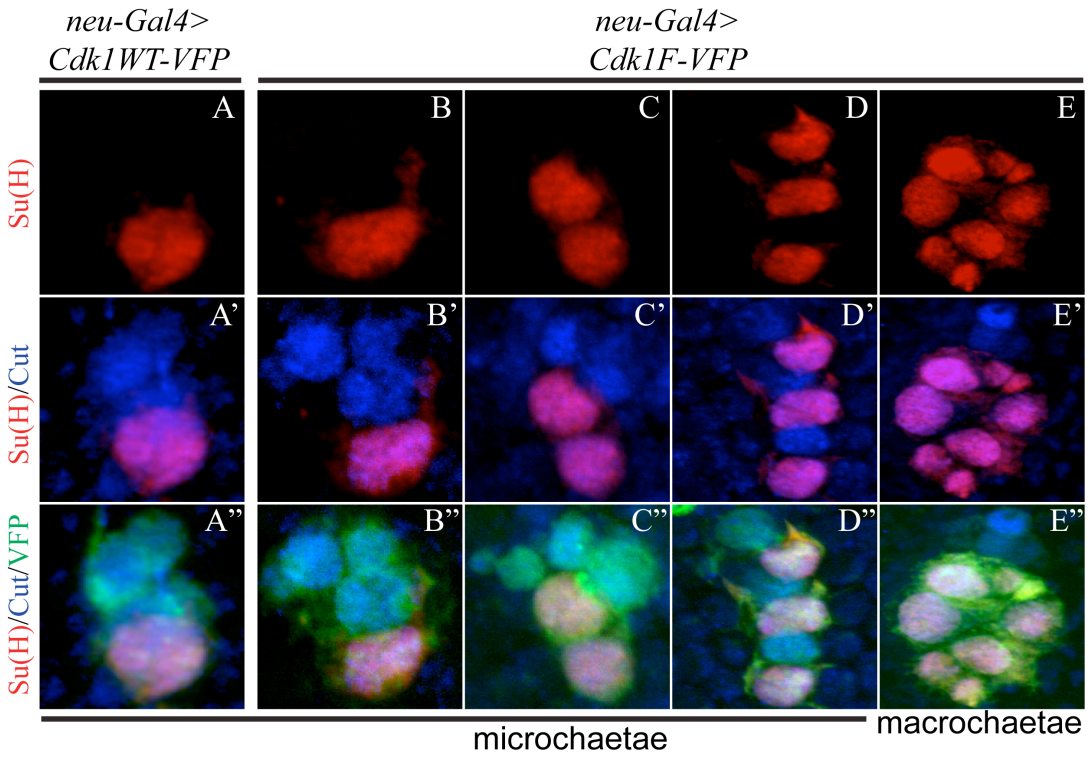

Figure 3-2: Supernumerary socket cells in SO lineage expressing Cdk1(Y15F)-VFP.

Drosophila microchaetae sensory organ lineage expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP was doubly stained in a 24-hr APF notum with anti-Su(H), which labels socket cells (red) and anti-Cut, which marks all the sensory cells (Blue). (A – A'') Normal microchaetae sensory organs expressing Cdk1(WT)-VFP contain a single Su(H) cell. In microchaetae lineage expressing Cdk1(Y15F)-VFP, we observed normal sensory organ containing one Su(H)-positive among 4 Cut-positive cells (B – B'') and mutant organs two (C – C'') or three (D – D'') Su(H)-positive cells among 5 or 6 Cut-positive sensory cells. (E – E'') Show an abnormal macrochaetae lineage expressing Cdk1(Y15F)-VFP containing 8 Su(H)-positive cells among 11 Cut-positive sensory cells

One possible explanation for the supernumerary socket cells in cells expressing Cdk1(Y15F)-VFP would be a pIIb-to-pIIa fate transformation, resulting in extra bristle and socket cells formed at the expense of neuron and sheath cells. To test this idea, we used anti-Elav antibodies which label neuron cells (Lin and Goodman, 1994) to label clusters expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP at 24 hours APF. Using Su(H) antibodies to identify clusters with more than one socket cell we examined whether these cells were associated with a neuron cell. In lineages expressing Cdk1(WT)-VFP, each Su(H)-positive cell was associated with one Elav-positive neuron cell in 100% of the cell clusters examined (Figure 3-3A – 3-3A’’’). In SO lineages expressing Cdk1(Y15F)-VFP we observed clusters with one Su(H)-positive cell (Figure 3-3B – 3-3B’’’) as well as aberrant cell clusters containing either 2 Su(H)-positive socket cells (Figure 3-3C – 3-3C’’’) or 3 Su(H)-positive socket cells (Figure 3-3D – 3-3D’’’), all of which were associated with a single Elav-positive cell. A similar result was seen in aberrant clusters of the macrochaetae lineage, for example one containing 7 Su(H)-positive socket cells with a single Elav-positive cell (Figure 3-3E – 3-3E’’’).

To determine whether each single neuron was associated with a sheath cell we doubly stained cells with Prospero (Spana and Doe, 1995) and Elav antibodies. In lineages expressing either Cdk1(WT)-VFP or Cdk1(Y15F), every Elav-positive neuron cell was associated with a Pros-positive sheath cell (Figure 3-4). These results verified the presence of pIIb descendant cells in aberrant clusters with multiple socket cells. Therefore, pIIb-to-pIIa cell fate transformation

is not directly responsible for supernumerary cells in Cdk1(Y15F)-VFP-expressing SO lineages.

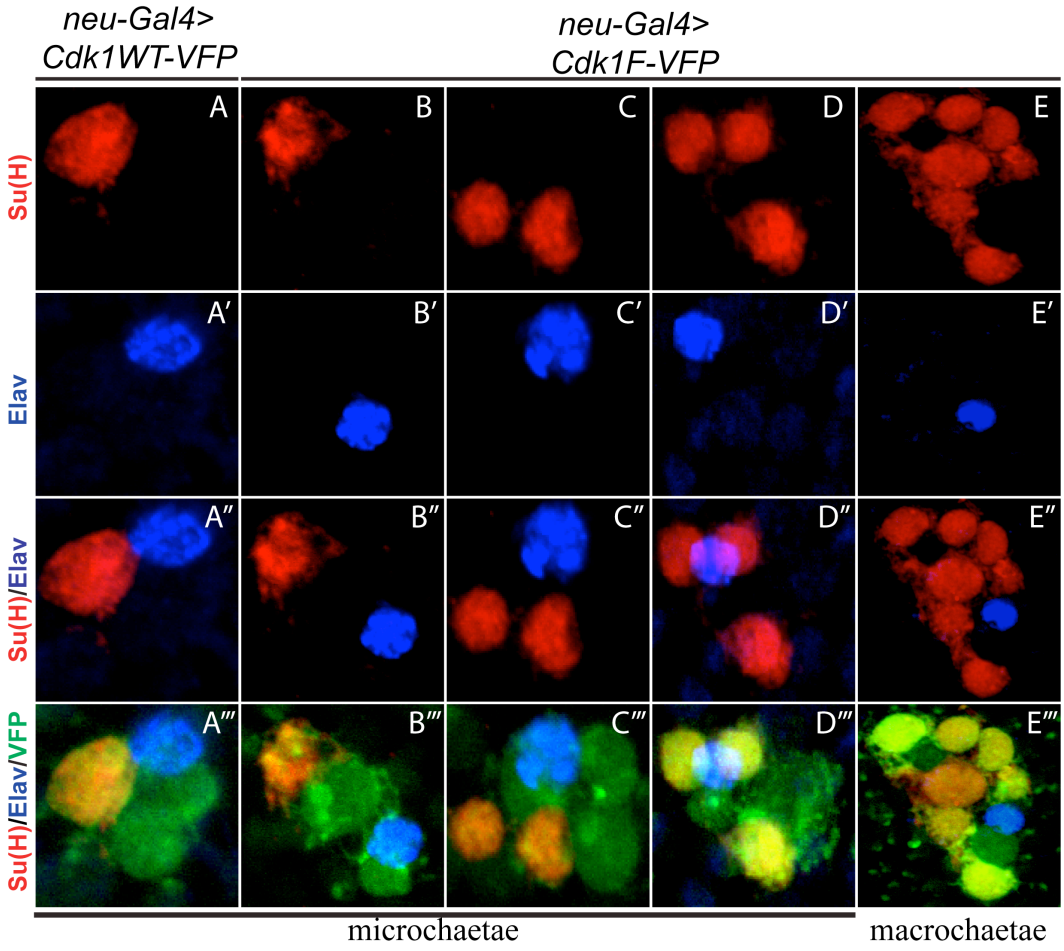

Figure 3-3: Each of the abnormal sensory cell clusters containing extra socket cells is associated with one neuron.

Sensory cells expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP at 24 hour APF are shown in green. Socket cells are detected by their specific accumulation of Su(H) (red) and neurons by ELAV (blue). (A – A’’’) Cdk1(WT)-VFP-expressing sensory organ has one socket cell and one neuron at 24 hours APF. (B – B’’’) Normal microchaetae lineage expressing Cdk1(Y15F)-VFP contains one socket and one neuron at 24 hours APF. Cdk1(Y15F)-VFP-expressing mutant sensory organs with 2-socket (C – C’’’) or 3-socket (D – D’’’) cells also contain one neuron each at 24 hours APF. (E – E’’’) A neuron cell is also observed in a Cdk1(Y15F)-VFP-expressing macrochaetae lineage containing 7 socket cells.

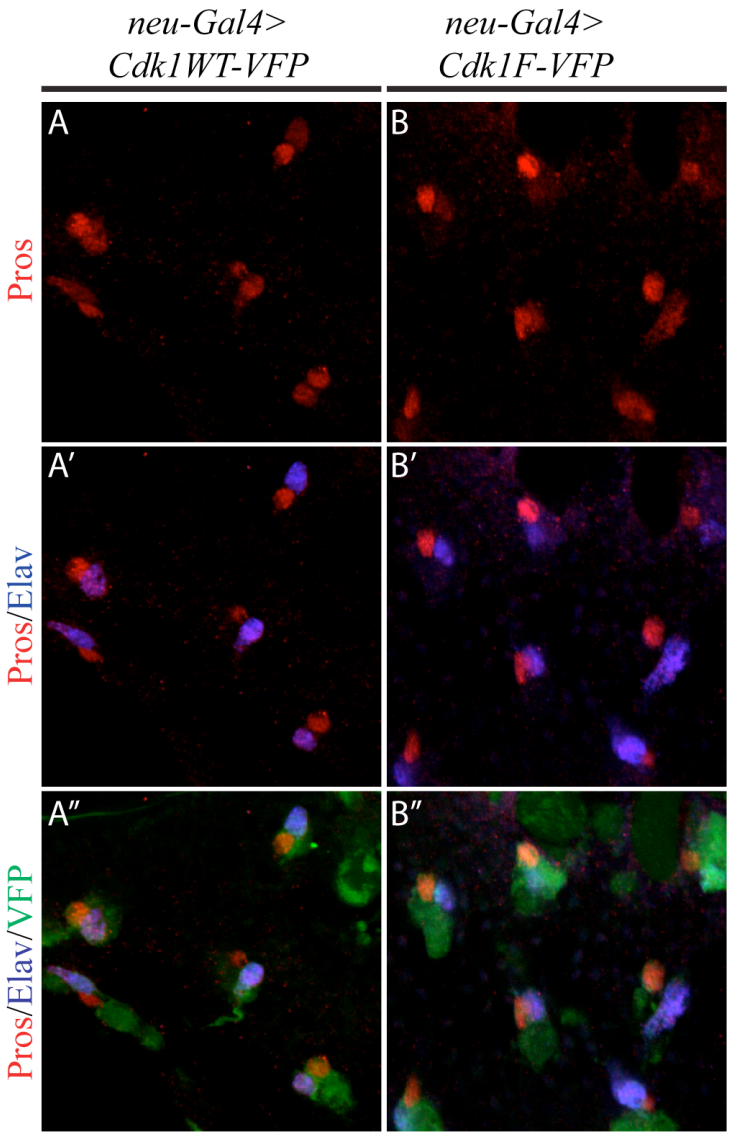

Figure 3-4: Each neuron cell is associated with a sheath cell in SO lineages ectopically expressing Cdk1(Y15F)-VFP

Sensory cells expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP at 24 hour APF are shown in green. Sheath cells are detected by their specific accumulation of Prospero (red) and neurons by ELAV (blue). (A – A'') Cdk1(WT)-VFP-expressing sensory organ has one sheath cell and one neuron at 24 hours APF. (B – B'') Similarly, all sensory organ lineages expressing Cdk1(Y15F)-VFP that were examined contains one sheath and one neuron at 24 hours APF.

Expression of Cdk1(Y15F)-VFP induced precocious mitosis in SOPs and its Pon-negative pIIa daughter cell

Another possible explanation for Cdk1(Y15F)-VFP expression causing supernumerary cells was that short-circuiting of G2 phase arrest in SOP cells might cause them to undergo multiple rounds of division before committing to a particular cell fate. To test this hypothesis we focused on the thoracic macrochaetae SOPs in wing imaginal discs that are born in third instar larvae but remain arrested in G2 phase until ~ 0-1 hours after puparium formation (APF) before undergoing differentiative asymmetric division (Hartenstein and Posakony, 1989). We used *neu-Gal4* to express Cdk1(WT)-VFP or Cdk1(Y15F)-VFP in these SOP cells and labeled the wing imaginal discs with phospho-histone H3 (PH3) antibodies to identify mitotic cells from 8 hours before puparium formation (BPF) to 1 hour APF. SOPs expressing Cdk1(WT)-VFP contained only 1 VFP-positive cell that was not labeled by PH3 antibodies when examined at 8 hours BPF, as expected for G2 phase-arrested cells (Figure 3-5A, see the inset, n=10). When examined at 1 hour APF, we noted that each SOP expressing Cdk1(WT)-VFP contained 1 VFP-positive cell that was also PH3-labeled (Figure 3-5A', see the insert, n=10). In contrast, Cdk1(Y15F)-VFP-expressing SOP cells were observed as clusters of 2 VFP-positive and/or PH3-labeled cells as early as 8 hours BFP (Figure 3-5B, see the inset, n=12). By 1 hour APF, Cdk1(Y15F)-VFP-expressing discs had cell clusters containing multiple VFP-positive and/or PH3-labeled cells (Figure 3-5B', see the inset, n=15). These results showed that the expression of Cdk1(Y15F)-VFP caused SOP cells to exit G2 phase quiescence

and divide prematurely.

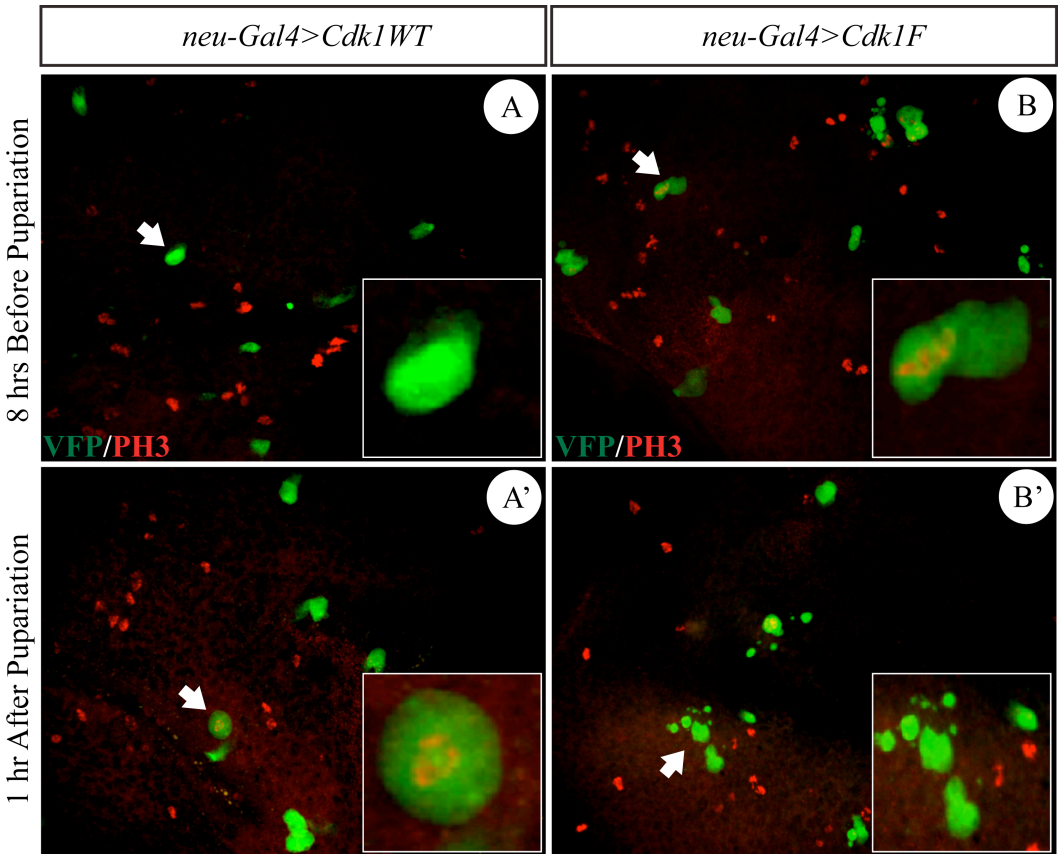

Figure 3-5: Non-inhibitable Cdk1 induced precocious mitosis in macrochaetae SOPs.

The VFP-tagged transgenes were expressed in SOPs using a *neu-Gal4* driver. Macrochaetae SOP in late third instar larval wing discs from 10 hours before pupa formation (BPF) through 1 hour after pupa formation (APF) were fixed and immunolabeled with PH3 antibodies (Red) to label mitotic cells. At 8 hours BPF, SOP expressing Cdk1(WT)-VFP (Green) had one VFP-labeled cell and were PH3-negative (A, see the insert), but at 1 hour APF, we noted that each VFP-positive was PH3-labeled cell (A', see the insert). At least 2 VFP-positive and/or PH3-labeled cells were seen in Cdk1(Y15F)-VFP-expressing SOPs at 8 hours BFP (B, see the inset). By 1 hour APF, we noted cell clusters containing multiple number of VFP-positive and/or PH3-labeled cells in the macrochaetae lineages expressing Cdk1(Y15F)-VFP (B', see the inset).

Having shown that the expression of Cdk1(Y15F)-VFP could induce premature mitosis in both the SOP and its pIIa daughter cell, we wondered whether the supernumerary socket cells reflected specific impact of the fusion proteins on the SOP cells or were due to secondary effects on the descendant cells. To address this possibility we expressed the Cdk1(WT)-VFP and Cdk1(Y15F)-VFP at different time point during the SO lineage and examined the compositions of the lineage at 24 hours APF using Su(H) antibodies. As shown in Figure 3-1, *neur-Gal4*-driven Cdk1(Y15F)-VFP resulted in extra socket cells phenotype. Here, we used $gal80^{ts}$ to turn off the expression *neur-Gal4*-driven transgenic proteins in SOP cells until pIIa and pIIb were born. SO lineages expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP in pIIa and pIIb cells produced normal cell clusters containing one Su(H)-positive cell (data not shown). Also, SO lineages expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP using *prospero-Gal4*, which is expressed only in pIIb, had no effect on SO cell clusters (Figure 3-6). Similarly, ectopically expressing Cdk1(WT)-VFP and Cdk1(Y15)-VFP using *elav-Gal4*, which is expressed only in pIIb descendant cells (i.e pIIIb), had no impact on the process of sensory organ development (Figure 3-6). Taken together, these data demonstrate that the supernumerary socket cells was due to specific effects of Cdk1(Y15)-VFP on SOP cells, not its descendant cells.

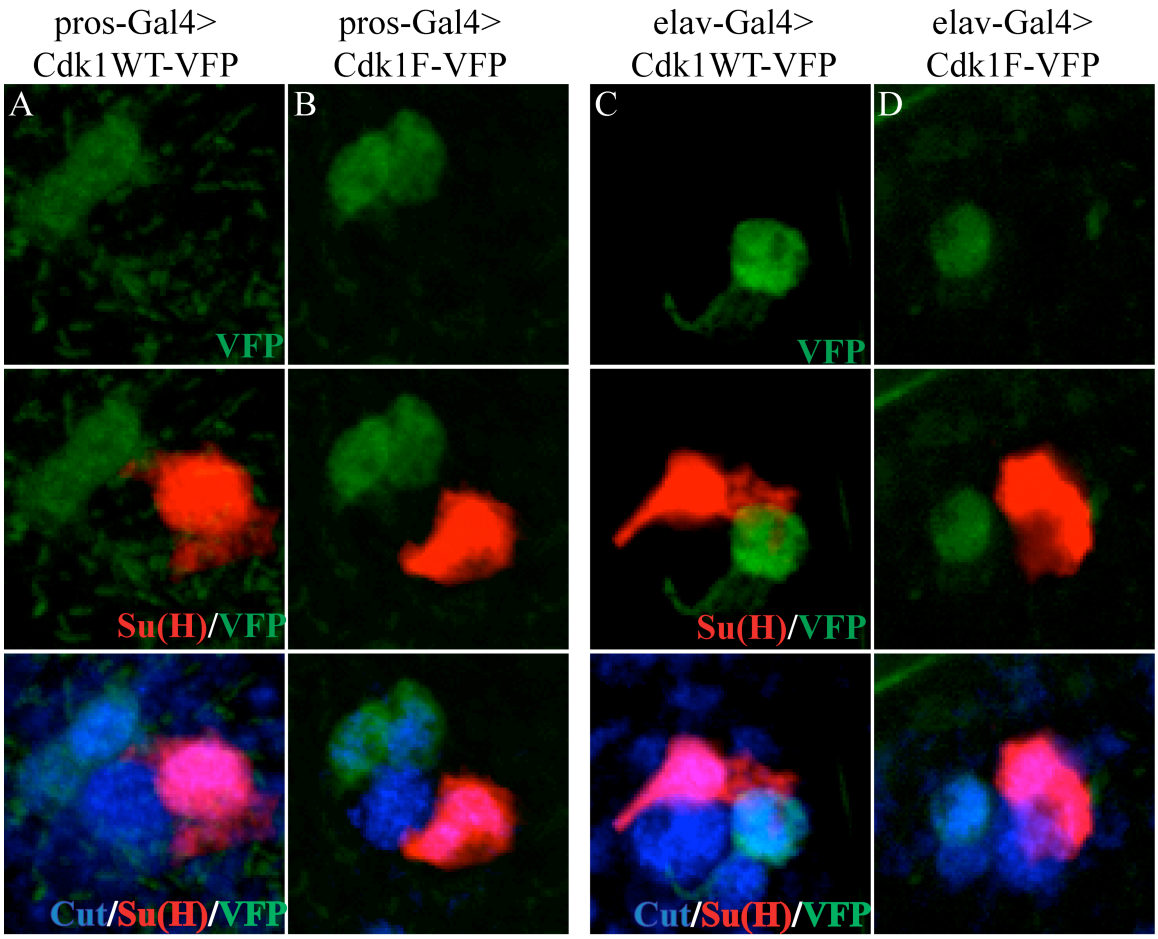

Figure 3-6: Expression of Cdk1(Y15F)-VFP has no effect on SO lineage after the birth of pIIa and pIIb daughter cells.

Sensory cells expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP under the control of *pros-Gal4* (which is expressed in pIIb cells) and *elav-Gal4* (which is expressed in pIIIb cells) at 24 hour APF are shown in green. Socket cells are detected by their specific accumulation of Su(H) (red). (A) SO lineages expressing Cdk1(WT)-VFP under the control of *pros-Gal4* has one socket cell and one neuron at 24 hours APF. (B) Expression of Cdk1(Y15F)-VFP using pros-Gal4 produced normal SO lineage containing one socket and one neuron at 24 hours APF. Similarly, SO lineages expressing Cdk1(WT)-VFP (C) and Cdk1(Y15F)-VFP (D) under the control of *elav-Gal4* produced normal lineage with one socket cell.

Cdk1(Y15F)-VFP expression does not cause disruption of SOP asymmetric cell division

Mis-segregation of cell fate determinants during SOP cell division results in extra socket/shaft phenotypes (Rhyu et al., 1994; Le Borgne and Schweisguth, 2003). Given that Cdk1(Y15F)-VFP-expressing SOP cells undergo premature cell division we considered whether insufficient time in G2 phase for synthesis or segregation of cell-fate determinants might cause the observed defects (O'Farrell and Kylsten, 2008). To address this possibility we co-expressed Pon::RFP, a marker of cellular asymmetry, with either Cdk1(WT)-VFP or Cdk1(Y15F)-VFP in SOPs using *neur-Gal4* driver and performed live imaging of the SO microchaetae lineage. In lineages expressing Cdk1(WT)-VFP, the SOP cells divided at 17 hr APF to produce an anterior Pon::RFP-positive pIIb cell and a posterior Pon::RFP-negative pIIa daughter cell (Figure 3-7A). A similar asymmetric localization of Pon::RFP was observed in the daughter cells of the Cdk1(Y15F)-VFP-expressing SOPs, however most of these cells had already divided earlier than 15 hr APF when this movie was made (Figure 3-7B).

In a normal SO lineage the plane of SOP division is perpendicular to the Pon crescent before anaphase (Lu et al., 1998a) and cell polarity defects that affect this relationship result in mis-specification of SOP daughter cell fate determination (Bellaiche et al., 2001). To examine whether the plane of cell division was affected by Cdk1(Y15F)-VFP expression we took advantage of the fact that the VFP-tagged Cdk1 brightly marked metaphase centrosomes and immunostained dividing SOPs with anti-Pon antibodies. We observed that the

plane of SOP division revealed by the metaphase centrosomes was perpendicular to the Pon crescent in both SOP expressing Cdk1(WT)-VFP (Figure 3-8A - A'', n=10) and Cdk1(Y15F)-VFP (Figure 3-8B – B'', n=15). These data showed that Cdk1(Y15F)-VFP expression did not affect either asymmetric segregation of cell fate determinants or the plane of SOP cell division, even though these cells divided at least 2 hours earlier than they normally would.

