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Functional analysis of *Drosophila wee1* using tagged transgenes

by

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ABSTRACT

Wee1-like kinases are cell cycle regulators, highly conserved from yeast to humans, which negatively regulate cell cycle progression by inhibiting Cdk1 through phosphorylation. After generating an *egfp-wee1* transgene under the UAS-GAL4 control, I was able to examine the localization and function of the tagged proteins during embryogenesis and oogenesis. Consistent with other organisms, *Drosophila* Wee1 proteins have a dynamic localization that showed cell type and cell cycle specificity. Syncytial embryos overexpressing Wee1 showed defects in both nuclear and cytoplasmic events, suggesting that Cdk1 is mis-regulated. Ectopic pseudo-cleavage furrows in these embryos also suggested a possible novel role of Cdk1 in regulating the actin cytoskeleton. During oogenesis, Wee1 overexpression promoted one extra round of cystocyte division. This unexpected observation argues against the current consensus that Wee1 is acting solely as a mitotic inhibitor and implies that the germline division counting mechanism is regulated by a novel process.

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List of abbreviations used in this thesis (in chronological order)

Cdk: cyclin-dependent-kinase

MPF: maturation promoting factor

cdc (mutants): cell-division-cycle

ORC: origin recognition complex

CAK: Cdk activating kinase

CKI: Cyclin-dependent-kinase inhibitor

Myt1: membrane-localized tyrosine/threonine-directed kinase

ER: endoplasmic reticulum

T14 (or thr 14): threonine 14 residue

Y15 (or tyr 15): tyrosine 15 residue

ATM: Ataxia Telangiectasia mutated

ATR: ATM related

Chk1 (or Chk2): checkpoint kinase 1 (or 2)

NLS: nuclear localization signal

EGFP: enhanced green fluorescent protein

UAS: upstream activating sequence

Hsp70: heat shock protein 70

IPTR: inositol 1, 4, 5-triphosphate receptor

HU: hydroxyurea

BrdU: 5-bromo-2-deoxyuridine

PH3: phospho-Histone H3

PBS: phosphate buffered saline

PBST: 0.3% TritonX-100 in 1XPBS or 0.1% TritonX-100 in 1XPBS or 0.02% Tween-20 in 1XPBS

NGS: normal goat serum

TE: 10 mM tris + 1 mM EDTA

PBSTM: PBST (Tween-20) + 5% skim milk

pT14-Cdk1: phosphorylated threonine 14 Cdk1

pY15-Cdk1: phosphorylated tyrosine 15 Cdk1

APC: anaphase promoting factor

Dhc: dynein heavy chain

KLP: kinesin-like protein

Ncd: non-claret disjunctional, a kinesin protein

CPE: cytoplasmic polyadenylation element

CPEB: cytoplasmic polyadenylation element binding protein

Chapter 1
Introduction

This thesis is focused on the analysis of the localization and function of *Drosophila* Wee1 using epitope tagged transgenes. Wee1-like kinases are highly conserved proteins that negatively regulate the cell cycle by phosphorylating Cdk1 (cyclin-dependent-kinase 1). In this chapter, I review our current knowledge on cell cycles and their control by Cdk1 and Wee1-like kinases. To familiarize the readers with the background information on my project, I also review what is known about the localization and function of Wee1 in different model organisms.

1.1 Cell cycle overview and cell cycle control

Cellular growth and division occur as a series of strictly ordered events, which is referred to as the cell cycle. There are variations in cell cycles in different organisms, or even within one organism, but a typical mitotic cell cycle consists of G1 phase, S phase, G2 phase and M phase. Only completion of one phase can lead to the start of the next. Cells have intrinsic cell cycle regulatory machinery to govern cell cycle progression. It has been demonstrated that cell cycles are controlled by the same basic machinery in species from the unicellular organism yeast to far more complex humans. The most important regulatory components are Cdk-Cyclin complexes that act coordinately to drive cell cycle progression.

1.1.1 Discovery of MPF (maturation promoting factor) and other Cdk-Cyclin complexes

The earliest cell cycle studies were done in *Xenopus* oocytes. These oocytes arrest in the prophase of meiosis I. Prior to being laid, steroid hormone progesterone is released by surrounding somatic cells and triggers the oocytes to mature. Oocytes will then progress into the metaphase of meiosis II. This process is defined as oocyte maturation. Masui and Markert (1971) found that a fraction of mature oocytes had the capacity to induce an immature oocyte to mature in the absence of hormone stimulation. This interesting factor was thus named MPF (maturation promoting factor), although at that time it was not clear what MPF was (Masui, 1974).

Subsequent cell cycle studies in the single-cell organism yeast shed light on what MPF was. Nurse *et al.* (1976) and Hartwell *et al.* (1974) pulled out a series of *cdc* (cell-division-cycle) mutants in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* respectively. *cdc2* in *S. pombe* and *CDC28* in *S. cerevisiae* were among them. These two genes were identified to be homologous to each other and could complement the mutation of each other in the two yeast species (Beach *et al.*, 1982; Russell *et al.*, 1989). It was also demonstrated that Cdc2/Cdc28 was required for progressing into mitosis (Reed and Wittenberg, 1990). Homologues of these two genes in higher eukaryotes were subsequently identified and cloned based on their ability to complement yeast *cdc2/CDC28* mutation (Lee and Nurse, 1987; Dunphy *et al.*, 1988; Labbe *et al.*, 1988; Lehner and O'Farrell, 1990; Colasanti *et al.*, 1991; Campbell *et al.*, 1995). Further genetic and biochemical studies in yeast and *Xenopus* oocytes revealed that purified MPF included a Cdc2/Cdc28 like protein (Arion *et al.*, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbe *et al.*, 1989). Around the same time, another component of MPF was identified to be a protein called Cyclin B (Evans *et al.*, 1983; Gautier *et al.*, 1990). Later biochemical studies revealed that Cdc2/Cdc28 was a protein kinase and that Cdc2/Cdc28 and Cyclin B formed a complex in which Cyclin B appeared to affect the kinase activity

of Cdc2/Cdc28 (Simanis and Nurse, 1986; Booher *et al.*, 1989; Draetta *et al.*, 1989). Therefore Cdc2/Cdc28 was named Cdk1 (cyclin-dependent kinase 1).

In the meantime, other Cdks and Cyclins were also discovered in yeast and then cloned in higher eukaryotes in a similar fashion as Cdk1 (Hagan *et al.*, 1988; Pines and Hunter, 1989). Cdks and cyclins are conserved proteins that have been found in all metazoans. Some known Cdks are Cdk1, Cdk2, Cdk4, Cdk5, Cdk6 and Cdk7. Known Cyclins are Cyclin A, Cyclin B, Cyclin D, Cyclin E, Cyclin H and other less characterized cyclins (Pines, 1993; Fisher and Nurse, 1995; Andrews and Measday, 1998). Cdks form a complex with their cyclin or regulatory partners. Cdk1 has been shown to form complexes with both Cyclin A and Cyclin B. Cdk2 binds to Cyclin A and Cyclin E. Cdk4 and Cdk6 both bind to Cyclin D. Cdk7 and Cyclin H form a complex that functions as a subunit of the Cdk activating kinase. Different Cdk-Cyclin complexes regulate different stages of the cell cycle. For example, Cdk2-Cyclin E and Cdk2-Cyclin A complexes promote the transition from G1 phase to S phase and the establishment of S phase. Cdk1-Cyclin A and Cdk1-Cyclin B complexes are involved in the transition from G2 phase to M phase. Sequential actions of various Cdk-cyclin complexes control the progression of cell cycles (Nasmyth, 1996).

1.1.2 Cdk1 function

Cdk1-Cyclin B complex is one major M-phase kinase, promoting important mitotic events such as chromosome condensation, nuclear envelope breakdown and spindle assembly (Lehner and O'Farrell, 1990). At the end of mitosis, Cyclin B is degraded by a ubiquitin-dependent pathway and Cdk1 kinase activity is thus downregulated leading to the exit of mitosis (Draetta *et al.*, 1989). Cdk1-Cyclin B was shown to have a variety of targets involved in various aspects of cell cycles. Known targets include ORC (origin recognition complex) proteins, yeast transcription-related proteins Whi5 and Ndd1, and proteins involved in regulating cytoplasmic events such as centrosome separation, spindle assembly and cytokinesis (Arion *et al.*, 1988; Carlson *et al.*, 2001; Ji *et al.*, 2002; DePamphilis, 2003; Jackman *et al.*, 2003; Liakopoulos *et al.*, 2003; Maekawa *et al.*, 2003; Jaspersen *et al.*, 2004; Litvak *et al.*, 2004; Mishima *et al.*, 2004). Cdk1 pairs with Cyclin A as well. Cdk1-Cyclin A complex has been demonstrated to function both in S and G2 phases although the exact roles of this complex are poorly understood (Nasmyth, 1996).

1.1.3 Regulation of Cdk1 activities

Cdk1 activity is regulated by several means. First of all, Cyclin B binding is critical for the kinase activity of Cdk1. When Cyclin B binds to Cdk1, it undergoes a conformational change that exposes the ATP-binding site in the kinase domain, which is required for its kinase activity (Morgan, 1995). Although the levels of Cdk1 remain constant throughout the cell cycle, Cyclin B levels show a cell cycle dependent oscillation (Evans *et al.*, 1983; Booher *et al.*, 1989; Pines and Hunter, 1989). Cyclin B starts to accumulate in G2 phase and gets degraded by ubiquitin-dependent pathway in anaphase. As a result, MPF activity oscillates in a cell cycle dependent manner (Murray *et al.*, 1989; Draetta *et al.*, 1989; Glotzer *et al.*, 1991).

Besides being regulated by Cyclin B levels, Cdk1 activity is also subjected to phosphorylation control, one of the most common mechanisms of regulating protein

activities. Wee1-like kinases phosphorylate Cdk1 in an inhibitory fashion (Lundgren *et al.*, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993), whereas Cdc25 phosphatase dephosphorylates Cdk1, resulting in its activation. This phosphorylation control targets the Cdk1-Cyclin B complex rather than Cdk1 alone (Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992).

Other factors regulate Cdk1 activities as well. For example, CAKs (Cdk activating kinases) activate Cdk1 by phosphorylating the threonine 161 residue on Cdk1 (threonine 167 in *S. pombe*, Gould *et al.*, 1991). CKIs (cyclin-dependent kinase inhibitors) block Cdk1 activity by binding to Cdk1-Cyclin B complexes. Known CKIs are the Cip/Kip family and the INK4 family in vertebrates, and *dacapo*, *roughex* in *Drosophila* (Xiong *et al.*, 1993; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994; Lane *et al.*, 1996; Foley *et al.*, 1999).

Cells regulate Cdk1 activities in response to different developmental cues or cellular signals (Moreno *et al.*, 1989). As mentioned earlier, Cyclin B levels fluctuate during the cell cycle, leading to an oscillation of Cdk1 activity as cells go through cell cycles. As a result, during those developmental processes where cell cycles need to be stopped or modified to accommodate the transition from one developmental stage to the next, Cdk1 activities are always held in check (Enoch *et al.*, 1992; Lew and Reed, 1993 and 1995; Sibon *et al.*, 1997; Sia *et al.*, 1998; Edgington *et al.*, 1999; Murakami *et al.*, 2004). For example, during gastrulation in *Xenopus* development, cells are arrested in a prolonged G2 phase to facilitate the movement of certain cells and cell fate determination. Cdk1 is inhibited during this process to ensure that cells will not enter mitosis prematurely (Murakami *et al.*, 2004). In addition, CKIs or Wee1-like kinases inhibit Cdk1 in response to DNA damage, thereby preventing entry into mitosis. In fact, lowered Cdk1 activity has been implicated in many cell cycle checkpoints where entry into mitosis is prevented (Rowley *et al.*, 1992; Walworth *et al.*, 1993; Kumagai *et al.*, 1995; Jin *et al.*, 1996; Lydall *et al.*, 1996; Blasina *et al.*, 1997; O'Connell *et al.*, 1997; Rhind *et al.*, 1997; Rhind and Nurse, 1998; Baber-Furnari *et al.*, 2000).

1.2 Wee1-like kinases

1.2.1 Discovery of Wee1-like kinases

wee1 was discovered in a screen looking for cells that were defective in cell size control in *S. pombe* by Nurse *et al.* (1975). *wee1* mutant cells divided at about half of the size of a normal cell. Measurement of cell cycle lengths indicated that *wee1* mutant cells have a shortened G2 phase. In similar screens, *cdc25* was discovered (Fantes and Nurse, 1977 and 1978; Fantes, 1981). *cdc25* ts (temperature sensitive) mutants failed to enter mitosis at the restrictive temperature (Russell and Nurse, 1986). Further genetic interaction studies showed that *wee1* and *cdc25* acted in an antagonistic way and both of them interacted with *cdc2* (Russell and Nurse, 1987; Moreno *et al.*, 1990). Futher biochemical research shed light on the properties of Wee1 and Cdc25. Wee1 was found to be a protein kinase and Cdc25 a phosphatase (Russell and Nurse, 1987; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Feathersone and Russell, 1991; Lee *et al.*, 1992; Parker *et al.*, 1992). They both target Cdk1 in the Cdk1-Cyclin B complex, but not Cdk1 by itself. Wee1 phosphorylates the tyrosine 15 residue on Cdk1, whereas Cdc25 dephosphorylates this site. This phosphorylation of Cdk1 was then shown to be inhibitory (Lundgren *et al.*, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993).

Dephosphorylation of Cdk1 by Cdc25 prior to M phase was demonstrated to be necessary for entry into mitosis because active Cdk1 is needed to promote mitosis in eukaryotes (Strausfeld *et al.*, 1991; Amon *et al.*, 1992; Izumi and Maller, 1993).

In *S. pombe*, there are two Wee1-like kinases: Wee1 and Mik1 (Lundgren, 1991; Lee *et al.*, 1994), whereas in *S. cerevisiae* there is only one Wee1 kinase: Swe1 (Booher, 1993). Several characterized metazoans have two Wee1-like kinases: Wee1 and Wee2 (sometimes referred to as Wee1A and Wee1B, Igarashi *et al.*, 1991; Honda *et al.*, 1995; Mueller *et al.*, 1995; Nakanishi *et al.*, 2000; Okamoto *et al.*, 2002; Leise and Mueller, 2002) however *Drosophila* only has one Wee1-like kinase, Wee (Campbell *et al.*, 1995). Nuclear Wee1 kinases in different organisms phosphorylate the tyrosine 15 residue on Cdk1 (or an analogous site).

Another kind of Wee1-like kinase was identified only in metazoans, and named Myt1 (membrane-localized tyrosine/threonine-directed kinase, Kornbluth *et al.*, 1994; Booher *et al.*, 1997; Liu *et al.*, 1997; Price *et al.*, 2002). As the name indicates, Myt1 is localized in the cytoplasm and associated with ER (endoplasmic reticulum) and Golgi bodies (different from the nuclear localization of Wee1). Myt1 can phosphorylate both the threonine 14 and the tyrosine 15 residues on Cdk1. Some evidence suggested that Myt1 has a preference for threonine 14 over tyrosine 15, but it still can phosphorylate both (Booher *et al.*, 1997). The differences in localization and phosphorylation site preference of Wee1 and Myt1 imply that they may serve distinct roles in cell cycle regulation. The current consensus is that Myt1 phosphorylates Cdk1 in the cytoplasm after it is paired with Cyclin B. Then Cdk1-Cyclin B is transported into the nucleus where it can be phosphorylated by Wee1 when Cdk1 is dephosphorylated prematurely by Cdc25. Thus, Myt1 and Wee1 act cooperatively to ensure that the cytoplasm and the nucleus are protected from prematurely activated Cdk1 (Figure 1-1 diagram).

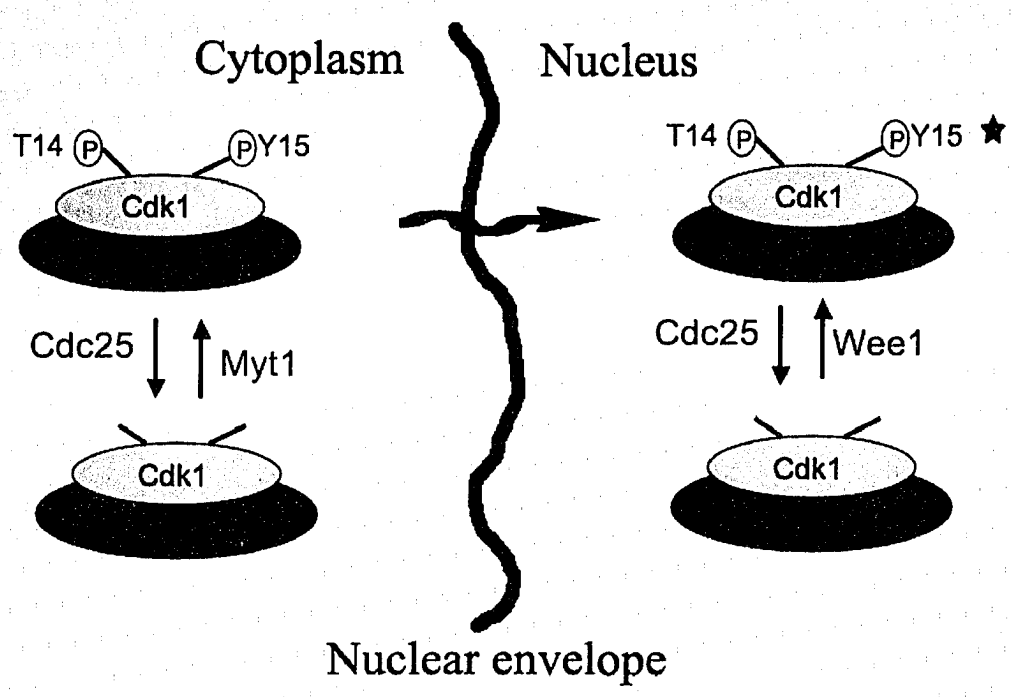

Figure 1-1 Current hypothesis on the regulation of Cdk1-Cyclin B by phosphorylation and dephosphorylation. Wee1-like kinases inhibit Cdk1 activities by phosphorylating threonine 14 (T14) and tyrosine 15 (Y15) residues on Cdk1, whereas Cdc25 phosphatase activates Cdk1 by removing the phosphorylation. Active Cdk1 is required for entry into mitosis. Wee1 only phosphorylates Y15, and Myt1 phosphorylates both T14 and Y15 (Booher *et al.*, 1997; Leise and Mueller, 2002). In the cytoplasm Cdk1 gets phosphorylated by Myt1 before translocating into the nucleus. In the nucleus Cdk1 gets phosphorylated by Wee1 on the Y15 if it is prematurely dephosphorylated by Cdc25. Cdc25 dephosphorylates Cdk1 on both T14 and Y15 leading to its activation both in the cytoplasm and the nucleus (Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992).

1.2.2 Wee1-like kinases and checkpoint control

To ensure that all events in the cell cycle occur properly, cells have evolved so-called checkpoints to examine whether it is ready for the next stage of the cell cycle and to respond when it is not yet ready. If one step of the cell cycle is disrupted, checkpoint pathways will be activated and transiently arrest the cell cycle ensuring that errors are repaired before cells enter the next stage. For example, when DNA replication is blocked, a DNA replication checkpoint will be activated. As a result, cells are arrested in S phase. Another example is the DNA damage checkpoint. When DNA is damaged either by environmental factors like ionizing radiation or by spontaneous DNA double strand breaks, the DNA damage checkpoint will be activated, leading to a delay in cell cycle progression, as well as activation of the DNA repair machinery. Loss of checkpoint controls often has serious consequences, such as cancer and apoptosis (Hartwell and Weinert, 1989; Dasso and Newport, 1990; Enoch and Nurse, 1990; Hartwel, 1992; Elledge, 1996; Bell *et al.*, 1999; de Klein *et al.*, 2000; Murakami and Nurse, 2000; Wang *et al.*, 2004). In order for the cell cycle to be arrested when checkpoints are activated, the activities of critical cell cycle regulators such as Cdk1 are modified during checkpoint responses. Studies demonstrated that Cdk1 is a critical factor in generating checkpoint responses (Rowley *et al.*, 1992; Walworth *et al.*, 1993; Kumagai *et al.*, 1995; Jin *et al.*, 1996; Lydall *et al.*, 1996; Blasina *et al.*, 1997; O'Connell *et al.*, 1997; Rhind *et al.*, 1997; Rhind and Nurse, 1998; Baber-Furnari *et al.*, 2000).