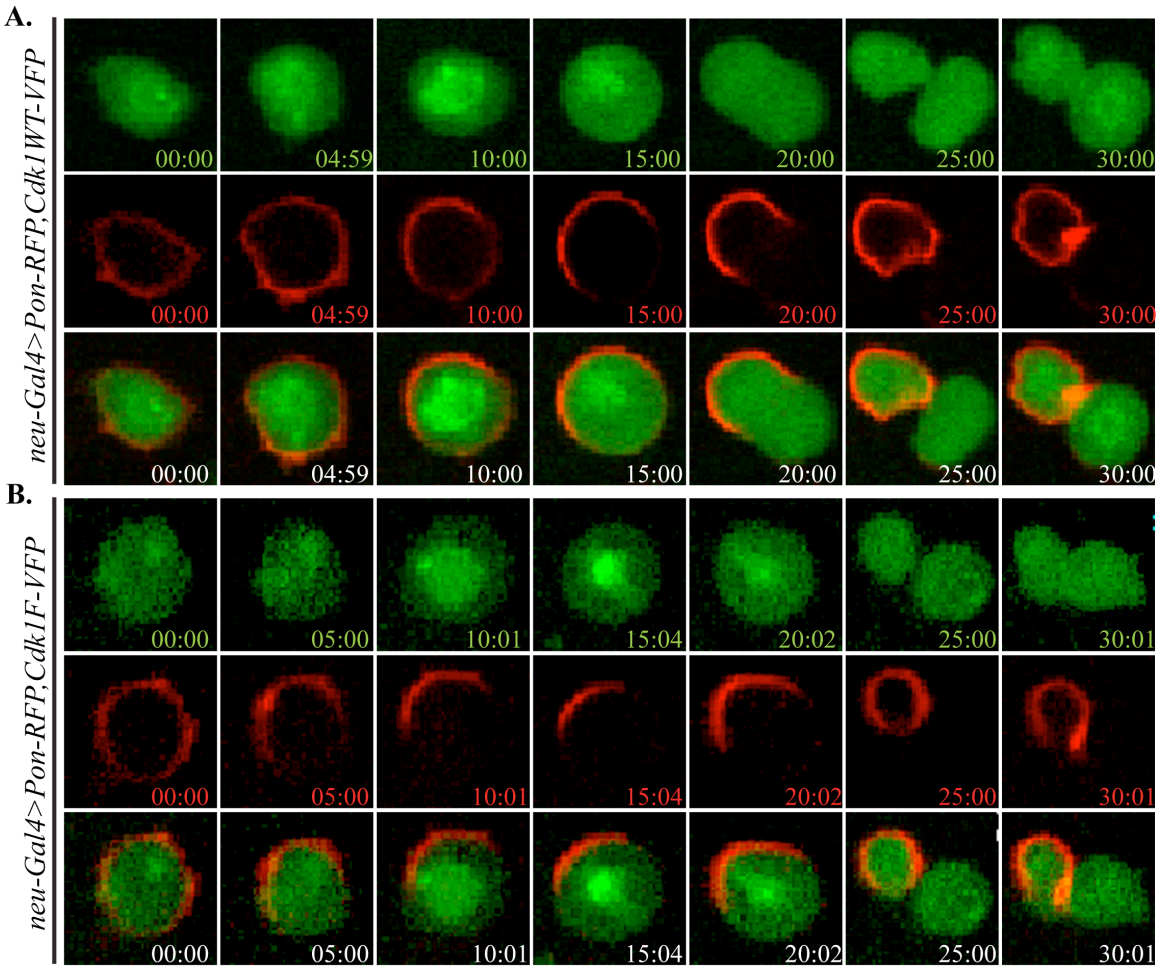

Figure 3-7: Cellular asymmetry is unperturbed in SOPs expressing non-inhibitable Cdk1.

Transgenic Cdk1(WT)-VFP or Cdk1(Y15F)-VFP (green) and Pon::RFP (red) are co-expressed in SOP cells using *neur-Gal4* and follows the SOP division using time-lapse imaging. The figure shows still images from the time-lapse imaging and the t = 0 represents the time of cell rounding. Pon::RFP is used as a marker of asymmetric division. In sensory organ lineages expressing (A) Cdk1(WT)-VFP and (B) Cdk1(Y15F)-VFP, Pon-RFP forms an anterior crescent in the pI cell ([A]: t = 15 mins; [B]: t = 15.04 mins) and asymmetrically segregates into the anterior pIIb daughter ([A]: t = 30 mins; [B]: t = 30.01 mins).

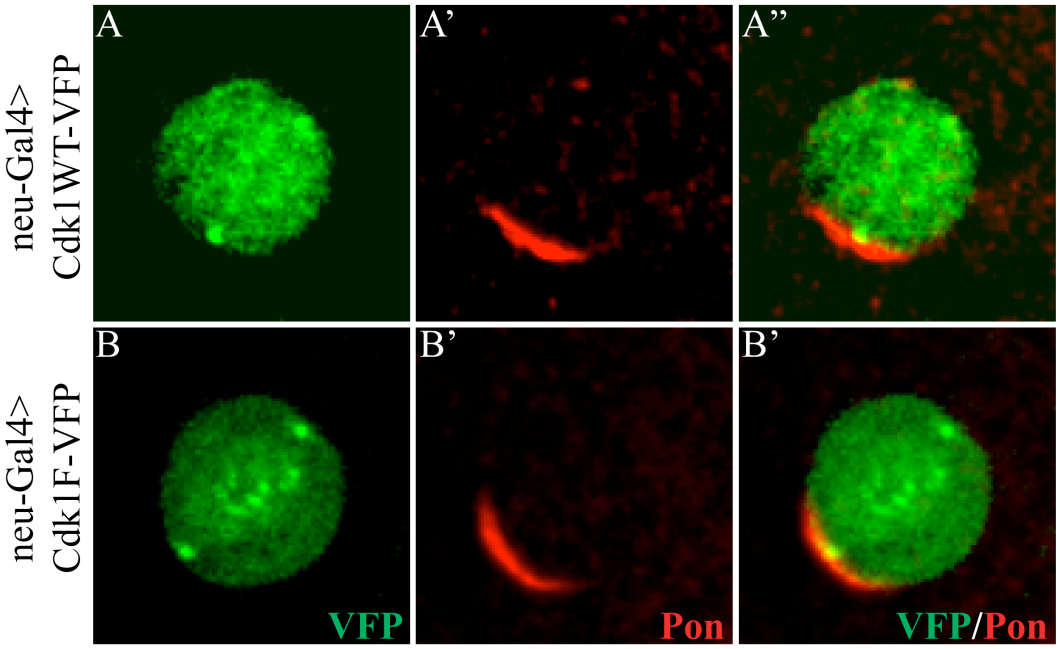

Figure 3-8: Expression of non-inhibitable Cdk1 does not disrupt the plane of SOP cell division.

The SOP cells expressing Cdk1(WT)-VFP (A – A'') or Cdk1(Y15F)-VFP (B – B'') were stained with anti-Pon antibodies and metaphase cells were identified by the appearance of the polar centrosomes as marked by VFP and characteristic Pon crescent at this stage. The two polar centrosomes are perpendicular to the Pon::RFP crescent in SOPs expressing either Cdk1(WT)-VFP (A – A'') or Cdk1(Y15F)-VFP (B – B''), indicating that the plane of cell division is unaltered.

Cdk1(Y15F)-VFP expression results in ectopic division of Pon-positive cells

Having established that Cdk1(Y15F)-VFP expression induced premature SOP cell division we wondered if extra cell divisions in the pIIa lineage were responsible for the supernumerary pIIa- descendant socket cells. To address this possibility, we examined the timing of cell division in pIIa daughter cells expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP. In a normal SO lineage, pIIb divides apico-basally ~ 2.5 hours after SOP division whereas the pIIa daughter cell divides anterior-posteriorly ~ 30 – 40 minutes later (Gho et al., 1999). We used Pon::RFP as a reporter for asymmetric cell-fate determinants and the movement of the VFP into the nucleus as a marker for mitotic entry to perform live imaging of SO lineages. In SO lineages expressing Cdk1(WT)-VFP, the Pros-negative pIIa daughter cell divided at 3 hours 30 minutes after SOP cell division, precisely 45 minutes after the division of the Pon-positive pIIb cell (Figure 3-9A). In contrast, in lineages expressing Cdk1(Y15F)-VFP the pIIa cells divided either simultaneously with or just before its Pon-positive sibling (Figure 3-9B). These data therefore showed that expression of Cdk1(Y15F)-VFP advanced the timing of pIIa cell division.

To determine if over-proliferation of the pIIa lineage was responsible for the supernumerary cells observed, we co-expressed Cdk1(WT)-VFP or Cdk1(Y15F)-VFP with Pon::RFP for live imaging of the entire lineage, from 15 hours APF through 24 hours APF. In Cdk1(WT)-VFP- expressing lineages, the Pon-positive pIIb daughter cell divided apico-basally ~ 2 hours 30 minutes after SOP cell division to produce an apical pIIIb cell and a posteriorly displaced Pon-

RFP-positive basal glia cell followed by a single pIIIb division and glia cell apoptosis (Movie 3-1). The Pon-negative pIIa daughter cell divided only once about 45 minutes after Pon-positive sibling (Movie 3-1). In contrast, in Cdk1(Y15F)-VFP-expressing lineages the Pon-positive "pIIb" and Pon-negative pIIa daughter cells divided simultaneously about 2 hours 20 minutes after SOP division (Movie 3-2). These results showed that even though the Cdk1(Y15F)-VFP-expressing Pon-negative pIIa daughter cell divided precociously, it did not undergo any additional cell division, excluding overproliferation of the pIIa lineage as an explanation for supernumerary socket cells.

Instead, Pon-positive "pIIb" cells of this aberrant Cdk1(Y15F)-VFP-expressing lineage underwent another cell division to produce 4 daughter cells (Movie 3-2). This, in addition to the two daughter cells produced by the Pon-negative pIIa cell division resulted in a cluster of 6 cells. No glia cell apoptosis was observed in these aberrant SO lineages, even after extended imaging (Movies 3-2). These results demonstrate that although the Pon-negative pIIa daughter cell did divide prematurely, it was an extra cell division (and absence of glial cell apoptosis) in the Pon-positive "pIIb" lineage that produced supernumerary cells. Thus, Cdk1(Y15F)-VFP expression in SOP cells resulted in asymmetric cell divisions that produced Pon-negative pIIa cells which followed a normal lineage and a Pon-positive sibling lineage capable of undergoing extra rounds of cell division.

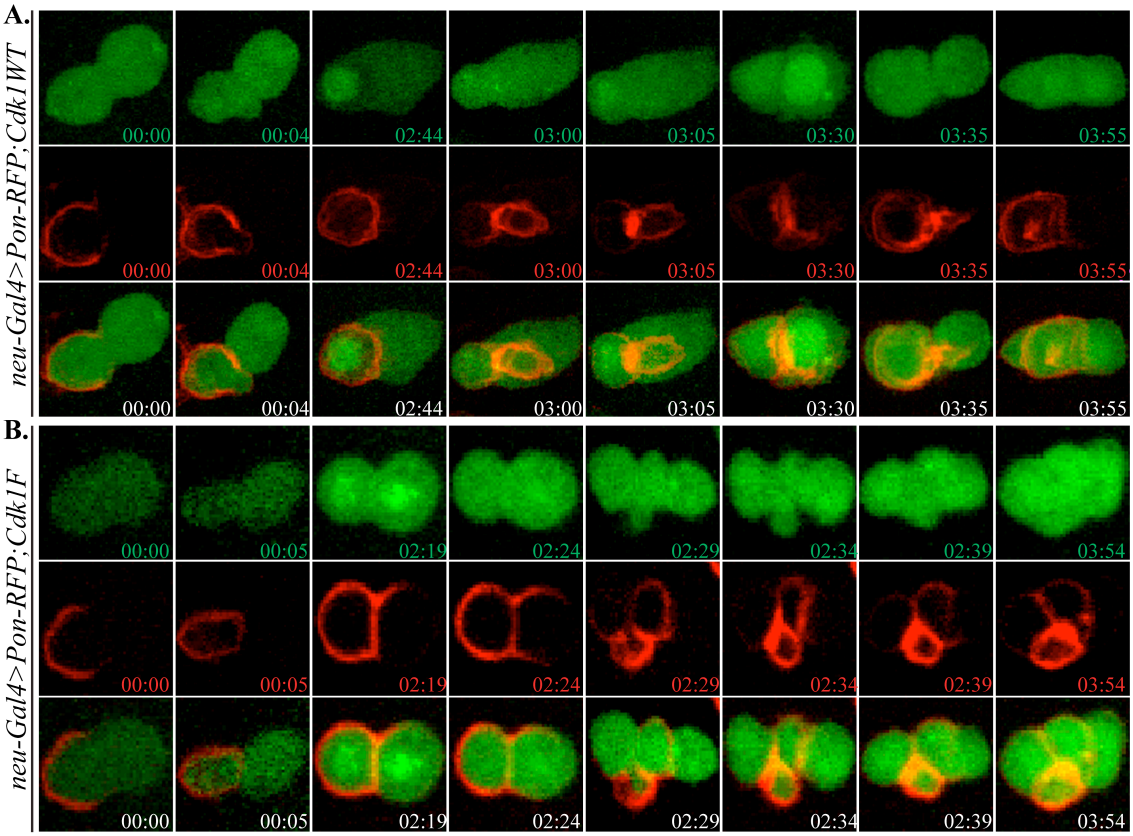

Figure 3-9: Expression of Cdk1(Y15F)-VFP induces precocious cell division in pIIa daughter cell.

Pon::RFP (red) is co-expressed with either Cdk1(WT)-VFP or Cdk1(Y15F)-VFP (green) in SOP using *neur-Gal4* and the SOP division is followed using time-lapse imaging. The figure shows still images from the time-lapse imaging and the t = 0 represents the SOP anaphase, indicating the birth time of pIIa and pIIb cells. Nuclear accumulation of the VFP tagged Cdk1 transgenic proteins marks mitotic initiation. (A) Sensory organ (SO) lineages expressing Cdk1(WT)-VFP showing that the Pon-positive pIIb cell enters mitosis about 2hrs 40mins after its birth, while the Pon-negative pIIa cell divides about 45 minutes after pIIb (3hrs 30mins after its birth). However, the Pon-negative pIIa daughter cell divides simultaneously with its Pon-positive sibling at 2hrs 20mins after their birth (B).

Expression of Cdk1(Y15F)-VFP short-circuits G2 quiescence forcing SOP cell to self-renew

Having established that Cdk1(Y15F)-VFP expression caused Pon-positive SOP daughter cells to produce extra cells, we wondered whether the SOP cell self-renewed to produce a normal pIIa cell and an abnormal sibling cell capable of undergoing a SOP-like cell division instead of terminally differentiating. In a normal SO lineage, the SOP cell divides anterior-posteriorly in the plane of the epithelium to produce an anteriorly located Pon-positive daughter cell that assumes neuronal cell fate due to the expression of the Prospero transcription factor (Manning and Doe, 1999; Reddy and Rodrigues, 1999b; Choksi et al., 2006). To assess if the mis-specified Pon-positive cells behave in a similar manner as SOP cells we co-expressed Cdk1(WT)-VFP or Cdk1(Y15F)-VFP with Pon::RFP to track the plane of asymmetric division, using live imaging to follow the entire SO lineage. Pon-positive pIIb cells expressing Cdk1(WT)-VFP divided apico-basally to produce an apical pIIIb and a posteriorly displaced Pon-positive daughter (glial) cell (Figure 3-10A). In contrast, aberrant Pon-positive cells expressing Cdk1(Y15F)-VFP divided within the plane of the epithelium (Figure 3-10B), generally anterio-posteriorly but with occasional spatial variation (Figure 3-10C & D). This data show that the mis-specified Pon-positive daughter cell divides anterior-posteriorly like the SOP or pIIa cells, rather than apico-basally as expected for a Pon-positive pIIb daughter cell. We therefore refer to this daughter cell as “pI-like”.

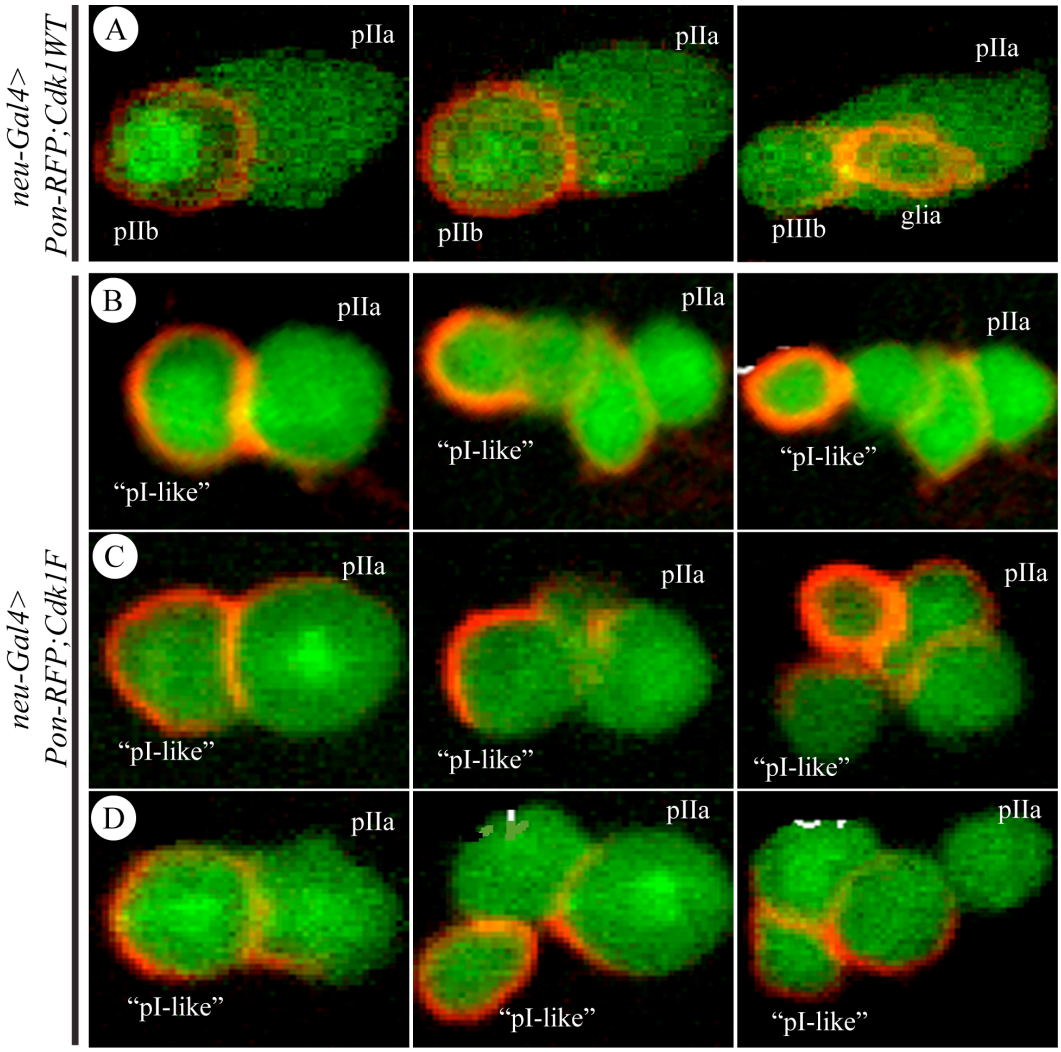

Figure 3-10: Mis-specified Pon-positive daughter cell undergoes a "pI-like" anterio-posterior division.

The planes of division in Pon-positive pIIb and Pon-negative pIIa daughter cells were examined in SO lineage expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP and Pon::RFP. The Pon-positive pIIb cells expressing Cdk1(WT)-VFP divided apico-basally to produce an apical pIIIb and a posteriorly displaced Pon-positive glia cells (A). On the contrary, the aberrant Pon-positive "pI-like" cells expressing Cdk1(Y15F)-VFP divided anterio-posteriorly within the plane of the epithelium like SOP and its Pon-negative pIIa sibling (B) and sometimes with slight degree of variations (C & D).

If the SOP self-renewal caused by the expression of Cdk1(Y15F)-VFP disrupted neuronal fate differentiation then we would expect that Prospero would not be expressed in the Pon-positive daughter cell immediately following SOP division. To test this idea we used Prospero antibodies (blue in Figure 3-10) to label cells of SO lineages expressing either Cdk1(WT)-VFP or Cdk1(Y15F)-VFP between 15 and 19 hours APF. Microchatae lineage SOPs do not express Pros at any stage of the cell cycle ((Manning and Doe, 1999); and data not shown). In SO lineages expressing Cdk1(WT)-VFP, we observed one Pros-positive cell in 100% of the 2-cell clusters examined at 17 hours APF (Figure 3-11, n=50) and two Pros-positive cells in 100% of clusters composed of three cells at 19 hours APF (Figure 3-11, n=80). In SO lineages expressing Cdk1(Y15F)-VFP, we observed no Pros-positive cell in 25% of the 2-cell and 30% of 3-cell clusters examined at 15 hours APF (Figure 3-11). At 17 hour APF, Cdk1(Y15F)-VFP-expressing SO lineages contained some 4-cell clusters with no Pros-positive cell and some with one Pro-positive cell (Figure 3-11). Although we still observed some clusters with one Pros-positive cell, most of the 5 or 6-cell clusters examined at 19 hours APF contained 2 Pros-positive cells (Figure 3-11, 78%). Collectively, these results indicate that the aberrant "pI-like" daughter cell undergoes additional rounds of SOP-like cell division prior to specifying neuronal cell fate. This suggests that Cdk1(Y15F) results in a disruption in the balance between cell division and neuronal lineage commitment in SO-lineages, and that this likely underlies the sensory bristle defects.

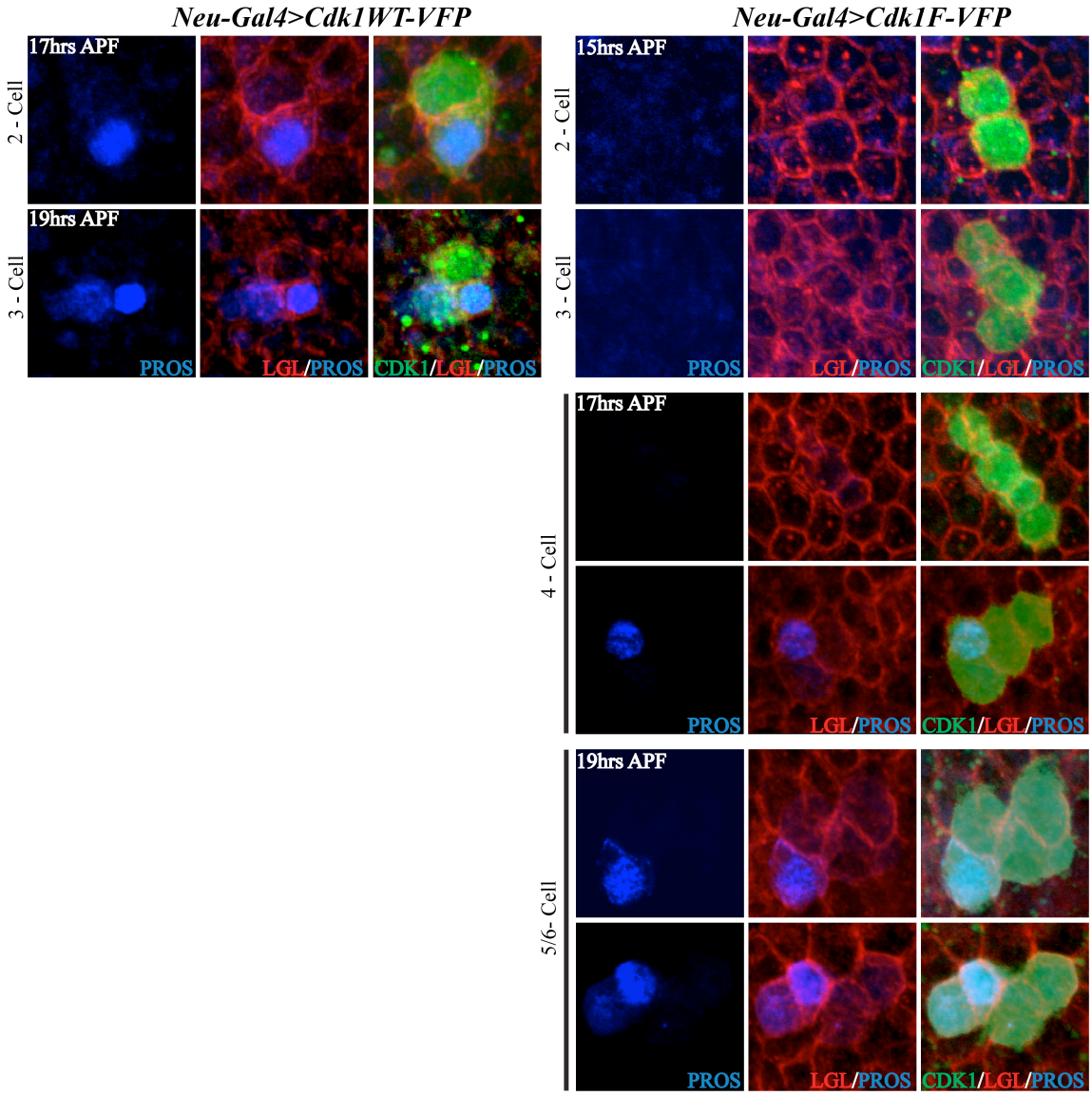

Figure 3-11: The expression of Cdk1(Y15F)-VFP disrupted neuronal fate specification in SO lineage.

Figure 3-11 Legend

Sensory cells expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP at 15 hours APF through 19 hours APF are shown in green. Acquisition of a neuronal fate by one of the daughter cells following SOP division was detected by accumulation of Prospero (blue), while Lgl (red) marked the cell cortex. At 17 hours APF, Cdk1(WT)-VFP-expressing sensory organ has two cells with one Pros-positive and one Pros-negative cells. By 19 hours APF, the Pros-positive daughter cells in Cdk1(WT)-VFP-expressing sensory lineages had divided, resulting in two Pros-positive cells and one Pros-negative cell. In SO lineages expressing Cdk1(Y15F)-VFP, clusters containing 2 or 3 cells with no Prospero were observed as early as 15 hours APF. At 17 hour APF, Cdk1(Y15F)-VFP-expressing SO lineages contained some 4-cell clusters with no Pros-positive cell and some with one Pros-positive cell . Although we still observed some clusters with one Pros-positive cell, most of the 5 or 6-cell clusters examined at 19 hours APF contained 2 Pros-positive cells.

The self-renewal division in Cdk1(Y15F)-VFP-expressing SOP cells occurs independently of the Prospero-mediated cell cycle regulation.

Prospero promotes terminal differentiation in neural precursor cells by repressing cell cycle genes such as *cyclin E*, which is required for self-renewal (Choksi et al., 2006; Berger et al., 2010) and overexpression of cyclin E confers self-renewing asymmetric division potential on otherwise terminally differentiating neural precursor cells (Bhat and Apsel, 2004). Therefore, the aberrant SOP cell self-renewal might be due to perturbation of the Prospero-dependent regulation of terminal differentiation as a consequence of Cyclin E overexpression. To test this idea, we asked whether lowering cyclin E levels in SOP cells expressing Cdk1(Y15F)-VFP could suppress the sensory bristle phenotype. We expressed Cdk1(WT)-VFP and Cdk1(Y15F)-VFP in hypomorphic *cyclin E* background (Brumby et al., 2004) and assessed adult flies for sensory bristle defects. The SO lineages hypomorphic for *cyclin E* (Figure 3-12A) or expressing Cdk1(WT)-VFP in hypomorphic *cyclin E* background produced normal sensory bristles (Figure 3-12B). Expression of Cdk1(Y15F)-VFP in hypomorphic *cyclin E* background failed to suppress the sensory bristle defects (Figure 3-12C).

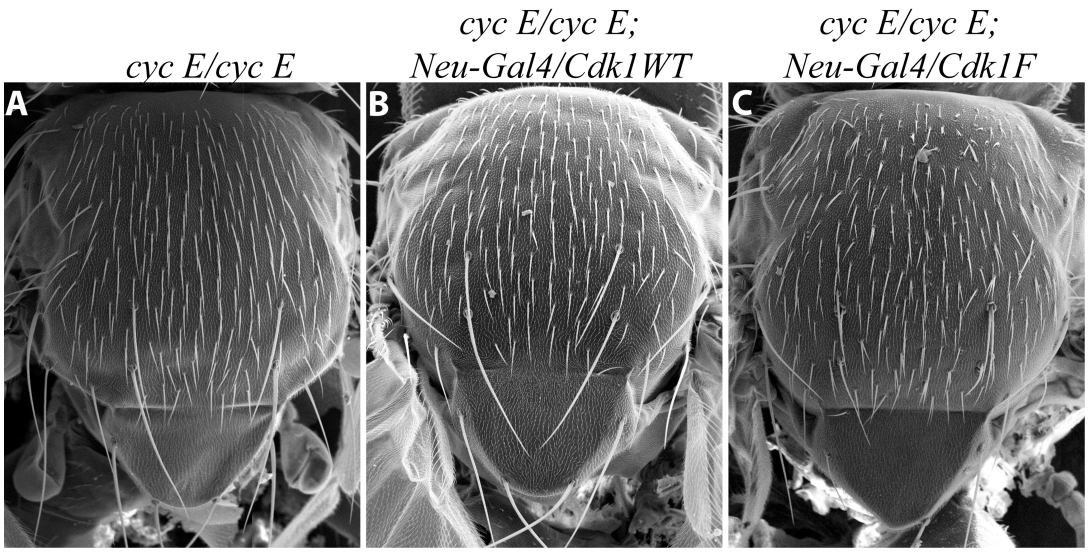

Figure 3-12: Lowering cyclin E level fails to suppress Cdk1(Y15F)-VFP associated sensory bristle defects.