Earlier checkpoint research was done in yeast, focusing on the DNA replication checkpoint (S/M checkpoint) and DNA damage checkpoint (G2/M checkpoint). Many screens were performed to identify genes whose loss of function confers sensitivity to drugs inhibiting DNA replication or ionizing radiation (causes DNA damage). A series of "*rad*" genes were pulled out in those screens (Weiner and Hartwell, 1988; Rowley *et al.*, 1992; Bentley *et al.*, 1996). The proteins encoded by these "*rad*" genes can sense stalled DNA replication or DNA damage, stop the cell cycle and activate repair machinery. Homologues of "*rad*" genes were found and cloned in higher eukaryotes. In fact these checkpoint genes are highly conserved from yeast to humans.

It has been thought that at least two parallel pathways play important roles in generating these two checkpoints. The yeast *tel1/TEL1* and *rad3/MEC1* genes, equivalent *Drosophila ATM* (Ataxia Telangiectasia mutated) and *mei-41* genes and mammalian *ATM* and *ATR* (ATM related) genes are thought to function at the early steps of such checkpoints (Sibon *et al.*, 1999). It has been suggested that ATM/ATR act as sensors of DNA damage or stalled DNA replication and transduce the signals downstream to Chk1/Chk2, two important checkpoint kinases (checkpoint kinase 1, checkpoint kinase 2, Walworth and Bernards, 1996; Boddy *et al.*, 1998; Brondello *et al.*, 1999). Current models for these two pathways are: ATM functions through Chk2 by phosphorylating Chk2, whereas ATR targets Chk1 by phosphorylation as well (Matsuoka *et al.*, 1998; Brown *et al.*, 1999). Chk1 and Chk2 further transduce the signals to downstream factors such as Wee1-like kinases and Cdc25 phosphatase (O'Connel *et al.*, 1997; Raleigh and O'Connell, 2000; Rhind and Russell, 2001). Chk1 has been shown to phosphorylate Wee1 leading to its activation. Meanwhile Chk1 can also phosphorylate Cdc25 resulting in its physical exclusion from the nucleus as well as a downregulation of its activity (Sanchez *et al.*, 1997; Zeng *et al.*, 1998; Funari *et al.*, 1999; Lopez-Girona *et al.*, 1999; Zeng and Piwnica-Worms, 1999; Uto *et al.*, 2004).

Cdk1 is a critical effect or target in such checkpoint responses (Rowley *et al.*, 1992; Walworth *et al.*, 1993; Kumagai *et al.*, 1995; Jin *et al.*, 1996; Lydall *et al.*, 1996; Blasina *et al.*, 1997; O'Connell *et al.*, 1997; Rhind *et al.*, 1997; Rhind and Nurse, 1998; Baber-Furnari *et al.*, 2000). Activated Wee1 can inhibit nuclear Cdk1 and absence of Cdc25 in the nucleus further prevents activation of nuclear Cdk1 (Heald *et al.*, 1993). Lowered levels of active Cdk1 in the nucleus will stop the cell cycle allowing time for repairs. Chk2 is thought to have similar functions as Chk1 in that it can phosphorylate and activate Wee1 while phosphorylating and inhibiting Cdc25, resulting in an inhibition of Cdk1 (Raleigh and O'Connell, 2000; Rhind and Russell, 2001; Figure 1-2 diagram). Other Cdk1 regulators are also activated or deactivated in response to checkpoints. For example, CKIs also play a role in inactivating Cdk1 in such checkpoint responses (Peter, 1997; Abal *et al.*, 2004).

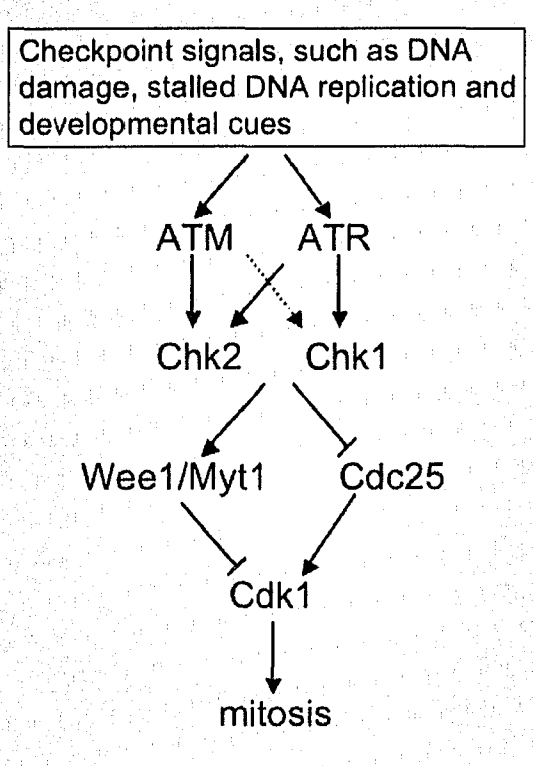

Figure 1-2 Conserved checkpoint pathways in response to signals such as DNA damage, stalled DNA replication and developmental cues. ATM/ATR are thought to act in the early steps of checkpoint responses. They transduce signals downstream to Chk1/Chk2 kinases via phosphorylation, which will in turn activate Wee1-like kinases and inhibit Cdc25 phosphatase. As a result, Cdk1 activities are downregulated, preventing cells from premature entry into mitosis. Whether ATM can phosphorylate Chk1 is unknown. Yeast genes are not represented in this diagram. However the checkpoint genes are conserved. *S. pombe tel*, *S. cerevisiae TEL1* are *ATM* homologues; *S. pombe rad3*, *S. cerevisiae MEC1* are *ATR* homologues; *S. pombe cds1*, *S. cerevisiae RAD53* are *Chk2* homologues. *S. pombe chk1/rad27*, *S. cerevisiae CHK1* are *Chk1* homologues. They perform similar functions as those in metazoans.

There are a few Wee1-like kinases in each species, and it is not entirely clear which Wee1-like kinase (s) is (are) required in an S/M or G2/M checkpoint. In *S. pombe*, *mik1* is required for such checkpoints, but there are still debates on whether *S. pombe wee1* is required (Amon *et al.*, 1992; Rowley *et al.*, 1992; Barbet and Carr, 1993; Lew and Reed, 1995; Boody *et al.*, 1998; Baber-Furnari *et al.*, 2000; Raleigh and O'Connell, 2000; Rhind and Russell, 2001). In different human cultured cell lines, *wee1* is either required or not required for such checkpoints (Furnari *et al.*, 1997; Li *et al.*, 2002; Chow *et al.*, 2003; Wang *et al.*, 2004). These results suggest that functional redundancy probably exists within members of Wee1-like kinases in a particular species.

1.2.3 Functions of Wee1 in early embryogenesis in *Drosophila*

Early embryogenesis in flies is a complex but highly regulated process, in particular the rapid, syncytial divisions. When a sperm fertilizes an egg, maternal nucleus and paternal nucleus fuse together and the new nucleus will start embryonic divisions (Foe and Alberts, 1983). The first 13 divisions occur without cytokinesis. All of the nuclei lie within the embryo. These 13 cycles consist of only alternating S and M phases. Cell cycles are rapid and synchronous. The average cell cycle time (before cycle 11) is about 8 minutes. Nuclei divide in the interior of the syncytium from cycle 1 to cycle 7. At cycle 7 to 9, the nuclei migrate to the cortex of the embryos and continue dividing. This nuclear migration process involves microtubules and microtubule-dependent motor proteins (Foe *et al.*, 1993). Subsequent cycles 11 to 13 slow down and show increased interphase lengths (cycle 11 to 13 interphase lengths respectively: ~10 minutes; ~12minutes; ~21 minutes). This increase in interphase length is coupled with the beginning of the first zygotic transcription (Edgar and O'Farrell, 1989 and 1990; Campbell *et al.*, 1995).

The fact that the interphase length of the earliest cycles is shorter than the length of mitosis suggests that maternal gene products have a profound impact on these cell cycles and are able to drive mitosis efficiently. Later, nuclear cycles slow down, presumably because maternal factors are titrated out by the rapid increase in the number of nuclei (Sibon *et al.*, 1997). There have been debates on whether checkpoints come into play during syncytial divisions. Originally, it was thought that checkpoints could not be functioning in such rapid cell cycles. But loss of *mei-41* (*ATR* in *Drosophila*) or *grapes* (*chk1* in *Drosophila*) or *wee1* all led to a similar mitotic catastrophe phenotype in that mutant embryos cannot progress beyond the syncytial divisions to cellularize (Forgarty *et al.*, 1997; Sibon *et al.*, 1999; Price *et al.*, 2000). These observations argue that an S/M checkpoint is essential during the rapid syncytial divisions of early embryogenesis. More recently, E. Homola in our lab confirmed that a Wee1-dependent Cdk1 targeting checkpoint does exist in syncytial embryos. Levels of Cdk1 tyrosine 15 inhibitory phosphorylation were increased when syncytial embryos were treated with aphidicolin, a drug inhibiting DNA replication, indicating a checkpoint response via Cdk1. In *wee1* mutants, this inhibitory phosphorylation on the tyrosine 15 residue was attenuated, but not lost (E. Homola, personal communication). These results indicate Myt1 may be functionally redundant to Wee1 and able to transduce checkpoint signals downstream to Cdk1 in syncytial embryos. Altogether, current evidence suggests checkpoints exist in syncytial embryos to ensure that S phase is completed before entering M phase. Common checkpoint pathways seem to be also utilized in this stage during the development.

After the first 13 cycles, interphase 14 is prolonged due to a 40 minute S phase. For the first time in *Drosophila* embryogenesis, a G2 phase appears. During this long interphase, the zygotic cell cycle machinery takes over and maternal products are actively degraded. At the same time, cellularization occurs, leading to the conclusion of the syncytial stage of development (Edgar and Datar, 1996). This important series of events is often referred to as the midblastula transition or maternal/zygotic transition. After this transition, cell cycles are not any longer synchronized. Instead, different mitotic domains are driven by pulses of zygotic cdc25 transcription and translation (Edgar and O'Farrell, 1989 and 1990). Gastrulation begins shortly after the midblastula transition.

Price *et al.* (2000) identified three *wee1* mutant alleles in *Drosophila* in a mutagenesis screen. They are the *DS1* allele, *ES1* allele and *ES2* allele. *ES1* allele contains an 8-bp deletion causing a frameshift followed by a stop codon. *ES2* allele contains a missense mutation in the kinase domain. *DS1* allele contains a missense mutation in the ATP-binding domain. The hemizygous mutants are all zygotic viable but maternal effect lethal (not fully penetrant in *DS1* allele). Homozygous zygotic mutants of all alleles are lethal due to a second site mutation induced during the process of mutagenesis. The eggs laid by hemizygous mothers have normal morphology but cannot cellularize. The *ES1* allele was used mostly in my thesis work. DNA abnormalities in mutant embryos can be detected as early as embryonic cycle 11, when nuclei fail to segregate properly and subsequently cluster together, a process analogous to the mitotic catastrophe observed in *wee1mik1* fission yeast mutants (Lundgren *et al.*, 1991; Price *et al.*, 2000).

Stumpff *et al.* (2004) did an analysis of *wee1* mutant phenotypes and found that cell cycle timing in *wee1* mutant embryos is disrupted. In wild type embryos, the interphase lengths from syncytial cycle 10 to 13 show a steady increase. In embryos laid by *wee1* mutant females, the interphase lengths did not show as much an increase as in wild type, consistent with *wee1* being a mitotic inhibitor.

E. Homola in our lab found some other interesting phenotypes in *wee1* mutant embryos (E. Homola, personal communication). Using antibodies directed against Wee1, she detected transient localization of Wee1 on the spindles at the onset of mitosis. Immunostaining also showed that mitotic spindles appeared to be shorter than those in wildtype. Pseudo-cleavage furrows seemed to be also affected in *wee1* mutant embryos. The pseudo-cleavage furrow is an actin rich network that surrounds the nucleus and its associated cytoplasm. It behaves similar to a cell membrane and is necessary for normal nuclear division during syncytial cycles. In *wee1* mutants, pseudo-cleavage furrows did not disassemble normally at the end of mitosis, in contrast to the wild type embryos. The regulation of pseudo-cleavage furrows is not well understood, but phosphorylated-tyrosine 15-Cdk1 was detected on the furrows, suggesting a possible and novel role of Cdk1 in regulating pseudo-cleavage furrows. It has already been shown in other systems that Cdk1 regulates cytoplasmic structures such as centrosomes and spindle pole bodies (Carlson *et al.*, 2001; Jaspersen *et al.*, 2004; Mishima *et al.*, 2004), although there is no evidence at present that Cdk1 regulates pseudo-cleavage furrows in *Drosophila* syncytial embryos. Thus, these observations provide clues to interesting new questions to answer in the future.

1.2.4 Genetic screens searching for interactions with Wee1-like kinases

A former graduate student in our lab, D. Price, found that the overexpression of Wee1 and Myt1 in the eye caused a rough eye phenotype (Price *et al.*, 2002). This was apparently because overexpression of Wee1 and Myt1 decreased the number of mitotic cells in the second mitotic wave in eye imaginal discs. In a screen looking for enhancers and suppressors of Wee1 and Myt1, *Notch* and *Delta* were identified as interacting genetically with *myt1* in addition to known interactors such as *cdc25*, *cdk1* and *tribbles*. Loss of *Delta* enhanced the overexpression of Myt1 caused rough eye phenotype, whereas loss of *Notch* suppressed it. *Notch* and *Delta* do not interact with the overexpression of Wee1 alone in the eye, however (Price, 2002). Notch/Delta signaling pathway has been implicated in the regulation of many cell cycle events as well as patterning events (Baonza and Garcia-Bellido, 2000; Lawrence *et al.*, 2000; Deng *et al.*, 2001; Torres *et al.*, 2003). For example, Notch/Delta signaling pathway is involved in regulating the switch from mitosis to endoreplication in follicle cells during oogenesis (Deng *et al.*, 2001). Another function for this pathway is regulating the wing patterning by regulating cell divisions and cell differentiation (Baonza and Garcia-Bellido, 2000; Lawrence *et al.*, 2000). However, there has not been any other report on the interaction of *myt1* with *Notch/Delta* (except Price's results) and how they might interact is not well understood.

1.2.5 Regulation of Wee1 kinases

Being important Cdk1 inhibitors, Wee1 kinases themselves are also regulated by other proteins. There are at least two reported means to regulate Wee1 proteins: protein degradation and phosphorylation. In yeast, Nim1/Cdr1 kinase was partially responsible for the phosphorylation and inactivation of Wee1 in accordance with cell cycle progression (Parker *et al.*, 1993; Wu and Russell, 1993). Nim1-related kinases (such as Hsl1 and Hsl7) and Cdc5/Polo kinase have also been shown to regulate Wee1 negatively by promoting Wee1 protein degradation (Barral *et al.*, 1999; McMillan *et al.*, 1999; Schulewitz *et al.*, 1999; Bartholomew *et al.*, 2001; van Vugt *et al.*, 2004; Asano *et al.*, 2005). Similar to many other cell cycle proteins, Wee1 is ubiquintinated by SCFbeta-TrCP in a Tome-1 dependent manner and then degraded (Ayad *et al.*, 2003; Watanabe *et al.*, 2004). This degradation occurs when Wee1 is phosphorylated (Kaiser *et al.*, 1998; Watanabe *et al.*, 2004). In *Xenopus* egg extracts, Wee1 protein was found to be hypo-phosphorylated during interphase, but hyper-phosphorylated in mitosis. Cdc2 and unknown kinases were responsible for this phosphorylation (Mueller *et al.*, 1995). In *Xenopus* and mammalian cells, Wee1 was also subjected to regulation by Chk1 and 14-3-3 (Wang *et al.*, 2000; Lee *et al.*, 2001). 14-3-3 binds to Wee1 during interphase, but not mitosis. This binding depends on the phosphorylation of a critical residue (Ser-549) on Wee1 by Chk1 kinase. After 14-3-3 binds to Wee1, the activity of the protein is decreased. Chk1 and Wee1 are known factors that function in several cell cycle checkpoints, so it is reasonable to propose that Chk1 phosphorylates Wee1 upon the activation of the checkpoint, facilitating the binding of 14-3-3 to Wee1 and leading to a stabilization of the protein. Wee1 will then inhibit Cdk1 causing the cell cycle to stop. Because Chk1, Wee1 and 14-3-3 are highly conserved proteins, similar interactions may also exist in *Drosophila*.

1.3 Using epitope tagged transgenes to study Wee1 protein localization and functions

1.3.1 Localization of Wee1 in different organisms

Wee1 proteins have a typical nuclear localization during interphase in many organisms including *Drosophila* (Heald *et al.*, 1993; Mitra and Schultz, 1996; Wu *et al.*, 1996; Nakanishi *et al.*, 2000; E. Homola, personal communication), but *Drosophila* Wee1 does not have a traditional NLS sequence (nuclear localization signal). There are two possible mechanisms for such nuclear localization. First of all, Wee1 may have an unconventional NLS, not detected by current online NLS prediction algorithms. Alternatively, the nuclear localization of this protein may be achieved through interaction with another protein (s). Evidence in *Xenopus* suggested that 14-3-3 interacts with Wee1 and this interaction has a profound influence on the localization of Wee1 within the nucleus. Mutations in *wee1* disrupting the interacting sites with *14-3-3* lead to an abnormal build-up of Wee1 proteins in unknown subnuclear structures (a dotted pattern, Lee *et al.*, 2001). So 14-3-3 may be playing a role in regulating the localization of Wee1.

Studies in human cultured cells demonstrated that the localization of human Wee1 proteins is dynamic and cell cycle dependent. With antibodies directed against human Wee1, Baldin and Ducommun (1995) found that Wee1 was localized in the nucleus during interphase with punctate dotted pattern inside the nucleus in an overexpression study. Those dots did not colocalize with structures stained by anti-BrdU or anti-PCNA, two antibodies that label the replication foci, which means that Wee1 was not localized to the replication foci. During mitosis, the majority of Wee1 was cytoplasmic, but some was associated with mitotic chromosomes, also in a punctate pattern. In late mitosis, Wee1 was found on the cleavage furrow as well as the mid body. During cytokinesis, Wee1 was in the nuclei of the two daughter cells as well as on the ends of the remains of mitotic spindles. The late-mitosis localization of Wee1 was shown to be dependent on microtubule assembly (Baldin and Ducommun, 1995). Their results indicated that Wee1 might be involved in regulating cytoskeletal structures.

Coincident with these results, Sakchaisri *et al.* (2004) reported that in *S. cerevisiae*, Swe1 was recruited to the septin-rich bud neck (where the daughter cell buds off the mother cell) in an Hsl l/Nim 1 dependent manner. At the bud neck, Swe1 was phosphorylated by Cla4/PAK and Cdc5/Polo, leading to a ubiquitin-mediated degradation of the protein. The downregulation of Swe1 at the bud neck was necessary for G2/M transition, but it is not fully understood why Swe1 is localized at the bud neck. It was assumed that negative regulation of Cdc28-Clb (Cdk1-Cyclin B) by Swe1 at the bud neck was important for G2/M transition, but the importance of Cdc28 function at the bud neck is not fully appreciated.

The above evidence raises some interesting questions such as what is the significance of the nuclear localization of Wee1 and how is the localization of Wee1 regulated, as recent evidence suggested that the mislocalization of a protein (for example, Cyclin A) does not necessarily interfere with its function (Dienemann and Sprenger, 2004).

1.3.2 Epitope tagging and UAS-GAL4 system

Wee1 is not an abundant protein during early embryogenesis (E. Homola, personal communication); therefore its detection requires immunofluorescent

amplification techniques. In order to better analyze the localization of Wee1, we decided to use epitopes to tag the protein to help visualize the protein. In addition, a genetic technique was used to increase the expression levels of the recombinant protein. The two tags chosen are: EGFP (*Aequorea Victoria* enhanced green fluorescent protein) and Myc tags (12 tandem copies of the Myc epitope). They have been widely used in *Drosophila* as well as other organisms to tag proteins of interest (Zhang *et al.*, 1996; Iacovoni *et al.*, 1999). Due to their small sizes, when tagged to another protein, they usually do not interfere with function or localization of that protein (Timmons *et al.*, 1997; Arnaud *et al.*, 1998; Verkhusha *et al.*, 1999; Terpe, 2003).