Expression of Cdk1(Y15F)-VFP Cdk1(WT)-VFP or Cdk1(Y15F)-VFP was expressed in hypomorphic *cyclin E* background and examined adult flies for sensory bristle defects. (A) Scanning electron micrograph showing normal bristles on adult thorax hypomorphic for *cyclin E*. (B) Shows normal sensory bristle on adult thorax expressing Cdk1(WT)-VFP in hypomorphic *cyclin E* background. (C) Shows thoracic sensory bristle defects on adult flies expressing Cdk1(Y15F)-VFP in hypomorphic *cyclin E* background.

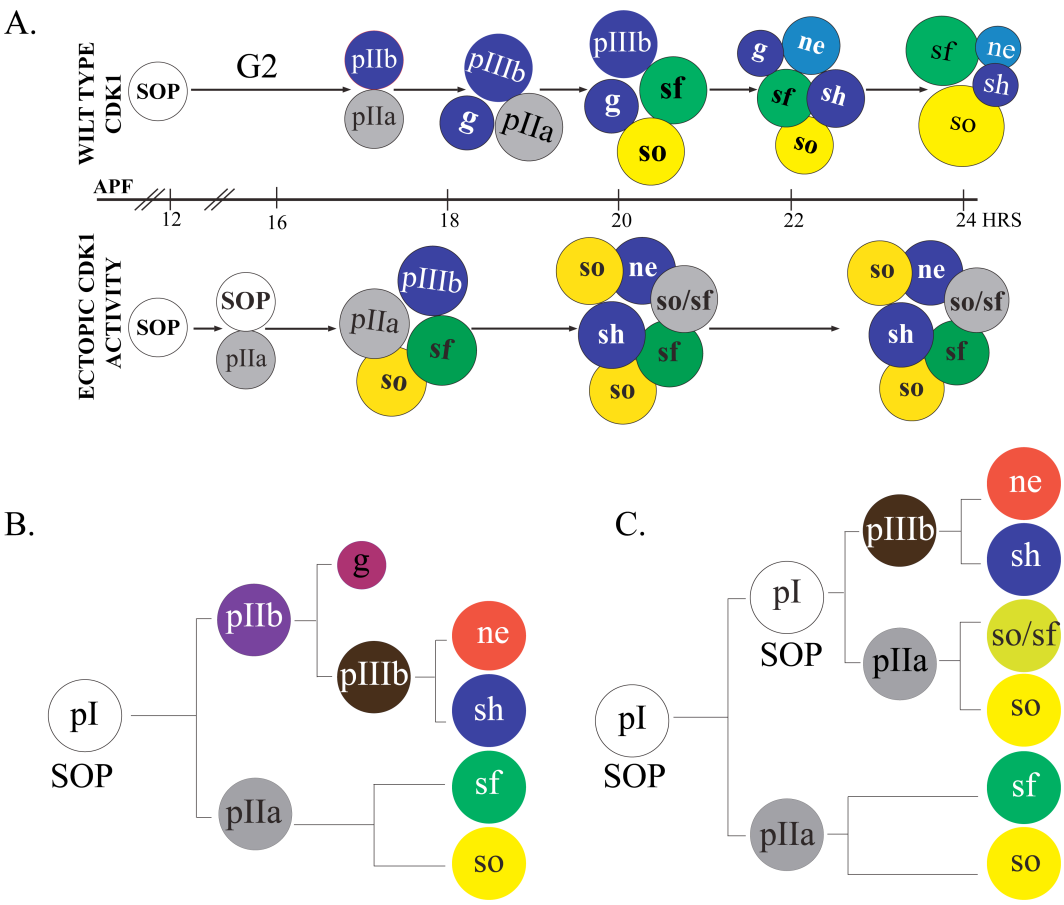

Figure 3-13: Cartoon summary of the SO lineages in Cdk1(WT)-VFP and Cdk1(Y15F)-VFP expressing SOP cells

Figure 3-13 Legend

(A) Following its birth at 12 hours APF, the SOP cell in a wild type SO lineage remains in a G2 phase quiescence but divides at about 17 hours APF. One of the resulting daughter cells, the pIIb cell, expresses Prospero (blue) and assumes neuronal fate, while the other cell (pIIa) assumes a structural fate and remains Pros-negative. At about 19 hours APF, the pIIb divides first to generate a pIIIb and a glia cell, and both descendant cells inherit Prospero (blue). The pIIa cell divides soon after (approximately 30 mins) to generate a shaft cell (green) and socket cell (yellow). Finally the pIIIb cell divides to produce a neuron cell and a sheath cell, with the sheath cell maintains a high level of Prospero (P), while the neuron has transient Prospero. At 17 hours APF, the mutant SO lineages expressing Cdk1(Y15F)-VFP contain four daughter cells (compared to two in wild type) with one Prospero positive cell, indicating that the SOP cells have divided multiple times prior to acquisition of neuronal fate. (B) Shows a cartoon summary of the wild type SO lineage with the SOP dividing to produce pIIa and pIIb; the pIIb then divides to produce pIIIb and glia (g); soon after the pIIa divide to form the shaft (sf) and socket (so) cells; this is followed by pIIIb division to produce neuron (ne) and sheath (sh) cells. (C) Shows the cartoon summary of the Cdk1(Y15F)-VFP expressing SO lineage with the mutant SOP cells dividing to produce a pIIa and a mis-specified SOP-like cell. The pIIa cell divides to produce a shaft cell (sf) and a socket cell (so), while the SOP-like cell divides simultaneously with the pIIa cell to produce another pIIa and a pIIIb cells; the new pIIa divides to produce extra socket and/or shaft cells, while the pIIIb finally divides to produce a neuron cell (ne) and a sheath cell (sh).

3.4 DISCUSSION

This study focused on the developmentally regulated G2 quiescence in SOP cells to assess how the timing of cell divisions is synchronized with cell fate determination during *Drosophila* sensory bristle development. Our data demonstrated that short-circuiting G2/M timing in the SOP cells via Cdk1(Y15F)-VFP expression caused ectopic cell divisions and aberrant cell fate determination, culminating in supernumerary sensory cells. We show that the expression of Cdk1(Y15F)-VFP truncates G2 phase quiescence in SOP cells resulting in ectopic sensory bristle cells without interfering with the process of asymmetric division. We excluded the possibility of cell fate transformation as the underlying cause of the supernumerary sensory cells. Instead, we provide evidence that premature exit from G2 quiescence confers a self-renewal potential on SOP cells that would otherwise terminally differentiate (Hartenstein and Posakony, 1990a; Posakony, 1994). Our data is consistent with a model that Cdk1(Y15F)-VFP-expressing SOP cells divide aberrantly to produce a normal pIIa daughter cell and an abnormal sibling capable of undergoing another round of SOP-like division to produce supernumerary sensory organ cells (Figure 3-13).

One of the most absorbing questions during eukaryotic development is the significance of the complexity of cell division in finely orchestrated morphogenetic processes such as cell fate determination (Edgar and O'Farrell, 1990; Harris and Hartenstein, 1991). Although previous observations suggest that the timing of mitosis in *Drosophila* embryogenesis and both the timing and number of divisions in Xenopus embryos were not necessary for morphogenetic

processes, including neuronal fate determination (Edgar and O'Farrell, 1990; Harris and Hartenstein, 1991), we present evidence indicating that precise regulation of mitotic entry timing and the number of divisions is critically important during the process of sensory bristle development. Altering the timing of mitotic entry by forcing G2 phase quiescent SOP cells into premature mitosis resulted in abnormal sensory organ with supernumerary socket cells. These data together with previous reports in *Drosophila* ventral furrow invagination demonstrate how mitotic entry must be coordinated with cell differentiation and fate determination during morphogenesis (Großhans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000).

Our analysis also revealed that truncation of G2 phase quiescence (shortening mitotic timing) resulted in a self-renewal cell division of SOP cells prior to their neuronal differentiation at a precise time during development. This is consistent with a proposal that there is a developmentally regulated window defined by the timing of G2 quiescence when SOP cells are competent to undergo self-renewal prior to neuronal differentiation. More pertinently, lengthening cell cycle sufficiently triggers differentiation of vertebrate neural progenitors (Calegari and Huttner, 2003) while truncating G2 phase arrest perturbs SOP cell differentiation (our data), suggesting that sufficient time in G2 phase arrest could be crucial for neuronal differentiation in neural precursor cells. Thus, providing evidence linking the timing of G2 quiescence in SOP cells to the critically important developmental choice of self-renewal versus terminal differentiation.

How might hasty or lengthened G2 quiescence influence cell fate choices remains a major conundrum, however. In light of our findings that insufficient time to properly segregate cell fate determinants or establish the plane of cell division does not underlie the observed cell fate disruption, the extended G2 quiescence could serve a unique developmental function, allowing cellular remodeling required for subsequent neural precursor cell differentiation (Hartenstein and Posakony, 1990b; Isshiki et al., 2001). Alternatively, the fact that cell division can be divorced from cell fate determination either by shortening G2 timing via induction of ectopic Cdk1 activity (our data) or by lengthening of G2 quiescence via Cdk1 inhibition (Hartenstein and Posakony, 1990b; Harris and Hartenstein, 1991; Fichelson and Gho, 2004), suggest the existence of temporal cue synchronizing the timing of neural precursor cell division and neuronal differentiation in SO lineages. An obvious consequence of such a temporal cue is that G2 quiescence exit and neuronal differentiation could be placed under the control of a common regulator, which would provide an efficient and effective way of making the two processes occur concomitantly during normal sensory organ formation (Myster and Duronio, 2000).

What synchronizes the timing of neural precursor mitosis with cell fate determination in SOP cells? An intriguing possibility is that the previously described mutual antagonistic interactions between Cyclin E, a component of cell cycle regulation and Prospero, a homeo-domain transcription factor which regulates the choice between stem cell self-renewal and differentiation (Reddy and Rodrigues, 1999b; Li and Vaessin, 2000; Choksi et al., 2006; Berger et al.,

2010), could serve as the cell cycle-dependent synchronizer of cell division and cell fate specification during neurogenesis. This intrinsic mechanism is refuted, however, at least in the current developmental context, by our genetic experiments that showed that a *cyclin E* hypomorph was unable to suppress the sensory bristle phenotypes associated with the Cdk1(Y15F)-VFP expression. Alternatively, the common molecular synchronizer might involve extrinsic developmental input such as hormonal regulation, acting independently to ensure that the String-mediated Cdk1 activation necessary for exiting the G2 quiescence occurs synchronously with the temporal cue controlling neuronal fate determination. Indeed, the steroid hormone ecdysone promotes *string* expression during the larval/pre-pupa transition in *Drosophila* histoblasts, triggering waves of proliferation following an extended period of G2 quiescence (Hayashi, 1996; Ninov et al., 2007; Ninov et al., 2009). In fact, the larval/pre-pupae ecdysone pulse occurs concomitantly with the first mitotic division of the SO lineage that produces progenitor cells with distinct cell fates (Sliter, 1989). Such developmentally regulated hormonal control of cell cycle and differentiation provides a novel perspective for addressing how differentiative and self-renewal asymmetric divisions are regulated in neural precursor cells.

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4. Cdc25^{Twe} dependent activation of Cdk1 is dispensable during meiotic entry in Drosophila Spermatocytes

4.1 INTRODUCTION

Entry into M-phase requires tight spatial and temporal regulation of Cyclin dependent kinase 1 (Cdk1) activity (Nurse, 1990). Control of Cdk1 activity relies on the balance of power between two highly conserved antagonistic regulatory forces; Cdc25 phosphatases and Wee-like inhibitory kinases (Wee1 and Myt1 kinases). Cdc25 opposes Wee-like kinase by removing the inhibitory phosphate on Cdk1 via dephosphorylation (Fantes, 1981). These core cell cycle machineries exist in both meiotic and mitotic M-phase, and are usually considered analogous. Yet, the cytological events of meiosis and mitosis are dramatically different. Unlike mitosis, meiotic division occurs in two phases (MI and MII) without an intervening S-phase and homologous chromosome separation, not sister chromatid segregation, occurs during meiotic G2/MI progression. As a result of these differences, the mechanism for regulating Cdk1 activity during mitosis must be adapted to account for the complex meiotic cytologies.

Drosophila has two Cdc25 phosphatases, String ($Cdc25^{Stg}$) and Twine ($Cdc25^{Twe}$), with distinct spatial patterns of expression. $Cdc25^{Stg}$ phosphatase is expressed exclusively in mitotically dividing cells (Edgar and O'Farrell, 1990), while $Cdc25^{Twe}$ phosphatase is expressed both in mitotically dividing pre-blastoderm embryos and meiotically dividing germ cells (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993; Di Talia et al., 2013; Farrell and O'Farrell, 2013). The only known function of both phosphatases is Cdk1 activation via dephosphorylation. Male spermatocytes carrying mutation in *cdc25^{twe}* gene blocked the G2/MI transition due to failure to activate Cdk1,

resulting in male sterility (Alphey et al., 1992; White-Cooper et al., 1993). Genetic interactions between Cdc25^{Twe} and a known G2 phase specific inhibitor of Cdk1, Roughex, show that the mechanism of Cdk1 activation during meiotic entry is more complicated. Increasing the gene dosage of *roughex* had no effect on the G2/MI transition but blocked the execution of MI/MII transition, while *roughex* mutants attempted an extra MII-like division, which was rescued by lowering the gene dosage of *cdc25^{twe}* (Gönczy et al., 1994; Sigrist et al., 1995). These data demonstrate the complexity of the process of meiotic G2/MI transition in spermatocytes via Cdk1 regulation.

The complex nature of the meiotic G2/MI transition was also demonstrated by data showing that some aspects of the meiotic G2/MI transition including chromosome condensation and nuclear envelope breakdown still occur in *cdc25^{twe}* mutants (Alphey et al., 1992; White-Cooper et al., 1993). Although these unexpected results could reflect the existence of residual Cdk1 activity in *cdc25^{twe}* mutant spermatocytes, the occurrence of chromosome condensation and nuclear envelope breakdown in *cdk1^{ts}* and *cdc25^{twe}*, *cdk1^{ts}* double mutant spermatocytes argue against this explanation (Sigrist et al., 1995). An alternative hypothesis is that the G2/MI transition in male meiosis could be mediated by a combination of Cdc25^{Twe}-dependent and -independent activation of Cdk1, thus in the *cdc25^{twe}* mutant, the Cdc25^{Twe}-independent steps can still occur (Sigrist et al., 1995). Consistent with this hypothesis, Cdc25-independent activation of Cdk1 has been reported for meiotic entry in *Xenopus* oocytes (Gaffre et al., 2011). This mechanism is thought to involve inhibition of Myt1 activity via rapid

accumulation of Cdk1/cyclin B, such that the increased Cdk1/Cyclin titrate Myt1 kinase activity, thereby escaping inhibitory phosphorylation (Gaffre et al., 2011). Another possibility is that the proposed Cdc25^{Twe}-independent mechanism may reflect a role for Cdc25^{Stg} phosphatase during the meiotic G2/MI transition in spermatocytes. Although the *Cdc25^{Stg}* mRNA was detected mainly in the mitotically dividing cells of the male germline (Alphey et al., 1992), the protein could persist at low levels in spermatocytes and play a dispensable role during meiotic entry, analogous to the functional redundancy of the two Cdc25 phosphatases in regulating Cdk1 activity during the mid blastula transition (MBT) in *Drosophila* embryo (Di Talia et al., 2013; Farrell and O'Farrell, 2013). Indeed, observations that Cdc25^{Twe} and Cdc25^{Stg} activities are regulated primarily post-transcriptionally via protein stability support the possibility that Cdc25^{Stg} may perdure for an extended period until meiotic G2/MI in spermatocytes (Di Talia et al., 2013; Farrell and O'Farrell, 2013).

In this study, we assessed the possibility that a Cdc25^{Twe}-independent activation of Cdk1 might play a role during meiotic G2/MI and MI/MII. We did this by expressing fluorescently tagged Cdk1 wild type (Cdk1WT-GFP) and phospho-acceptor mutant (Cdk1(Y15F-GFP) proteins in *cdc25^{twe}* mutant testes, using a spermatocyte specific promoter. Taking the advantage of the fact that endogenous Cdk1 proteins are not activated in *cdc25^{twe}* mutant spermatocytes, we demonstrated that Cdc25^{Twe}-dependent activation of Cdk1 was largely dispensable during meiotic G2/MI but absolutely required for the MI/MII transition. We therefore concluded that the Cdc25^{Twe}-independent activation of

Cdk1 during meiotic G2/MI transition could be a conserved feature of meiosis.

4.2 MATERIALS AND METHODS

Immunocytochemistry

For both whole mount and squashed testes preparations, 1 to 2- day old adult male flies were dissected in freshly prepared testis buffer (10mM Tris, 183 mM KCL, 47mM NaCl) at room temperature and processed by standard protocols (Bonaccorsi, 1998 #30). The tissue preparations were then transferred to poly-lysine coated slides and squashed with siliconized cover slips, frozen immediately in liquid nitrogen and moved to chilled ethanol (-20° C). Tissues were fixed in 4% paraformaldehyde (freshly prepared from 16% stock from EM Grade, Electron Microscopy Sciences), quickly washed once with PBT (PBS+0.1% TritonX) and then permeabilized in PBT+ sodium deoxycholate for one hour. After this, tissue samples were washed in PBT and blocked with PBTB for 1 hour. Finally, the samples were incubated in primary antibodies as follows: rabbit anti-Phospho-Histone H3 (Upstate; 1:2000 dilution) to label mitotic chromosomes, mouse anti-spectrin obtained from the Developmental Studies Hybridoma Bank (DSHB; 1:500) to label fusomes, rabbit anti-gamma-tubulin (1:100) as a centrosome marker, mouse anti-beta-tubulin to label centrosome and spindle, and mouse anti-Cyclin A (DSHB 1:10) to stage the meiotic G2/M transition. After incubation in primary antibody overnight at 4° C, tissue preparations were washed in PBT 2X (15min each time) and then incubated in the appropriate fluorescent secondary antibodies (anti-rabbit, anti-mouse, anti-rat secondary antibodies coupled with Alexa-488 and Alexa-568 from Molecular Probes used at 1:1000 dilutions) for 2 hours at room temperature. The preparations were then washed in PBT twice

(15min each time) at room temperature and then mounted in Vectashield (Vector Laboratories).

Sterility assay:

Single males of the appropriate genotypes were kept for 24 hours in a vial along with 5 age-matched *yw* control virgin flies. The adult flies were successively transferred into new vials and the number of progeny from each vial was counted as a measure of male fertility. For each genotype, 20 single male crosses were set up. The *yw* single males were crossed with 5 *yw* virgins as the control. The average number of progeny per male, along with standard deviation, was calculated for each genotype.

Western Blot analysis

One to two day old adult testes of the appropriate genotype were dissected in PBS and placed in freshly prepared 1X PBS on ice. For each sample, 5 pairs of testes were homogenized in SDS–PAGE sample buffer containing 2 mm vanadate and 10 mm NaF as phosphatase inhibitors, and boiled for 5 min. The proteins were separated by electrophoresis on a 10% acrylamide gel, then transferred to a Hybond P membrane blot (Amersham). The blot was probed with mouse antibodies (1:1000) directed against phospho-mitotic epitopes (MPM2, Cell Signaling Technology) over night at 4°. The blot was reprobed with antibodies against the Cdk1 conserved PSTAIRE motif (1: 5000) and Actin (1: 5000; Chemicon). Proteins were detected using anti- rabbit or anti-mouse secondary

antibodies conjugated to horseradish peroxidase diluted 1: 5000 (Amersham) and a GE Healthcare ECL Plus chemiluminescence kit.

4.3 RESULTS

Centrosome elongation and migration occurs normally in late G2 phase *cdc25*^{twe} mutant spermatocytes

It was previously shown that *cdc25*^{twe} mutant spermatocytes failed to assemble spindles (Alphey et al., 1992; White-Cooper et al., 1993; Sigrist et al., 1995), suggesting a failure in a Cdk1/Cyclin facilitated but centrosome-mediated process of spindle assembly (Blangy et al., 1997; Nigg, 2001; Kramer et al., 2004; Crasta et al., 2006). To further characterize this aspect of the *cdc25*^{twe} mutant phenotype we examined centriole dynamics during the prolonged G2 phase in wild type and *cdc25*^{twe} mutant spermatocytes by immunostaining using γ-tubulin antibodies that mark the centriole. *Drosophila* spermatocytes normally undergo a 4-day developmental G2 phase arrest, followed by meiotic entry (G2/MI). The prolonged G2 phase arrest can be conceptually divided into 6 stages based on the nuclear morphology (Cenci et al., 1994), with S1 - S4 being early to mid- G2 phase and S5 – S6 considered late G2 phase.

As a control, we first examined centriole dynamics in *yw* spermatocytes throughout the G2 phase leading up to the meiotic spindle formation. Centrioles appeared as duplicated foci during early G2 phase, then became elongated as the centrosomes migrated apart during late G2 phase and formed a rounded pericentriolar mass at the pole during pro-metaphase I and anaphase I (Figure 4-1). Next, we examined wild type and *cdc25*^{twe} mutant spermatocytes concurrently. Centriole dynamics in wild type spermatocytes were similar to the control described in Figure 4-1, appearing as duplicated foci during early G2 phase

(Figure 4-2A), that became elongated as the centrosomes migrated apart during the late G2 phase (Figure 4-2C) and formed a rounded pericentriolar mass at the pole during pro-metaphase I (Figure 4-2E). In *cdc25*^{twe} mutant spermatocytes, the duplicated centrioles also separated, elongated and migrated to the poles (Figure 4-2B & D). The pericentriolar mass was not properly formed in *cdc25*^{twe} mutants, however (Figure 4-2F). These data indicate that Cdk1 activation via Cdc25^{Twe} phosphatase may not be required for this aspect of meiotic G2/MI entry.

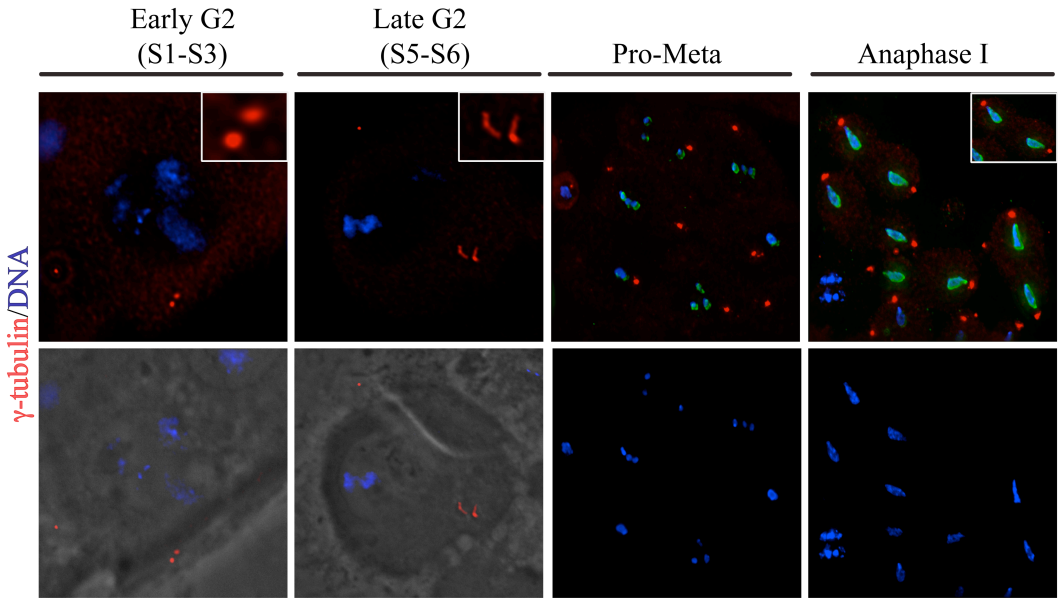

Figure 4-1: Dynamic behavior of centrioles in *Drosophila* spermatocytes.

Gamma-tubulin marks centrioles (red) and anti-PH3 labels M-phase antigens (green), while the DNA is shown in blue. Centrioles appear as paired structures in early G2 phase, but become elongated during the late G2 phase and start migrating to the opposite poles. At pro-metaphase, centrioles at the poles exhibit a peri-centriolar mass. Each secondary spermatocyte subsequently inherits a pair of centrioles following anaphase I.

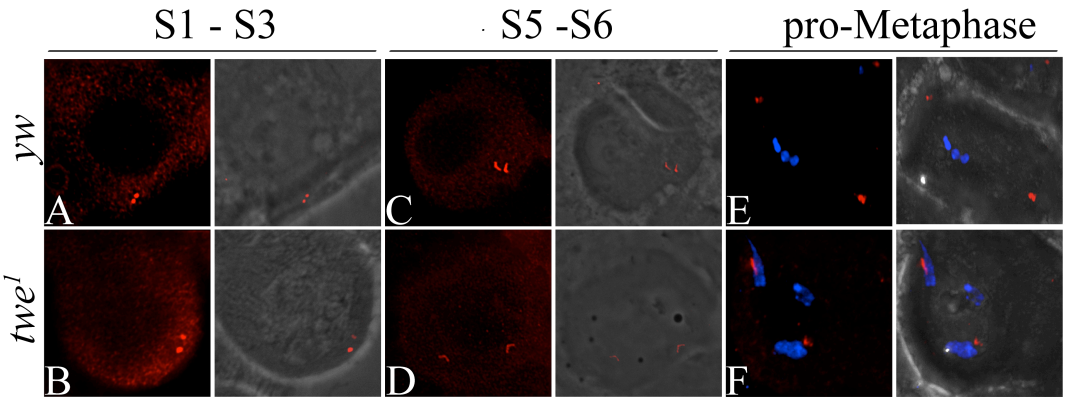

Figure 4-2: Centriole elongation and migration occur unpertubed in *cdc25^{twe}* mutant spermatocytes.

γ-tubulin antibodies (red) were used to aessess cenriole dynamics in fixed samples of *cdc25^{twe}* mutant spermatocytes. (A, C and E) Centriole dynamic in *y,w* control spermatocytes. During early G2 (S1 – S3), centrioles appear as duplicated foci (A). Later during G2 phase (S5 – S6), centrioles appear as a pair of elongated structures (C) and finally become a mass of centrosomes at each pole during pro-metaphase. Similar centriole dynamics is observed in *cdc25^{twe}* mutant spermatocytes (B, D and F). Duplicated centrioles (B) also elongate (D) and migrate to the poles (F) in *cdc25^{twe}* mutant spermatocytes.