The UAS-GAL system is also a widely used genetic technique that offers the opportunity to overexpress genes of interest in desired tissues at desired developmental stages (Verkhusha *et al.*, 1999; Mollereau *et al.*, 2000; Goto *et al.*, 2003). GAL4 is a yeast transcription factor that can be activated by the UAS (upstream activating sequence) sequence. In a typical UAS-GAL4 system, the gene of interest is usually cloned into a UAS vector and the GAL4 is expressed under control of a promoter (or enhancer) of a particular gene. UAS and GAL4 bind to each other and activate transcription of the transgene in the same pattern as the gene whose promoter is associated with GAL4 (Phelps and Brand, 1998). Bloomington *Drosophila* Stock Center has a wide collection of GAL4 lines, which greatly facilitates the experiments of overexpression of transgenes. By introducing EGFP or $(Myc)_{12}$ tags into this system, proteins of interest are easily detectable by fluorescence or antibody staining against the tags. This technique is particularly useful for proteins of low abundance (such as Wee1) or when lacking antibodies against the proteins. For most part of my thesis, I overexpressed tagged Wee1 fusion proteins and studied their localization patterns and functions.

1.4 Conclusion

In this thesis I focus mostly on analyzing the localization and functions of Wee1 by using tagged fusion proteins and the UAS-GAL4 system during embryogenesis and oogenesis in *Drosophila* development. I show that overexpression of tagged Wee1 fusion proteins is a valuable system for studying its function and localization when coupled with corresponding mutant analysis. I also describe another *wee1* mutant phenotype detected in the nervous system in late adulthood. In addition, I made an attempt to understand the reason why *wee1* mutants are sensitive to hydroxyurea.

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Chapter 2
wee1 mutant adults exhibit progressive locomotor defects

2.1 Introduction

Locomotor activity is a complex yet highly coordinated behaviour that requires many types of neurons and muscles to function together. In insects, locomotor activity can be measured by climbing tests, walking tests and other behavioural tests (Fernandez *et al.*, 1999; Martin *et al.*, 1999; Martin, 2003; Martin, 2004). There have been many studies in *Drosophila* on the characterization and regulation of locomotor activities. It has been demonstrated that locomotor activity, especially climbing activity, is a good indication of fly aging (Fernandez *et al.*, 1999). Flies gradually show decreased climbing activity as they age, presumably due to a decreased rate of metabolism (Fernandez *et al.*, 1999). Some groups also characterized the defined pattern of fly walking (Martin, 2004).

One aspect of the *wee1* mutant phenotype is an apparent climbing problem, suggestive of a locomotor defect. We observed *wee1* mutant flies that were unable to climb or fly as well as wild type flies do, resulting in mutant flies becoming stuck on the grape juice plate at the bottom of a cage. To further examine this phenotype and to determine if *wee1* mutants do indeed exhibit a significant locomotor defect, climbing assays were performed. This allows the climbing ability of *wee1* mutants to be quantified and compared to that of the wild type.

One major question that this phenotype presents is what role a cell cycle regulator, such as Wee1, plays in locomotor function? One possible explanation is that neurons are affected in *wee1* mutants. Neurons play important roles in initiating and regulating various aspects of behaviour, including locomotor activity. A common paradigm for neural development is that stem cell divisions produce neural progenitor cells that divide asymmetrically and some but not all of the daughter cells differentiate into neurons (Chia *et al.*, 2001). Neurons are permanently differentiated cells that normally never enter the cell cycle again once they have completed differentiation (Hayes *et al.*, 1991; Ino *et al.*, 1993; Espanel, 1997; Chia *et al.*, 2001). Since neurogenesis and cell cycle controls are intricately connected (Lu *et al.*, 2000) and Wee1 plays an important role in cell cycle control as the negative regulator of Cdk1, it is reasonable to speculate that neurogenesis may be defective in *wee1* mutants due to abnormal Cdk1 regulation. At least two outcomes could occur in *wee1* mutants: first of all, functional neurons may not be able to form properly due to a disruption in early neurogenesis; secondly at later stage, mature neurons may re-enter the cell cycle due to a failure of cell cycle arrest. Both of these two possibilities could potentially result in compromised neuronal function, thus a loss of normal locomotor activity. Although there was no previous report suggesting Wee1 is involved in neural function in *Drosophila*, there has been one report in humans suggesting that downregulation of Wee1 activity caused abnormal Cdk1 activity in affected neurons in patients with the neurological disorder Alzheimer's disease (Tomashevski *et al.*, 2001). The interpretation of these results was that in normal neurons, constitutive Wee1 activity ensures inactive Cdk1 and prevents neurons from re-entering the cell cycle (Tomashevski *et al.*, 2001). This study brings about an interesting question whether *Drosophila* Wee1 has a similar function in the nervous system as observed in humans.

If Wee1 has a function in the nervous system, another possible target could be Cdk5. Cdk5 is a Cdk that is only active in the nervous system and has a unique regulatory partner, designated p35 (Hellmich *et al.*, 1994; Sauer *et al.*, 1996; Poon *et al.*, 1997). Cdk5 is very similar to Cdk1 with respect to amino acid sequence and more importantly,

the tyrosine 15 residue targeted by Wee1 and Myt1 inhibitory phosphorylation is also conserved in Cdk5 (Hellmich *et al.*, 1994; Sauer *et al.*, 1996; Poon *et al.*, 1997). So far, there has been no evidence suggesting that Cdk5 has a role in regulating the cell cycle. Instead, it has been implicated in controlling axonal guidance, patterning and neuronal growth (Shuang *et al.*, 1998; Connel-Crowley *et al.*, 2000; Ledda *et al.*, 2002). The phosphorylation profile of Cdk5 is also different from Cdk1. In an *in vitro* study, human Wee1 was unable to phosphorylate the Y15 residue of human Cdk5 (Poon *et al.*, 1997). Another more recent study showed that c-Abl could phosphorylate the Y15 residue of Cdk5 in human tissue culture cells to activate Cdk5-p35 complexes (Zukerberg *et al.*, 2000), a result that raises a serious question whether *Drosophila* Wee1 might regulate *Drosophila* Cdk5 *in vivo* at all. To answer this question, I performed genetic interaction assays between *wee1* and *Cdk5-p35* and found suppression by Wee1 overexpression of the phenotypes caused by the overexpression of Cdk5-p35 complexes, suggestive of a possible genetic interaction. But these results do not rule out the possibility that the locomotor defect could be caused by Cdk1 misregulation in *wee1* mutants. Figure 2-1 summarizes the hypotheses raised above.

A third possibility to explain the *wee1* mutant locomotor defects is that the muscle formation or function in the mutants is disrupted. However, considering our overexpression results that showed axonal localization of EGFP-Wee1 (refer to Chapter 4), we are currently more interested in the neural fuction of Wee1.

In this chapter, I demonstrate that *wee1* mutants exhibit a progressive locomotor defect in adulthood, indicating that Wee1 is required to maintain normal locomotor activity in *Drosophila*. Whether this neural function of Wee1 is mediated by Cdk1 or Cdk5 needs further research.

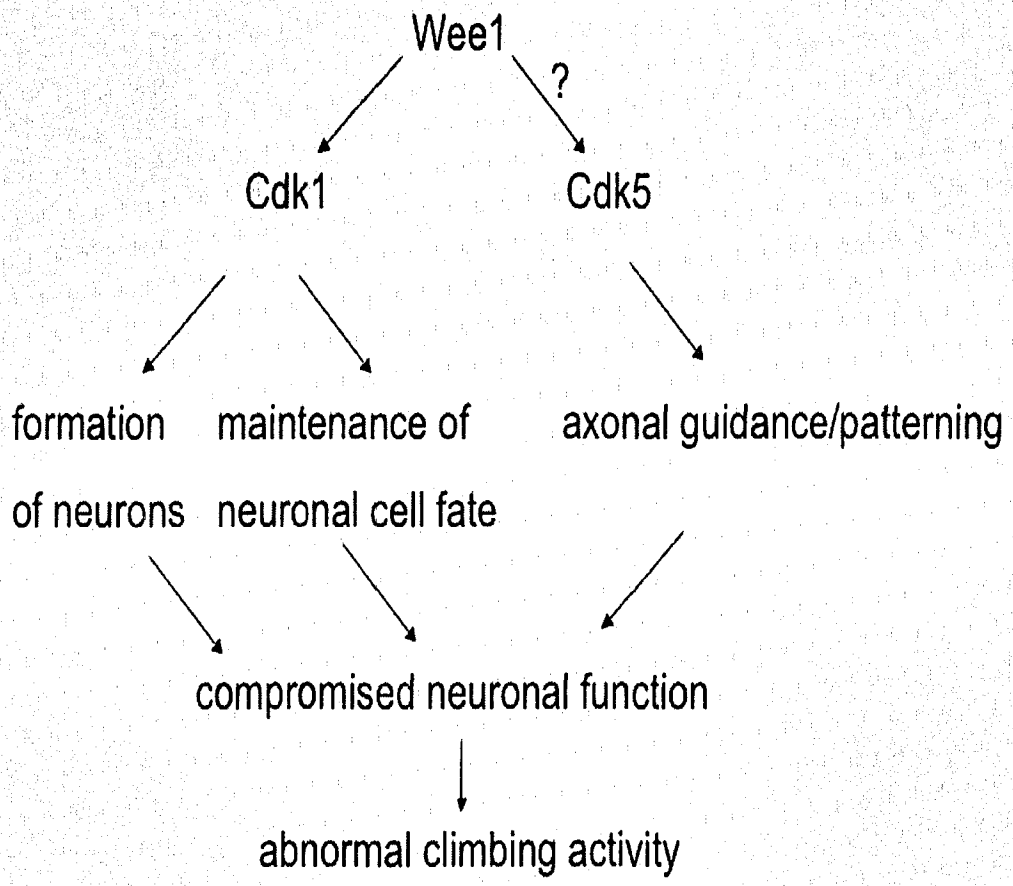

Figure 2-1 Possible roles of Wee1 in maintaining normal locomotor activities in *Drosophila*. Cdk1 and Cdk5 are two possible candidates for its neural functions. Cdk1 as a key cell cycle regulator is involved in the formation of neurons as well as the maintenance of the cell cycle arrest in permanently differentiated neurons. Either process, when disrupted, can lead to compromised neuronal function, resulting in abnormal climbing activity. Cdk5, on the other hand, is involved in regulating axonal guidance and patterning. If Wee1 regulates Cdk5, then misregulation of Cdk5 in *wee1* mutants can lead to a loss of normal neuronal function, therefore loss of normal locomotor activity.

2.2 Materials and methods

2.2.1. *Drosophila* stocks for climbing tests

yw flies were used as a control; *w*; *wee1*^{ES1}*/CyO, P{act-GFP}* and *w*; *Df(2L) WO5/CyO, P{act-GFP}* flies were crossed to generate *w*; *wee1*^{ES1}*/Df(2L) WO5* (*wee1* hemizygote) flies. The genotypes used for comparison both had straight wings, so differences in climbing ability were not due to differences in wing functions. Flies were raised on standard fly media at 25°C.

2.2.2. Climbing tests

Newly eclosed *yw* (wild type control) and *w*; *wee1*^{es1}*/Df(2L) WO5* flies were collected and transferred into vials with fresh food. There were thirty males or females per vial and twelve vials per genotype (six vials of males and six vials of females for each genotype). The climbing test was done in a glass cylinder made with two glass vials taped together. Half the length of each glass vial was marked with a red line to serve as a reference point. For each individual test, flies from one vial were transferred into the glass cylinder without anesthetization and then given two minutes to get used to the new environment. Then the flies were allowed 18 seconds to climb after being gently tapped down to the bottom. 18 seconds was determined by S. Tiong (a previous member of our lab) to be the time required for newly eclosed wild type flies to climb above the reference point. At the end of 18 seconds, flies that were able to climb above the reference point were scored as climbers, whereas those that were below the reference point were scored as non-climbers. The numbers of each category were recorded. The climbing test was repeated three times for each vial and every sample vial of flies was measured in the same way. The temperature of the room where climbing tests were performed was ~22°C (room temperature); however, the relative humidity was not controlled. All tests were carried out under constant lighting conditions. These tests were repeatedly done twice a week at the same time during the day (to avoid any fluctuations in fly daily activity levels) over a period of a month. The first test was done one day after flies newly eclosed. Before each test, vials were checked for dead flies and numbers of dead and live flies were recorded. After each test, flies were transferred into vials with fresh food.

2.2.3. Longevity tests

After the climbing tests, flies were kept for another two months to test for their life span. The numbers of dead and live flies were recorded twice a week until eventually all of the flies died. During this period, flies were transferred into vials with fresh food after each measurement to ensure that food quality and humidity do not influence the results.

2.2.4. Data collection and statistical analysis

All data were compiled using Microsoft Office Excel software. With the help of Dr. P. Hurd from the department of Psychology at the University of Alberta, I performed a series of analysis of covariance tests to determine the mean proportion of flies successfully climbing to the reference point as a function of age, sex and genotype.

2.2.5. Tests for genetic interaction between *wee1* and *cdk5*

Genetic crosses were set up to test for the interaction between *wee1* and *Cdk5-p35*. The following stocks were used: *yw*; *UAS-egfp-wee1* (homozygous on the third chromosome, for more information about the stock please refer to Chapter 4), *w*; *UAS-cdk5*, *UAS-p35/TM3, Sb* (a gift from Dr. Giniger at the Axon Guidance and Neural Connectivity Unit of NIH) and *P{w[+mW.hs]=GawB}elav^{C155}* (Bloomington Stock Center #458; *elav-GAL4* on the X chromosome). GAL4, a yeast transcription factor, can bind to UAS sequences in the genome and drive overexpression of target genes linked to these UAS sequences (Verkhusha *et al.*, 1999; Mollereau *et al.*, 2000; Goto *et al.*, 2003). Overexpression systems were chosen for these experiments because a Cdk5 fly mutant is not yet available. Overexpression of Cdk5 and its regulatory unit p35 in the *Drosophila* eye using the *GMR-GAL4* line has been previously shown to cause an adult rough eye phenotype (Connell-Crowley *et al.*, 2000). Because the GMR-GAL4 line by itself shows a slight rough eye phenotype, I used elav-GAL4 line in place of *GMR-GAL4* to avoid this problem (*elav-GAL4* flies do not show a rough eye phenotype). *elav-GAL4* drives overexpression of target genes in the same compartment (posterior to the morphogenic furrow) of the eye tissue as *GMR-GAL4* does (Lin and Goodman, 1994; Freeman, 1996), so I expected to see a similar rough eye phenotype when overexpressing Cdk5-p35 with *elav-GAL4*. If there were a rough eye phenotype associated with overexpression of Cdk5-p35, I would look for any suppression or enhancement of the phenotype when overexpressing EGFP-Wee1 and Cdk5-p35 together. The *elav-GAL4* line and overexpression of EGFP-Wee1 alone served as controls.

2.3 Results

2.3.1. *wee1* mutants exhibit progressive adult locomotor defects

To quantify the locomotor defect, I repeatedly measured the climbing ability of 360 wild type control flies and 360 *wee1* mutant flies over time. With this data, I was able to generate profiles of climbing activities for each genotype, depicted in Figure 2-2 (males) and Figure 2-3 (females). At the beginning of their life span, both the controls and the mutants had comparable climbing ability. Two weeks after eclosion, however, *wee1* mutant flies started to show a decrease in climbing activity compared to the controls. This difference became more noticeable as the flies aged. At the end of one month, almost no mutant flies could climb, even though the majority of the controls (~70%) were still able to climb above the reference point. A statistical analysis of this data showed that over time the climbing ability of *wee1* mutants became significantly different relative to the controls, as supported by a p value of 5.47e-06 for males (wild type versus *wee1* mutant) and 3.31e-07 for females (wild type versus *wee1* mutant). It was previously shown that wild type flies lost climbing activity gradually as they aged (Fernandez *et al.*, 1999). Therefore, the control data from this experiment was consistent with previous results. Both in the controls and *wee1* mutants, the sex of the flies did not seem to affect their ability to climb (p value was 0.93992 for control males versus females, 0.521 for mutant males versus females).

Because dead flies were recorded before each climbing test and for another two months after the climbing tests were completed, profiles of longevity for each genotype were also generated, as depicted in Figure 2-4. *wee1* mutant flies did not show any obvious difference in life span compared to the controls, as the majority of the flies died

by the age of two months, with very few surviving until three months of age. Previous results showed a life span of w^{1118} (commonly used as wild type in laboratory conditions) to be 40-45 days for flies kept in groups (Fernandez *et al.*, 1999). My data showed a longer life span in both in *yw* controls and *wee1* mutants. In their experiment 100 flies were kept together in a chamber (Fernandez *et al.*, 1999) and in my experiment 30 flies were kept in a vial. Thus it is possible that in my experiment, flies had less stress and competition with regard to food and territory, thereby prolonging their lifespan. Another possible explanation is that there are differences in the fly food between this study and those from other labs that could conceivably affect fly longevity.

A summer student, S. Newell, performed the same climbing tests with a different *wee1* allele ($wee1^{ES2}$) that showed results consistent with those seen with the $wee1^{ES1}$ allele (Figure 2-5, 2-6 and 2-7). Mutants started to show a climbing defect two weeks after eclosion. The life span of the mutants did not seem to differ from that of the controls (the control female data was not representative, due to a small sample size). This was an important result, as it makes the possibility of the *wee1* mutant climbing defects being due to spurious genetic background effects less likely. Based on the data we collected, we concluded that *wee1* mutants develop a progressive locomotor defect, implying that Wee1 is required for maintaining normal locomotor activities in *Drosophila*.

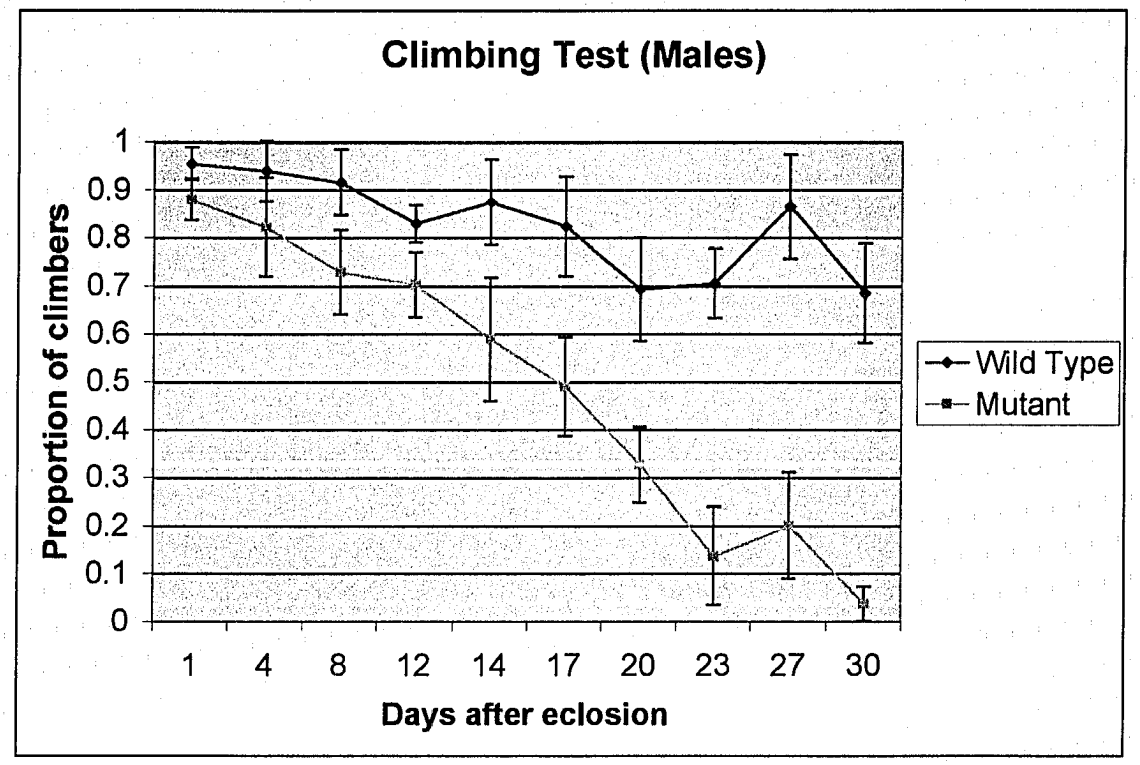

Figure 2-2: *wee1* mutant males showed a faster decrease in climbing ability compared to that of the wild type. The blue curve represents wild type males and the red curve represents *wee1* mutant males (*w; wee1^{ES1}/Df(2L) WO5*). At day 1, both genotypes showed a similar proportion of climbers. At day 30, the proportion of climbers in the *wee1* mutants dropped to close to zero, whereas that of the wild type only dropped to ~70%. Standard deviations calculated for every point are shown as vertical lines.