Differential localization of Cdk1-GFP proteins expressed in *cdc25^{twe}* mutant spermatocytes

The observations that chromosome condensation and nuclear envelope breakdown could still occur in *cdc25^{twe}* mutant spermatocytes (White-Cooper et al., 1993; Sigrist et al., 1995) suggest that Cdc25^{Twe}-dependent activation of Cdk1 is only required for certain aspects of meiotic G2/MI transition. To assess this possibility, we generated GFP-tagged *Cdk1* transgenes under the control of spermatocyte specific β-Tubulin (tv3) promoter, allowing us to stably express transgenic Cdk1 fusion proteins in a *cdc25^{twe}* mutant background. We used four constructs: *tv3-Cdk1WT-GFP*, *tv3-Cdk1(T14A)-GFP*, *tv3-Cdk1(Y15F)-GFP* and *tv3-Cdk1(T14A,Y15F)-GFP*. Males expressing Cdk1(T14A,Y15F)-GFP in a wild type background were sterile and excluded from this study. This was consistent with previous data showing that completely non-inhibitable Cdk1 is not a suitable tool for *in vivo* analysis of the G2/M transition because it causes extensive genomic damage in addition to abrogating the checkpoint (Ayeni et al., 2013).

The only known role for Cdc25^{Twe} is Cdk1 activation via dephosphorylation and Cdk1 activity facilitates its own nuclear translocation at prophase (Gavet and Pines, 2010). To examine whether this aspect of meiotic G2/MI was Cdc25^{Twe}-dependent we assessed the spatial localization of our fluorescent Cdk1 fusion proteins in *cdc25^{twe}* mutant spermatocytes. During late G2 phase (S5 - S6), the Cdk1WT-GFP and Cdk1(T14A)-GFP proteins were localized predominantly in the cytoplasm of *cdc25^{twe}* mutant spermatocytes, whereas Cdk1(Y15F)-GFP was both cytoplasmic and nuclear in this background

(Figure 4-3A). By pro-metaphase, however, Cdk1WT-GFP and Cdk1(T14A)-GFP as well as Cdk1(Y15F)-GFP proteins were predominantly nuclear in *cdc25*^{twe} mutant spermatocytes (Figure 4-3B). These observations suggested that Y15 inhibitory phosphorylation was required to prevent pre-mature nuclear accumulation of Cdk1 during late stages of the prolonged G2 phase. Importantly, our results showed that the nuclear translocation of Cdk1 proteins during meiotic G2/MI was not affected in a *cdc25*^{twe} mutant background.

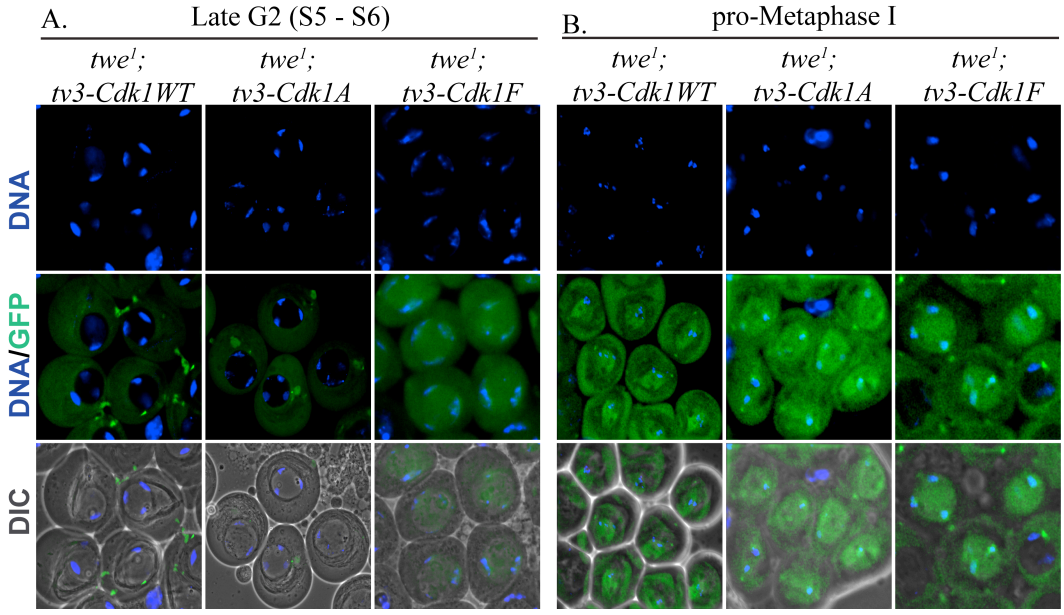

Figure 4-3:Cdk1 phospho-form lacking inhibitable Y15 residue accumulates prematurely in spermatocytes nucleus during the late G2 phase.

Fluorescence microscopy of unfixed squashed preparations from *cdc25*^{twe} mutants expressing Cdk1(WT)-VFP, Cdk1(T14A)-GFP or Cdk1(Y15F)-GFP, and stained with live DNA marker (blue). (A) Both Cdk1(WT)-VFP and Cdk1(T14A)-GFP were mainly cytoplasmic during the late G2, while Cdk1(Y15F)-GFP had become predominantly nuclear. (B) During pro-metaphase, however, all the three transgenic proteins were primarily in the nucleus, indicating that the cells had entered into M-phase.

Apart from the nucleo-cytoplasmic re-distribution of Cdk1 proteins, we also noted a characteristic fusome localization of the transgenic Cdk1 proteins. Fusomes are germline-specific membrane-bound organelles related to endoplasmic reticulum that are thought to be important for differentiation-dependent vesicle trafficking (Lin and Spradling, 1995; Lilly et al., 2000). To explore this novel observation, we labeled fusomes using antibodies against its structural component Hts (Zaccai and Lipshitz, 1996). In both the *yw* control and *cdc25*^{twe} mutant spermatocytes, the fusomes appear as an elaborately branching structure (Figure 4-4). During G2 phase (S1 – S6) the transgenic Cdk1WT-GFP proteins localized to the Hts-labeled branching fusome structures, which appeared to become fragmented during M-phase when Cdk1WT-GFP proteins become nuclear (Figure 4-5A). The elaborate branching fusome structures later re-appeared during the spermatid stage, but this time without Cdk1WT-GFP protein localization (Figure 4-5A). Similar fusome localization was observed in *cdc25*^{twe} mutant spermatocytes expressing Cdk1(T14A)-GFP (data not shown). In contrast, the fusome appeared fragmented throughout G2 phase and M-phase in *cdc25*^{twe} mutant spermatocytes expressing Cdk1(Y15F)-GFP (Figure 4-5B). Remarkably, however, a branching fusome structure did appear during the spermatid stage of *cdc25*^{twe} mutants expressing Cdk1(Y15F)-GFP (Figure 4-5B). These results showed for the first time that Cdk1 localizes on the fusomes, suggesting that inhibitory phosphorylation of Cdk1 could be required for maintaining this structure during G2 phase.

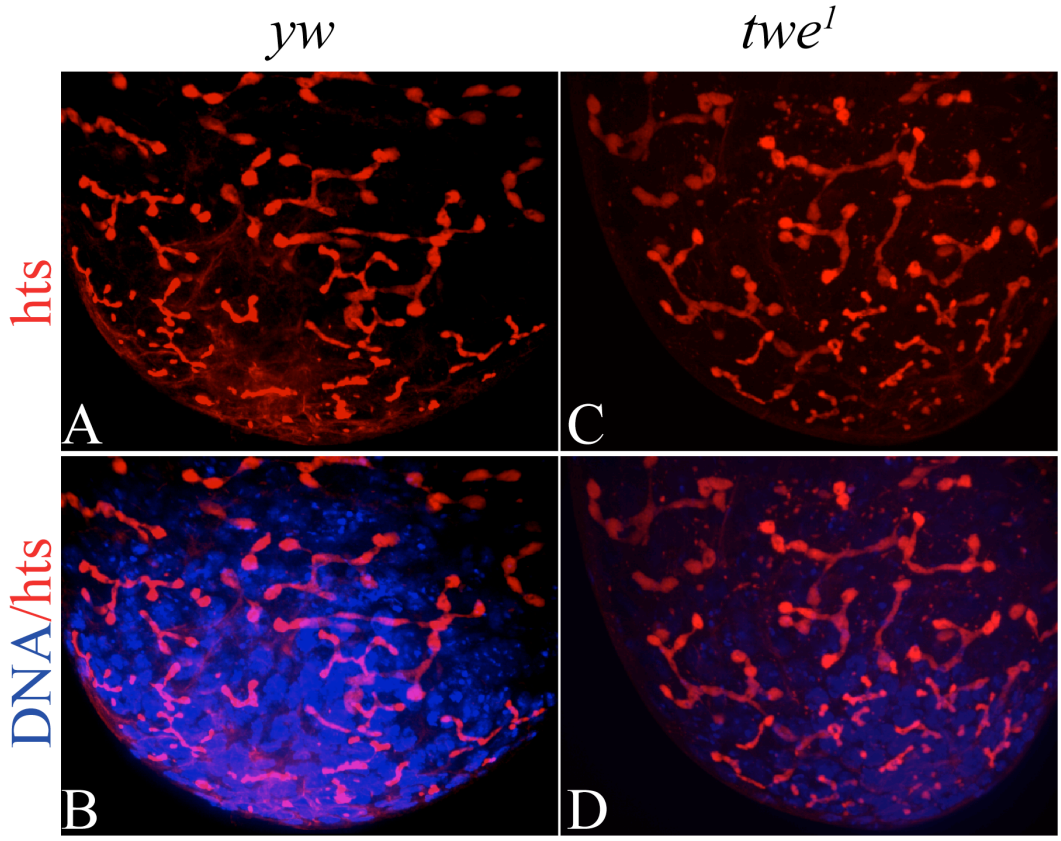

Figure 4-4: Fusome structures are not affected in *cdc25^{twe}* mutant spermatocytes.

The fusomes are marked in red by Hts, A & C and DNA staining is shown in blue, B & D. (A and B) In *yw* control, fusomes (red) appears as elaborate branching structures. (C and D) Similar branching fusome structures are present in *cdc25^{twe}* mutant spermatocytes.

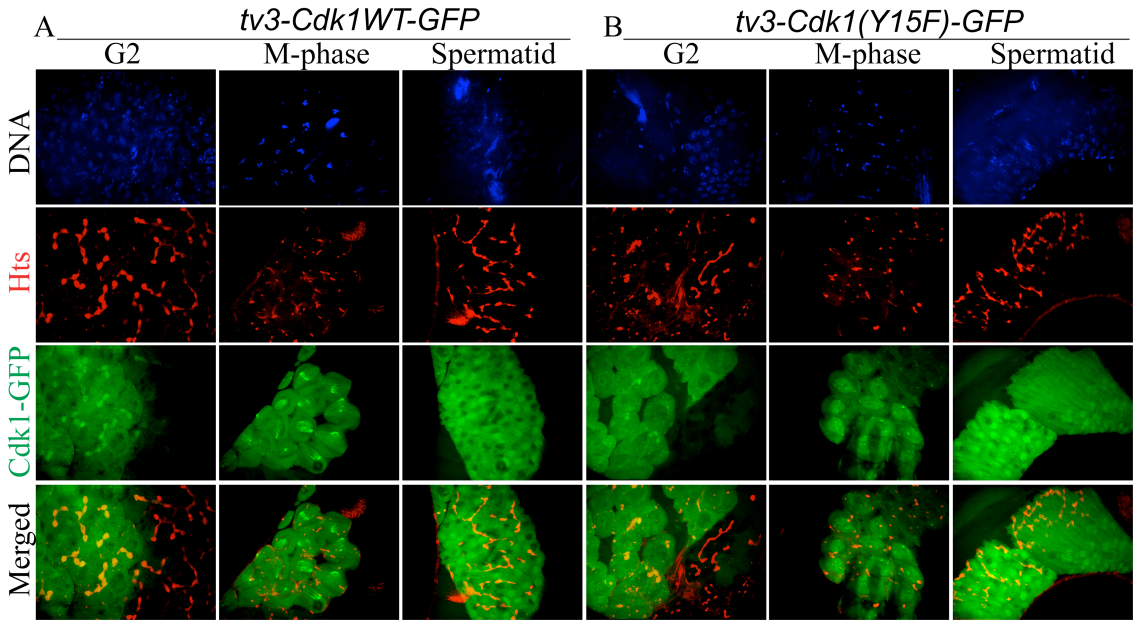

Figure 4-5: Cdk1WT-GFP proteins localize to the fusome in spermatocytes.

Cdk1-GFP protein is shown in green, while the fusomes are marked in red by Hts and the DNA staining, shown in blue, is used to stage the cysts. (A) In *cdc25*^{twe} mutants expressing Cdk1(WT)-GFP, fusomes appear as elaborate branching structures in G2 phase that co-localize with Cdk1, become fragmented at M-phase when Cdk1 proteins are predominantly nuclear and re-appear during spermatid differentiation. (B) In contrast, fusome fragmentation was observed throughout G2 phase in *cdc25*^{twe} mutant expressing Cdk1(Y15F)-GFP, persisted through M-phase but the elaborate branching fusomes re-appeared during spermatid stage.

Expression of wild type Cdk1 restores G2/MI progression in *cdc25^{twe}* mutant spermatocytes

Having established a suitable system for stably expressing transgenic Cdk1 fusion proteins in *cdc25^{twe}* mutant background, we explored the possibility of a Cdc25^{Twe}-independent mechanism during G2/MI transition. We expressed Cdk1WT-GFP, Cdk1(T14A)-GFP and Cdk1(Y15F)-GFP in the *cdc25^{twe}* mutant background and analyzed progression through meiotic G2/MI using anti-Phospho histone H3 (PH3) to label condensed MI chromosomes (red), anti-β tubulin to reveal meiotic spindle formation (green) and anti-cyclin A as a marker for the G2/MI transition. Consistent with previous reports, chromosome condensation was observed in *cdc25^{twe}* mutant spermatocytes but neither spindle formation (green) nor PH3 (red) staining occurred (Figure 4-6A and 6B). In *cdc25^{twe}* spermatocytes expressing wild type Cdk1WT-GFP, however, a bipolar spindle was formed and the cells were PH3 positive (Figure 4-6C and 6D). Similar results were observed when Cdk1(T14A)-GFP (Figures 4-6E and 4-6F) or Cdk1(Y15F)-GFP (Figure 4-6G and 4-6H) were expressed in a *cdc25^{twe}* mutant background. These results demonstrate that expression of transgenic Cdk1 proteins could promote specific aspects of the meiotic G2/MI that were lacking in *cdc25^{twe}* mutant spermatocytes.

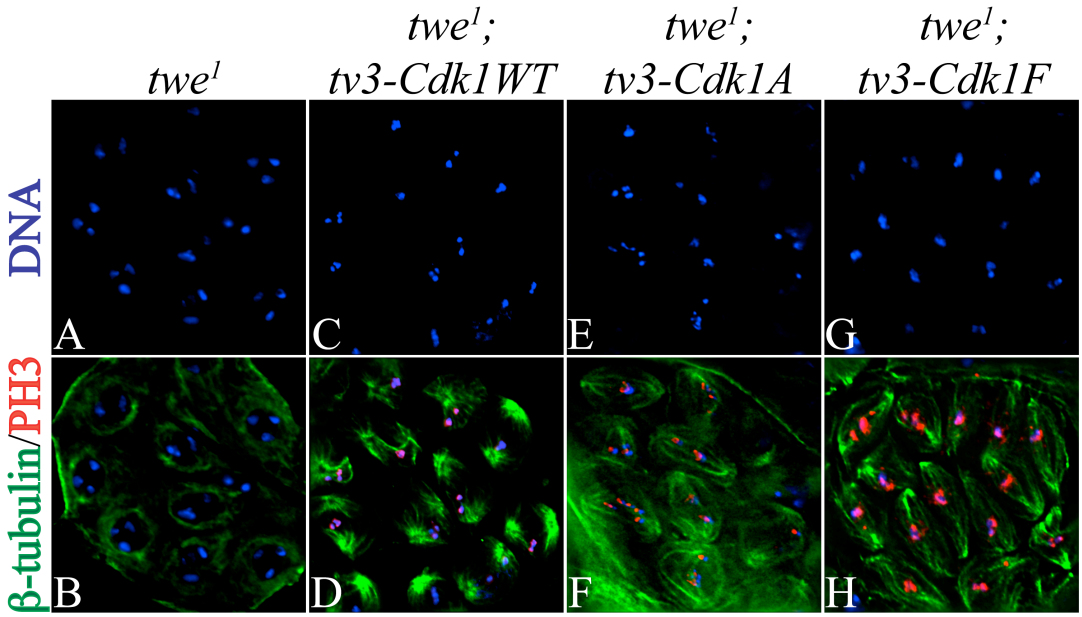

Figure 4-6: Expression of transgenic Cdk1 phospho-forms restores key aspects of meiotic entry in *cdc25^{twe}* mutants.

Dividing spermatocytes were stained with β-tubulin (green in B, D, F and H), PH3 (red in B, D, F and H) and DNA (blue). Neither spindle formation nor PH3 staining was observed in *cdc25^{twe}* mutant spermatocytes (A and B). Meiotic entry in spermatocytes expressing Cdk1WT-GFP (C and D), Cdk1(T14A)-GFP (E and F) or Cdk1(Y15F)-GFP (G and H) in *cdc25^{twe}* mutants as shown by spindle formation and PH3 incorporation.

Next, we examined the G2/M transition in *cdc25^{twe}* mutants by ectopically expressing Cdk1 fusion proteins to assess cyclin A localization during G2 phase and pro-metaphase. Cyclin A localizes predominantly in the cytoplasm during G2 phase but then translocates into the nucleus where it is subsequently degraded at metaphase I, when the chromosomes appear as three highly condensed DNA clumps, indicating a successful G2/MI transition (Lin et al., 1996). In *cdc25^{twe}* mutants, cyclin A was predominantly cytoplasmic at pro-metaphase I and persisted in the 16-cell spermatids (Figure 4-7A), indicating a failure of both nuclear translocation and degradation typical of MI phase. In *cdc25^{twe}* mutant spermatocytes expressing Cdk1WT-GFP, cyclin A was predominantly nuclear during pro-metaphase I as revealed by the DNA condensation and nuclear localization of the GFP-tagged transgenic Cdk1 proteins which later disappeared during metaphase I (Figure 4-7B). Expression of Cdk1(T14A)-GFP (Figure 4-7C) or Cdk1(Y15F)-GFP (Figure 4-7D) in *cdc25^{twe}* mutant spermatocytes also produced nuclear cyclin A at pro-metaphase I, which was rapidly degraded during metaphase I. Collectively, these results demonstrate that expression of wild type Cdk1 restored aspects of meiotic entry that were otherwise missing in *cdc25^{twe}* mutant spermatocytes, independent of Cdc25^{Twe} phosphatase activity. These data also showed that elevating Cdk1 in *cdc25^{twe}* mutant spermatocytes restored cyclin A degradation in a *cdc25^{twe}* mutant background. Taken together, these results suggest the existence of mechanism(s) other than Cdc25^{Twe} for activating Cdk1 during meiotic G2/M I transition.

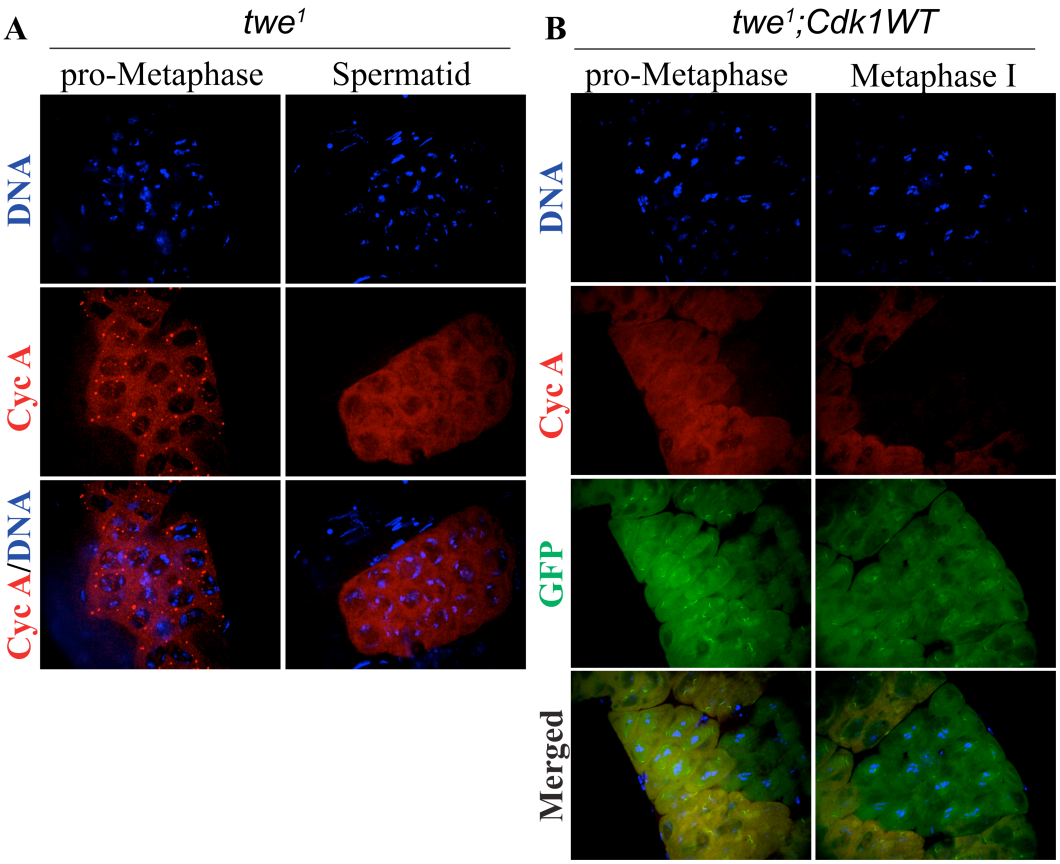

Figure 4-7: Cyclin A degradation reveals meiotic entry in *cdc25^{twe}* mutants expressing transgenic Cdk1 proteins.

Cyclin A is shown in red, DNA in blue and the transgenic Cdk1 is in green. (A) Cyclin A remained cytoplasmic in pro-metaphase and persisted until spermatid differentiation in *cdc25^{twe}* mutants. In cdc25^{twe} mutants expressing Cdk1WT-GFP (B), Cdk1(T14A)-GFP (C) or Cdk1(Y15F)-GFP (D), cyclin A was evenly distributed throughout the cell during pro-Metaphase as marked by nuclear accumulation of Cdk1 proteins but disappeared at metaphase as expected for dividing cells. (NB: Refer to the next page for Figure 4-7C & D).

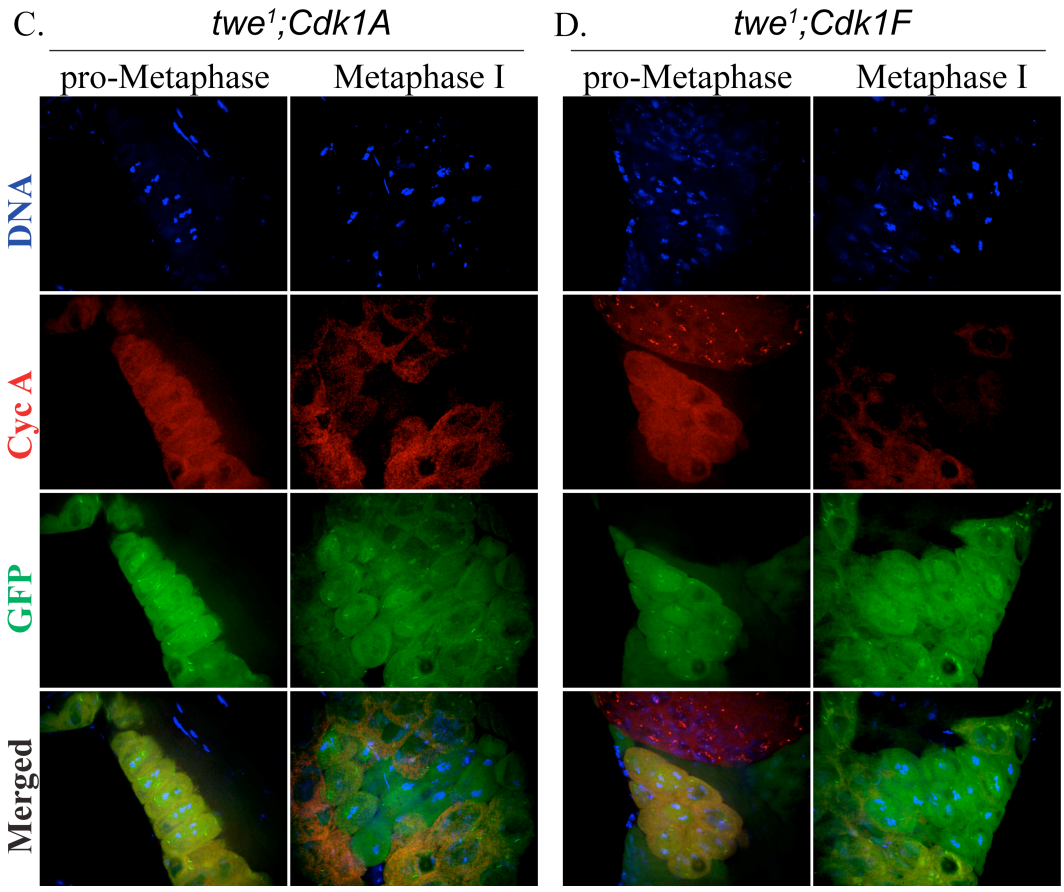
C.
twe1;Cdk1A
D.
twe1;Cdk1F
pro-Metaphase
Metaphase I
pro-Metaphase
Metaphase I
DNA
Cyc A
GFP
Merged

Figure 4-7 C & D

Expression of Cdk1WT-GFP in *cdc25^{twe}* spermatocytes restored the appearance of M-phase phosphorylated epitopes

Meiotic G2/MI is characterized by the appearance of phosphorylated epitopes on Cdk1 mitotic substrates that are recognized by MPM2 monoclonal antibodies (Davis et al., 1983; Vandre et al., 1984; Hecht et al., 1987). These MPM2 reactive epitopes are absent in *cdc25^{twe}* mutant spermatocytes (White-Cooper et al., 1993). If the loss of MPM2 epitopes in a *cdc25^{twe}* background is due to failure of Cdk1 activation, then expression of transgenic wild-type Cdk1 protein would not be expected to change this phenotype. To test this prediction, protein samples from testes expressing Cdk1WT-GFP, Cdk1(T14A)-GFP or Cdk1(Y15F)-GFP in *cdc25^{twe}* mutant spermatocytes were resolved on SDS-PAGE and probed with MPM2 antibodies.

Using Actin as a loading control, we observed MPM2-reactive proteins in *yw* control testes that were markedly reduced in *cdc25^{twe}* mutants (Figure 6). In *cdc25^{twe}* mutant spermatocytes expressing Cdk1WT-GFP, MPM2 reactive epitopes were detected at similar levels as the *yw* control (Figure 6). Similar levels of the MPM2 reactive epitopes were also observed in *cdc25^{twe}* testes expressing Cdk1(T14A)-GFP or Cdk1(Y15F)-GFP (Figure 4-8). Taken together, these data showing that expression of Cdk1WT-GFP, Cdk1(T14A)-GFP or Cdk1(Y15F)-GFP restored the Cdk1 phosphorylated epitopes, implies the existence of a Cdc25^{Twe}-independent mechanism for activating Cdk1 during the meiotic G2/MI transition.