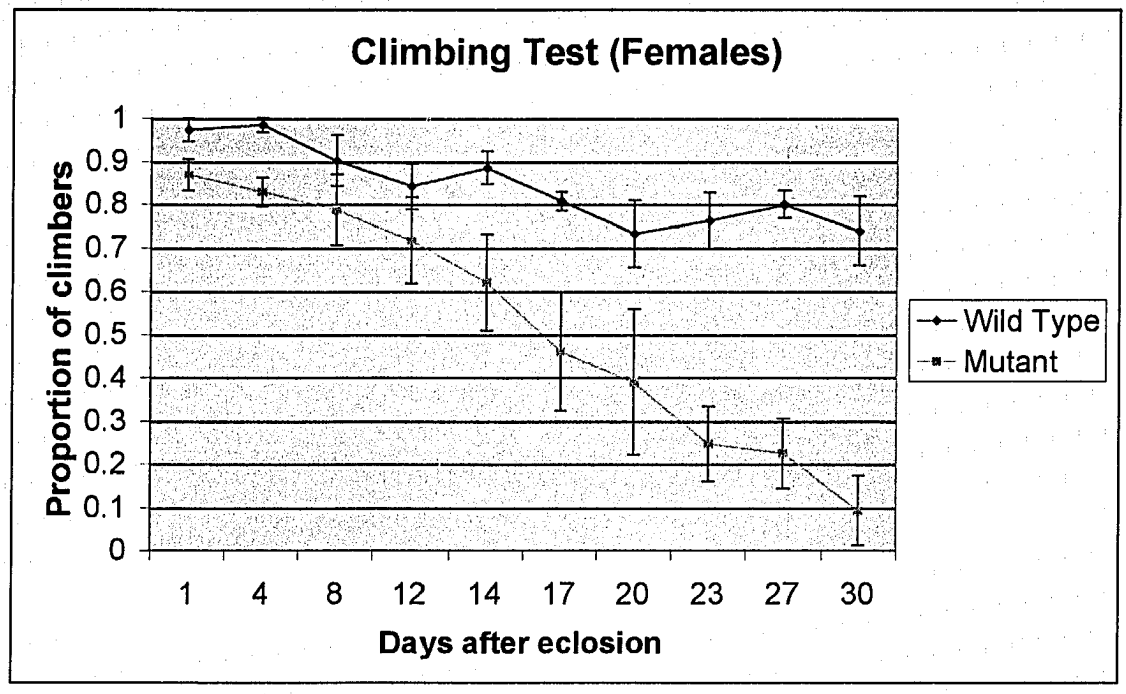

Figure 2-3: *wee1* mutant females showed a faster decrease in climbing ability compared to that of the wild type. The blue curve represents wild type females and the red curve represents *wee1* mutant females (*w; wee1^{ES1}/Df(2L) WO5*). At day 1, both genotypes showed a similar proportion of climbers. At day 30, the proportion of climbers in the *wee1* mutants dropped to ~10%, whereas that of the wild type only dropped to ~75%. Standard deviations calculated for every point are shown as vertical lines.

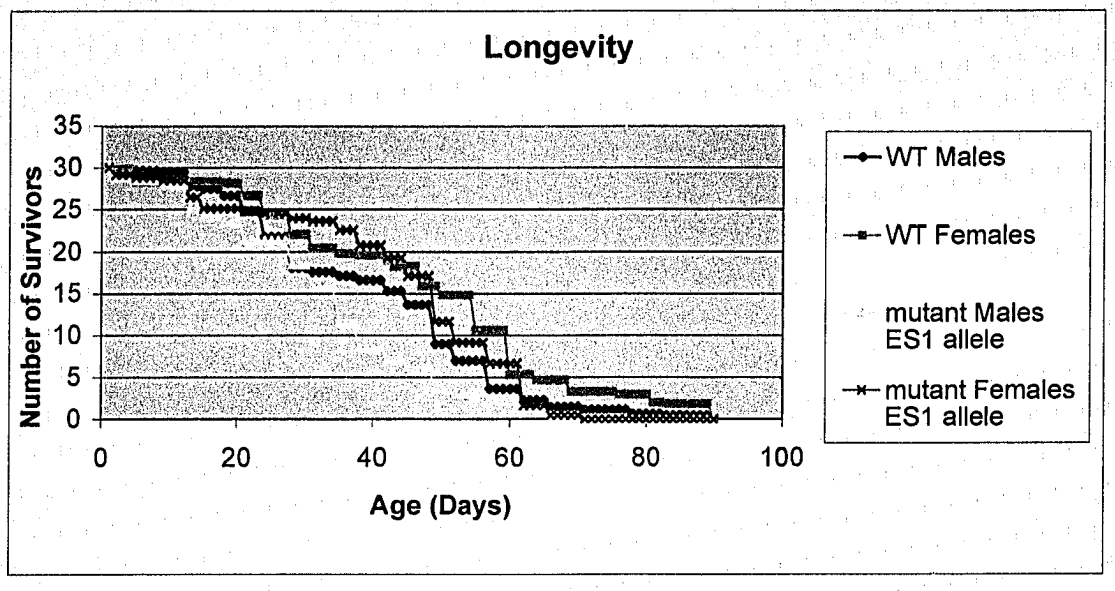

Figure 2-4: Similar longevity of wild type flies and *wee1* mutant flies. (*w; wee1*^{ES1}*/Df(2L) WO5*). The blue curve represents wild type males and the red curve represents wild type females. The yellow curve represents mutant males and the cyan curve represents mutant females. There was no obvious difference between the life span of wild type and the *wee1* mutants, as the majority of the flies died around 60 days with very few of them surviving till ~90 days.

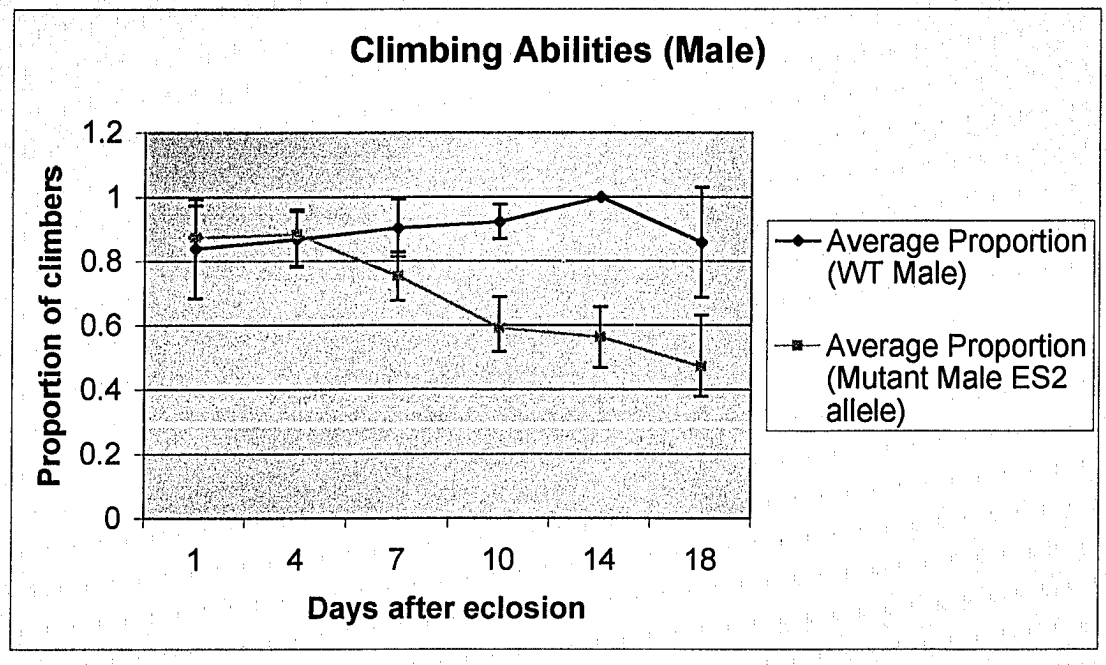

Figure 2-5: *wee1* mutant males showed a faster decrease in climbing ability compared to that of the wild type. The blue curve represents wild type males and the red curve represents *wee1* mutant males (a different allele *w; wee1^{ES2}/Df(2L) WO5*). At day 1, both genotypes showed a similar proportion of climbers. At day 18, the proportion of climbers in the *wee1* mutants dropped to ~50%, whereas that of the wild type remained the same as younger flies. Standard deviations calculated for every point are shown as vertical lines.

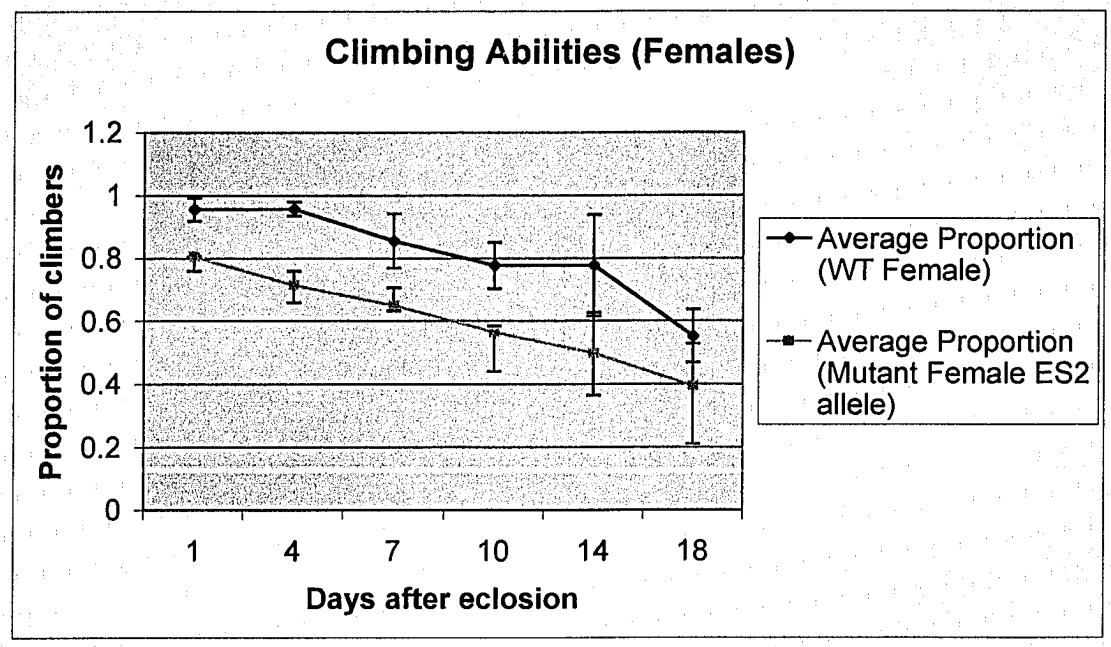

Figure 2-6: *wee1* mutant females showed a faster decrease in climbing ability compared to that of the wild type. The blue curve represents wild type males and the red curve represents *wee1* mutant males (a different allele *w; wee1^{ES2}/Df(2L) WO5*). At day 18, the proportion of climbers in the *wee1* mutants dropped from ~80% to ~40%, whereas that of the wild type dropped from ~98% to ~55%. Standard deviations calculated for every point are shown as vertical lines. The data for wild type females were not representative as only one vial of flies was tested.

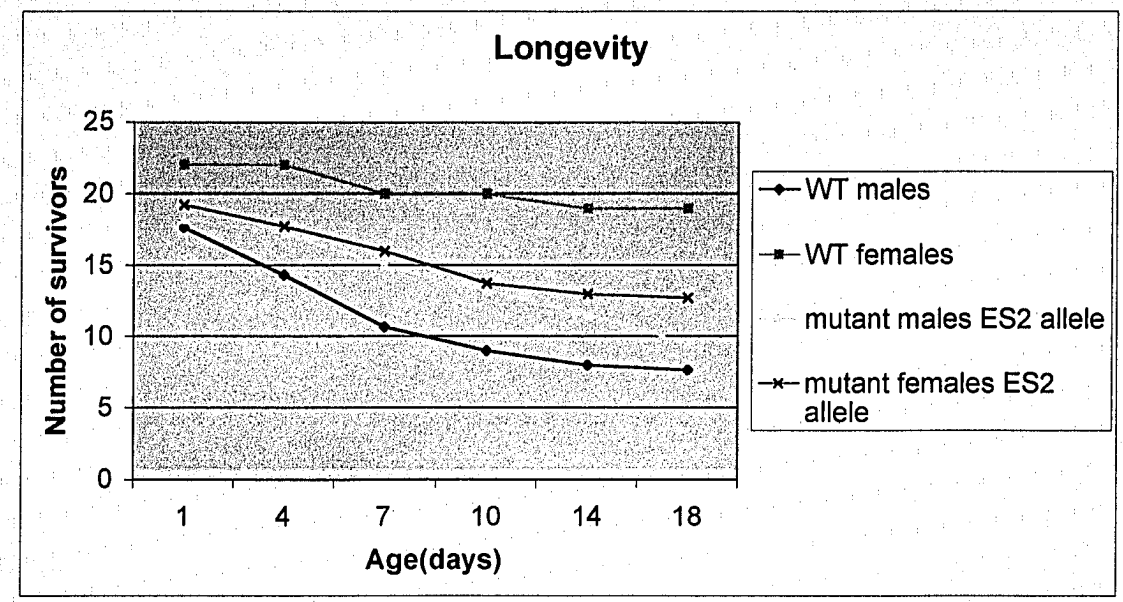

Figure 2-7: Similar longevity of wild type flies and *wee1* mutant flies. (a different allele *w; wee1^{ES2}/Df(2L) WO5*). The blue curve represents wild type males and the red curve represents wild type females. The yellow curve represents mutant males and the cyan curve represents mutant females. There was no obvious difference between the life span of wild type and the *wee1* mutant males. The data for wild type females were not representative as only one vial of flies was tested.

2.3.2. Genetic interactions between *wee1* and *Cdk5* in an overexpression system

In order to test if the loss of locomotor activity in *wee1* mutants could be caused by misregulation of *Cdk5-p35*, genetic interaction tests were performed using the UAS-GAL4 overexpression system. When overexpressed alone, EGFP-Wee1 did not cause a rough eye phenotype. Cdk5-p35 however, when overexpressed alone, caused rough patches on the eyes in ~7% of the flies (n=115). When Wee1 was co-expressed with Cdk5-p35, all of the flies had normal eyes, suggesting a suppression of the rough eye phenotype caused by Cdk5-p35 overexpression. Although the rough eye phenotype caused by Cdk5-p35 overexpression under *elav-GAL4* was weaker than the one under *GMR-GAL4* (Connell-Crowley *et al.*, 2000) and not fully penetrant, this weak phenotype was completely suppressed by overexpressing EGFP-Wee1, suggesting that Wee1 might regulate Cdk5-p35 in an inhibitory manner.

2.4 Discussion

2.4.1. Wee1 may be involved in maintaining neuronal cell cycle arrest

Using a climbing assay, we examined the climbing ability of *wee1* mutants and observed a locomotor defect beginning around two weeks after eclosion, a defect that progressively worsened as they aged. Because loss of *wee1* only affected locomotor activity in older adults, I reason that loss of *wee1* might affect the maintenance of neuronal cell fate. Because differentiated neurons exit the cell cycle permanently, the cell cycle machinery needs to be regulated to maintain this arrest. In this process, Wee1 could be required to prevent neurons from inappropriate Cdk1 activation and subsequent re-entry into the cell cycle. It remains a possibility that Wee1 might also function during early neurogenesis, resulting in subtle defects that become progressively worse over time. For example, loss of *wee1* may lead to the formation of fewer functional neurons, or some of them may be defective. In order to distinguish between these possibilities, we would need to section the adult brains to identify any abnormality in newly eclosed *wee1* mutants. Preliminary examination of young *wee1* mutant brains by our collaborator did not reveal any obvious defects in the central nervous system (M. Feany, Harvard Medical School, personal communication). These observations suggest that the possibility of Wee1 serving a role in maintaining neuronal cell fate is more likely.

Our results are interesting because they suggest possible neural functions for *wee1*, an idea supported by a previous report in humans that Wee1 activity was down regulated in Alzheimer's disease-affected neurons (Tomashevski *et al.*, 2001). In that system, the affected neurons then underwent apoptosis or necrosis. Although patients with Alzheimer's disease do not usually show a loss of coordinated movement, for a much simpler nervous system in *Drosophila* it is possible that through a similar mechanism, loss of *wee1* leads to the cell death of certain neurons, and affects a wider range of behaviour including locomotor activities. Future research should focus on whether Cdk1 plays a role in this phenotype and if so what function the regulation of Cdk1 by Wee1 has in maintaining normal locomotor activity.

It still remains a possibility that loss of *wee1* could lead to defects in the muscles, leading to a loss of climbing ability. However, we detected stabilized EGFP-Wee1 proteins in the axons of the embryonic central nervous system in an overexpression study.

This result makes the neural function of Wee1 more preferable for the analysis at this stage.

2.4.2. Is Cdk5 a possible target of *wee1* in the nervous system?

Genetic interaction studies using an overexpression assay showed an interaction between *wee1* and *Cdk5-p35*. This preliminary data, which still needs to be further tested, suggested that Wee1 might regulate Cdk5-p35 in the nervous system in an inhibitory manner. Although a previous report demonstrated that human Wee1 was not capable of phosphorylating the conserved Y15 residue on human Cdk5 *in vitro* (Poon *et al.*, 1997), it did not rule out the possibility that these two proteins interact in other means. In fact another report discovering that c-Abl positively regulates Cdk5-p35 through the phosphorylation of Y15 *in vivo* seemed to suggest that even if Wee1 does interact with Cdk5, it does not have to be Y15 phosphorylation (Zukerberg *et al.*, 2000). If *wee1* regulates *Cdk5*, then misregulation of Cdk5 could result in defects in processes regulated by Cdk5, such as axonal guidance and patterning, which may well cause defects in locomotor activities (Connel-Crowley *et al.*, 2000). It will be interesting to further examine these interactions in the future, by examining the phenotype of overexpression of Cdk5-p35 in a *wee1* mutant background and by studying Cdk5 activities in a *wee1* mutant using antibodies against Cdk5 (recently commercially available). When Cdk5 mutants with defects in the nervous system are reported, genetic interaction studies can be done to test the interaction using *wee1* and *Cdk5* mutants.

Collectively my results suggest that *Drosophila* Wee1 is required for maintaining normal locomotor activity in adulthood and that *wee1* and *Cdk5* interact genetically in an overexpression assay. Detailed analysis of mutant interactions for these genes should help to clarify the neural functions of Wee1.

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Chapter 3
Effects of hydroxyurea on *wee1* mutant larval brains and wing imaginal discs

3.1 Introduction

Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase, an enzyme involved in the synthesis of deoxyribonucleotides. By effectively reducing the amount of dNTPs, HU inhibits DNA replication (Koc *et al.*, 2004). When cells are exposed to HU during S phase, the presence of incomplete DNA replication typically initiates a DNA replication checkpoint (Enoch and Nurse, 1990). Arrested cells will not enter mitosis until DNA replication is complete (Weinert *et al.*, 1994; Desany *et al.*, 1998). Such a checkpoint is important because it prevents cells with incompletely replicated DNA from dividing and thereby compromising genomic stability. Because of these properties, HU has been widely used to study DNA replication checkpoint functions in many model organisms (Rhind and Russell, 2000). This checkpoint is also called the S/M checkpoint, because cells become arrested in S phase upon treatment with HU (Enoch and Nurse, 1990).