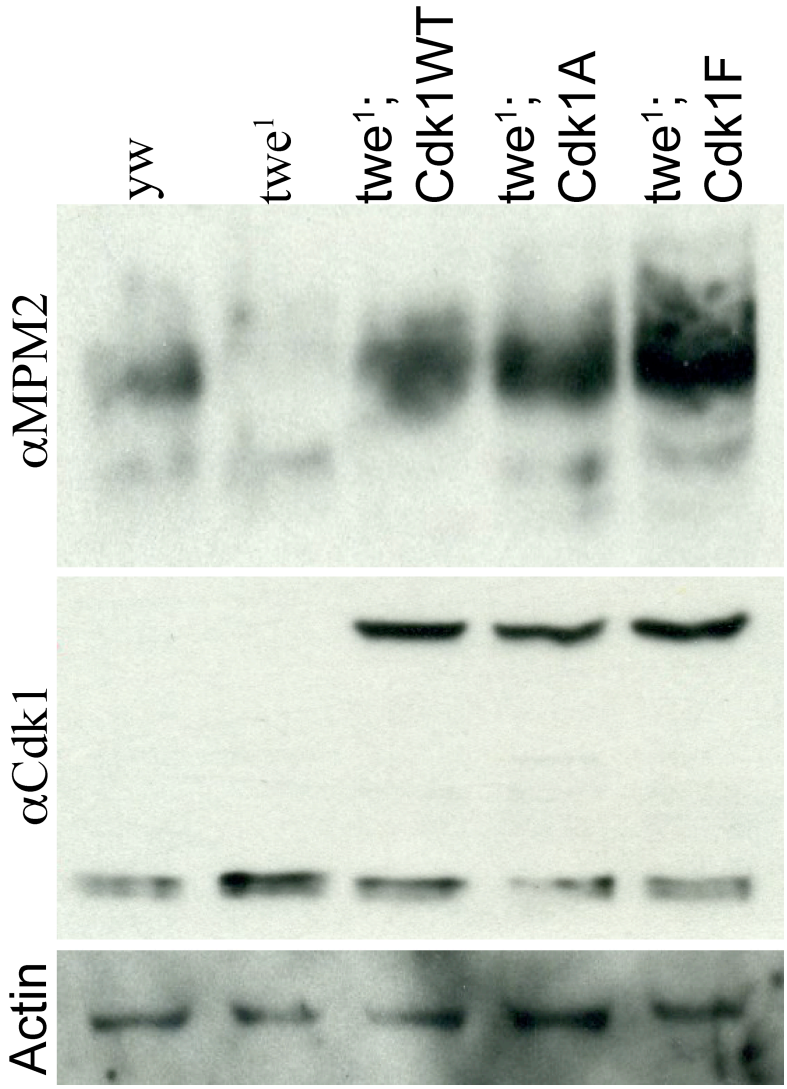

Figure 4-8: Restoration of M-phase phosphorylated epitopes in *cdc25^{twe}* mutants expressing transgenic Cdk1 proteins.

Protein extract from testes of different genotypes were subjected to western blotting using MPM2 antibodies. Extracts from wild-type testes show normal MPM2 levels, whereas *cdc25^{twe}* mutants had drastically reduced MPM2 phosphoepitopes. The MPM2 immunoreactive epitopes were restored in *cdc25^{twe}* mutant expressing Cdk1(WT)-GFP, Cdk1(T14A)-GFP or Cdk1(Y15F)-GFP. Actin was used as a loading control.

cdc25^twe^ spermatocytes expressing Cdk1WT-GFP successfully complete meiotic G2/MI but not the MI/MII transition.

Meiosis has two phases of division, G2/MI and MI/MII phases. Cysts with 32 primary spermatids result from successful MI division whereas cysts with 64 secondary spermatids result from MII division (Sigrist et al., 1995). In *cdc25^twe^* mutant spermatocytes expressing Cdk1WT-GFP, 76% of the cysts contained 32 spermatids (38/50) with the remaining cysts containing 16 spermatids (Figure 4-9A), demonstrating partial restoration of meiotic G2/MI progression. Similarly, 78% of *cdc25^twe^* mutants expressing Cdk1(T14A)-GFP contained 32 spermatids (36/45), with the remaining having 16 spermatids (Figure 4-9B). On the contrary, almost all cysts scored in *cdc25^twe^* mutant expressing Cdk1(Y15F)-GFP contained 64 spermatids (48 out of 50 i.e 96%), demonstrating proper execution of both meiotic G2/MI and MI/MII (Figure 4-9C). The remaining 4% (2 out of 50) were difficult to characterize as either 32 or 64 spermatids. Thus, transgenic wild type Cdk1 proteins were only able to promote completion of G2/MI, whereas Cdk1(Y15F)-GFP expression was sufficient for restoring both G2/MI and MI/MII. These results suggest that Cdk1 activation via Y15-specific dephosphorylation is specifically required for the MI/MII transition of male meiosis.

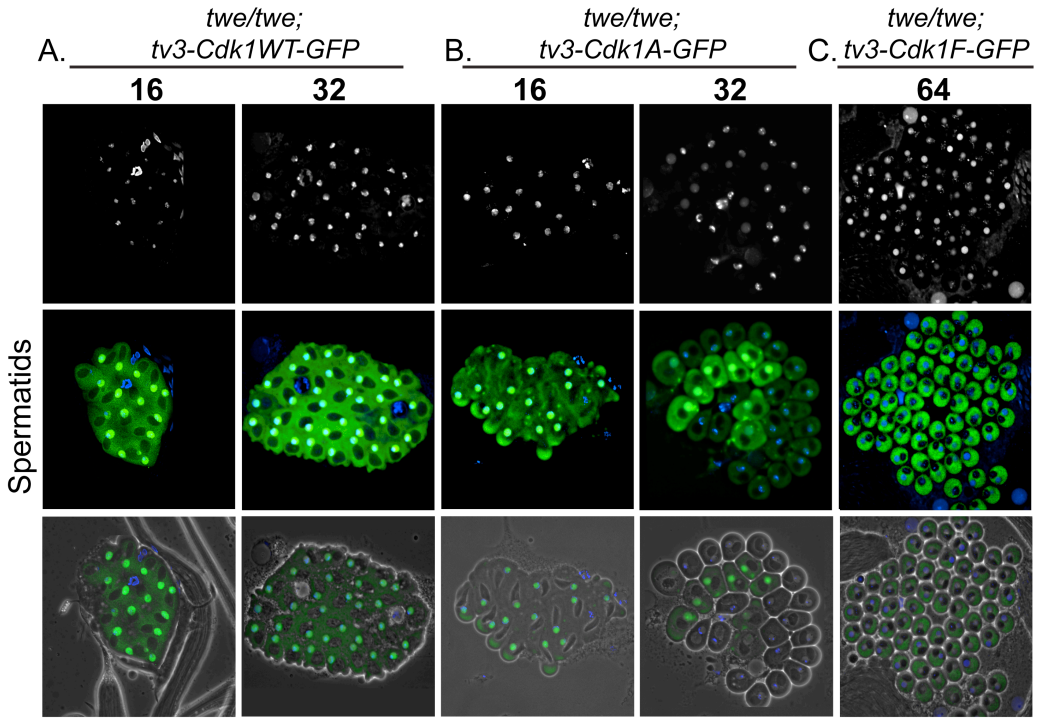

Figure 4-9: *cdc25*^{twe} spermatocytes expressing Cdk1WT-GFP successfully executed G2/MI but not second meiotic division.

Fluorescence microscopy examination of spermatids in unfixed squashed preparations from *cdc25*^{twe} mutants expressing Cdk1(WT)-GFP, Cdk1(T14A)-GFP or Cdk1(Y15F)-GFP, showing nuclear accumulation of Cdk1 (green). (A) *cdc25*^{twe} mutants expressing Cdk1(WT)-GFP contained mostly 32-spermatid cysts (76% of the cysts counted) and occasionally 16-spermatid cysts, indicating the occurrence of only one meiotic division. (B) Similar results were obtained in *cdc25*^{twe} mutants expressing Cdk1(T14A)-GFP with 78% of the cysts containing 32-spermatids while the rest was 16-spermatid cysts. (C) On the contrary, *cdc25*^{twe} mutants expressing Cdk1(Y15F)-GFP contained wild type 64-spermatid cysts.

Expression of Cdk1(Y15F)-GFP proteins restores fertility in *cdc25^{twe}* mutant testes

Having shown that expression of Cdk1(Y15F)-GFP could apparently rescue both MI and MII progression in *cdc25^{twe}* mutant spermatocytes, we wondered if the resulting haploid cells would produce functional sperm. To test sperm functionality, we performed sterility assays using previously described protocols (Regan and Fuller, 1988; Zhang et al., 2004). As expected, *cdc25^{twe}* mutant flies produced zero progeny, whereas heterozygous *cdc25^{twe}* /+ control males produced an average of 122±19 progeny per male (Figure 4-10). In *cdc25^{twe}* mutants expressing Cdk1WT-GFP or Cdk1(T14A)-GFP, there were an average of 2±10 and 3±6 progeny produced per male, respectively (Figure 4-10). In *cdc25^{twe}* male flies expressing Cdk1(Y15F)-GFP, however, we observed 90±19 progeny per male (Figure 4-10). These results showing that Cdk1(Y15F)-GFP rescued *cdc25^{twe}* male sterility confirm that Cdk1 dephosphorylation of the Y15 residue was required for producing functional sperm. Collectively, we have provided evidence for the existence of both Cdc25^{Twe}-dependent and independent regulatory mechanisms for Cdk1 activation during male meiosis. Surprisingly, the Cdc25^{Twe}-dependent mechanism appears to be dispensable for meiotic G2/MI but absolutely required for MI/MII progression, whereas Cdc25^{Twe}-independent mechanisms can play a compensatory role that facilitates the G2/MI transition in the absence of Cdc25^{Twe} activity.

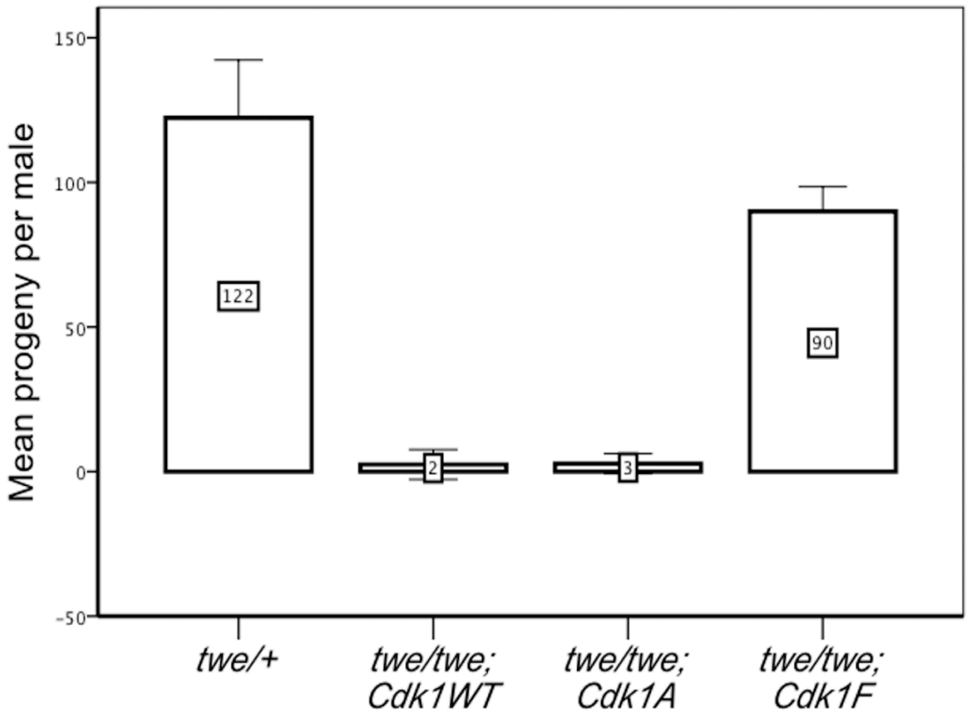

Figure 4-10: Expression of Cdk1(Y15F)-GFP in *cdc25^{twe}* mutant spermatocytes rescues male sterility.

Individual males of indicated genotypes were crossed to 5 *yw* females and the number of progeny from the cross was counted. For each genotype, at least 20 males were tested. As shown in the graph, an average of 122±19 progeny per male was obtained in the *cdc25^{twe}*/+ control, whereas *cdc25^{twe}* mutants expressing *tv3-Cdk1WT-GFP* or *tv3-Cdk1(T14A)-GFP*, produced an average of 2±10 and 3±6 progeny per male, respectively. An average of 90±19 progeny per male was obtained in *cdc25^{twe}* mutants expressing *tv3-Cdk1(Y15F)-GFP*.

4.4 DISCUSSION

In this study, we analyzed the spatial localization of transgenic Cdk1 fusion proteins and the regulatory mechanisms for Cdk1 activation during the complex meiotic G2/MI and MI/MII transitions. We found that transgenic Cdk1 proteins that could not be phosphorylated on the Y15 residue prematurely accumulated in the nucleus during the prolonged spermatocyte G2 phase arrest, indicating an important role for Y15 phosphorylation to inhibit Cdk1 as a regulatory mechanisms that prevents precocious access of active Cdk1 to nuclear substrates (Ookata et al., 1992; Heald et al., 1993; Wells et al., 1999; Gavet and Pines, 2010). The Cdk1 fusion proteins also localized to the fusome, a branched, membrane-associated cytoskeletal structure that inter-connects the spermatocytes of each cyst, in *Drosophila*. Wild type Cdk1 transgenic proteins expressed in *cdc25^{twe}* mutant spermatocytes promoted spindle nucleation, generated MPM2 and phospho-histone H3 epitopes. The resulting cysts contained 32 spermatids, indicating completion of meiotic G2 and MI phases. Thus, simply increasing Cdk1 levels was sufficient for meiosis I, in the absence of Cdc25^{Twe} phosphatase activity. Wild type Cdk1 expression was incapable of promoting MI/MII in *cdc25^{twe}* mutant testes, however. Remarkably, *cdc25^{twe}* spermatocytes expressing Cdk1(Y15F)-VFP mutant proteins successfully executed both G2/MI and MI/MII phases, producing fully functional sperm. These data demonstrate for the first time that Cdk1 activation via Cdc25^{Twe} phosphatase is largely dispensable during meiotic G2/MI transition, but required for promoting the MI/MII transition in *Drosophila* spermatocytes.

How could expression of wild type Cdk1 be driving meiotic G2/MI in the absence of Cdc25^{Twe} phosphatase? One possible explanation is that the level of Cdk1 activity required for the meiotic G2/MI transition may depend on a balance between endogenous Cdk1/cyclin and its G2 phase regulators such as Roughex and Myt1. Expression of Cdk1WT-VFP could raise levels of Cdk1 proteins beyond some threshold where they could stoichiometrically titrate the negative regulators, allowing Cdk1 activation. This possibility would be consistent with data from studies of *Xenopus* oocytes, where the key mechanism for regulating meiotic entry involves the balance between cyclin B synthesis and Myt1 activity, instead of Cdc25 activity (Gaffre et al., 2011). Indeed, a similar high level of Cdk1 was also reported to facilitate meiotic entry in rat spermatocytes (Godet et al., 2000).

Our data suggest that meiotic G2/MI can be regulated by both Cdc25^{Twe}-dependent and -independent mechanisms. Multiple data from the current study support the proposed Cdc25^{Twe}-independent mechanism. Ectopically expressing wild type Cdk1 in spermatocytes lacking *cdc25^{twe}* activity promoted prompt cyclin A degradation during pro-metaphase I, nucleated the meiotic spindle, facilitated the appearance of MPM2 and PH3 reactive epitopes and resulted in successful completion of meiotic G2/MI. One possible explanation for this Cdc25^{Twe}-independent mechanism could be a meiotic role for Cdc25^{Stg} phosphatase, at least in this context. An important question, however, is how does Cdc25^{Stg} activity elude detection during meiotic G2/MI? Although several explanations could account for this, the simplest one is that the Cdc25^{Stg} protein

may be present at low levels during meiotic G2/MI but is obscured by peaking of $Cdc25^{Twe}$ proteins in meiotic cells. This proposal would be consistent with the recently described overlapping roles of both Stg and Twe phosphatases during the MBT of *Drosophila* embryogenesis (Di Talia et al., 2013; Farrell and O'Farrell, 2013). Alternatively, the differential role for Cdk1 regulation by $Cdc25^{Twe}$ during meiotic G2/MI and MI/MII could reflect an intrinsic difference between meiotic chromosome behaviors. Indeed, analysis of meiotic division in grasshopper spermatocytes reveals that the information for separating homologous chromosomes during the G2/MI reductional division is inherent within the homologous chromosomes themselves, whereas the MI/MII equational division depends on the spindle property (Paliulis and Nicklas, 2000). Such chromosome-dependent mechanism acting during meiotic G2/MI may rely on a $Cdc25^{Twe}$ independent mechanism.

Besides addressing the role of $Cdc25^{Twe}$ phosphatase in meiotic entry, we also uncovered a novel spatial localization of Cdk1 proteins on fusome structures in *Drosophila* spermatocytes. In light of the previous observations that other key regulators of G2/MI (cyclin A and cyclin B) localize to the fusome in *Drosophila* spermatocytes and oocytes (Eberhart et al., 1996; Lilly et al., 2000; Mathieu et al., 2013), our results showing that Cdk1 is present on the fusome structure validates the proposal that fusome structures act as a center for precisely timed Cdk1 activation in a wave-like manner, to promote synchronous meiotic entry (Lin and Spradling, 1995; Lilly et al., 2000). Therefore, the absence of the fusome branching structures in spermatocytes expressing transgenic Cdk1(Y15F)-VFP

proteins suggests that maintaining the fusome structure depends on proper regulation of Cdk1 activity. Indeed, loss of *myt1* encoding an important Cdk1 inhibitory kinase caused a similar disruption of fusome branching structures in spermatocytes, consistent with this proposal (Jin et al., 2005). Intriguingly, despite disruption of fusome structures in $cdc25^{twe}$ mutant spermatocytes expressing Cdk1(Y15F)-GFP and *myt1* mutant spermatocytes, we did not observe evidence for loss of spermatocyte synchrony, implying that the role of fusome structures may not be crucial for male meiotic divisions. Moreover, the reappearance of a fusome branching structure during spermatid differentiation in $cdc25^{twe}$ mutants expressing Cdk1(Y15F)-GFP suggests that this enigmatic organelle may serve novel functions during spermatid differentiation and/or sperm individualization.

Importantly, our results indicating that the expression of transgenic Cdk1(Y15F)-VFP rescues $cdc25^{twe}$ mutant males sterility could be of interest beyond *Drosophila*. Failure of meiotic division is a common cause of sterility in humans, responsible for infertility affecting over 6% of patients (Chaganti and German, 1979; Cantú et al., 1981; Luetjens et al., 2004; Bonilla and Xu, 2008; Massart et al., 2012). Human azoospermic phenotype is associated with dysfunction of meiotic division and it is astonishingly similar to the meiotic G2/MI arrest in *Drosophila* (Sigrist et al., 1995; Maines and Wasserman, 1999; Xu et al., 2003). Given that the genes involved in meiotic division are broadly conserved in metazoans (Carani et al., 1997; Houston et al., 1998; Maines and Wasserman, 1999; Reijo et al., 2000; Xu et al., 2001), it will be important to determine whether heterologous expression of Cdk1(Y15F) in other organisms

could restore fertility associated with meiotic arrests in azoospermic males, which will be of potential clinical interest.

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5. CONCLUSIONS AND PERSPECTIVES

5.1 BACKGROUND

Studies in yeast, *Drosophila*, *Xenopus* and mammalian systems indicate the critical importance of Cdk1 inhibitory phosphorylation in diverse pre-mitotic checkpoints. The regulatory checkpoints ensure that mitosis is not attempted while DNA replication is going on, following DNA damage, during cell morphogenesis and in response to developmental signals that spatially and temporally coordinate cell proliferation. Unicellular eukaryotes rely on inhibitory phosphorylation of Cdk1 on Y15 for proper mitotic timing and DNA damage-induced pre-mitotic checkpoints (Gould and Nurse, 1989; Rhind et al., 1997), while metazoans utilize two inhibitory sites (T14 and Y15) on Cdk1 to regulate its activity (Draetta et al., 1988; Lehner and O'Farrell, 1990; Krek and Nigg, 1991a; Krek and Nigg, 1991b; Norbury et al., 1991). The T14 residue of Cdk1 is also conserved in yeasts but it does not appear to be a major regulatory site, although Wee1 can phosphorylate the site under certain unusual circumstances. Thus, inhibitory phosphorylation on both T14 and Y15 sites on Cdk1 is a distinctive feature of cell cycle regulation in multicellular eukaryotes, the relevance of which is presently unclear. Why dual phosphorylation evolved in metazoans has eluded scientists for decades and the impact of specific phosphorylated residues on Cdk1-mediated checkpoint mechanisms has remained unclear.

In this study I examined the developmental relevance of single and dual phosphorylation of Cdk1 during *Drosophila* adult development. I undertook tissue specific expression analysis of the genetically engineered Gal4-inducible VFP-tagged wild-type Cdk1, as well as three different Cdk1 phospho-inhibition mutants:

Cdk1(T14A)-VFP, Cdk1(Y15F)-VFP and Cdk1(T14AY15F)-VFP. I presented evidence that different Cdk1 inhibitory phospho-isoforms exhibit distinct functional properties and demonstrated that metazoans evolved a more elaborate Cdk1 regulatory mechanism to orchestrate morphological development. Profound developmental differences were associated with expression of different Cdk1 mutants, indicating that inhibitory phosphorylation of T14 and Y15 residues are non-redundant regulatory mechanisms, with only Y15 phosphorylation being necessary and sufficient for developmental G2 phase arrest. The study suggested rationales for how biochemical differences in Cdk1 inhibitory phosphorylation mechanisms are used to regulate specific stages of animal development.

5.2 G2/M CHECKPOINT ASSAYS: CDK1(Y15F) VERSUS CDK(T14A,Y15F)

A strategy widely employed for studying the role of Cdk1 inhibitory phosphorylation in G2/M checkpoint regulation is to mutate Y15 or both Y15 and T14 inhibitory phosphorylation sites (Gould and Nurse, 1989; Krek and Nigg, 1991b; Norbury et al., 1991; Jin et al., 1996; Blasina et al., 1997; Rhind et al., 1997; Rhind and Russell, 1998). Transient expression of Cdk1(T14A,Y15F) has been used as a tool for assaying G2/M checkpoint. For example, Cdk1(T14A,Y15F) expression induced precocious or catastrophic mitosis when expressed in mammalian cells (Blasina et al., 1997; Heald et al., 1993; Krek and Nigg, 1991b). Similar results were observed with expression of Cdk1(T14A,Y15F) in HeLa cells at levels equivalent to endogenous Cdk1 (Jin et al., 1996). Overexpression of either Cdk1(Y15F) or Cdk1(T14A,Y15F) also

abrogated responses to ionizing radiation in human cell lines, emphasizing the role of Y15 inhibitory phosphorylation in G2 phase checkpoint arrest (Blasina et al., 1997; Fletcher et al., 2002; Jin et al., 1996). Here, however, I provided a relevant developmental context for the *in vitro* observations and demonstrated that Cdk1(Y15F) is the appropriate tool for assaying G2/M checkpoint. Consistent with the *in vitro* observations, my results showed that both Cdk1(T14A,Y15F) and Cdk1-(Y15F)-VFP expression bypassed developmental and DNA damage G2 phase checkpoints. Cdk1(T14A,Y15F) expression, however, was associated with extensive cellular, chromosomal and developmental aberrations, the hallmark of genomic instability, that were not seen with Cdk1(Y15F) expression. These observations demonstrated, for the first time, that Cdk1(Y15F) is the suitable transgenic tool for G2/M checkpoint assays without compromising genome instability.

5.3 DEVELOPMENTAL ROLE FOR Y15 SPECIFIC INHIBITORY PHOSPHORYLATION OF CDK1

It has long been established that inhibitory phosphorylation of Cdk1 is critical for pre-mitotic checkpoint mechanisms in all eukaryotes but the role that dual Cdk1 phosphorylation plays in metazoans remain unclear. Although dWee1-mediated Y15 inhibitory phosphorylation of Cdk1 is indispensable for completing the rapid nuclear divisions of early *Drosophila* embryogenesis, Wee1 activity is dispensable later in development because of partial functional redundancy with dMyt1 (Price et al., 2000; Stumpff et al., 2004; Jin et al., 2008). Thus, the question of whether Cdk1-Y15 phosphorylation serves specific developmental

roles later in development remained unanswered. This thesis examined the role of this regulatory mechanism in the context of *Drosophila* development, by characterizing functional properties of different Cdk1 phospho-inhibited isoforms. I presented evidence that tissue-specific expression of a Cdk1 mutant lacking Y15 phosphorylation could by-pass developmentally or DNA-damage-induced G2/M cell cycle checkpoint arrest and cause apoptosis and cell proliferation defects.

Surprisingly, my data also showed that specific loss of Y15 phosphorylation did not translate to adult developmental defects, suggesting that compensatory regulatory mechanisms regulating cell proliferation and cell death can regenerate the tissue (Neufeld et al., 1998; Foley et al., 1999; Abrams, 2002; Reis and Edgar, 2004; Bandura and Edgar, 2008). One mechanism is Myt1 phosphorylation of Cdk1 on T14 of Cdk1(Y15F), which was previously shown to be independent of Y15 phosphorylation, *in vitro* (Kornbluth et al., 1994; Mueller et al., 1995; Fattaey and Booher, 1997; Liu et al., 1997; Jin et al., 2008). This proposed mechanism is consistent with my observation that T14 phosphorylation also affected Cdk1 activity, as histone H1 kinase activity of Cdk1(Y15F) was higher than the Cdk1WT transgenic protein, but markedly less than the completely non-inhibitable Cdk1(T14AY15F) variant.

5.4 SINGLE VERSUS DUAL INHIBITORY PHOSPHORYLATION OF CDK1

Cdk1 inhibitory phosphorylation on T14 and Y15 has been proposed to be an ordered reaction, with phosphorylation of one site depending on the other (Krek and Nigg, 1991a; Krek and Nigg, 1991b). This proposal was negated by my observations that single Cdk1 phospho-mutants were still phosphorylated on the remaining residue, indicating these protein modifications do not follow an obligate order. What distinguishes the functional properties of singly phosphorylated Cdk1 isoforms *in vivo* remains an open question. Differential phosphorylation of T14 and Y15 residues may affect Cdk1 localization or its ability to interact with specific mitotic substrates (Holt et al., 2009; Koivomagi et al., 2011). The regulation of such properties may explain the specific role of dMyt1 during development (Jin et al., 2005; Jin et al., 2008). In rapidly cycling syncytial embryos, Wee1-dependent S/M checkpoint mechanisms prevent mitotic catastrophe by Y15 phosphorylation of Cdk1 (Fogarty et al., 1997; Price et al., 2000; Stumpff et al., 2004). Later in development however, Myt1 seems to be the major Cdk1 inhibitory kinase (Jin et al., 2008). The enhanced severity of phenotypic defects induced by expression of Cdk1(T14A,Y15F)-VFP relative to Cdk1(Y15F)-VFP suggest that Myt1-mediated T14 phosphorylation could also be important at other stages of the cell cycle besides G2 phase (Sprenger et al., 1997). For example, recent studies of mammalian cells showed that Cdk1 inhibitory phosphorylation prevented re-initiation of mitosis during G1 phase and failure of this mechanism caused caspase-dependent cell death (Potapova et al.,

2009). Defects in either of these regulatory mechanisms could therefore be responsible for the chromosome defects observed in neuroblasts expressing Cdk1(T14A,Y15F)-VFP.

Myt1-mediated T14 phosphorylation of Cdk1 may have evolved as a mechanism for accumulating dually-inhibited Cdk1-Cyclin B complexes during S-phase and prolonged G2 phase. Such a role is suggested by the requirement for Myt1 activity during oocyte maturation in organisms with a prolonged pre-meiotic G2 phase arrest. Indeed, studies in *X. laevis*, *C. elegans*, and *M. musculus* specifically implicate dual phosphorylation of Cdk1 by Myt1 kinase in developmentally regulating G2 phase arrest of pre-meiotic oocytes (Palmer et al., 1998; Nakajo et al., 2000; Okumura et al., 2002; Peter et al., 2002; Schmitt and Nebreda, 2002; Burrows et al., 2006; Ruiz et al., 2010; Gaffre et al., 2011). The differences in catalytic activity between singly and dually phosphorylated Cdk1 isoforms and the more severe phenotypic defects associated with expression of completely non-inhibitable Cdk1 are also consistent with this idea. Although dMyt1 is not essential for female meiosis, *Drosophila* oocytes do not undergo a prolonged pre-meiotic G2 phase so these findings are not inconsistent with a specific role for dMyt1 in G2 phase arrest (Jin et al., 2005). Indeed, *Drosophila* Myt1 is required in primary spermatocytes, which do undergo a prolonged pre-meiotic G2 phase arrest, as well as for G2/M checkpoint responses to ionizing radiation in wing imaginal discs (Jin et al., 2005; Jin et al., 2008).

Additionally, T14 phosphorylation could potentiate Y15 phosphorylation, whereby Y15 phosphorylation alone has a significant inhibitory effect on Cdk1

activity but phosphorylation of T14 in addition to Y15 enhances the inhibition. Other possible explanations cannot be discounted, however. For example, the combined T14A,Y15F mutation may increase the hydrophobicity of the ATP binding pocket to make the enzyme more easily activated, or influence interactions with specific substrates to allow premature Cdk1 activation, explaining more severe effects than those observed with Cdk1(Y15F) mutants. Alternatively, Cdk1 singly phosphorylated on Y15 or T14 may be differentially susceptible to de-phosphorylation. One appealing idea is that singly phosphorylated Y15 is more refractory to de-phosphorylation than singly phosphorylated T14, consistent with biochemical evidence from cultured mammalian cells (Borgne and Meijer, 1996). This constraint could explain phenotypic differences associated with Cdk1(T14A)-VFP and Cdk1(Y15F)-VFP expression. Although many questions remain regarding the exact molecular mechanisms, this study provides new insights suggesting that regulation of Cdk1 inhibitory phosphorylation has evolved for coordinating cell proliferation with critical processes of animal development.

5.5 DEPENDENCY OF T161 ACTIVATORY PHOSPHORYLATION OF CDK1 ON ITS INHIBITORY PHOSPHORYLATION

Analysis of the role of T14 inhibitory phosphorylation was complicated by the fact that the Cdk1(T14A)-VFP mutant exhibited a reduction in T161 activatory phosphorylation *in vivo*. This observation is consistent with a recent study of cultured mammalian cells suggesting that T161 phosphorylation is favoured by T14 inhibitory phosphorylation, in part because Y15 phosphorylation

seemed to de-stabilize Cdk1-cyclin complexes (Coulonval et al., 2011). This relationship has interesting developmental implications that may explain the specialized role of Myt1 in developmentally G2-phase arrested cells (Nakajo et al., 2000; Okumura et al., 2002; Schmitt and Nebreda, 2002; Jin et al., 2005; Burrows et al., 2006). The dependency of T161 activatory phosphorylation on T14 inhibitory phosphorylation could play a significant role in ensuring a gradual accumulation of Cdk1 that may be crucial for spatially and temporally coordinating cell division with critical development processes. The relatively low levels of T161 phosphorylation in Cdk1(T14A) mutant proteins and the observation that this mutant version of Cdk1 rescued *cdk1* lethality would also support a hypothesis that Y15 inhibitory phosphorylation, in the absence of phosphorylatable T14, could have an antagonistic effect on T161 activatory phosphorylation by destabilizing Cdk1/Cyclin complexes. These proposals would be consistent with *in vitro* studies on Cdk2, showing that the loss of T160 activatory phosphorylation significantly lowered the initial activation of the kinase activity and resulted in slow acting enzyme but the overall enzyme kinetic was not extinguished (Holmes and Solomon, 2001; Coulonval et al., 2003; De Vivo et al., 2006). In fact, structural analysis of the closely related kinase Cdk2 demonstrates that Y15 phosphorylation alone negatively impacts the enzyme alignment and substrate binding due to steric hindrance (Bartova et al., 2004). Such structural changes caused by Y15 phosphorylation of Cdk1(T14A)-VFP may reduce the efficiency of T161 phosphorylation below a detection threshold of my assay.

The proposed antagonistic role of Y15 phosphorylation, if in fact true, could be of significance during fast dividing syncytium embryonic development, explaining why barely detectable levels of Y15 phosphorylation are sufficient for regulating Cdk1 activity during the rapidly cycling cell cycles, despite the fact that T161 does not appear to be significantly dephosphorylated until around nuclear cycle 10 (Edgar et al., 1994). Indeed, the oscillation of T161 activatory phosphorylation of Cdk1 was first detectable beginning in interphase of cycle 10 and became progressively greater in successive interphases such that by S phase of cycle 13 phosphorylated T161 was undetectable (Edgar et al., 1994). These T161 oscillations coincide with the detection of Y15-phosphorylated Cdk1 during interphase of cycle 10-12 (Stumpff et al., 2004), reinforcing the idea of antagonistic relationship between Y15 and T161 phosphorylation. One way to test this idea is by comparing wild type and *wee1* mutant extracts from interphase embryos (10 – 12) for oscillation in T161-phosphorylated Cdk1 (by gel mobility shift and phospho-specific antibody). The aim of the experiment would be to determine if loss of *wee1* activity affects the normal interphase oscillations of T161 phosphorylation during interphase 10 -12.

5.6 DOES THE TIMING OF NEURAL CELL DIVISION INFLUENCE CELL FATE DETERMINATION?

Developmentally regulated cell cycle quiescence in neural precursor cells is fundamental for neurogenesis, yet it remains one of the least mechanistically explored processes in the field of cell cycle regulation. In particular, investigations of disease biogenesis have focused primarily on over-proliferation

of neural precursor cells, in spite of growing evidence linking disruption of neural quiescence to neurodegenerative disease and tumorigenesis. Neural precursor cells undergo either a self-renewal asymmetric division producing itself and a daughter cell of different fate or a differentiative asymmetric division to produce two terminally differentiating daughter cells (Gho and Schweisguth, 1998; Gho et al., 1999; Lu et al., 1999; Schober et al., 1999; Kaltschmidt et al., 2000; Lu et al., 2000). Deregulation of cellular asymmetry in neural precursor cells is associated with severe developmental outcomes and cancer formation (Woods et al., 1996; Bilder et al., 2000; Pardal et al., 2003; Fomchenko and Holland, 2005; Passegue, 2006).

The *Drosophila* mechanoreceptor sensory organ precursor (SOP) has been studied extensively as a model for understanding the process of neural development (Hartenstein and Posakony, 1989; Gho et al., 1999; Reddy and Rodrigues, 1999; Fichelson and Gho, 2004). The SOPs are selected from clusters of equipotent cells that are arrested at the G2-phase of the cell cycle (Usui and Kimura, 1992; Kimura et al., 1997) and subsequently divide asymmetrically in a precisely timed manner to produce the pIIa and pIIb cells. The pIIb adopts neural fate and goes through two rounds of division to generate the internal neuron and sheath cells, while the pIIa cell divides slightly after the pIIb division to produce two cells that will later differentiate to form the external support hair and socket cells (Bodmer et al., 1989; Hartenstein and Posakony, 1989; Gho et al., 1999).

With my transgenic Cdk1 tools I was able to investigate how short-circuiting G2/M timing affects the process of sensory organ development. Having

shown that Cdk1F caused a bypass of G2/M checkpoint arrest without causing genome instability, I used this genetic tool to analyze the impact of forced mitosis on cell fate during mechanosensory organ development (thoracic micro and macrochaetae). The results showed that manipulating the timing of neural precursor re-entry from quiescence into a proliferative state is developmentally coordinated with the process of cell fate specification. Phenotypic analysis and time-lapse imaging of the sensory organ lineages showed that: (1) expression of Cdk1(Y15F)-VFP exclusively in sensory organ precursor (SOP) cells produces supernumerary pIIa descendant cells, without causing pIIb-to-pIIa cell fate transformation (2) ectopic Cdk1 activity terminates G2-phase quiescence forcing the SOPs into premature mitosis without disrupting the plane of SOP division or asymmetric segregation of cell fate determinants and (3) precocious transition from quiescence to proliferation forces SOPs to self-renew prior to undergoing neuronal differentiation, resulting in supernumerary cells. Collectively, these data suggest that G2 phase quiescence via Cdk1 inhibitory phosphorylation is essential for developmentally synchronizing the timing of SOP division with the acquisition of neuronal cell fate specification. I therefore concluded that the G2 quiescence of SOP cells serves as a developmental window when the choice of neuronal differentiation versus self-renewal is made during sensory bristle development.

5.7 CDC25^{TWE}-DEPENDENT AND INDEPENDENT ACTIVATION OF CDK1 DURING MALE MEIOSIS

Unlike mitosis, meiotic division involves complex cytological changes that occur in two phases, G2/MI and MI/MII transitions. The burst in Cdk1 activity orchestrated by Cdc25-like phosphatase dephosphorylation is required during prophase to promote crucial events of meiotic entry, including chromosome condensation, spindle nucleation and chromosome segregation. The apparent execution of the key meiotic events in spermatocytes lacking Cdc25^{Twe} phosphatase activity raises the question of whether Cdk1 activation via dephosphorylation is absolutely necessary for meiotic entry in spermatocytes (Alphey et al., 1992; White-Cooper et al., 1993; Sigrist et al., 1995). In this study, I assessed the requirement for Cdc25^{Twe}-dependent activation of Cdk1 in male meiosis by analysing GFP-tagged wild type and phospho-acceptor mutant Cdk1 proteins expressed in *cdc25^{twe}* mutant spermatocytes. Analysis of live spermatocytes implicated Y15-specific inhibition of Cdk1 as part of the regulatory mechanisms for preventing precocious access of active Cdk1 to its nuclear substrates. I demonstrated, for the first time, that Cdk1 localized to the fusome structures during G2 phase and presented evidence indicating that non-inhibitable Cdk1 compromised the stability of the enigmatic fusome structures.

Immunofluorescence microscopy and biochemical analysis revealed that spermatocytes could undergo meiotic G2/MI independently of Cdc25^{Twe} activity when transgenic Cdk1 WT was expressed, but the meiotic MI/MII was completely stalled in the absence of Cdc25^{Twe} activity. These observations suggest

that Cdk1 dephosphorylation by Cdc25^{Twe} phosphatase is required for the second phase of meiotic division but largely dispensable for the meiotic G2/MI transition. My data further revealed that the expression of a mutant form of Cdk1 that bypassed the requirement for Cdc25^{Twe}-dependent dephosphorylation of the Y15 residue rescued male sterility in *cdc25^{twe}* mutants. Collectively, I presented evidence for the existence of both Cdc25^{Twe}-dependent and –independent regulation of Cdk1 activity in male meiosis. Although, I have provided evidence that Cdk1 abundance may be the key mechanism regulating Cdk1 activity during meiotic entry, future biochemical and genetic analysis will also be required to determine if other regulatory players are involved in the Cdc25^{Twe}-independent activation of Cdk1 during meiotic G2/MI transition. Potential candidates may include Cdc25^{Stg}, a mitotic homolog of Cdc25^{Twe}, having a role in male meiosis.

Alternatively, the Cdc25^{Twe}-independent mechanism may involve differential regulation of Cdk1/cyclin A and Cdk1/cyclin B complexes during the prolonged G2 phase or the G2/M1 meiotic transition. It is already known that Cdk1/cyclin A complexes appear early during G2 phase. It is possible that these may not be regulated normally via inhibitory phosphorylation, hence are not able to drive meiotic G2/MI. On the other hand, Cdk1/cyclin B activity could be strictly regulated via inhibitory phosphorylation and required mainly for orchestrating the meiotic MI/MII transition. Consistent with this proposal, levels of cyclin A progressively increase through the extended G2-phase and disappear at pro-metaphase, whereas cyclin B accumulation does not occur until shortly before the onset of the first meiotic division and peaks during MI/MII transition,

even though *cyclin A* and *cyclin B* mRNAs are both expressed in primary spermatocytes at the onset of meiosis (Gonczy et al., 1994; Lin et al., 1996; White-Cooper et al., 1998).

5.8 THE ROLE OF Y15 INHIBITORY PHOSPHORYLATION OF CDK1 AS A NOVEL LOCALIZATION MECHANISM

Cdk1 is known to shuttle between the nucleus and cytoplasm, with inactive Cdk1 predominantly accumulating in the cytoplasm during interphase before active complexes abruptly move *en masse* into the nucleus in early prophase (Hagting et al., 1998; Jackman et al., 2002). This behavior conveniently prevents Cdk1 complexes from prematurely interacting with nuclear substrates during G2 phase and is associated with nuclear Wee1 activity (Heald et al., 1993) and Myt1 tethering of mitotic cyclinB/Cdk1 complexes in the cytoplasm (Liu et al., 1999; Wells et al., 1999). Recently, it was shown that Cdk1 actively promotes its own nuclear translocation during the G2/M transition (Gavet and Pines, 2010a; Gavet and Pines, 2010b). Thus, this study examined whether Y15 inhibitory phosphorylation might have a direct effect on Cdk1 localization during interphase, in addition to inhibiting Cdk1 catalytic activity. When expressed in *cdc25*^{twe} mutant spermatocytes, Cdk1(Y15F)-GFP relocated into the nucleus at a stage when Cdk1(WT)-GFP proteins were still mainly cytoplasmic. These results implicated Y15-specific inhibition of Cdk1 as part of the regulatory mechanisms for preventing precocious access of active Cdk1 to its nuclear substrates. This hypothesis was further corroborated by the transgene expression studies in endocycling salivary glands, which suggested that Y15 inhibitory phosphorylation

of Cdk1 prevented accumulation of transgenic Cdk1/Cyclin B complexes in the nucleus. Indeed, this interpretation could explain the specific requirements for Cdk1-Y15 inhibitory phosphorylation in G2/M checkpoint arrested cells.

The observation that loss of Cdk1-Y15 phosphorylation affected localization of transgenic Cdk1 in both meiotically dividing spermatocytes and endocycling salivary gland cells demonstrated that Cdk1 localization could be modified by Y15 inhibitory phosphorylation, or lack thereof. This novel regulatory mechanism could act either by Y15 phosphorylation inhibiting Cdk1 nuclear import or, alternatively, Cdk1 nuclear export could be stimulated by Y15 inhibitory phosphorylation (Figure 5-1). To distinguish between these possibilities, future studies will need to examine how Cdk1 nuclear import and export mechanisms are affected by Y15 phosphorylation and to resolve whether observations made in *Drosophila* salivary glands or spermatocytes can be generalized to other types of cells.

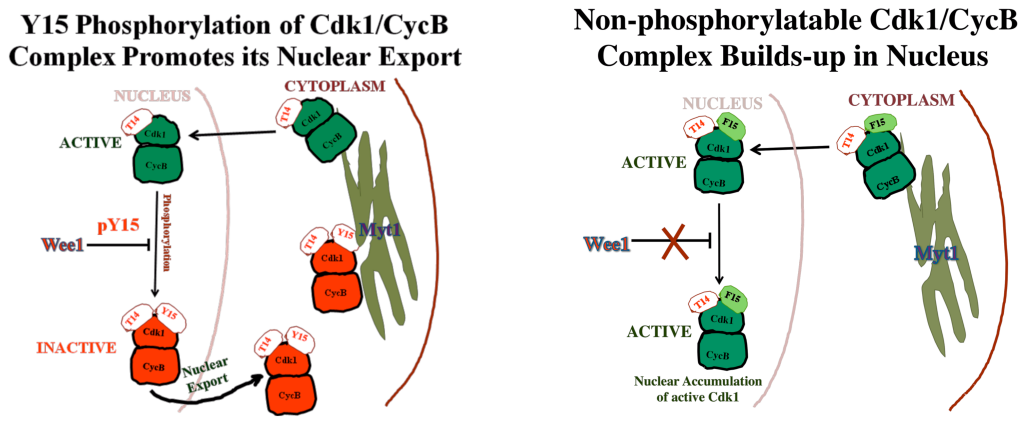

Figure 5-1: Model indicating the role of Y15 inhibitory phosphorylation in regulating subcellular localization of Cdk1/Cyclin B.

Cdk1/Cyclin B sub-cellular localization could be influenced by a Wee1-mediated Y15 inhibitory phosphorylation facilitating Cdk1 nuclear export or via a Myt1-mediated Y15 inhibitory phosphorylation of Cdk1 directly preventing nuclear import or, indirectly influencing nuclear import via Y15-dependent Myt1-mediated cytoplasmic sequestration of Cdk1. This model will be compatible with the lack of inhibitable Y15 residue on Cdk1(Y15F)-VFP resulting in nuclear accumulation of Cdk1/Cyclin B protein in the endocycling salivary gland cells.

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Appendix A1: Inhibitory phosphorylation of Cdk1 can occur independently of cyclin binding in interphase cells.

INTRODUCTION

Several reversible protein phosphorylations, acting independently or cooperatively, promote unidirectional progression through the eukaryotic cell division cycle. Particularly, transitioning from interphase to M-phase in dividing eukaryotic cells relies on tight regulation of cyclin dependent kinase 1 (Cdk1) activity via phosphorylation. Cdk1 activity exits in interphase "OFF" and mitosis "ON" states. In interphase, Cdk1/cyclin complexes are recognized by multiple protein kinases, resulting in phosphorylation on Y15, T14 and T161 residues. Cdk1/cyclin phosphorylation on T161 by Cdk-activating kinase activates the complexes, but is held inactive by inhibitory phosphorylation on Y15 and/or T14 residue by Wee1 and Myt1 kinases (McGowan and Russell, 1995; Mueller et al., 1995; Parker et al., 1995; Booher et al., 1997). This is later followed by dephosphorylation on Y15 and T14 residues, but not T161 phosphorylation, to facilitate mitotic entry (Edgar and O'Farrell, 1989; Edgar et al., 1994; Borgne and Meijer, 1996; Lehman et al., 1999). In fact, expression of Cdk1AF (with Y15F and T14A mutations) or Cdk1F (with Y15F mutation) in eukaryotic cells is associated with premature mitosis (Gould and Nurse, 1989; Krek and Nigg, 1991; Norbury et al., 1991; Jin et al., 1996; Fletcher et al., 2002).

The above model for keeping Cdk1/cyclin inactive during interphase raises a fundamental question involving the obligate sequence of activatory and inhibitory phosphorylation of Cdk1/cyclin during interphase. Importantly, how does a cell coordinate inhibitory and activatory phosphorylations to keep Cdk1/cyclin complexes in an inactive state to prevent premature mitotic events in

interphase cells? This question is further clouded by the fact that stable formation of Cdk1/cyclin complexes requires T161 phosphorylation (Larochelle et al., 1998) and unlike Cdk2 where inhibitory phosphorylation is independent of previous cyclin binding and precedes activating T160 phosphorylation (Coulonval et al., 2003), Cdk1 activatory and inhibitory phosphorylations are thought to depend on cyclin binding (Solomon et al., 1990; Meijer et al., 1991; Parker et al., 1991; Liu et al., 1997). Concomitantly, inhibitory phosphorylation of Cdk1 must either occur before or simultaneously with the T161 activatory phosphorylation. Alternatively, Cdk1 monomer could be phosphorylated prior to cyclin binding, with T161 activatory phosphorylation only acting to prime the activatable complexes for full activation via dephosphorylation.

Attempts to address this issue in proliferating cells have suffered from several complications including a lack of consistent sensitive assays, competition between endogenous and transgenic Cdk1 proteins due to limited cyclin availability, non-homogenous cell populations and lack of cell synchrony. In this study, we focused on re-assessing the sequence of regulatory events involved in controlling Cdk1 activity via inhibitory and activatory phosphorylations during interphase by ectopically expressing Cdk1 fusion proteins in *Drosophila* salivary gland cells, as an alternative system for addressing the question. These cells are constitutively in interphase but do not express Cdk1 or mitotic Cyclins A, B or B3, thereby providing a unique physiologic system in which experimental results would not be complicated by potential interactions with the endogenous proteins (Stern et al., 1993; Hayashi and Yamaguchi, 1999). Salivary gland cells offer

another advantage for analyzing the sub-cellular localization of proteins, in that they are much larger than most cells in *Drosophila*. Additionally, endoreplicating salivary gland cells will further provide an opportunity to re-evaluate whether the mechanism by which the ectopic expression of Cdk1/cyclin complexes inhibit endoreplication in salivary gland cells depends on its phosphorylation status (Hayashi and Yamaguchi, 1999) and provide an insight into endoreplication as an escape strategy utilized by cancer cells to avoid mitotic catastrophe in response to anti-mitotic drugs (Erenpreisa et al., 2005).

MATERIALS AND METHODS

Immunostaining of salivary gland

Salivary glands from staged mid to late third instar larvae were dissected in 1X phosphate-buffered saline (PBS). To synchronize the larvae, Drosophila cultures were maintained at 22^{o}C on standard Drosophila media supplemented with 0.05% bromophenol. The time point corresponding to mid to late third instar larvae was determined by disappearance of bromophenol blue from the gut and initiation of wandering behavior (Maroni and Stamey, 1983). Larvae whose guts were partially blue and had started wandering were selected for these studies. The dissected salivary glands were fixed for 15 minutes in 4% paraformaldehyde at room temperature, washed three times for 5 minutes each in PBT (1X PBS and 0.3% TritonX) and blocked for 30 minutes with 5% bovine serum albumin in 1X PBT. Primary antibodies used were mouse anti-Cyclin B antibodies (DSHB, 1:10) and anti-Cyclin E antibodies (a gift from H. Richardson; 1:200). Secondary antibodies conjugated Alexa Fluor-568 (Molecular Probes) was used at a 1:1000

working dilution.

Western Blot Analysis of Protein Extracts

Salivary glands were dissected from staged third instar larvae and kept on ice before further processing. Protein samples were made from two pairs of salivary glands per sample by homogenizing the tissues in SDS-PAGE sample buffer. The extracts were then separated on 10% SDS-PAGE mini-gels and electrophoretically transferred to a Hybond P PVDF membrane (Amersham). The membrane was blocked with 5% bovine serum albumin in TBST buffer (10 mM Tris-HCl, pH 8.0, 150mM NaCl and 0.1% Tween-20) for 1 hr and incubated with the appropriate primary antibodies over night at 4^{o}C. Proteins were detected using anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Amersham), with a GE Healthcare ECL Plus chemiluminescence kit. The primary antibodies used were rabbit anti-pT161-Cdk1 (1:1000), pT14-Cdk1 (1:500) and pY15-Cdk1 (1:1000; Cell Signaling Technology), mouse anti-GFP (1:5000; Clontech), and mouse anti-Actin (1:5000; Chemicon).

Quantitation of Salivary Gland Cells

Z-stack images of salivary glands labeled by fluorescent microscopy were captured on a Zeiss Axioskop equipped with a Retiga CCD camera. The number of cells in each salivary gland was counted manually using the counting tool in the Volocity software. For each genotype, the total number of cells per salivary gland from seven different salivary glands was counted and the data was combined to determine the average number of cells per gland, and standard error.

RESULTS

Transgenic Cdk1 proteins are stably expressed in salivary gland cells

The absence of Cdk1 and mitotic cyclin in salivary gland cells might reflect a global mechanism for downregulating mitotic regulators during endoreplication, for example APC/C-catalyzed proteolysis. We first assessed whether the transgenic proteins could be inducibly expressed in salivary gland cells by expressing Cdk1WT-VFP and Cdk1(Y15F)-VFP using the *sd-Gal4* driver, which induces expression in salivary glands as well as wing discs (Fitzpatrick et al., 2002). As shown by the VFP signal, Cdk1WT-VFP and Cdk1(Y15F)-VFP were stably expressed throughout the salivary gland cells (Figure A1-1A & B, respectively). To assay total protein levels, we performed western blots analysis of protein extracts from salivary glands expressing Cdk1WT-VFP and Cdk1(Y15F)-VFP probed with anti-Cdk1 (PSTAIR) antibodies. Ectopically expressed transgenic Cdk1 variants of the appropriate size (61kDa) were detectable in extracts probed with PSTAIRE antibodies (Figure A1-1C**)**. These data indicate that the transgenic Cdk1 fusion proteins are stably expressed and demonstrate a lack of a persistent mechanism for downregulating Cdk1 in salivary gland cells.

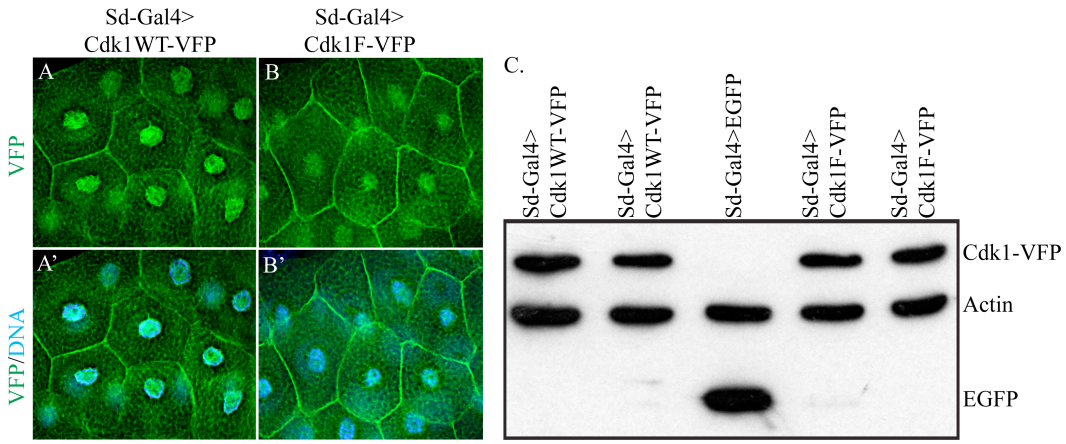

Figure A1 - 1: Ectopically expressed transgenic Cdk1 variants were stably expressed salivary gland cells.

The green channel shows the transgenic protein and the blue channel represent DNA dye. Transgenic Cdk1WT-VFP (A and A') or Cdk1(Y15F)-VFP (B and B') expressing using *sd-Gal4* driver localize to both cytoplasm and nucleus. (C) Blot of protein extracts from salivary gland of indicated genotypes probed with anti-GFP antibodies revealed the expected size (61kDa) of Cdk1WT-VFP and Cdk1(Y15F)-VFP fusion proteins. The duplicate Cdk1WT-VFP and Cdk1(Y15F)-VFP on the blot represent different transgenic lines in each case. Actin was used as the loading control, while EGFP expression served as a positive control.