Many studies have investigated the response to HU at the cellular level in different systems (Walworth *et al.*, 1993; Allen *et al.*, 1994; Murakami and Okayama, 1995; Ye *et al.*, 1996; Francesconi *et al.*, 1997; Uchiyama *et al.*, 1997; Boddy *et al.*, 1998; Rhind and Russell, *1998*; Zeng *et al.*, 1998; Taylor *et al.*, 1999; Rhind and Russell, 2000; Zarzov *et al.*, 2002). It is now thought that the DNA replication checkpoint is primarily regulated by the ATR-Chk1 checkpoint pathway in metazoans (Rhind and Russell, 2000), whereas in yeast, Rad3/MEC1 (ATR homologues in *S. pombe* and in *S. cerevisiae*)) and Cds1/RAD53 (Chk2 homologues in *S. pombe* and in *S. cerevisiae*) seem to be more important. When DNA replication forks are stalled, either spontaneously or due to HU treatment, ATR (Rad3/MEC1 in yeast) is though to become active and proceed to phosphorylate checkpoint targets (Bentley *et al.*, 1996; Hekmat-Nejad *et al.*, 2000; Zhao and Piwnica-Worms, 2001). Studies have demonstrated that upon activation of the replication checkpoint, ATR in higher eukaryotes phosphorylates Chk1 (Walworth *et al.*, 1993; Tanaka *et al.*, 2001; Zhao and Piwnica-Worms, 2001) and in yeast Rad3/MEC1 phosphorylates Cds1/RAD53. Once this occurs, Chk1 and Cds1/RAD53 are believed to activate Wee1 (and possibly also the Wee1 paralogue Myt1) and inactivate Cdc25 through distinct phosphorylation events as well as by translocation of the protein out of the nucleus (Francesconi *et al.*, 1997; Boddy *et al.*, 1998; Rhind and Russell, 1998; Zeng *et al.*, 1998; Furnari *et al.*, 1999; Rhind and Russell, 2001; Uto *et al.*, 2004). Together, activation of Wee1 and inhibition of Cdc25 results in the downregulation of Cdk1, thereby preventing mitotic entry as long as DNA replication remains inhibited. Inhibitory phosphorylation of the Y15 residue of Cdk1 by Wee1-like kinases has been shown to be required for S/M checkpoint arrest in *Schizosaccharomyces pombe* (Rhind and Russell, 1998). For a summary of the DNA checkpoint response, refer to Figure 3-1.

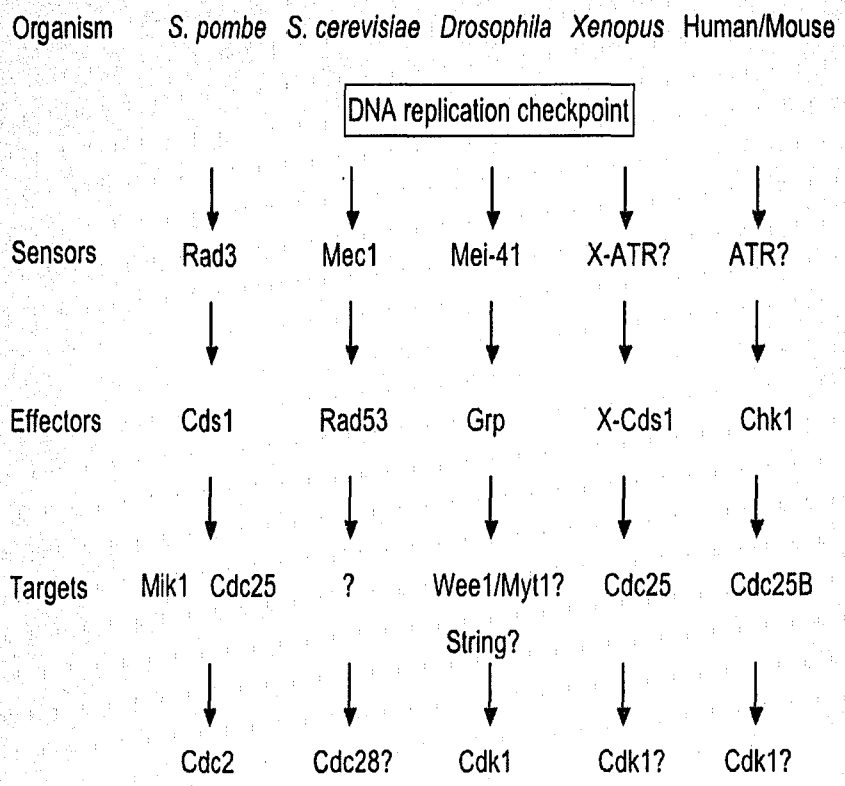

Figure 3-1 DNA replication checkpoint pathways in various model organisms. Adapted from Rhind and Russell, 2000. Arrows do not differentiate regulation in an inhibitory or activating fashion. A question mark following a protein indicates that there is only circumstantial evidence for its involvement in the checkpoint. A question mark alone indicates that the identity of the protein(s) is currently unknown.

Research on the DNA replication checkpoint has involved various model organisms, ranging from budding yeast (*S. cerevisiae*), fission yeast (*S. pombe*) to human cultured cells (Rhind and Russell, 2000), but it has not been done extensively in *Drosophila*. The *Drosophila* genome encodes homologues of all the components of the previously described DNA replication checkpoint pathway, including *mei-41* (*atr*), *grapes* (*chk1*) and *wee1*. Although the detailed molecular mechanisms involved in the DNA replication checkpoint response in *Drosophila* have not yet been reported, there are good reasons to believe that *Drosophila* utilizes similar mechanisms as other organisms (Sibon *et al.*, 1999; Krause *et al.*, 2001). Krause *et al.* (2001) demonstrated that wild type larval brain cells responded to hydroxyurea treatment by preventing entry into mitosis, therefore showing a lower mitotic index (proportion of mitotic cells). Sibon *et al.* (1999) found that both *mei-41* and *grp* mutant embryos failed to delay the cell cycle upon treatment with aphidicolin, another DNA replication inhibitor, as measured by live cell cycle timing. Recently, Stumpff *et al.* (2004) reported similar mitotic timing defects for *wee1* mutants, which also undergo mitotic catastrophe at late stages of syncytial development. Collectively, these results indicate that the DNA replication checkpoint is functional in flies and that this response is likely to be regulated by the ATR-Chk1 signaling pathway activating Wee1 and thereby inhibiting Cdk1. Another study done previously in our lab used aphidicolin treatment and demonstrated that wild type embryos showed elevated levels of tyrosine 15-phosphorylated Cdk1 in the presence of aphidicolin. Although *wee1* mutants also showed a slight increase of tyrosine-phosphorylated Cdk1, this response was significantly attenuated (E. Homola, personal communication). The remaining response to aphidicolin in the *wee1* mutants was probably due to the presence of Myt1, a redundant Cdk1 inhibitory kinase. These results indicated that the DNA replication checkpoint is functional in *Drosophila* syncytial embryos and that both Wee1 and Myt1 can inhibit Cdk1 during this response. The involvement of Mei-41/Grp/Wee1 in the DNA replication checkpoint in flies was further confirmed by two previously published studies done in our lab. Price *et al.* (2000) and Radcliffe *et al.* (2002) demonstrated that *wee1* mutants as well as *mei-41* and *grapes* mutants are sensitive to HU with respect to viability. The objective of this aspect of my thesis was to examine the mechanisms of the death of *wee1* mutants in the presence of HU. Specifically, I wanted to determine if there is a link between *wee1* lethality caused by exposure to HU and a defect in the DNA replication checkpoint response.

In order to determine whether there was a defective DNA replication checkpoint in *wee1* mutants, wild type and *wee1* mutant larvae were exposed to HU and the checkpoint response was examined using standard methods. Reagents used for these experiments were anti-BrdU (5-bromo-2-deoxyuridine) and anti-PH3 (phospho-Histone H3). BrdU is an analog of thymidine and can be incorporated into DNA during replication. Anti-BrdU antibody staining therefore identifies cells that were replicating when they were exposed to BrdU. Histone H3 is specifically phosphorylated in mitosis; therefore anti-PH3 antibody marks cells that are in mitosis (Hendzel *et al.*, 1997). Because one cell cycle is ~10 hrs in wing imaginal discs and ~2 hours in developing larval brains, treating larvae with HU for 12 hours should theoretically arrest all the non-synchronous wild type cells in S phase (Neufeld *et al.*, 1998; Cohen, 1993). A functional replication checkpoint response to HU in wild type cells should result in cells arresting in S phase unable to replicate DNA or divide, therefore less BrdU and PH3 staining is

expected. If *wee1* mutants cannot generate a functional checkpoint in response to HU, the cells would be expected to continue cell cycle progression instead of arresting, resulting in more cells replicating their DNA (increased amount of anti-BrdU staining) and more cells in mitosis (increased amount of anti-PH3 staining), than in the wild type.

The second goal of this project was to determine why the viability of *wee1* mutants is affected by HU exposure. One possibility is that a defective DNA replication checkpoint leads to progression through the cell cycle despite the presence of incompletely replicated DNA. If this is the case, genomic stability would be compromised and the cells may respond by triggering apoptosis. In order to determine if *wee1* mutants have increased apoptosis when exposed to HU, I marked apoptotic cells by antibody staining. Caspase 3 is one caspase that is cleaved in response to apoptotic signals. Once cleaved, caspase 3 becomes an active protease promoting apoptosis (Nicholson *et al.*, 1995). Hence, anti-cleaved Caspase 3 antibodies label apoptotic cells. If the lethality of *wee1* mutants following HU exposure was due to ectopic cell death, I would expect to see increased caspase-3 staining in larval tissues following HU treatments.

Using the above methods to examine the wild type controls and *wee1* mutants after HU treatments, I found that both wild type and mutant cells were able to arrest before entry into mitosis and *wee1* mutants did not show an obvious checkpoint defect. But after a 7-day HU treatment, *wee1* mutants exhibited smaller tissue mass and an increase in cell death, suggesting that cumulative effects of the treatment were preferentially promoting cell death and possibly also blocking regenerative cell proliferation.

3.2 Materials and Methods

3.2.1. *Drosophila* stocks used for the analysis

w; $wee1^{ES1}$/*T(2;3) CyO; TM6B, Tb* and *w*; *Df(2L) WO5/T(2;3) CyO; TM6B, Tb* flies were crossed to select *w*; $wee1^{ES1}$/*Df(2L) WO5* hemizygotes (selecting against the Tubby phenotype: *Tb* larvae are shorter and thicker than wild-ype). *w*; $wee1^{ES1}$/*CyO, P{act-GFP}* larvae were used as heterozygous controls.

3.2.2. HU treatment

Wandering third-instar larvae of heterozygous controls and *wee1* hemizygous mutants were selected and transferred into food containing 8 mM HU. This media was prepared by adding a stock solution of HU to fresh food and allowing it to equilibrate for one day. The concentration of HU used (8mM) was determined by previous studies to represent a threshold for the viability of *wee1* mutants (Radcliffe *et al.*, 2002). The following time points were chosen to assess cell cycle parameters in wing imaginal discs and brains (4/8/12/24-hour HU treatment for BrdU; 12-hour HU treatment for PH3 staining; 7-day HU treatment for Caspase 3 staining).

3.2.3. Immunofluorescence

Four sample groups were examined. They were: heterozygous controls without HU treatment; controls with HU treatment; *wee1* mutants without HU treatment and *wee1* mutants with HU treatment. My protocol was modified from previously published protocols (Shermoen, 2000). Larval wing discs and brains were dissected out of

wandering third instar larvae (in HU experiments, right after the HU treatment) and incubated with a 1:100 BrdU solution (dissolved in phosphate buffered saline (PBS), BrdU working concentration was 75 μg/ml, Sigma), for 20 minutes. Samples were then fixed in PBS with 3.7% formaldehyde for 20 minutes, followed by a treatment with 6N hydrochloric acid for 30 minutes. Samples were then washed with PBST (0.3% TritonX-100 in PBS) three times for 5 minutes each time and then blocked with PBST+10% NGS (normal goat serum) for an hour at room temperature and incubated with anti-BrdU in PBST overnight at 4°C. For other antibody staining, larval wing discs and brains were dissected out of wandering third instar larvae and fixed in PBS with 3.7% formaldehyde for 20 minutes, and then washed with PBST three times for 5 minutes each time. Samples were also blocked with PBST+10% NGS for an hour at room temperature and incubated with primary antibody in PBST overnight at 4°C. The concentrations of antibodies used were: mouse anti-BrdU at 1:200 (Developmental Studies Hybridoma Bank), rabbit anti-PH3 at 1:2,000 (Upstate) and anti-cleaved Caspase3 at 1:500 (Cell Signaling). The next day, samples were washed with PBST four times for 15 minutes each time, and then incubated with secondary antibodies in PBST at room temperature for an hour. The secondary antibodies used were: 1:1,000 goat anti-mouse or goat anti-rabbit Alexa Fluor 488 (green signal, from Molecular Probes), 1:1,000 goat anti-mouse or goat anti-rabbit Alexa Fluor 568 (red signal, from Molecular Probes). After secondary antibody incubation, samples were washed with PBST three times for 15 minutes each time and then stained with Hoechst 33258 in PBS at a final concentration of 5 μg/ml for 5 minutes. After a final wash in PBST for 10 minutes, samples were ready to mount in anti-fade mounting media (9 parts glycerol+1 part 10XPBS containing 10 mg/ml 1, 4-phenylenediamine, Sigma).

3.2.4. Confocal microscopy and data processing

Leica confocal microscope (Model: TCS SP2) was used to gather data from samples. The Leica confocal microscope software and Adobe Photoshop CS software were used to compile images.

3.3 Results

Before carrying out any checkpoint experiments, it was important to confirm that 8mM of HU was sufficient to cause a reduced viability phenotype in *wee1* mutants. As expected, heterozygous control larvae did not die upon 8mM HU treatment, although their development was delayed in that it took more days for them to eclose (data not shown). The *wee1* mutant larvae all died ($n>50$) with HU treatment, as expected. These results suggested that the HU treatment was efficient in my experiments.

3.3.1. *wee1* mutants did not show an obvious defect in generating a DNA replication checkpoint

Using anti-BrdU and anti-PH3 antibody staining, I was able to examine cell cycle program changes in the larvae that were treated with HU. My initial hypothesis was that there would less cells replicating their DNA after HU treatment in control cells, which should be shown by a decrease in the amount of anti-BrdU staining. However, control cells both in the wing discs and the brains showed similar amount of anti-BrdU staining before and after HU treatment (Figure 3-2A and B and Figure 3-3A and B), implying that

they were still able to incorporate normal levels of BrdU after various lengths of HU treatment (4/8/12/24 hours). Similar observations were made with *wee1* mutants (Figure 3-2C and D and Figure 3-3C and D). I also expected a decrease in the amount of mitotic cells due to the arrest in S phase. In this case I observed that the amount of M phase cells in the wild type controls did show a decrease after a 12-hour HU treatment (Figure 3-4A and B). *wee1* mutant cells also showed a decreased number of mitotic cells (Figure 3-4C, D), indicating that mutant cells were able to delay entry into mitosis. These results suggested that the replication checkpoint was probably functional in spite of lack of obvious differences in DNA replication.

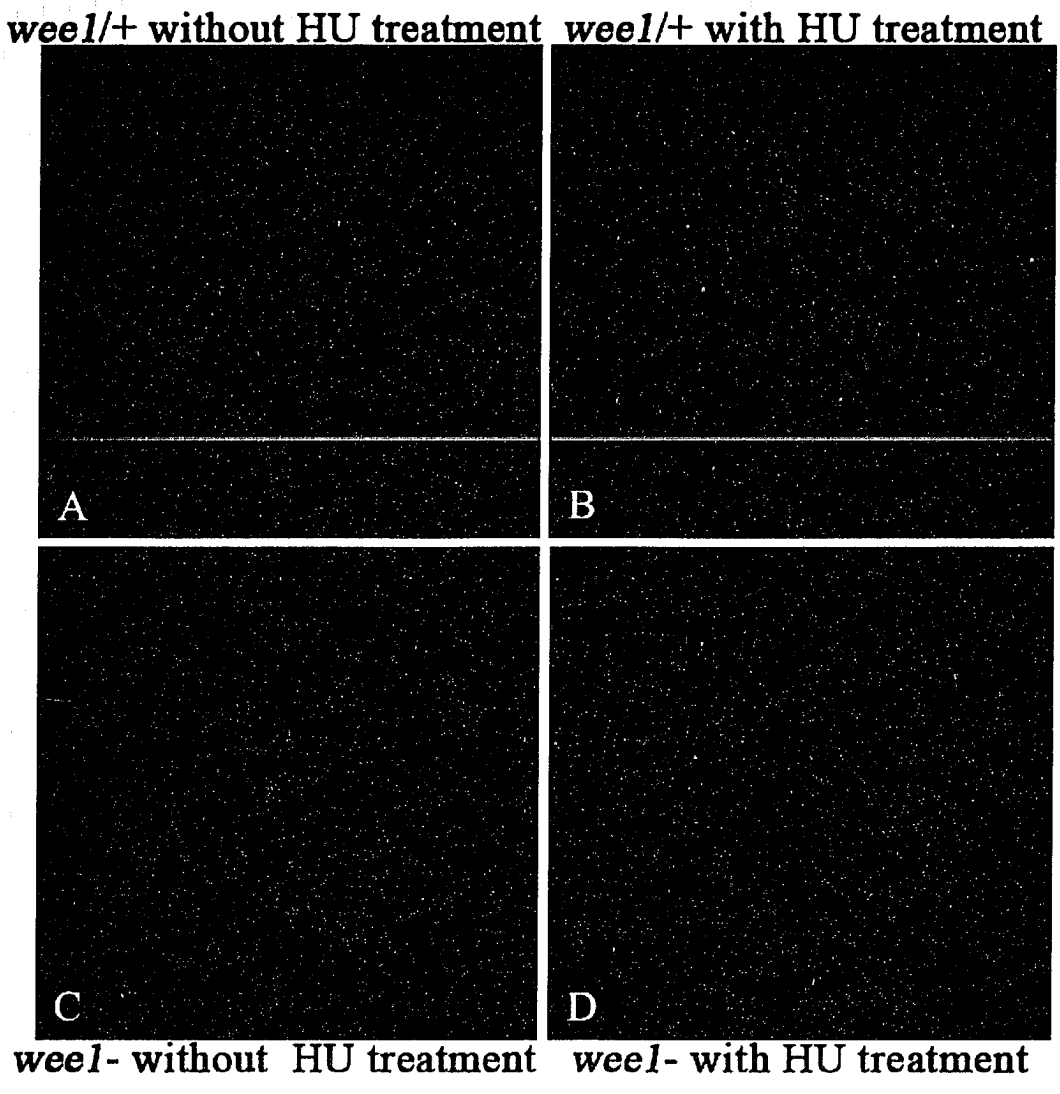

Figure 3-2 The number of replicating cells in wing imaginal discs did not change in either the controls or in *wee1* mutants in response to hydroxyurea (HU) treatment. 24-hour HU treatment. (A) Anti-BrdU staining (labels replicating cells) in a control wing disc without HU; (B) Anti-BrdU staining in a control wing disc with HU; (C) Anti-BrdU staining in *wee1* mutant wing disc without HU; (D) Anti-BrdU staining in *wee1* mutant wing disc with HU. There was no discernible difference between A and B or C and D, which suggests that both the wild type control and the mutant wing disc cells were able to incorporate BrdU after hydroxyurea treatments.

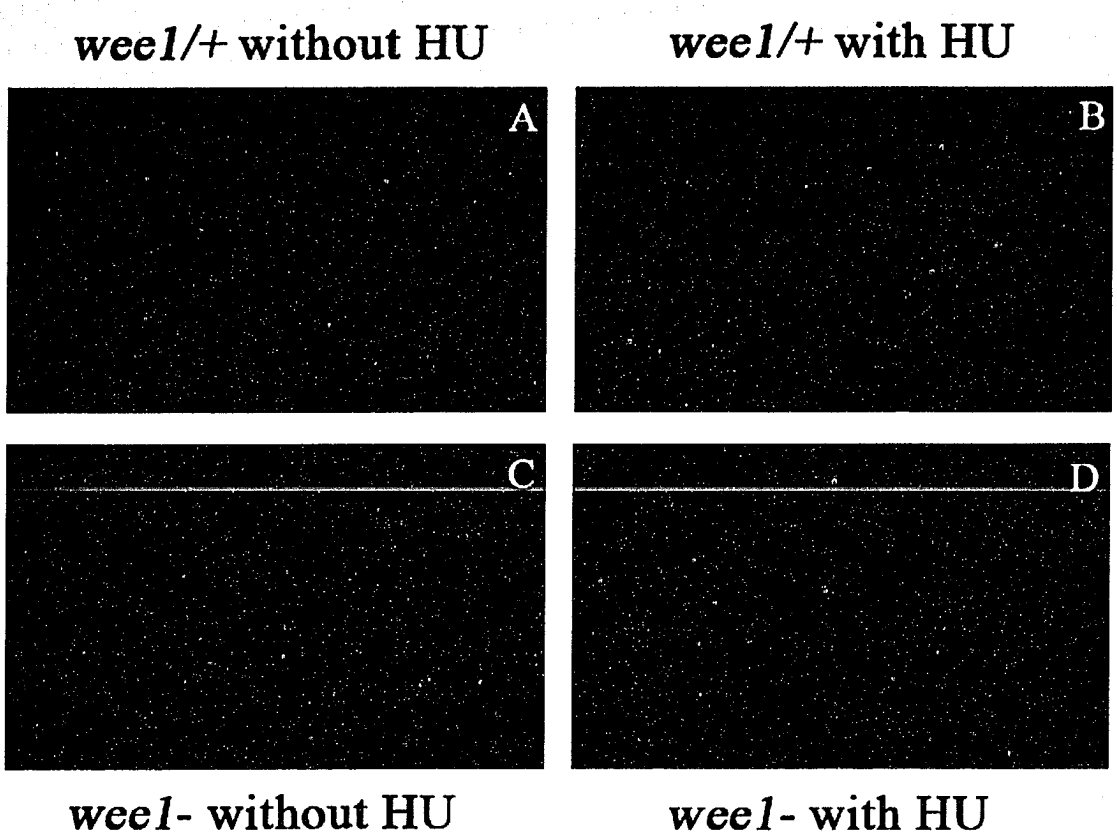

Figure 3-3 The number of replicating cells in proliferating brain lobes did not change in either the controls or in *wee1* mutants in response to hydroxyurea (HU) treatment. 24-hour HU treatment. (A) Anti-BrdU staining (labels replicating cells) in a control brain without HU; (B) Anti-BrdU staining in a control brain with HU; (C) Anti-BrdU staining in *wee1* mutant brain without HU; (D) Anti-BrdU staining in *wee1* mutant brain with HU. There was no discernible difference between A and B or C and D, which suggests that both the wild type control and the mutant brain cells were able to incorporate BrdU after hydroxyurea treatments.

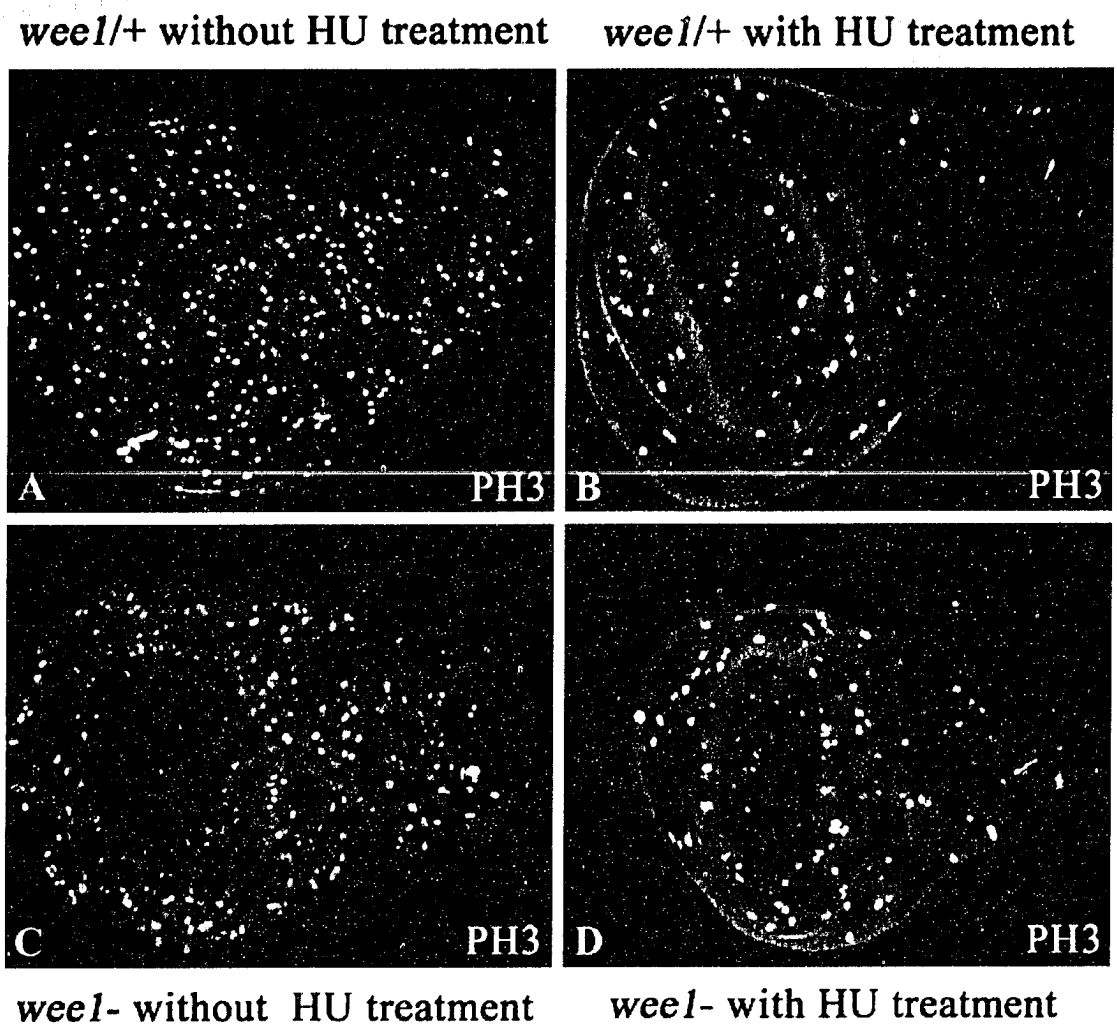

Figure 3-4 The number of mitotic cells in wing discs decreased in both the controls and *wee1* mutants in response to hydroxyurea (HU) treatment. 12-hour HU treatment. (A) Anti-PH3 staining (labels mitotic cells) in a control wing disc without HU; (B) Anti-PH3 staining in a control wing disc with HU; (C) Anti-PH3 staining in *wee1* mutant wing disc without HU; (D) Anti-PH3 staining in *wee1* mutant wing disc with HU. The number of mitotic cells decreased both in the control and mutant wing disc cells after hydroxyurea treatments.

3.3.2. *wee1* mutants exhibited increased levels of apoptosis after HU treatment

To determine if the lethality of *wee1* mutants exposed to HU was due to increased rates of apoptosis during the larval stages, experiments were carried out using anti-cleaved caspase 3 antibodies to mark apoptotic cells. This tool was used to examine the relative numbers of cells undergoing apoptosis before and after HU treatment. An increase of apoptotic cells was observed in the mutants after a 7-day HU treatment, suggesting that there were more cells undergoing Caspase 3-dependent apoptosis after HU treatment than before treatment (Figure 3-5A and B for discs, C and D for brains). I also observed that *wee1* mutant wing discs and brains were smaller after a 7-day HU treatment than those before treatments. This is consistent with more apoptosis resulting in a loss of tissue mass. Occasionally (~10% of the population, n>50) black tumor-like tissues were seen in *wee1* mutants after prolonged HU treatment (7 days), which was also never observed in the controls.

Collectively my results showed that *wee1* mutant did not seem to have a defective DNA replication response in larval wing discs or brains. The lethality of the mutants caused by HU is associated with increased levels of apoptosis.

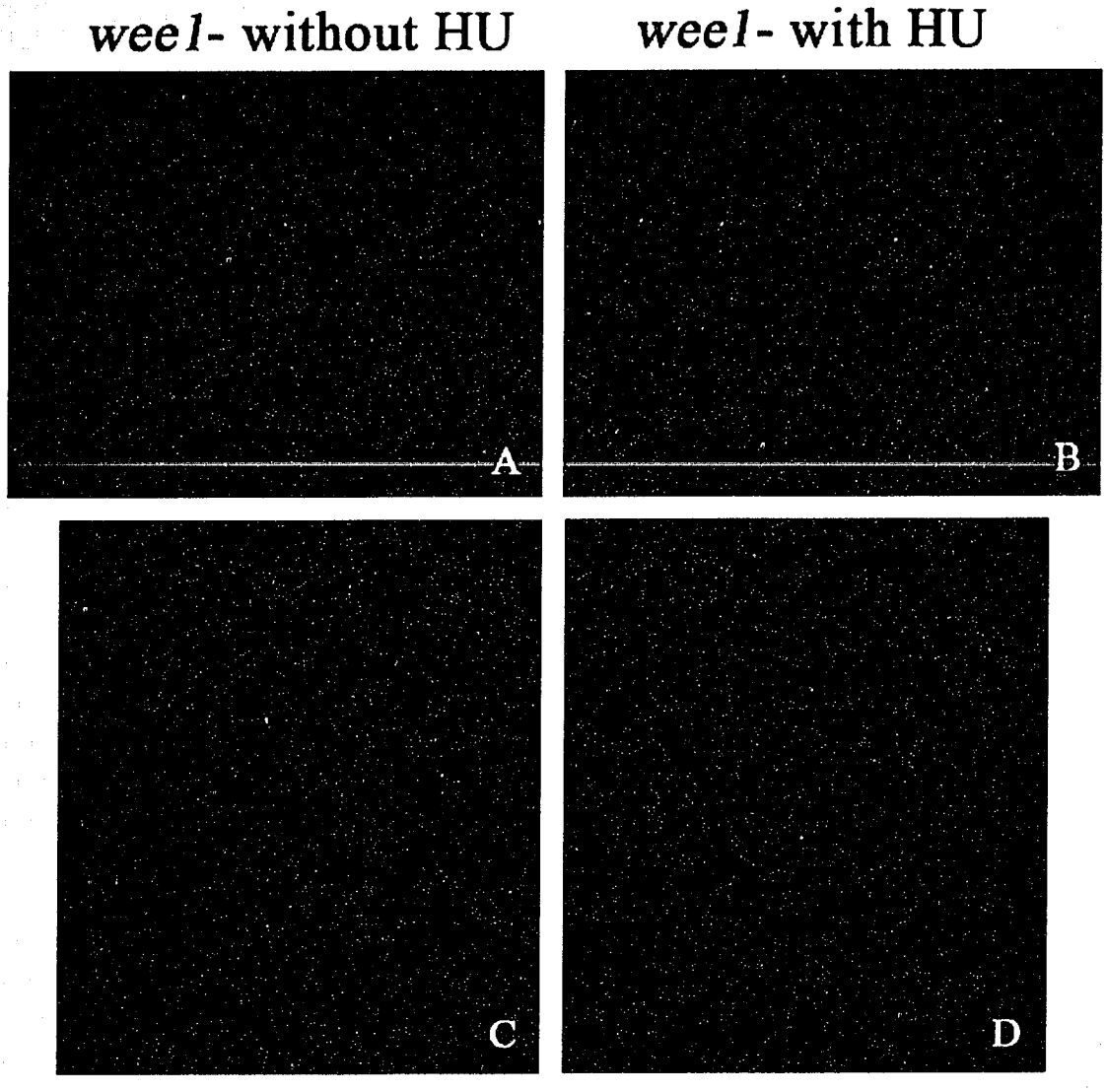

Figure 3-5 The number of apoptotic cells increased in *wee1* mutants after HU treatment. 7-day HU treatment. (A) Anti-Caspase 3 staining (labels apoptotic cells) in a *wee1* mutant wing disc without HU; (B) Anti-Caspase 3 staining in *wee1* mutant wing disc with HU; (C) Anti-Caspase 3 staining in *wee1* mutant brain without HU; (D) Anti-Capase 3 staining in *wee1* mutant brain with HU. Note that there was an increase in the numbers of apoptotic cells after HU in the mutants, which was particularly notable in the highly proliferative optic lobes of the brain.

3.4 Discussion

3.4.1. BrdU was not a good indicator of an S phase arrest

I found it surprising that both the wild type and mutant cells were still able to replicate their DNA after a 24-hour HU treatment, which theoretically should have blocked all cells even in a non-synchronous population from replicating DNA (because their cell cycles are much shorter than 24 hours). Judged from my experiments, BrdU was not a good indicator for a DNA replication checkpoint arrest in this system.

3.4.2. Is Wee1 essential for the DNA replication checkpoint in *Drosophila*?

The first goal of this part of my thesis was to examine if loss of *wee1* leads to a defective DNA replication checkpoint arrest. Contrary to my hypothesis, both the wild type and mutant wing disc cells showed a decrease in the number of mitotic cells after a 12-hour HU treatment, suggesting that cells were prevented from entry into mitosis in both genetic backgrounds. Thus, loss of *wee1* did not seem to cause a defect in generating a replication checkpoint response. We can exclude the trivial explanation for this discrepancy: that the drug was not effective in this experiment, given that there was a marked developmental delay in both controls and *wee1* mutants, yet only the mutants were inviable. However, my results are consistent with previous results obtained in our lab indicating that *wee1* mutant embryos were still able to respond to another DNA replication inhibitor, aphidicolin, by elevated Cdk1 inhibition, although to a lesser extent than in normal embryos (E. Homola, personal communication). Considering the likely functional redundancy between Wee1 and Myt1, it seems probable that Myt1 was responsible for generating the pre-mitotic checkpoint responses that I observed in wing disc cells. Taken together, these results suggest that under normal circumstances, Wee1 and Myt1 may be redundantly required for the DNA replication checkpoint response in *Drosophila*.

3.4.3. The lethality of *wee1* mutants upon HU treatment is associated with increased apoptosis

The second goal of this part of my project was to determine if the lethality associated with HU treated *wee1* mutants was due to ectopic apoptosis. Consistent with this hypothesis, I have demonstrated that more anti-caspase 3 staining was observed following HU treatment in *wee1* mutants than prior to HU treatment. The fact that I observed smaller wing discs and brains in mutants after prolonged HU treatment compared with those from untreated larvae, suggested an additional explanation: that there may have been less proliferation in the *wee1* mutant cell populations exposed to HU than in the absence of HU.

One interesting phenotype I noticed in mutants treated with HU for 7 days was that there were sometimes black tumor-like tissues in the larvae. These were not observed in the control larvae. These black tissues could have been melanotic tumors, which often occur as a result of disrupted immune system (Rodriguez *et al.*, 1996). These results are suggestive of an impaired immune system in *wee1* mutants. Since it is still not well understood how cells metabolize HU, this phenotype could also be explained, as break down products of HU are able to induce the formation of melanotic tumors. More studies are necessary to confirm whether melanotic tumors occur consistently in *wee1* mutants after HU treatments.

Collectively, my results demonstrated that Wee1 is not essential for generating a pre-mitotic DNA replication checkpoint response in larval wing discs. This is probably because Myt1 is redundantly required for the checkpoint response. I could also conclude that the observed inviability of *wee1* mutant flies upon exposure to HU is in part due to increased apoptosis.

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Chapter 4
Analysis of Wee1 localization and function using epitope tags

4.1 Introduction

The localization of Wee1 has previously been demonstrated to be dynamic and cell cycle dependent in two systems: budding yeast and human cells (Baldin and Ducommun, 1995; Sakchaisri *et al.*, 2004). Therefore, we wanted to study the localization pattern of *Drosophila* Wee1. In order to visualize the protein, we have previously made a peptide antibody against part of the Wee1 N-terminal non-conserved region and have optimized this antibody for use in western blots and immunofluorescence (E. Homola and S. Campbell, personal communication). Due to either inaccessability of the antibody to Wee1 or else low levels of endogenous Wee1 proteins, it has been necessary to amplify the fluorescent signal with a tyramide system (Molecular Probes) to get detectable signals. In order to better characterize and visualize the localization of Wee1, we decided to make epitope tagged Wee1 fusion proteins. Two previously characterized tags were chosen: one that incorporates 12 tandem copies of the Myc epitope (Iacovoni *et al.*, 1999) and another that incorporates a modified GFP (enhanced green fluoresecent protein; Zhang *et al.*, 1996). These epitopes have been widely used in many organisms including *Drosophila*, to tag a variety of proteins, without affecting their localization or function to our knowledge (Timmons *et al.*, 1997; Amaud *et al.*, 1998; Verkhusha *et al.*, 1999; Terpe, 2003). A former graduate student, Z. Jin, had previously made $(Myc)_{12}$ and EGFP tagged Myt1 constructs and transgenic lines. In these lines, the fusion proteins were functional in that they rescued the adult bristle and male sterility defects (Jin, 2005). They also showed the expected localization to the endoplasmic reticulum and Golgi apparatus, assessed either by fluorescence (EGFP-tagged construct) or by using antibodies against the epitope tags (Jin, 2005).

In order to express tagged Wee1 at levels suitable for immunofluorescence, the UAS-GAL4 system was used to overexpress the tagged fusion proteins. The Bloomington *Drosophila* Stock Center has a large collection of GAL4 drivers (from now on I will be using the term "driver" to refer to GAL4 lines) that can be used to express UAS-linked genes in specific tissues and at specific developmental stages. To take advantage of these resources, I generated epitope-tagged Wee1 constructs in a UASp transposon vector. The following constructs were made: *UASp-egfp-wee1* (cDNA) and *UASp-*$(myc)_{12}$*-wee1*(cDNA), as controls. By crossing GAL4 driver lines with transgenic flies carrying these constructs, the Wee1 fusion proteins can be expressed in a variety of cell types.

After testing the functionality of the transgenes, I examined the localization and function of the tagged proteins during embryogenesis and oogenesis. Consistent with other organisms, *Drosophila* Wee1 proteins have a dynamic localization that showed cell type and cell cycle specificity. Syncytial embryos overexpressing Wee1 showed defects in both nuclear and cytoplasmic events, suggesting that Cdk1 was misregulated. Ectopic pseudo-cleavage furrows in these embryos also suggested a possible novel role of Cdk1 in regulating the actin cytoskeleton. During oogenesis, overexpressing Wee1 promoted one extra round of cystocyte division, which will be discussed in detail in Chapter 5.

4.2 Materials and Methods

4.2.1. Cloning of *UASp-egfp-wee1* and *UASp-*$(myc)_{12}$*-wee1* constructs

UASp is a modified version of a UAST vector, which can be selected in bacteria by Ampicillin resistance (original vector described in Rorth, 1998, the multiple cloning

site was modified by C. Walker in our lab to incorporate different restriction sites for cloning purposes). Four enzyme sites were used in my subcloning experiments. In the order from 5' end to 3' end, the enzyme sites used were: KpnI, EcoRI, BamHI and XbaI. *UASp-(myc)*$_{12}$ (previously made in our lab by Z. Jin). The *myc* sequence (12 copies) was flanked by KpnI and EcoRI. To generate a *UASp-egfp* vector for cloning, the *UASp-(myc)*$_{12}$ plasmids were digested with KpnI and EcoRI (Gibco, Invitrogen) and treated with Alkaline Phosphatase (Gibco, Invitrogen) to generate UASp empty vectors. *egfp* fragments were isolated by digestion with enzymes KpnI(5') and EcoRI(3') from *pBS-egfp* (generated by Z. Jin) and then subcloned into UASp empty vectors to generate *UASp-egfp* using T4 DNA ligase (Gibco, Invitrogen). Then, *wee1* cDNA was amplified by PCR using restriction site-tagged primers: 5'-CAGGGATCCATGGCATTCCGCCAG TCG-3', 5'-GCTGGCTCTAGAACGCCAAGCTCGAAATTAAC-3'. The resulting PCR fragments had a BamHI site (GGATCC) at the 5' end and an XbaI site (TCTAGA) at the 3' end. The template used was *pBSK-wee1* (*wee1* cDNA; Campbell *et al.*, 1995). In each 20 µl PCR reaction, there was 1-5 pmol of template, 5 pmol of primers, 1 µl Pfu (a gift from Dr. Pickard in the Department of Biological Sciences at University of Alberta), 10 nM dNTP, 1X Pfu buffer. The reactions were performed using the following conditions: denaturing temperature of 95°C for 1 minute, annealing temperature of 55°C for 1 minute, and elongation temperature of 72°C for 2.5 minutes for 22 cycles. The resulting PCR products were digested with BamHI and XbaI (Gibco, Invitrogen) to generate DNA adhesive ends compatible with the destination vector. The destination vectors *UASp-egfp* and *UASp-(myc)*$_{12}$ were also digested with BamHI and XbaI and then treated with Alkaline Phosphatase to prevent self-annealing. The PCR-amplified insert DNA was then ligated to each of the respective vectors using T4 DNA ligase, to generate *UASp-egfp-wee1* and *UASp-(myc)*$_{12}$*-wee1* (Figure 4-1 for a diagram of the plasmid). For these manipulations, DNA was purified from agarose electrophoresis gels using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences).