Cdk1 Inhibitory phosphorylation occurs independently of mitotic cyclin

Wee1 and Myt1 inhibitory kinases are thought to only phosphorylate mitotic cyclin-bound Cdk1 (Solomon et al., 1990; Meijer et al., 1991; Parker et al., 1991; Liu et al., 1997). To re-evaluate whether Cdk1 can be phosphorylated in the absence of mitotic cyclins, we took advantage of the fact that salivary gland cells do not normally express mitotic cyclins (Stern et al., 1993; Hayashi and Yamaguchi, 1999) and our observation that the transgenic Cdk1 proteins can be stably expressed in these cells (see Figure A1-1). We assayed the phosphorylation status of the transgenic Cdk1 proteins by performing western blot analysis on extracts from salivary glands expressing these proteins. Non-transgenic *yw* strain salivary gland extracts were used as a negative control. Using PSTAIRE antibodies to detect total Cdk1 (phosphorylated and unphosphorylated), we observed that the *yw* control extracts did not show detectable levels of endogenous Cdk1 protein (Figure A1-2A), as expected (Stern et al., 1993; Hayashi and Yamaguchi, 1999). Using phospho-specific Cdk1 antibodies (pY15-Cdk1 and pT14-Cdk1), we found that Cdk1WT-VFP expressed in salivary glands was phosphorylated on both Y15 and T14 (Figure A1-2A), indicating that transgenic wild type Cdk1 can be phosphorylated by Wee1 and Myt1 kinases in the absence of mitotic cyclin. Cdk1(Y15F)-VFP was only phosphorylated on T14 and Cdk1(T14A,Y15F)-VFP was not phosphorylated at either site (Figure A1-2A), demonstrating the specificity of the inhibitory phosphorylation of ectopically expressed Cdk1 proteins.

Previous studies have shown that T161 activatory phosphorylation occurs

only when Cdk1 is bound to a mitotic cyclin (Solomon et al., 1992; Larochelle et al., 1998). To examine whether phosphorylation of the transgenic Cdk1 proteins on the T161 residue required cyclin interaction, we expressed Cdk1WT-VFP and Cdk1(Y15F)-VFP alone or together with transgenic cyclin B in salivary gland using *sd-Gal4* driver. Western blot analysis was performed on the protein extracts from the salivary glands of different genotypes. Using antibodies that detect the presence of Cdk1 phosphorylation on the T161 activating residue (pT161-Cdk1), we found that Cdk1WT-VFP and Cdk1(Y15F)-VFP were not phosphorylated on the T161 residue when expressed alone (Figure A1-2B), implying that they were inactive catalytically. The T161 residue became phosphorylated when Cdk1WT-VFP and Cdk1(Y15F)-VFP were co-expressed with cyclin B in salivary gland cells however (Figure A1-2B), implying that the Cdk1/cyclin interaction is a precondition for T161 activatory phosphorylation. While this interpretation is consistent with data from other systems (Solomon et al., 1992; Larochelle et al., 1998), in our case it appears that only the T161 activatory phosphorylation requires Cdk1/cyclin interaction, whereas Y15 and/or T14 inhibitory phosphorylation occurs independently of cyclin binding.

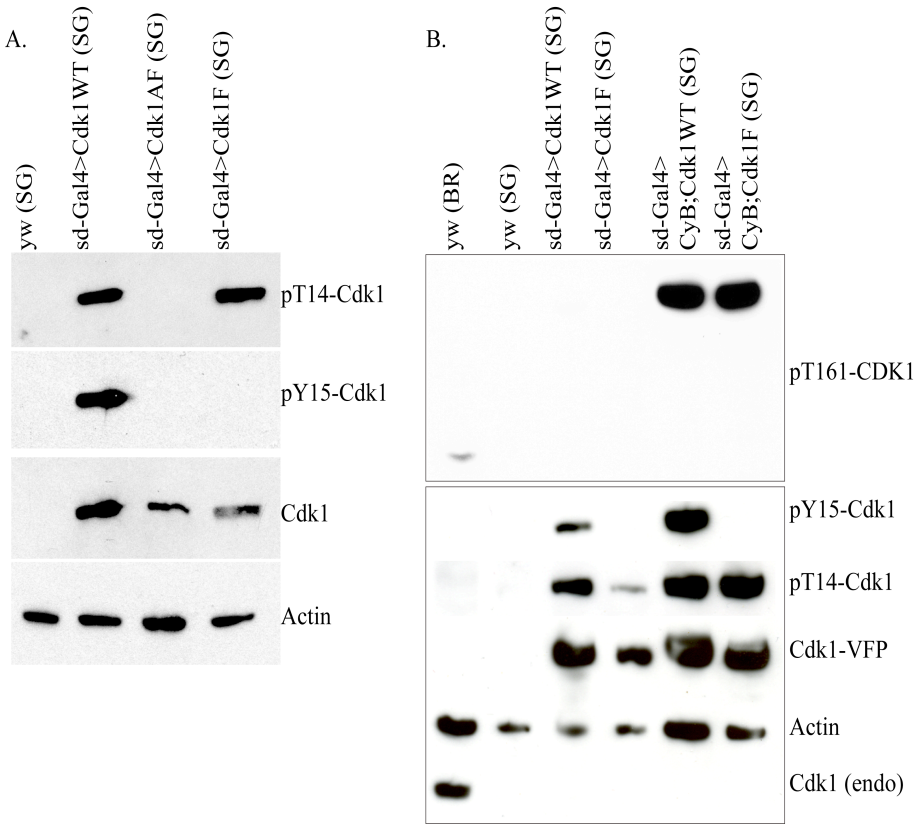

Figure A1 - 2: Cdk1 inhibitory phosphorylation can occurr independently of mitotic cyclin interaction.

Western blot of salivary gland extracts of indicated genotype were probed with antibodies specific to the Cdk1, pT14-Cdk1, pY15-Cdk1, pT161-Cdk1 and Actin as a loading control. (A) Cdk1WT-VFP extract was immunolabeled for both pT14Cdk1 and pY15Cdk1 (see also Figure A1-1B). In Cdk1(Y15F)-VFP extract the pT15Cdk1 was completely absent but pT14Cdk1 was detected, whereas both pT14Cdk1 and Y15Cdk1 were not detected in Cdk1(T14A,Y15F)-VFP extract. When re-probed with PSTAIRE antibodies endogenous Cdk1 (34 kDa) was not detected both in the *yw* control extract and ectopic protein extracts (see also Figure A1-1B), but the ectopic fusion proteins (61kDa) were detected in the salivary gland extracts from transgenic sample. (B) Consistent with the absence of Cdk1 in salivary gland, T161 phosphorylation was not detected in extract from *yw* salivary glands (SG). When Cdk1WT-VFP or Cdk1(Y15F)-VFP proteins was expressed alone, T161 phosphorylation did not occur, but when co-expressed with

cyclin B the transgenic fusion proteins were fully phosphorylated on T161 residue. (NB: BR means brain and SG represents salivary gland).

Co-expression of transgenic Cdk1 and cyclin B inhibit endoreplication independently of the phosphorylation status.

In light of the observation that T161 activatory phosphorylation occurred when transgenic Cdk1 and cyclin B were ectopically co-expressed, we wondered if this expression might infringe on the mechanism for regulating constitutive interphase in salivary gland cells. To address this possibility we again expressed the transgenes alone or with cyclin B in salivary gland cells using *sd-Gal4*. Compared with staged *yw*, non-transgenic salivary glands (Figure A1-3A and 3B), expression of Cdk1WT-VFP alone did not produce defects in the average number of cells, or the size or morphology of third instar salivary gland polytene chromosome (Figure A1-3A & 3B, see also Figure 1A). Similarly, expression of Cdk1(Y15F)-VFP alone also produced normal salivary glands (Figure A1-3A & 3B, see also Figure A1-1A).

Expression of cyclin B alone, as a negative control, had no effect on morphology, size or number of cells per salivary gland, presumably because there was no available Cdk1 to interact with (Figure A1-3A and 3B). When we co-expressed Cdk1WT-VFP with Cyclin B however, we noted a dramatic reduction in the size of the salivary glands polytene chromosome (Figure A1-3B). The average number of cells per salivary gland was not affected in this case (Figure 3A), however the cells were much smaller (Figure A1-3B). These defects suggested a failure of endoreplication, similar to defects previously reported for cyclin A expressed alone (Weiss et al., 1998) or co-expressed with transgenic, untagged wild type Cdk1 (Hayashi and Yamaguchi, 1999). Salivary glands co-

expressing cyclin B and Cdk1(Y15F)-VFP were also markedly reduced in size (Figure A1-3B), compared with glands expressing either cyclin B or Cdk1(Y15F)-VFP alone. Similar to salivary glands co-expressing cyclin B and Cdk1WT-VFP, there was no significant effect on the average number of cells in each salivary gland (Figure A1-3A). Cells co-expressing cyclin B and Cdk1(Y15F)-VFP were much smaller than normal (Figure A1-3B), but were larger than cells co-expressing cyclin B and Cdk1WT-VFP, however, suggesting that Cdk1(Y15F)-VFP was less effective in blocking endoreplication than Cdk1WT-VFP.

We wondered how Cdk1 and cyclin B cooperatively inhibited endoreplication in this experiment. Pulses of cyclin E expression drive endoreplication in salivary glands, whereas continuous cyclin E expression inhibits endoreplication (Sauer et al., 1995; Follette et al., 1998; Weiss et al., 1998). We therefore reasoned that ectopically expressed Cdk1/cyclin B complexes might be inhibiting endoreplication by stabilizing cyclin E levels in salivary gland cells. Indeed, in addition to inhibition of endoreplication in salivary gland cells co-expressing cyclin and Cdk1WT-VFP, we observed higher levels of cyclin E protein (Figure A1-4B & 4B'). Similar high level of cyclin E expression was observed throughout the salivary gland cells co-expressing cyclin B and Cdk1(Y15F)-VFP (Figure A1-4C & 4C').

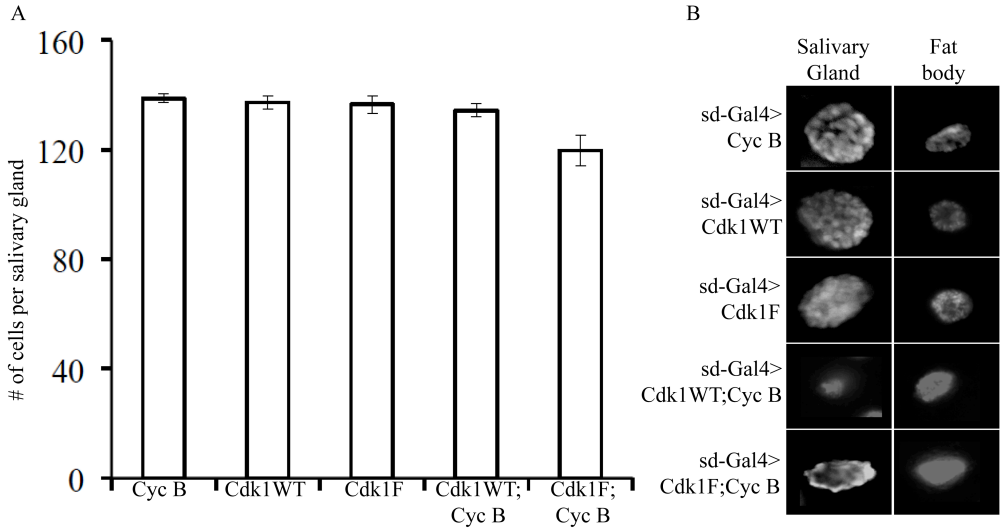

Figure A1 - 3: Co-expression of cyclin B with each of Cdk1WT-VFP and Cdk1(Y15F)-VFP inhibits growth of salivary gland nuclei.

(A) Graphical representation of the average number of cells per salivary gland for each indicated genotype. The error bars show the standard deviation. (B) The panels show the relative size of salivary gland nuclei and fat body nuclei from larvae expressing the indicated ectopic proteins in endoreplicating salivary gland cells under the control of *sd-Gal4*. This *Gal4* driver does not express in fat body, judged by VFP fluorescence.

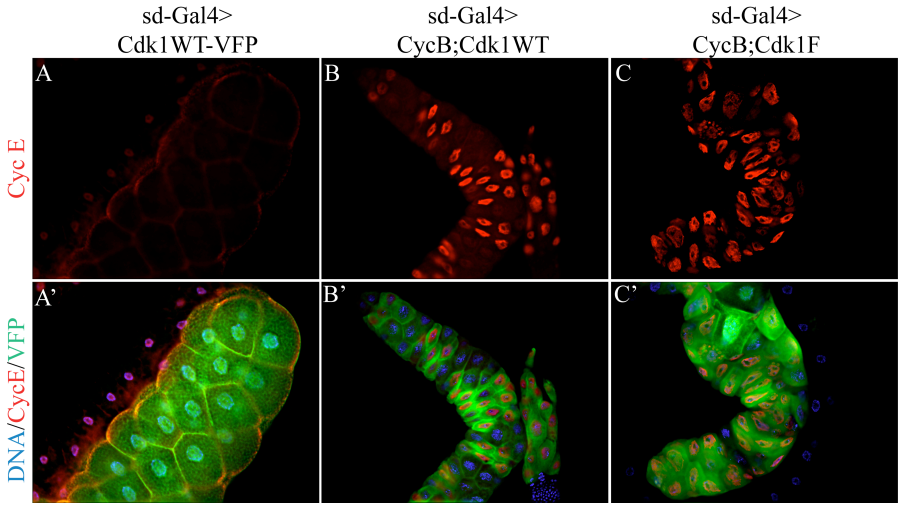

Figure A1 - 4: Ectopic co-expression of Cdk1 and cyclin B results in accumulation of cyclin E in salivary gland cells.

The red channel represents cyclin E immunostaining, the blue channel is DNA and the green channel represents VFP fluorescence from the Cdk1 fusion proteins. (A) Cyclin E is barely detectable in salivary glands expressing Cdk1WT-VFP alone (or Cdk1(Y15F)-VFP alone, not shown). (B) Ectopic co-expression of cyclin B and Cdk1WT-VFP in salivary gland cells results in accumulation of cyclin E. (C) Similarly, co-expression of cyclin B and Cdk1(Y15F)-VFP causes cyclin E accumulation in salivary gland cells.

Y15 Inhibitory Phosphorylation affects sub-cellular localization of Cdk1 protein

We also observed striking differences in the subcellular localization of transgenic Cdk1 proteins co-expressed with Cyclin B. As shown in Figure 1, when Cdk1WT-VFP or Cdk1(Y15F)-VFP was expressed alone, the fluorescent fusion protein was visible in both the cytoplasm and nucleus of the salivary gland cells. Interestingly, when Cdk1WT-VFP was co-expressed with Cyclin B, the VFP-tagged proteins were predominantly in the cytoplasm, predominantly on some undefined structure (Figure A1-5A). This behavior recapitulates what is normally observed for Cdk1/cyclin B complexes in interphase cells (Heald et al., 1993; Kao et al., 1999). In contrast, when Cdk1(Y15F)-VFP was co-expressed with Cyclin B, the fluorescent proteins appeared predominantly in the nucleus (Figure A1-5B). We conclude from these results that Y15 inhibitory phosphorylation of Cdk1/cyclin B can influence sub-cellular localization of the complex.

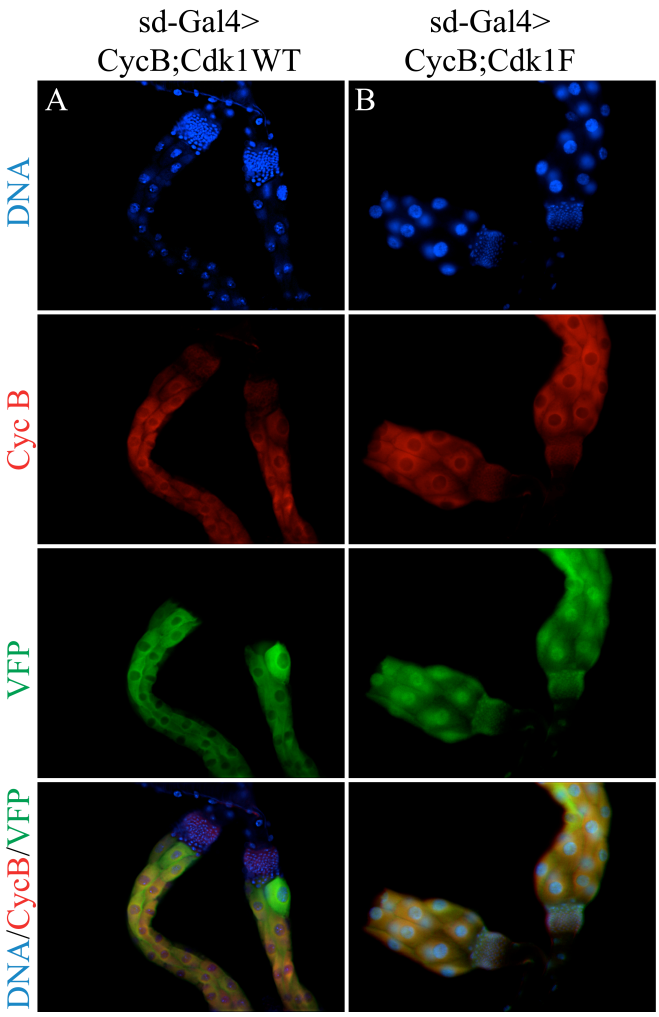

Figure A1 - 5: Co-expression of cyclin B and transgenic Cdk1 proteins caused differential sub-cellular localization of the fusion proteins.

The DNA is marked in blue, cyclin B in red and the fusion proteins in green. When co-expressed with cyclin B, the transgenic Cdk1WT-VFP localized predominantly in the cytoplasm (A), whereas co-expression of cyclin B with Cdk1(Y15F)-VFP preferentially localized the fusion protein majorly in the nucleus (B), suggesting a role for Y15 inhibitory phosphorylation of Cdk1 in targeting Cdk1/cyclin complexes to a specific cell compartment.

DISCUSSION

Inhibitory phosphorylation is not restricted to mitotic cyclin-bound Cdk1

In this study we re-assessed how mitotic cyclins influence Cdk1 inhibition and activation, seeking to understand the relationship between T161 and Y15/T14 phosphorylation of Cdk1 in interphase. Biochemical analysis of the transgenic Cdk1 proteins ectopically expressed in constitutively interphase salivary gland cells, lacking Cdk1 and mitotic cyclins, revealed that T161 activatory phosphorylation of Cdk1 occurred only when it was co-expressed with cyclin B. These results therefore confirm the *in vivo* dependence of T161 phosphorylation upon prior mitotic cyclin binding (Solomon et al., 1992). We also found that ectopically expressed Cdk1 WT fusion proteins in constitutively interphase salivary gland cells were phosphorylated on both Y15 and T14 residues. Unlike the T161 activatory phosphorylation of Cdk1, these results demonstrate that inhibitory phosphorylation of the ectopically expressed Cdk1 was independent of prior mitotic cyclin-binding.

These results contradict the previous conclusions that Wee1 and Myt1 kinases do not phosphorylate monomeric Cdk1 proteins (Meijer et al., 1991; Parker et al., 1991; Solomon et al., 1992; Liu et al., 1999; Wells et al., 1999) but are consistent with the situation in Cdk2 where Y15 inhibitory phosphorylation occurred independently of cyclin binding and preceded activatory phosphorylation (Coulonval et al., 2003). If these processes were exclusively cyclin-dependent, how would an interphase cell deal with the potential lagging time between T161 activatory phosphorylation and Y15/T14 inhibitory

phosphorylation when Cdk1/cyclin B is active? Our results provide a simple solution to this conundrum; prior inhibitory phosphorylation of monomeric Cdk1 could serve as an important regulatory mechanism for ensuring that once cyclin-bound and subsequently phosphorylated on T161, the Cdk1/cyclin complexes remain inactive but are primed to be activated via Cdc25-mediated Y15/T14 dephosphorylation.

Our study also provides insight into the mechanism of inhibition of endoreplication in salivary gland cells ectopically co-expressing mitotic cyclin and Cdk1 proteins (Hayashi and Yamaguchi, 1999). Although cyclin E expression was barely detectable in salivary gland ectopically expressing Cdk1 alone, we found high level of cyclin E expression in salivary gland cells ectopically co-expressing cyclin B with Cdk1WT-VFP or Cdk1(Y15F)-VFP. These observations suggest that ectopic expression of Cdk1/cyclin inhibits endoreplication by stabilizing or maintaining continuous cyclin E expression and demonstrate that inhibition of endoreplication is independent of Cdk1 inhibitory phosphorylation status. This is consistent with previous data showing that continuous cyclin E expression via ectopic expression of cyclin E protein inhibited endoreplication (Follette et al., 1998; Weiss et al., 1998).

How does ectopic Cdk1/cyclin stabilize or maintain continuous cyclin E expression? One possibility is that the ectopic Cdk1 proteins may interact with and stabilize cyclin E, thereby preventing timely oscillation of cyclin E proteins required for endoreplication. The fact that the ectopic expression of Cdk1 alone did not inhibit endoreplication argued against this possibility, however, suggesting

instead that the formation of cyclin/Cdk1 and/or its T161 activatory phosphorylation is required for inhibiting endoreplication. Alternatively, the ectopically expressed Cdk1/cyclin complexes could directly or indirectly perturb the formation of E2F-Rb repressive complexes required for ensuring proper oscillation of cyclin E expression in endoreplicating cells (Weng et al., 2003). Indeed, ectopic Cdk1 co-expressed with cyclin A in salivary glands was previously shown to interact with E2F, *in vivo* (Hayashi and Yamaguchi, 1999). Further studies are needed to distinguish these alternative explanations and to define exactly how ectopic co-expression of mitotic cyclins and Cdk1 in salivary gland cells inhibits endoreplication. Such studies could provide insight into how cancer cells that undergo endoreplication escape mitotic catastrophe when exposed to anti-mitotic drugs (Erenpreisa et al., 2005).

The role of Y15 Inhibitory Phosphorylation as a novel localization mechanism

Inactive Cdk1/cyclin complexes predominantly accumulate in the cytoplasm during interphase before active complexes move into the nucleus in early prophase (Hagting et al., 1998; Jackman et al., 2002). This localization behavior therefor prevents Cdk1 complexes from prematurely interacting with nuclear substrates, during G2 phase. This mechanism is associated with nuclear Wee1 activity (Heald et al., 1993) and Myt1 tethering of mitotic cyclin B/Cdk1 complexes in the cytoplasm (Liu et al., 1999; Wells et al., 1999). Recently, it was shown that Cdk1 actively promotes its own nuclear translocation during the G2/M transition (Gavet and Pines, 2010a; Gavet and Pines, 2010b). Thus, we considered

whether Y15 inhibitory phosphorylation might have a direct effect on Cdk1 localization during interphase, in addition to inhibiting Cdk1 catalytic activity. Transgene expression studies in endocycling salivary glands suggest that Y15 inhibitory phosphorylation of Cdk1 prevents accumulation of transgenic Cdk1/Cyclin B complexes in the nucleus. Such a novel regulatory mechanism could act either by Y15 phosphorylation inhibiting Cdk1 nuclear import or, alternatively, Cdk1 nuclear export could be stimulated by Y15 inhibitory phosphorylation. To distinguish between these possibilities, future studies will need to examine how Cdk1 nuclear import and export mechanisms are affected by Y15 phosphorylation to resolve whether observations made in salivary glands can be generalized to other types of cells.

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Appendix A2: Preliminary characterization of the phenotypic consequences of expressing Cdk1 mutant with non-inhibitable Y15F residue during syncytial embryonic development

INTRODUCTION

Eukaryotic cells depend on reversible inhibition of Cdk1 activity via activation of Wee1-like Cdk1 inhibitory kinases (Wee1 and Myt1) and inhibition of Cdc25 phosphatases that activate Cdk1 as an important G2 phase checkpoint mechanism, which prevents M-phase entry in response to incomplete DNA replication, unrepaired DNA damage or developmental signals (Elledge, 1996; Kastan and Bartek, 2004; Perry and Kornbluth, 2007). Breakdown in this regulatory mechanism could lead to genome instability and cancer as well as neurodegenerative diseases (Castedo et al., 2002; Masaki et al., 2003; Rimkus et al., 2008; Iorns et al., 2009). When phosphorylated by Wee1 on Y15 residue or Myt1 on both the Y15 and/or T14 residue, Cdk1 becomes inactivate (McGowan and Russell, 1993; Mueller et al., 1995; Parker et al., 1995; Booher et al., 1997; Fattaey and Booher, 1997). This regulatory mechanism is largely conserved in both somatic and embryonic cells, even though the cell division cycles are significantly different. Chromosome replication (S phase) and genome division (M phase) in somatic cells are separated by gap phases (G1 and/or G2 phase), functioning as crucial arrest points following DNA damage. In normal cells, abrogation of the G2 phase arrest can lead to mitotic catastrophe and eventual apoptosis (Castedo et al., 2004; Kawabe, 2004; Bucher and Britten, 2008). Wee1 is implicated as a G2 phase arrest gatekeeper, protecting cells from mitotic catastrophe and apoptosis, and it is found to be un-regulated in different forms of human cancers (Masaki et al., 2003; Blenk et al., 2008; Iorns et al., 2009).

Unlike most somatic cells, early *Drosophila* embryonic cells undergo rapid cleavage cycles in which the gap phases are omitted (O'Farrell et al., 2004). Specifically, *Drosophila* embryonic development begins with 13 rapid syncytium and synchronous S/M nuclear divisions, lacking gap phase, which are followed by patterned G2/M regulated cell divisions that set up the basic body plan during gastrulation. Remarkably, inhibitory phosphorylation of Cdk1 is barely detectable in early *Drosophila* embryos (Edgar et al., 1994), and for a long time formed the basis for the conclusion that early embryos did not have an active cell cycle checkpoint mechanism. Subsequent genetic and biochemical analysis have, however, demonstrated that Wee1-mediated Y15 inhibitory phosphorylation of Cdk1 is in fact essential for regulating pre-mitotic checkpoint during early *Drosophila* and mammalian embryonic cell cycles (Price et al., 2000; Stumpff et al., 2004; Tominaga et al., 2006). Maternal *wee1* mutants develop relatively normally until cycle 11 or 12, when nuclei start to undergo a brief but violent "mitotic catastrophe" that soon consumes the entire embryo, indicating that maternal dWee1 is required to ensure faithful execution of mitosis by preventing mitotic catastrophe in the rapid S/M cycles (Stumpff et al., 2004). The execution of normal cycle 1-10 in *dwee1* embryo is puzzling and triggers an important question on the mechanism of regulating Cdk1 activity prior to syncytium cycle 11.

The occurrence of *wee1* phenotypes at cycle 11 or 12 onward corresponds to the emergence of late replications during syncytial embryonic cycles, which are thought to be responsible for the progressive lengthening of S phase (Shermoen et

al., 2010). This raises an interesting question on whether dWee1 regulation of the embryonic cell cycles occurs strictly via Y15 phosphorylation of Cdk1 or by another mechanism in addition to or unrelated to Cdk1 inhibitory phosphorylation. These questions are particularly relevant given the fact that endogenous dWee1 protein is difficult to detect in the early embryo. Understanding how the barely detectable endogenous dWee1 engages the pre-mitotic checkpoint during the rapid S/M embryonic cell division could provide an insight into checkpoint regulation in somatic cells. This preliminary study examined the role of Y15 by analyzing the consequence of the maternal expression of transgenic Cdk1 wild type (Cdk1WT-VFP) and mutant isoforms (Cdk1(Y15F)-VFP), in which the Y15 residue is mutated to non-phosphorylatable phenylalanine. The preliminary results demonstrate that maternal expression of transgenic Cdk1(Y15F)-VFP in *Drosophila* embryo phenocopies the loss of *dwee1* function.