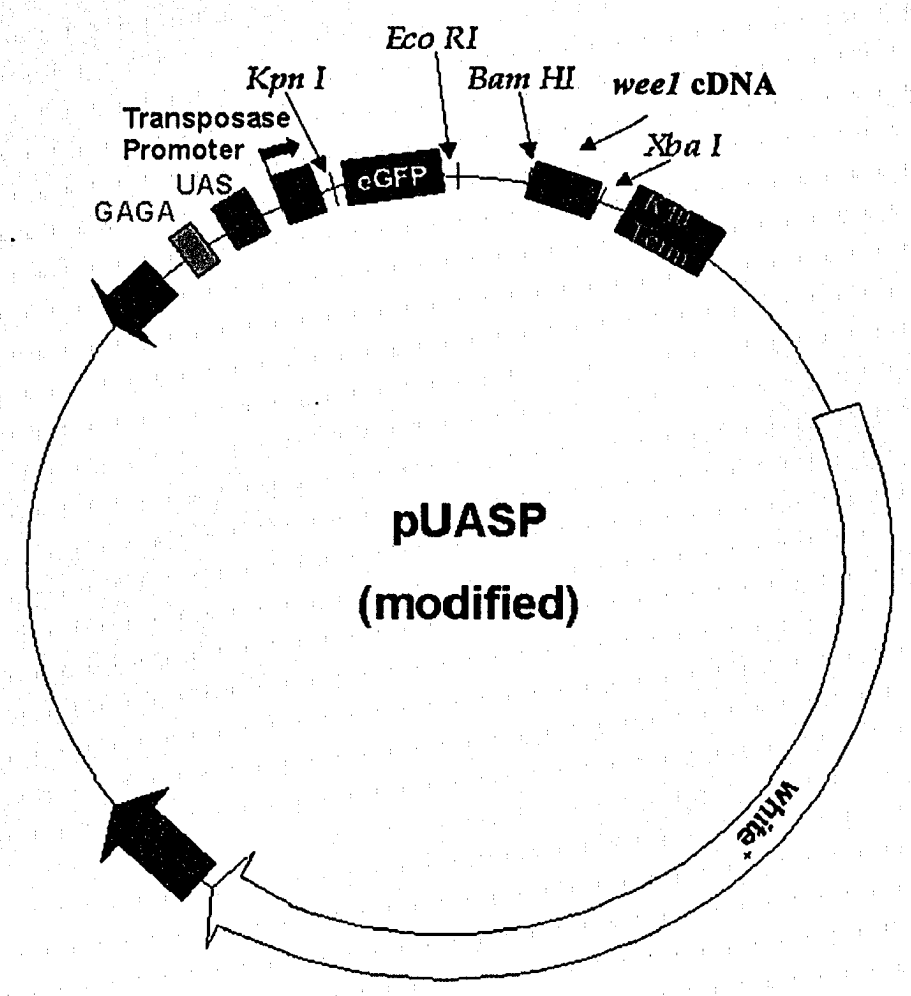

Figure 4-1 The end product of *UASp-egfp-wee1* (cDNA). Plasmid UASp has P-elements (P5' and P3' that are sites where insertion into the fly genome occurs), *white* gene (to facilitate the identification of red-eyed transformants), GAGA sites, UAS sequence, transposase promotor and K10 terminator (terminator of the *E. coli* K10 gene). *egfp* is subcloned with KpnI and EcoRI sites, followed by *wee1* cDNA sequence with BamHI and XbaI sites. *UASp-(myc)$_{12}$-wee1* is very similar to this plasmid, only with *myc* sequences replacing *egfp*. Not drawn on scale and only important restriction sites and elements are shown.

4.2.2. Sequencing constructs

All constructs were sequenced using DYEnamic ET kit (Amersham Biosciences). For each 20 µl reaction, there was 1-5 pmol of template, 5 pmol of primer, 4 µl of sequencing reagent premix and 4 µl of sequencing buffer. Primers used are listed in Table 4-1. The PCR sequencing reactions were: 95°C for 20 seconds, then 50°C for 15 seconds and 60°C for 60 seconds. This cycling program was repeated for 25 cycles. After PCR was completed, 2 µl of sodium acetate/EDTA buffer (provided in the kit) and 80 µl of 95% ethanol were added to each reaction to precipitate the DNA. Each tube was mixed well on a vortex mixer and then centrifuged for 15 minutes at ~12,000 rpm. The supernatant was removed by aspiration and the DNA pellets were washed with 70% ethanol. After a brief centrifugation, the ethanol was removed by aspiration and DNA pellets were allowed to air-dry. The products of these sequencing reactions were then analyzed on sequencing gels run by the MBSU (Microbiology Service Unit in the department of Biological Sciences at University of Alberta). Using the ABI PRISM (Model 373 Version 3.4.1 SemiAdaptive Version 3.3.1b2) sequencer, electronic sequencing data were obtained and analyzed in Genetool 2.0 to ensure that no errors were present in the constructs.

DNA fragments to be sequenced	Primers (name + sequence)
egfp	141: 5'GAACTTCAGGGTCAGCTTGC3' 494: 5'ACTTCAAGATCCGCCACAAC3' KpnI-EGFP: 5'CTGGGTACCATGGTG AGCAAGGGCGAG3' EGFP-EcoRI: 5'AATGAATTCCTTGTACAGCTCGTCCAT3'
$(myc)_{12}$	KpnI-Myc: 5'GAATTGGGTACCGTGTGCTGGAATGCGGCT3' Myc-XhoI: 5'GACCTCGAGTCCGTTCAAGTCE3'
wee1	BamHI-wee1: 5'CAGGGATCCATGGCATTCCGCCAGTCG3' #101: 5'GCGGAGGACGATCATATGCTG3' #104: 5'GACAAGAGGCCCACTTCGCAG3' #107: 5'ATGGCTCACAATAAGAAGCGC3' #108: 5'AAGCGCAGGCTTAACGCACGC3' #109: 5'GTCTACCAACGACGTGACGC3' #110: 5'CAGTTTCAGTAGTAGCCGCT3' #113: 5'TCGCCGATGATGACTTTGAC3' #116: 5'ATATGCGATTAGAAAGAGTAAGAAGC3' 201F: 5'CAATATCTGATAACTCGCAA3' Wee2: 5'GAGAACCTCAGTTGTCG3' Wee-XbaI: 5'GCTGCCTCTAGAACGCCAAGCTCGAAATTA AC3' 1038: 5'GGAAAATATGTCGGCCTTGA3' 1222: 5'TCGTCATCTTTGGTCTGCTG3' 1241: 5'CAGCAGACCAAAGATGACGA3' 1399: 5'ATTGTTTGGGTGAGGCAGAG3'

Table 4-1 List of sequencing primers

4.2.3. Generating fly transformants

Germline transformation was performed according to standard protocols (Santamaria, 1986). S. Hannan (in the department of Biological Sciences at University fo Alberta) helped me with the injections. We modified the standard protocol as follows: Each construct was mixed with Δ2-3 helper plasmid to promote P element insertion into the genome (Robertson *et al.*, 1988) at a ratio of 5 μg: 1 μg in 10 μl TE buffer (10 mM tris+1 mM EDTA). The solution of DNA was centrifuged at maximum speed for 30 minutes just before loading four injection needles with approximately 1-2 μl of DNA solution each. A cage of *yw* flies was set up several days earlier, so that embryos would be laid on the grape juice plate at the bottom of the cage. Grape plates were prepared using the following recipe: 10 g Bacto Agar was thoroughly dissolved in 255 ml of distilled water on a heated plate. Then heat was removed and a quarter of a can of Welch's grape juice concentrate was added to it. After the solution cooled down to approximately 60°C, the pH was adjusted to 6.4 and 18 g dextrose and 9 g of sucrose were added. The agar solution was then poured into plates (Falcon Petri Dish 35X10 mm, only the lids were used as these fit the fly cages). Embryos of *yw* flies were collected over 40 minute periods, then dechorionated for 46 seconds with a 50% fresh bleach solution (Javex bleach) and washed with a generous amount of distilled water. 60-100 dechorionated embryos were lined up on a slide with heptane glue (heptane + Scotch double sided tape mixed overnight). The embryos were dehydrated slightly in a desiccator for 7 minutes and 45 seconds before heavy oil (Halocarbon HC-700) was added to cover them and prevent dehydration. The embryos were then injected at the posterior region where the pole cells will form, providing the DNA with an opportunity to become incorporated into the genome of germline cells. Slides were then covered with more heavy oil and incubated at 18°C for 48 hours. Newly hatched larvae were picked up and transferred into a vial with instant food (potato flakes+distilled water). Eclosed males were crossed to *yw* virgin females to select transformants. *w+* (red-eyed) F1 flies were selected as transformants.

4.2.4. Mapping the transformants

Each single male transformant (identified by *w+* eye color) was crossed to *w; T(2;3) ap ^{xa}, ap ^{xa} / CyO; TM3, Sb*. F1 generation males with *w+*, *CyO* and *TM3, Sb* were then crossed to *yw* virgin females. F2 generation phenotypes were then analyzed. If *CyO* and *w+* segregated from each other in the F2 generation, the transgene was on the second chromosome. If *TM3, Sb* and *w+* segregated from each other in the F2 generation, the transgene was on the third chromosome. If only F2 females expressed *w+*, the transgene was on the X chromosome. The chances of getting an insertion on the fourth chromosome are negligible and can be ignored. In my experiments, all of the transgenes were mapped to the X, second or the third chromosomes. A list of transgenic strains that I isolated is outlined in Table 4-2.

Transgene	Name	Chromosome
UASp-(*myc*)$_{12}$-*wee1*	MW2°#1, MW2°#2, MW2°#3	second
UASp-(*myc*)$_{12}$-*wee1*	MW3°#1, MW 3°#2, MW 3°#3	third
UASp-(*myc*)$_{12}$-*wee1*	MW X	X
UASp-*egfp*-*wee1*	EW2°#1, EW 2°#2, EW 2°#3, EW 2°#4	second
UASp-*egfp*-*wee1*	EW 3°#1	third

Table 4-2 List of transgenes

4.2.5. GAL4-driven expression of epitope-tagged Wee1

I obtained a number of different GAL4 driver lines from Bloomington *Drosophila* Stock Center, as well as from our own collection, to test the transgenic lines I have created. The GAL4 lines tested in these experiments are listed in Table 4-3. Transgenic lines carrying tagged Wee1 were crossed to different GAL4 drivers, so that both elements would be present in the progeny of the cross. UASp-*egfp* (obtained from the Bloomington Stock Center and Dr. Rorth lab at the EMBL, Heidelberg) lines were each crossed to the same drivers, to serve as controls. Only five of the drivers showed strong and easily detectable expression of the transgenes. They were: *engrailed-GAL4*, *nanos VP16-GAL4*, *maternal tubulin-GAL4* and two GAL4 lines with unknown promoters: one that drives expression in the embryonic neurons (Bloomington Stock Center #2689 w^{1118}; *P{GawB} v85*) and one expressed in follicle cells surrounding the oocyte after stage 12 during oogenesis respectively (Bloomington Stock Center #3751 w^{1118}; *P{GawB}c204/TM3, Ser1*).

Bloomington Stock number	Notes
1874	w[*]; P{w[+mW.hs]=GawB}389 GAL4 in embryonic CNS
1822	y[1] w[*]; P{w[+mC]=GAL4-Hsp70.PB}31-1/T(2;3)B3, CyO: TM6B, Tb[1] GAL4 in embryonic CNS&PNS
3751*	w[1118]; P{w[+mW.hs]=GawB}c204/TM3, Ser[1] GAL4 in follicle cells over oocyte after stage 12
4442	w[*]; P{w[+mC]=GAL4-nos.NGT}40 nos GAL4 (weak)
4937*	w[1118]; P{w[+mC]=GAL4::VP16-nos.UTR}MVD1 nanos GAL4 VP16 primordial germ cell marker
5910	w[1118]; P{w[+mC]=EcR.GET-BD-GAL4}1 GAL4 in larval EcR-A-expressing neurons destined for apoptosis at metamorphosis
6800	w[*]; P{w[+mC]=nrv2-GAL4.S}3 GAL4 in nervous system
6798	w[*]; P{w[+mC]=Cha-GAL4.7.4}19B GAL4 in cholinergic neurons
N/A*	y[1] w[*]; P{w[+mC]=GAL4-engrailed} engrailed GAL4
2689*	P{w[+mW.hs]=GawB}V85, w[*] GAL4 in all embryonic neurons
N/A*	w[*]; P{w[+mC]=mata4-GAL-VP16} maternal tubulin-GAL4,V32a line is on the 2nd chromosome, V37P line is on the 3rd chromosome.

Table 4-3 List of GAL4 lines. * marks the drivers that gave detectable expression of the transgenes.

4.2.6. Testing for the functionality of epitope-tagged Wee1 by genetic rescue

To test the functionality of the *UASp-egfp-wee1* transgene, I performed genetic rescue experiments. *wee1* mutants have a maternal embryonic lethal phenotype, therefore overexpression of EGFP-Wee1 using the *maternal tubulin-GAL4* driver in a *wee1* mutant background should rescue this phenotype if the transgene is functional. To test this idea, I performed genetic crosses to generate flies with both *UASp-egfp-wee1* and *maternal tubulin-GAL4* in *wee1* (*w; wee1^{ES1}/Df(2L) WO5*) mutant background and then examined the viability of embryos laid by those females. Another GAL4 line, *heat-shock GAL4* was also used to perform the genetic rescue experiment to induce moderate maternal overexpression of EGFP-Wee1. For this experiment, genetic crosses were performed to generate flies with both *heat-shock GAL4* and *UASp-egfp-wee1* in a *wee1* (*w; wee1^{ES1}/wee1^{ES2}*) mutant background. Females of this genotype were heat shocked for 30 minutes each day for four days. Each day, embryos laid by these females that developed beyond cycle 14 were scored. The percentages of embryos older than cycle 14 before and after heat shock were compared to evaluate whether the transgene is functional.

4.2.7. Immunofluorescent analysis of embryos expressing tagged Wee1

a). Embryo staining:

Embryos from the following crosses were analyzed: 1st group: *engrailed-GAL4* crossed to Myc-Wee1 2°#1 (for details of each tagged Wee1 line, refer to Table 4-2); 2nd group: *embryonic neuron-GAL4* crossed to EGFP-Wee1 2°#1; 3rd group: eggs laid by F1 females from cross *maternal tubulin-GAL4* crossed to EGFP-Wee1 2°#4, with the control being eggs laid by F1 females from cross *maternal tubulin-GAL4* crossed to *UASp-gfp* or *UASp-egfp*.

For embryo collections, adult flies of the desired genotypes were put in a collection cage. Embryos were collected, dechorionated with 50% bleach and washed with an ample amount of distilled water. Fixation methods varied for different antibodies. There were three different fixatives used: firstly, the standard 3.7% formaldehyde fixative (1 part 3.7% formaldehyde in 1XPBS: 1 part heptane) for 20 minutes, suitable for most antibodies; secondly, 37% formaldehyde fixative (1 part 37% formaldehyde stock solution: 1 part heptane) for 5 minutes, suitable for anti-Tubulin antibodies; thirdly, 1 part methanol: 1 part heptane fixative, suitable for western blotting experiments. After fixation, embryos were then devitellinized with methanol, then washed and stored in methanol at -20°C until required. To prepare the embryos for immunofluorescent analysis, they were rehydrated in PBST (PBS+0.1% TritonX-100) for 15 minutes and then washed in PBST three times for 5 minutes each time. Embryos were then blocked in PBST+10% NGS (normal goat serum) for an hour at room temperature. The samples were then incubated (with agitation) in primary antibody staining solution at 4°C overnight. Primary antibodies were diluted in PBST: 1:500 for rabbit anti-GFP (BD Science); 1:500 for rabbit anti-Myc (Cell Signaling); 1:2,000 for rabbit anti-PH3 (Upstate); 1:200 for mouse anti-alpha/beta Tubulin (Sigma and Hybridoma Bank); 1:100 for rabbit anti-pY15 Cdk1 (Cell Signaling); 1:500 for rabbit anti-Centrosomin (a gift from Kaufman Lab at HHMI Indiana University); 1:1,000 for mouse anti-nuclear Lamin (Developmental Studies Hybridoma Bank). The next day, samples were washed in PBST four times for 15 minutes each time and incubated with secondary antibodies for one hour at room temperature. The dilutions used for secondary antibodies were: 1:1,000 for anti-

rabbit Alexa Fluor 488 or 568, 1:1,000 for anti-mouse Alexa Fluor 488 or 568 (all secondary antibodies were from Molecular Probes). After secondary antibody incubation, samples were washed in PBST four times for 15 minutes each time and then stained with Hoechst 33258 (1:100 in PBST, to obtain the final working concentration of 5 μg/ml) for ten minutes. A final wash was performed to remove unbound Hoechst stain and the samples were mounted in anti-fade mounting media (9 parts glycerol: 1 part 10XPBS, containing 10 mg/ml 1, 4-phenylenediamine).

b). Ovary staining

The F1 females from the following crosses were examined: *follicle cell-GAL4* crossed to EGFP-Wee1 2°#4, with the control being *follicle cell-GAL4* crossed to *UASp-egfp*. Newly eclosed F1 females from these crosses were transferred into vials with fresh food and yeast paste in the presence of sibling males. After three to five days, females were dissected and their ovaries were teased apart using tungsten needles, to increase the accessibility of the tissue to antibodies. The ovaries were then fixed in standard fixative (1 part [PBS+0.05% NP-40+4% formaldehyde]: 3 parts heptane) for 20 minutes, then washed in PBST (PBS+0.1% TritonX-100) three times for 5 minutes each time. Subsequent procedures were the same as described for embryo staining, for blocking, primary/secondary antibody incubation, washes and mounting. Rabbit anti-GFP (BD Science) antibody was used at a dilution of 1:500.

c). Confocal microscopy and data collection

All samples were examined under Leica confocal microscope (Model: TCS SP2). Data were collected and compiled using Leica confocal software and Adobe Photoshop CS software.

4.2.8. Time-lapse microscopy

A cage of EGFP-Wee1 2°#1/*maternal tubulin-GAL4* flies (from cross: *maternal tubulin-GAL4* X EGFP-Wee1 2°#1) was set up to collect embryos with maternally expressed EGFP-Wee1. Embryos were collected and hand dechorionated by rolling them on Scotch double-sided tape. For each experiment, 10 embryos were transferred to a coverslip (24 mm X 50 mm) with a stripe of heptane glue at the center and then covered with heavy oil. GFP fluorescence was then continuously examined using a Leica confocal microscope over a period of 1 hour to 2 hours.

4.2.9. Western blotting experiments

Embryos laid by F1 females from the cross: *maternal tubulin-GAL4* X EGFP-Wee1 2°#1, as well as *maternal tubulin-GAL4* X *UASp-egfp* (or *UASp-gfp*) were collected, dechorionated and fixed in methanol, then stored at -20°C until use. Embryos were rehydrated in Embryo Buffer (Su, 2000), and stained with Hoechst 33258 to stage them with respect to developmental stage under a Zeiss fluoresescent microscope. Syncytial stage embryos that were fixed prior to cycle 14 were individually selected, then transferred into Laemmli sample buffer (Bio-Rad Laemmli premix sample buffer+fresh b-mercaptoethanol) and boiled for 10 minutes. For anti-GFP blotting, the extracts were normalized so that a final concentration of 1 embryo extract/μl was used. For the phospho-Cdk1 blots, the extracts were normalized to a final concentration of 1 embryo

extract/10 µl. The samples were resolved on SDS-PAGE mini-gels, and then transferred to Hybond -P membrane (Amersham Biosciences). Each well in the gels was loaded with 10 µl of sample or protein molecular weight standards ladder (Bio-Rad). Membranes were stored at 4°C. For blotting, membranes were briefly wet with methanol and immediately washed in PBST (PBS+0.02% Tween-20) three times for 5 minutes each time. After a further wash of 15 minutes in PBST, membranes were blocked in PBSTM (PBST + 5% skim milk) for an hour at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C. Dilutions for primary antibodies were: 1:1,000 mouse anti-GFP (BD Science); 1:2,000 mouse anti-alpha or anti-beta-Tubulin (Sigma and Developmental Studies Hybridoma Bank); 1:1,000 rabbit anti-pY15 Cdk1 (from Cell Signaling); 1:1,000 rabbit anti-pT14 Cdk1 (developed in our lab). The next day, membranes were washed in PBST six times for 10 minutes each time, followed by secondary antibody incubation for one hour at room temperature. Anti-mouse or anti-rabbit Horseradish Peroxidase-conjugated secondary antibodies were used at a dilution of 1:10,000 (Amersham Biosciences). Another series of washes were performed four times for 15 minutes each time. Bound antibodies were detected using an ECL Plus Western Blotting Detection kit (Amersham Biosciences) according to the manufacturer's instructions.