MATERIALS AND METHODS

Live imaging using confocal microscope

Embryos maternally expressing Cdk1WT-VFP or Cdk1(Y15F)-VFP and mCherry-Tubulin were collected for 30 min, aged for 30 min, dechorionated in 50% bleach, and mounted on coverslips in halocarbon oil. Live analysis of the timing of mitosis and interphase was then performed on the embryos. Data for fluorescent time-lapse movies were acquired with an inverted microscope (IX81; Olympus; 60×, NA 1.42 oil objective) equipped with a spinning-disc confocal head (CSU10; Yokogawa). Image capture with a CCD camera (ORCA-R2;

Hamamatsu Photonics) was controlled by MetaMorph software (Molecular Devices). Mitosis was defined as the time between nuclear envelope breakdown to nuclear envelope formation, while interphase was considered as the time interval between nuclear envelope formation and breakdown (Stumpff et al., 2004).

RESULTS AND DISCUSSIONS

Maternal expression of Cdk1(Y15F)-VFP caused embryonic lethality

To determine whether the Wee1-mediated Y15 inhibitory phosphorylation is the primary mechanism of Cdk1 regulation during the rapid syncytium embryonic cell cycle, we assess the developmental impact of maternal expression of Cdk1(Y15F)-VFP on embryo development. Transgenic Cdk1WT-VFP and Cdk1(Y15F)-VFP were expressed maternally in embryos using two Gal4 drivers with different expression levels, *T279-Gal4* or *nos-Gal4* drivers; *T279-Gal4* producing stronger expression levels than *nos-Gal4-Gal4*. Embryos expressing Cdk1(WT)-VFP under the control of either *T279-Gal4* or *nos-Gal4* drivers developed normally and produced progeny, indicating that the transgenic Cdk1WT-VFP is compatible with embryonic development (data not shown). In contrast, expression of Cdk1(Y15F)-VFP using *T279-Gal4* or *nos-Gal4* driver resulted in embryonic lethality (data not shown), indicating that being able to regulate Cdk1 via Y15 phosphorylation is critical during early embryogenesis and consistent with the maternal effect of the loss of *wee*1 function.

Embryo expressing Cdk1(Y15F)-VFP undergo premature mitosis

In *wee1* mutant embryos, earlier syncytia cycles occur normally until cycle 11 and 12 when the interphase length becomes progressively shorter, nuclei advance prematurely into mitosis with aberrant spindle and accumulated DNA damage, and subsequently under undergo mitotic catastrophe (Price et al., 2000; Stumpff et al., 2004). To further characterize the phenotype associated with the expression of Cdk1(Y15F)-VFP, live imaging was performed with embryos

expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP and mCherry-Tubulin, as a marker for nuclear envelope breakdown and formation, under the control of the *T279-Gal4*. There were no apparent defects in cell cycle progression during syncytial cycles in embryos expressing Cdk1(WT)-VFP when compared to a previously report (Stumpff et al., 2004); Movies A2-1). Interphase 12 and 13 took 8.02±0.02 mins and 12.69±0.54 mins, respectively (Table A2-1 & Movie A2-1) and the mitotic spindles were unperturbed (Figure A2-1) in embryos expressing Cdk1(WT)-VFP. Similar results were observed when Cdk1(WT)-VFP and mCherry-Tubulin were co-expressed using maternal *nos-Gal4* driver; interphase 12 and 13 took 7.8±0.57 mins and 14.20±0.85 mins, respectively (Table A2-2 & Movie A2-3).

In embryos expressing Cdk1(Y15F)-VFP under the control of a maternal *T279-Gal4* driver, the timing of interphase 12 was comparable to embryos expressing Cdk1(WT)-VFP, taking about 8.30±0.05 mins. Cycle 13 in these embryos had a significantly shorter interphase than embryos expressing Cdk1(WT)-VFP however (Table 1 and Movie A2-2). Other aberrations such as monopolar spindles also became evident in some nuclei during mitosis 12 (Figure A2-1) as well as spindle interactions during mitosis 13 (Figure A2-2 and Movie A2-2). Similar shortening of interphase length was observed starting from interphase 12 when Cdk1(Y15F)-VFP was expressed using the maternal *nos-Gal4* driver (Table A2-2 & Movie A2-4). These results are consistent with the requirement for dWee1 activity for control of early embryonic nuclear division,

confirming that Y15 inhibitory phosphorylation of Cdk1 is important for mitotic timing during the later part of syncytium embryonic cell cycles.

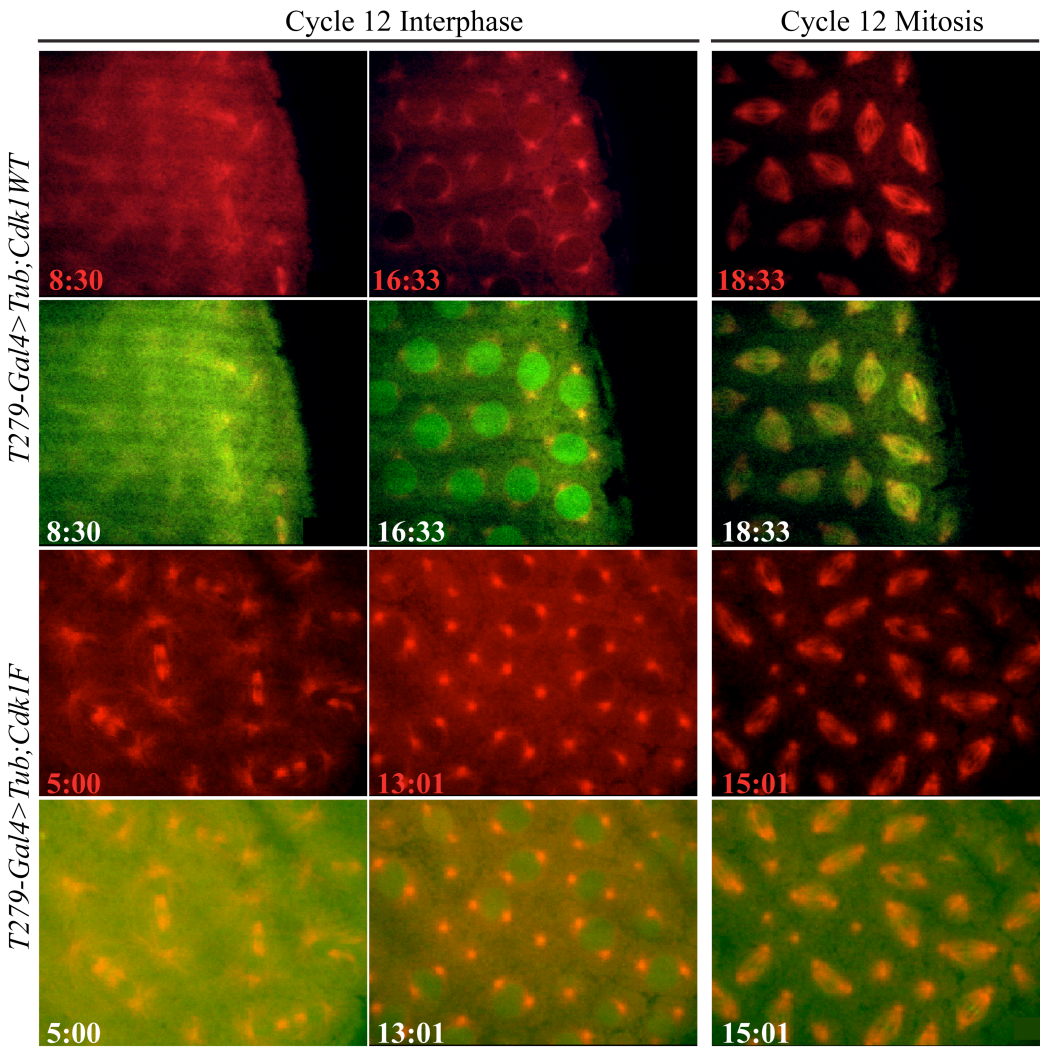

Figure A2 - 1: Monopolar spindle aberrations observed in embryos expressing Cdk1(Y15F)-VFP during mitotic cycle 12.

Live embryo expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP with mCherry-Tubulin were analyzed using spin disk confocal microscopy. The length of cycle 12 interphase, defined by the time between nuclear formation and nuclear breakdown as marked by Tubulin (Red), was the same for embryos expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP. Monopolar spindles were observed in embryos expressing Cdk1(Y15F)-VFP starting from cycle 12 mitosis.

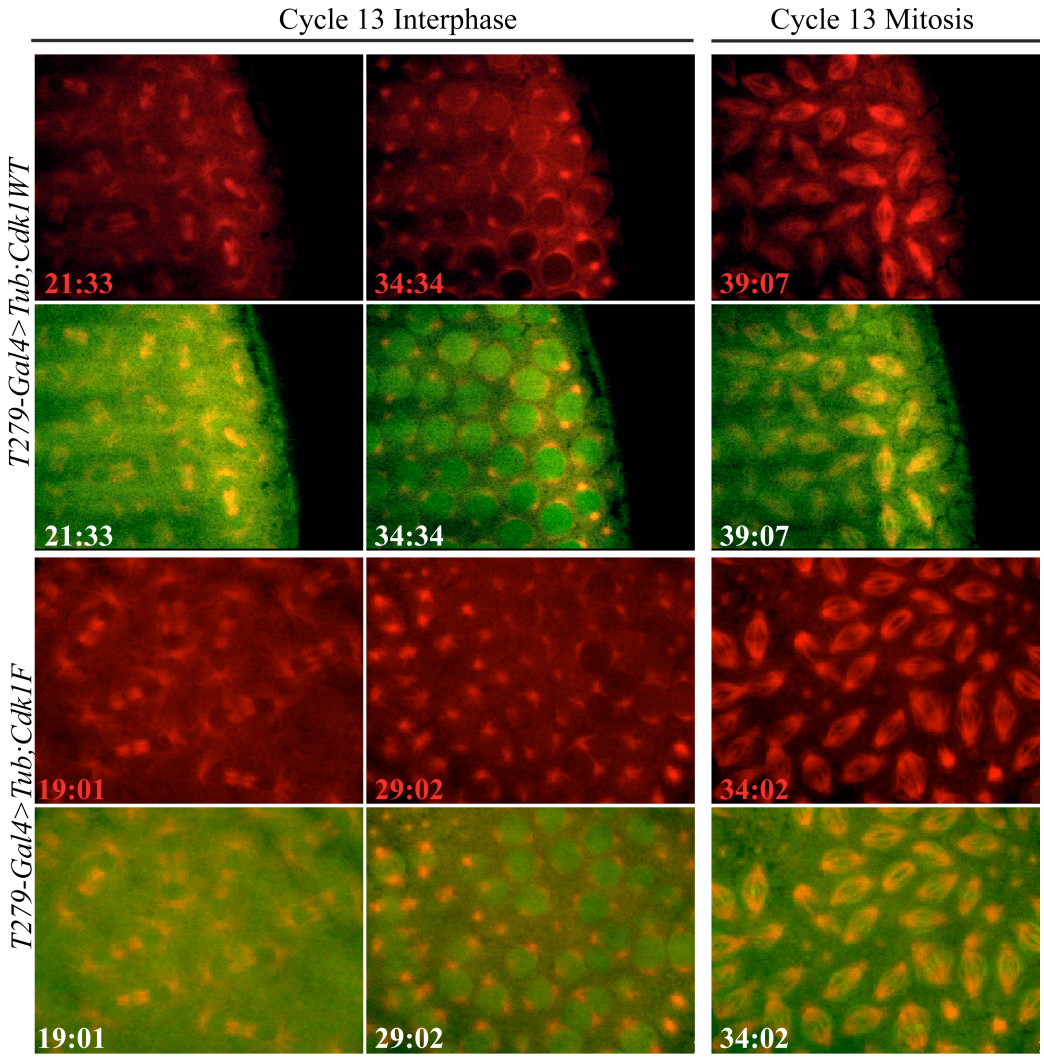

Figure A2 - 2: Expression of Cdk1(Y15F)-VFP shortens interphase length and causes spindle aberrations.

Live embryo expressing Cdk1(WT)-VFP or Cdk1(Y15F)-VFP with mCherry-Tubulin were analyzed using spin disk confocal microscopy. Compared to the embryos expressing Cdk1(WT)-VFP, the length of cycle 13 interphase was significantly shorter in embryos expressing Cdk1(Y15F)-VFP. Other aberrations such as spindle interactions and monopolar spindle were also evident during cycle 13 mitosis in embryos expressing Cdk1(Y15F)-VFP.

Table A2 -1: Cell Cycle Times in Embryos Maternally Expressing Transgenic Cdk1 and mCherry-Tubulin under the Control of T279-Cdk1Drivers

Cycle	Cdk1WT (min) (n = 2)	Cdk1F (min) (n = 2)
Mitosis 11	4.31±0.01	Not available
Interphase 12	8.02±0.02	8:31±0:05
Mitosis 12	4.65±0.50	5.65±0.05
Interphase 13	12.69±0.54	10.03±0.02

Syncytial embryos expressing Cdk1WT-VFP or Cdk1(Y15F)-VFP and mCherry-Tubulin under the control of maternal *T279-Gal4* driver were subjected to live imaging and the lengths of interphase and mitosis were determined as previously described (Stumpff et al., 2004). The mCherry-Tubulin was used to mark nuclear envelope breakdown (movement of tubulin into the nucleus) and nuclear envelope formation (tubulin exclusion from the nucleus). Mitosis was defined as the time between nuclear envelope breakdown to nuclear envelope formation, while interphase was considered as the time interval between nuclear envelope formation and breakdown (Stumpff et al., 2004); (NB: n=2).

Table A2 - 2: Cell Cycle Times in Embryos Maternally Expressing Transgenic Cdk1 and mCherry-Tubulin under the Control of *nos-Gal4* Driver

Cycle	Cdk1WT (min) (n = 2)	Cdk1F (min) (n = 2)
Mitosis 11	5.10±0.14	6.10±0.14
Interphase 12	7.80±0.57	6.10±0.14
Mitosis 12	5.90±0.70	6.05±0.07
Interphase 13	14.20±0.85	9.10±0.14

Syncytial embryos expressing Cdk1WT-VFP or Cdk1(Y15F)-VFP and mCherry-Tubulin under the control of maternal *nos-Gal4* driver were subjected to live imaging and, the lengths of interphase and mitosis were determined as previously described (Stumpff et al., 2004). The mCherry-Tubulin was used to mark nuclear envelope breakdown (movement of tubulin into the nucleus) and nuclear envelope formation (tubulin exclusion from the nucleus). Mitosis was defined as the time between nuclear envelope breakdown to nuclear envelope formation, while interphase was considered as the time interval between nuclear envelope formation and breakdown (NB: n = 2).

Movie A2-1: *Drosophila* embryo expressing Cdk1WT-VFP and mCherry-Tubulin under the control of maternal *T279-Gal4* driver. The mCherry-Tubulin was used to mark nuclear envelope breakdown (movement of tubulin into the nucleus) and nuclear envelope formation (Tubulin exclusion from the nucleus). Stacks of 6 planes at 0.5 micron intervals were collected every 30 seconds and the embryo is undergoing mitosis 11 (M11) at the start of the.

Movie A2-2: *Drosophila* embryo expressing Cdk1(Y15F)-VFP and mCherry-Tubulin under the control of maternal *T279-Gal4* driver. The mCherry-Tubulin was used to mark nuclear envelope breakdown (movement of tubulin into the nucleus) and nuclear envelope formation (Tubulin exclusion from the nucleus). Stacks of 6 planes at 0.5 micron intervals were collected every 30 seconds and the embryo is undergoing mitosis 11 (M11) at the start of the movie.

Movie A2-3: *Drosophila* embryo expressing Cdk1WT-VFP and mCherry-Tubulin under the control of maternal *nos-Gal4* driver. The mCherry-Tubulin was used to mark nuclear envelope breakdown (movement of tubulin into the nucleus) and nuclear envelope formation (Tubulin exclusion from the nucleus). The embryo is undergoing mitosis 11 (M11) at the start of the movie.

Movie A2-4: *Drosophila* embryo expressing Cdk1(Y15F)-VFP and mCherry-Tubulin under the control of maternal *nos-Gal4* driver. The mCherry-Tubulin was used to mark nuclear envelope breakdown (movement of tubulin into the nucleus) and nuclear envelope formation (Tubulin exclusion from the nucleus). The embryo is undergoing mitosis 11 (M11) at the start of the movie.

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Appendix B: List of stocks used for the experiments and short descriptions where possible.

#	NAME	ORIGIN	DESCRIPTION
1	*UAS-Cdk1(T14A)-VFP*	Campbell Lab	Transgenic strain expressing Cdk1 mutant with threonine-14 to alanine mutation.
2	*UAS-Cdk1(Y15F)-VFP*	Campbell Lab	Transgenic strain expressing Cdk1 mutant with tyrosine-15 to phenylalanine mutation.
3	*UAS-Cdk1(T14A,Y15F)-VFP*	Campbell Lab	Transgenic strain expressing completely non-inhibitable Cdk1 mutant, threonine-14 to alanine and tyrosine-15 to phenylalanine mutations.
4	*UAS-Cdk1(WT)-VFP*	Campbell Lab	Transgenic strain expressing wild-type Cdk1.
5	*w;UAS-CycB;UAS-Cdk1(T14A,Y15F)-VFP*	Campbell Lab	Recombinant stock
6	*w;UAS-CycB;UAS-Cdk1(WT)-VFP*	Campbell Lab	Recombinant stock
7	*w;UAS-CycB;UAS-Cdk1(Y15F)-VFP*	Campbell Lab	Recombinant stock
8	*w;UAS-CycB;UAS-Cdk1(T14A)-VFP*	Campbell Lab	Recombinant stock

9	*w;UAS-EcR^{RNAi}/CyO;UAS-Cdk1(WT)-VFP/TM6,Tb*	Campbell Lab	Recombinant stock
10	*w;UAS-EcR^{DN}/CyO;UAS-Cdk1(WT)-VFP/TM6,Tb*	Campbell Lab	Recombinant stock
11	*w;UAS-EcR^{RNAi}/CyO;UAS-Cdk1(Y15F)-VFP/TM6,Tb*	Campbell Lab	Recombinant stock
12	*w;UAS-EcR^{DN}/CyO;UAS-Cdk1(Y15F)-VFP/TM6,Tb*	Campbell Lab	Recombinant stock
13	*w;cdc2^{E1-E24}/CyO;UAS-Cdk1(WT)-VFP*	Campbell Lab	A recombinant stock that can be used to express wild-type Cdk1 in a temprature sensitive *cdc2* mutant background.
14	*w;cdc2^{E1-E24}/CyO;UAS-Cdk1(T14A)-VFP*	Campbell Lab	A recombinant stock that can be used to express Cdk1A in a temprature sensitive cdc2 mutant background.
15	*w;cdc2^{E1-E24}/CyO;UAS-Cdk1(Y15F)-VFP*	Campbell Lab	A recombinant stock that can be used to express Cdk1F in a temprature sensitive cdc2 mutant background.
16	*w;cdc2^{E1-E24}/CyO;UAS-Cdk1(T14A,Y15F)-VFP*	Campbell Lab	A recombinant stock that can be used to express Cdk1AF in a temprature sensitive cdc2 mutant background.
17	*w;cdc2^{B47};neur-Gal4,gal80ts/TM6,Tb,Hu*		

18	*w;cdc2^{B47};actin-Gal4/TM6,Tb,Hu*		
19	*w;cdc2^{B47};tubulin-Gal4/TM6,Tb,Hu*		
20	*w^{-};p{UAS-CycB}*	Bloomington	
21	*y,w;Sco/CyO,act-GFP,w^{+};wor-gal4^{2}/TM6,Tb,Hu,e*		
22	*w;{en-Gal4,w^{+}}/CyO;myt1^{R6}/TM6,Tb,Hu*		
23	*w;UAS-Cdk1(WT)-VFP;myt1^{R6}/TM6,Tb,Hu*		
24	*w;UAS-Cdk1(Y15F)-VFP;myt1^{R6}/TM6,Tb,Hu*		
25	*w;UAS-Cdk1(T14A,Y15F)-VFP;myt1^{R6}/TM6,Tb,Hu*		
26	*y,w;UAS-Notch-mCherry/TM3,Sb,Ser*	Shigeo Hayashi's Lab	

27	*w-;p[{UAS-Notch-mCherry},{UAS-Cdk1WT-VFP}]/TM6,Tb,Hu*		7 independent recombinant lines were made between UAS-Notch-mCherry and UAS-Cdk1WT-VFP and their expressions were very in salivary gland cells.
28	*w-;p[{UAS-Notch-mCherry},{UAS-Cdk1(Y15F)-VFP}]/TM6,Tb,Hu*		8 independent recombinant lines were made between UAS-Notch-mCherry and UAS-Cdk1(Y15F)-VFP and their expressions were very in salivary gland cells.
29	*w-;p[{UAS-Notch-mCherry},{UAS-Cdk1(T14A)-VFP}]/TM6,Tb,Hu*		6 independent recombinant lines were made between UAS-Notch-mCherry and UAS-Cdk1(T14A)-VFP and their expressions were very in salivary gland cells.
30	*w;P{UAS>mRFP-Pon[LD]} (2nd Chromosome)*	Knoblich's Lab	
31	*w/y,w;;P{UAS>mRFP-Pon[LD]}[1.2]/TM3,Sb*	Knoblich's Lab	
32	*w;;[neur-Gal4],P{UAS>mRFP-Pon[LD]}[1.2]/TM3,Sb*	Knoblich's Lab	
33	*w/y,w;;P{UAS>Lgl-mCherry}[10.1]/TM3,Sb*	Knoblich's Lab	
34	*w/y,w;P{UAS>Lgl-mCherry}[6.1]/CyO*	Knoblich's Lab	
35	*w;P{UAS>Lgl-mCherry}/CyO;UAS-Cdk1WT-VFP/TM6,Tb*	Campbell Lab	

36	*w;P{UAS>Lgl-mCherry}/CyO;UAS-Cdk1(Y15F)-VFP/TM6,Tb*	Campbell Lab	
37	*w;P{UAS>Lgl-mCherry}/CyO;UAS-Cdk1(T14A)-VFP/TM6,Tb*	Campbell Lab	
38	*w-;P{UAS-Wee1^{KD}-RFP/CyO};UAS-Cdk1WT-VFP/TM3,Sb*	Campbell Lab	Wee1^{KD} is a kinase dead transgenic strain generated in Campbell lab. The recombinant stock for co-expressing Cdk1WT with the kinase dead version of Wee1.
39	*w-;P{UAS-Wee1^{KD}-RFP/CyO};UAS-Cdk1(Y15F)-VFP/TM3,Sb*	Campbell Lab	Recombinant stock for co-expressing Cdk1F with the kinase dead version of Wee1.
40	*w-;P{UAS-Wee1^{KD}-RFP/CyO};UAS-Cdk1(T14A)-VFP/TM3,Sb*	Campbell Lab	Recombinant stock for co-expressing Cdk1A with the kinase dead version of Wee1.
41	*w-;P{UAS$_T$-mCherry-Wee1};UAS-Cdk1(T14A,Y15F)-VFP/TM6,Tb,Hu*	Campbell Lab	The UAS$_T$-mCherry-Wee1 was a gift from Eric Wiechaus Lab. A recombinant stock for simultaneously expressing Wee1 and Cdk1AF.
42	*w-;P{UAS$_T$-mCherry-Wee1};UAS-Cdk1(Y15F)-VFP/TM6,Tb,Hu*	Campbell Lab	A recombinant stock for simultaneously expressing Wee1 and Cdk1F.
43	*w-;P{UAS$_T$-mCherry-Wee1};UAS-Cdk1WT-VFP/TM6,Tb,Hu*	Campbell Lab	A recombinant stock for simultaneously expressing Wee1 and Cdk1WT.
44	*w;tv3-Cdk1WT-GFP/CyO*	Campbell Lab	#2 is viable, #6 is lethal and #9 is X-Chromose. Transgenic stock expressing Cdk1WT under the control of spermatocyte-specific tubulin promoter.
45	*w;tv3-Cdk1WT-GFP/TM6,Tb,Hu*	Campbell Lab	#5 is viable, #1 and #7 are lethal.

46	*w;tv3-Cdk1(T14A)-GFP/CyO*	Campbell Lab	#1 and #3 are lethal on 2nd chromosome. Transgenic stock expressing Cdk1A under the control of spermatocyte-specific tubulin promoter.
47	*w;tv3-Cdk1(T14A)-GFP/TM6,Tb,Hu*	Campbell Lab	#4 and #6 are 3rd chromosome viable.
48	*w;tv3-Cdk1(Y15F)-GFP/CyO*	Campbell Lab	#1 and #2 are homozygous lethal on 2nd chromosome. Transgenic stock expressing Cdk1F under the control of spermatocyte-specific tubulin promoter.
49	*w;tv3-Cdk1(Y15F)-GFP/TM6,Tb,Hu*	Campbell Lab	#4 is viable on the 3rd chromosome
50	w^{+};twe^{1},cn,bw/CyO	Bloomington	Homozygous male sterile *twine* mutant.
51	w^{-};twe^{1},cn,bw/CyO;tv3-Cdk1WT-GFP/TM6,Tb,Hu	Campbell Lab	Recombinant stocks that can be used to express wild-type Cdk1 in *twine* mutant spermatocytes.
52	w^{-};twe^{1},cn,bw/CyO;tv3-Cdk1(T14A)-GFP/TM6,Tb,Hu	Campbell Lab	Recombinant stocks that can be used to express Cdk1A in *twine* mutant spermatocytes.
53	w^{-};twe^{1},cn,bw/CyO;tv3-Cdk1(Y15F)-GFP/TM6,Tb,Hu	Campbell Lab	Recombinant stocks for expressing Cdk1F in *twine* mutant spermatocytes.
54	$cycE^{JP}$	H. Richardson	Hypomorphic *cycE* alleles. Flies homozygous for these allele show rough eyes phenotype. Five different alleles were generously provided by H. Richardson Lab.

55	*w⁻;cyc E^{JP};neur-Gal4/TM6,Tb,Hu*	Campbell Lab	Recombinant stock
56	*w⁻;cyc E^{JP};UAS-Cdk1WT-VFP/TM6,Tb,Hu*	Campbell Lab	Recombinant stock
57	*w⁻;cyc E^{JP};UAS-CdkI(Y15F)-VFP/TM6,Tb,Hu*	Campbell Lab	Recombinant stock
58	*scabrous-Gal4*	Not sure	A pan-neuronal gal4 that drives the expression of its reporter in the neuroectoderm, as well as early neuroblasts and SOP cells.
59	*sgs3-Gal4*	Not sure	Expresses in wondering 3rd instar larval salivary gland cells.
60	*scalloped-Gal4 (sd-Gal4)*	Not sure	It drives expression throughout presumptive wing margin during wing imaginal development.
61	*elav-Gal4*	Not sure	Neuron specific gal4 driver. It is for panneuronal expression of its reporter genes
62	*engrailed-Gal4 (en-Gal4)*	Not sure	*en-GAL4* driver is specifically expressed in the posterior compartment of the wing disc tissue reflecting the expression pattern of the engrailed (en) gene.
63	*prospero-Gal4 (pros-Gal4)*	Gift from Chris Doe's Lab	Drives expression in embryonic CNS, 3rd instar neuroblast, pupal external sensory organ precursor cell pIIb and in sub type of adult neurons and glia cells.
64	*neur-Gal4/TM6,Tb,Hu*	Bloomington	*neur-Gal4* drives expression in SOP cells and their descendant cells.

65	*neur-Gal4,gal80^{ts}/TM6,Tb,Hu*	Gho's lab	gal80^{ts} is used for temporally controlling transgene expression. At permissive temprature, gal80 directly bind to and transcriptionally represses gal4 activity.
66	*eyeless-Gal4*	Not sure	Eye-specific gal4 driver. It drivers the exprssionof its reporter gene in the dividing cells of eye imaginal discs.