4.3 Results

After making the constructs, I generated transgenic lines carrying the *UASp-egfp-wee1*, and *UASp-(myc)$_{12}$-wee1* constructs and obtained independent lines for each. Because there were already available *UAS-egfp* lines at the Bloomington *Drosophila* Stock Center and from other labs, I was able to use them as controls. After mapping what chromosomes the transgenes were on (Materials and Methods) and establishing homozygous stocks for each of the transgene insertions, I crossed the stocks to a collection of lines carrying different GAL4 drivers (listed in Table 4-3). The following GAL4 lines drove relatively strong expression of the transgenes in early embryos: *engrailed-GAL4*, *nanos-GAL4*, *maternal tubulin-GAL4*, *embryonic neuron-GAL4* (*P{GawB}v85*), *follicle cell-GAL4* (*P{GawB}c204*) (* in Table 4-3). By means of western blotting, immunofluorescence and time-lapse microscopy described above (Materials and Methods), I was able to examine the localization and function of overexpressed tagged Wee1. I found that Wee1 was dynamically localized in different cell types at different developmental stages. I also observed that ectopic expression of tagged Wee1 during early embryogenesis and oogenesis both caused severe phenotypes. The syncytial embryos showed defects in both nuclear and cytoplasmic events. Such defects were consistent with misregulated Cdk1 activity. Defects in oogenesis will be discussed in detail in Chapter 5.

4.3.1. Test for genetic rescue with EGFP-Wee1

Before undertaking any experiments with the *wee1* transgenes, it was very important to determine if the fusion proteins were functional. To address this issue, I performed genetic rescue experiments. Because loss of maternal Wee1 leads to a mitotic catastrophe phenotype in early embryogenesis and the mutant embryos cannot develop past cycle 14 (Price *et al.*, 2000), therefore my rationale for the rescue experiment was that if the fusion protein was functional it should rescue the embryonic lethal phenotype

in *wee1* mutants. Flies containing both *UASp-egfp-wee1* and *maternal tubulin-GAL4* transgenes in a *wee1* mutant background were generated using standard genetic crosses. Overexpression of EGFP-Wee1 maternally failed to rescue the loss of maternal Wee1 because none of the embryos had cellularized, implying that they were not capable of developing further than cycle 14. However, those embryos showed very similar phenotypes as overexpressing EGFP-Wee1 in a wild type background alone (refer to part 4.3.4 of this section for details of the phenotypes). My interpretation is that overexpression of EGFP-Wee1 under the maternal GAL4 driver was so strong that the overexpression phenotype overwhelms any effect of rescuing the maternal loss of function phenotype. As a result, overexpression of EGFP-Wee1 in a wild type background and in a *wee1* mutant background showed similar phenotypes. An alternative strategy I tried was to use a different driver with lower maternal expression. For this purpose I used a *heat-shock GAL4* driver, allowing overexpression levels to be modulated by the timing and periodicity of heat shocks delivered to the mothers. This driver was successfully used previously for a similar experiment involving a non-tagged version of Wee1 to drive weak overexpression in early embryos (Price *et al.*, 2000). Before the heat shock treatment, $wee1^{ES1}/wee1^{ES2}$ mutants showed a partially penetrant maternal embryonic lethal phenotype (22.2% cellularized embryos, n=45). The first day after the heat shock treatment, mild expression of EGFP-Wee1 increased the proportion of cellularized embryos to 36.7% (n=30). The second day after the heat shock treatment, the proportion of cellularized embryos further increased to 40.6% (n=101). The third day after the heat shock treatment, this proportion dropped back to 23.2% (n=112). Taking together, my results are consistent with previously published results (Price *et al.*, 2000) in that heat shock driven expression of the transgene maternally led to a partial rescue of the syncytial embryonic lethal phenotype and the second day after heat shock the expression levels of the transgene seemed to be the highest. However, it is unclear why the proportion of rescued embryos dropped on the third day. Taken together, these results provide initial evidence suggesting that the fusion protein EGFP-Wee1 is functional.

4.3.2. Biochemical assays to test the functionality of EGFP-Wee1

Since the genetic rescue experiment only showed a partial rescue by EGFP-Wee1, I decided to further test the functionality of EGFP-Wee1 by assaying its biochemical activity *in vivo*. First of all, the fusion protein could be detected by western blotting, using an antibody directed against GFP in an embryo overexpressing EGFP-Wee1 under the maternal driver (Figure 4-2A). Because Cdk1 is the only known substrate of Wee1 (Stumpff *et al.*, 2004), I examined the phosphorylation status of the tyrosine 15 residue of Cdk1 in the presence of EGFP-Wee1 overexpression. Western blotting assays showed that as expected, the levels of phosphorylated tyrosine 15-Cdk1 were much higher in embryos overexpressing EGFP-Wee1 than in the controls. In extracts made from a single control embryo, the level of tyrosine-15 Cdk1 phosphorylation was below detection, however it was very apparent in an embryo overexpressing EGFP-Wee1 (Figure 4-2B). These results indicate that the fusion protein is functional as a Cdk1 inhibitory kinase.

Interestingly, Cdk1 threonine 14 phosphorylation levels were also increased in the presence of EGFP-Wee1 overexpression, as shown in the western blot (Figure 4-2B). Previous results in our lab demonstrated that threonine 14 phosphorylation levels were decreased in *wee1* mutants, in addition to a decrease in tyrosine 15 (E. Homola, personal

communication). These two pieces of evidence suggest that Wee1 affects T14 phosphorylation. In metazoans Wee1 has not been reported to be capable of phosphorylating the T14 residue on Cdk1. Instead Myt1 has been shown to phosphorylate T14, as well as Y15 (Booher, 1997). Therefore our results raised a possibility that *Drosophila* Wee1 may in fact be capable of phosphorylating T14 or that Wee1 activity is indirectly affecting T14 phosphorylation carried out by Myt1. These questions may be resolved by examining T14 phosphorylation levels in a *myt1* mutant embryo in the future.

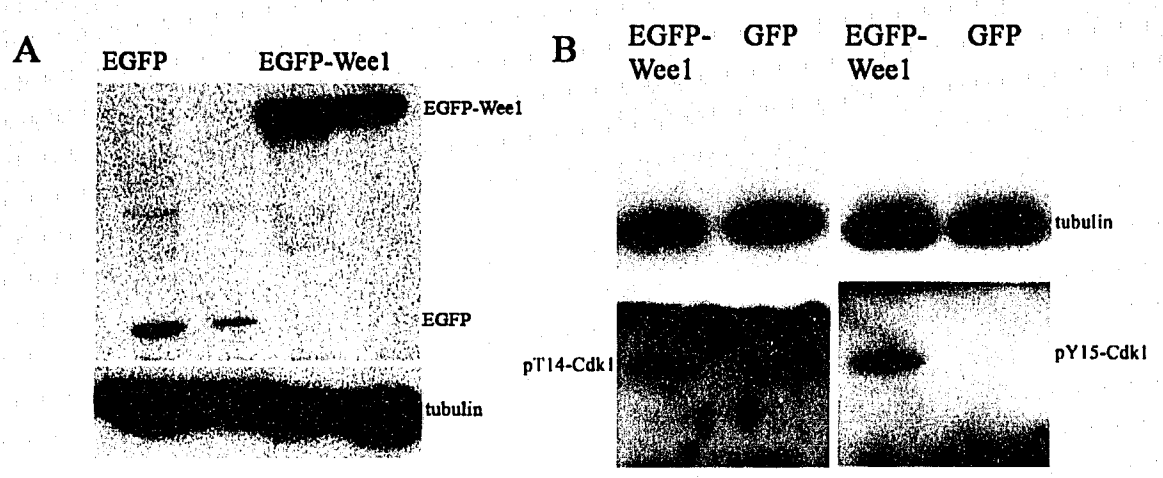

Figure 4-2 Overexpression of the EGFP-Wee1 fusion proteins stimulated Cdk1 inhibitory phosphorylation. (A) Expression of the EGFP-Wee1 fusion proteins. The first two lanes were loaded with 10 embryos laid by females of the genotype: *maternal-GAL4>UAS-egfp*. The next two lanes were loaded with 10 embryos laid by females of the genotype: *maternal-GAL4>UAS-egfp-wee1*. The membrane was blotted with anti-GFP antibody. The control shows a ~30KD EGFP band, whereas the EGFP-Wee1 fusion protein is around ~105KD, as predicted. (B) The phosphorylation status of Cdk1 on T14 and Y15 residues in the presence of excessive EGFP-Wee1. The first two lanes were loaded with a single embryo each laid by females of genotypes: *maternal-GAL4>UAS-egfp-wee1* and *maternal-GAL4>UAS-egfp* respectively. This blot was blotted against the anti-pT14-Cdk1 antibody. In the control, T14 phosphorylation was too low to be detected in a single embryo. But in an embryo overexpressing EGFP-Wee1, T14 was heavily phosphorylated. The next two lanes were loaded with a single embryo each laid by females of genotypes: *maternal-GAL4>UAS-egfp-wee1* and *maternal-GAL4>UAS-egfp* respectively as well. This blot was blotted against the anti-pY15-Cdk1 antibody. In the control, Y15 phosphorylation was also too low to be detected in a single embryo. In an embryo overexpressing EGFP-Wee1, however, Y15 was heavily phosphorylated as expected. This result demonstrates that the fusion protein is functional and that surprisingly, overexpression of EGFP-Wee1 affected the phosphorylation of the T14 residue on Cdk1 in *Drosophila* syncytial embryos.

4.3.3. Dynamic localization of tagged Wee1

Giving that Wee1 localization in other systems is highly dynamic and cell cycle dependent (Baldin and Ducommun, 1995; Sakchaisri *et al.*, 2004), I studied the localization of *Drosophila* Wee1. Due to technical problems with the previously made anti-Wee1 antibody, I chose to use tagged Wee1 and an overexpression system to look at Wee1 localization. Overexpression of EGFP-Wee1 and Myc-Wee1 always showed identical localization patterns in these experiments, indicating that the tag itself was not determining subcellular localization. For convenience, only data for one of the tagged proteins is presented here for each driver.

engrailed-GAL4 drove overexpression of Myc-Wee1 in a segmental pattern during late embryogenesis, a developmental stage when segmentation of the embryo along the anterior-posterior axis occurs. Anti-Myc and anti-nuclear Lamin double staining demonstrated that tagged-Wee1 was localized in the nucleus in these cells, consistent with Wee1 being localized to nuclei during interphase as has been reported in other systems (Figure 4-3B. Heald *et al.*, 1993; Mitra and Schultz, 1996; Wu *et al.*, 1996; Nakanishi *et al.*, 2000).

Another driver *embryonic neuron-GAL4* drove overexpression of tagged Wee1 in embryonic neurons. GFP fluorescence revealed that EGFP-Wee1 was localizing in the central commissure region (Figure 4-3A, the insert shows a higher magnification), which implies that the EGFP-Wee1 protein could be stabilized in the axoplasm of non-mitotic neurons.

Similarly, a *follicle cell-GAL4* driver overexpressed EGFP-Wee1 in endoreplicating somatic follicle cells after stage 12 of oogenesis. EGFP-Wee1 localized to the cytoplasm, as visualized by anti-GFP antibody staining (Figure 4-4D, E and F). The EGFP alone control showed a non-specific localization with a slight preference for the nucleus, indicating that the tag itself was not responsible for this localization (Figure 4-4A, B and C).

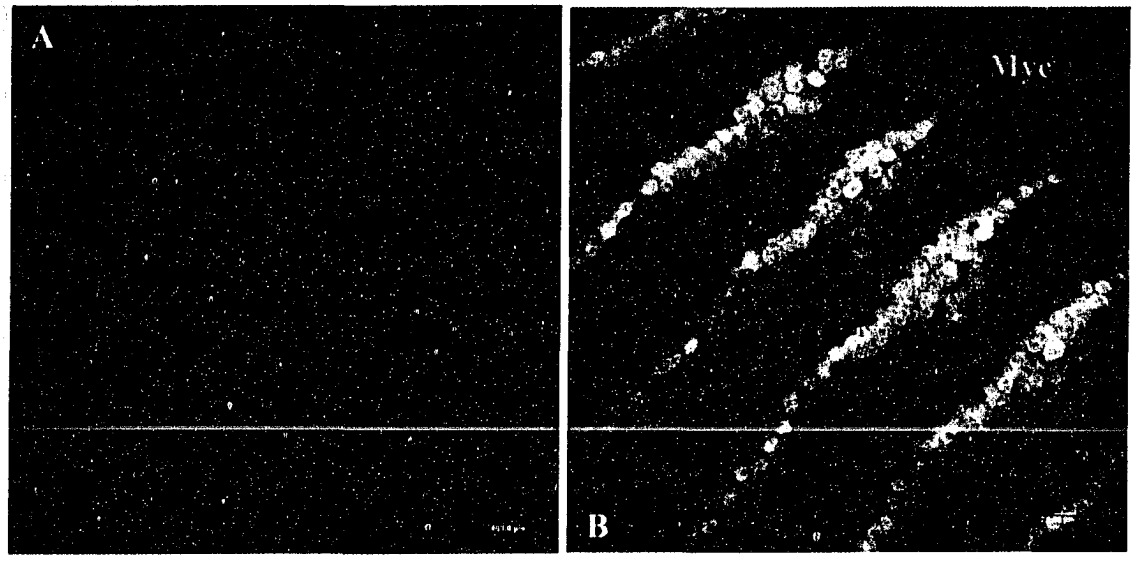

Figure 4-3 Cytoplasmic and nuclear localization of EGFP-Wee1 when overexpressed with two different GAL4 lines: *embryonic neuron-GAL4* and *engrailed-GAL4*. (A) Overexpression of EGFP-Wee1 in the central nervous system under an *embryonic neuron-GAL4* line, with GFP fluorescence shown in green. The inset shows a higher magnification of the axoplasmic localization of EGFP-Wee1. (B) Overexpression of Myc-Wee1 during late embryogenesis when segmentation occurs, under control of the *engrailed-GAL4* line. Myc-Wee1 is localized in the nucleus. Nuclear Lamin (green) marks the nuclear envelope and the Myc-Wee1 signal is shown in red.

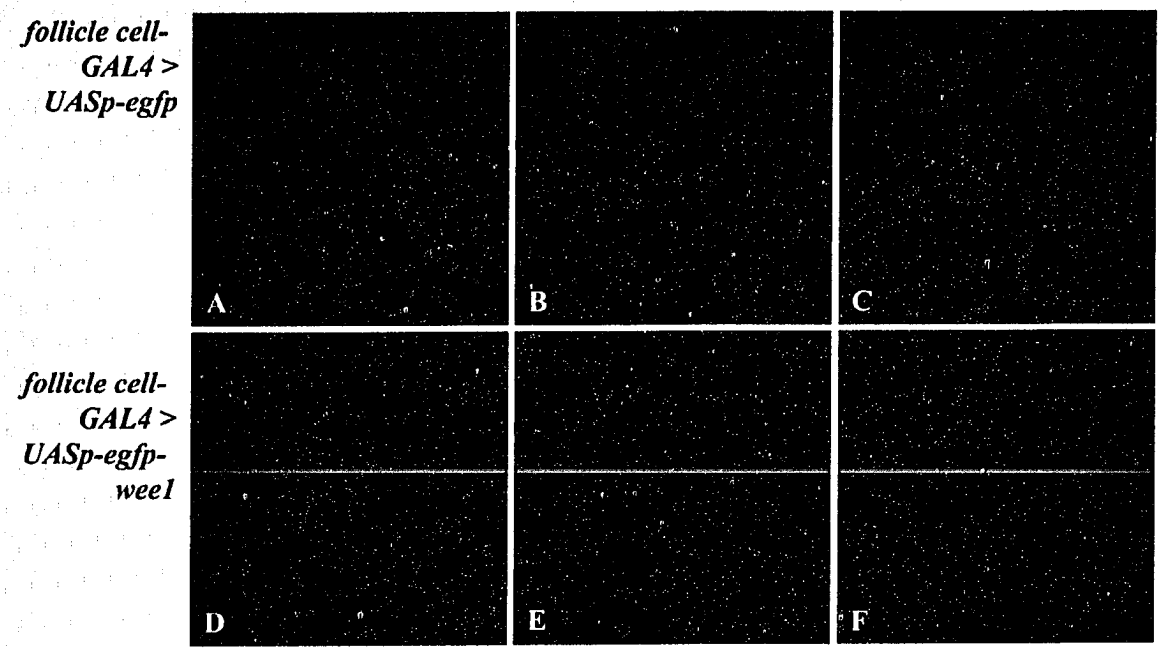

Figure 4-4 Cytoplasmic localization of EGFP-Wee1 in follicle cells at stage 12 during oogenesis under the control of *follicle cell-GAL4*. GFP expression is shown in green and DNA is shown in blue. Panels A-C show overexpression of EGFP alone. Note that EGFP is both in the nucleus and the cytoplasm, but showed a preference for the nucleus. (A) EGFP signal, (B) DNA, (C) Merged image. Panels D-F show overexpression of EGFP-Wee1 under control of the same driver. Note that EGFP-Wee1 is localized in the cytoplasm. (D) EGFP-Wee1, (E) DNA, (F) Merged image.

The *maternal tubulin-GAL4* driver caused expression of EGFP-Wee1 in embryos laid by mothers carrying both the driver and the *UASp-egfp-wee1*. The EGFP-Wee1 fusion protein could be detected by western blotting as mentioned earlier, using an antibody directed against GFP (Figure 4-2A). Immunostaining of embryos with this antibody showed that EGFP-Wee1 localized in the nucleus during interphase and was enriched in undefined subnuclear structures (dots in the nuclei, Figure 4-5A and B). During mitosis, the EGFP-Wee1 signal was dispersed throughout the cytoplasm, however. We could also see some accumulation apparently on metaphase chromosomes in a small fraction of the fixed embryos (~5% of the embryos, n=100), which may be due to transient colocalization (Figure 4-5C to F). Live analysis of these embryos confirmed the nuclear localization of EGFP-Wee1 during interphase, and at the onset of mitosis, the majority of the protein dispersed quickly into the cytoplasm. Live analysis also provided support for a small fraction of the protein being transiently localized to structures presumed to be chromosomes and mitotic spindles (E. Homola and S. Campbell, personal communication, Figure 4-6). The EGFP nuclear dots observed during interphase in fixed embryos were also observed in the live movies (Figure 4-6 interphase panels, the insert in the first image shows subnuclear dots). We have not yet been able to resolve the localization of EGFP-Wee1 to the spindles during mitosis in fixed embryos because this association is apparently very transient, judged by live analysis. We will address this aspect in more detail in future experiments.

Collectively, the above results indicate that the EGFP-tagged Wee1 (or Myc tagged Wee1) localized to different subcellular compartments in different cell types (summarized in Table 4-4), and that the tags did not interfere with such localization. Similar to Swe1 in *S. cerevisiae* and Wee1 in humans, overexpressed EGFP-Wee1 showed a dynamic localization during early embryogenesis and revealed a colocalization with cytoskeletal structures: mitotic spindles in *Drosophila* embryos, budding neck in *S. cerevisiae*, and cleavage furrow/mid body in human cultured cells (Baldin and Ducommun, 1995; Sakchaisri *et al.*, 2004). The localization of overexpressed Wee1 to subnuclear structures of unknown origin during interphase was also consistent with results in human and *Xenopus* cells (Lee *et al.*, 2001).

maternal-GAL4>UASp-*egfp-wee1*

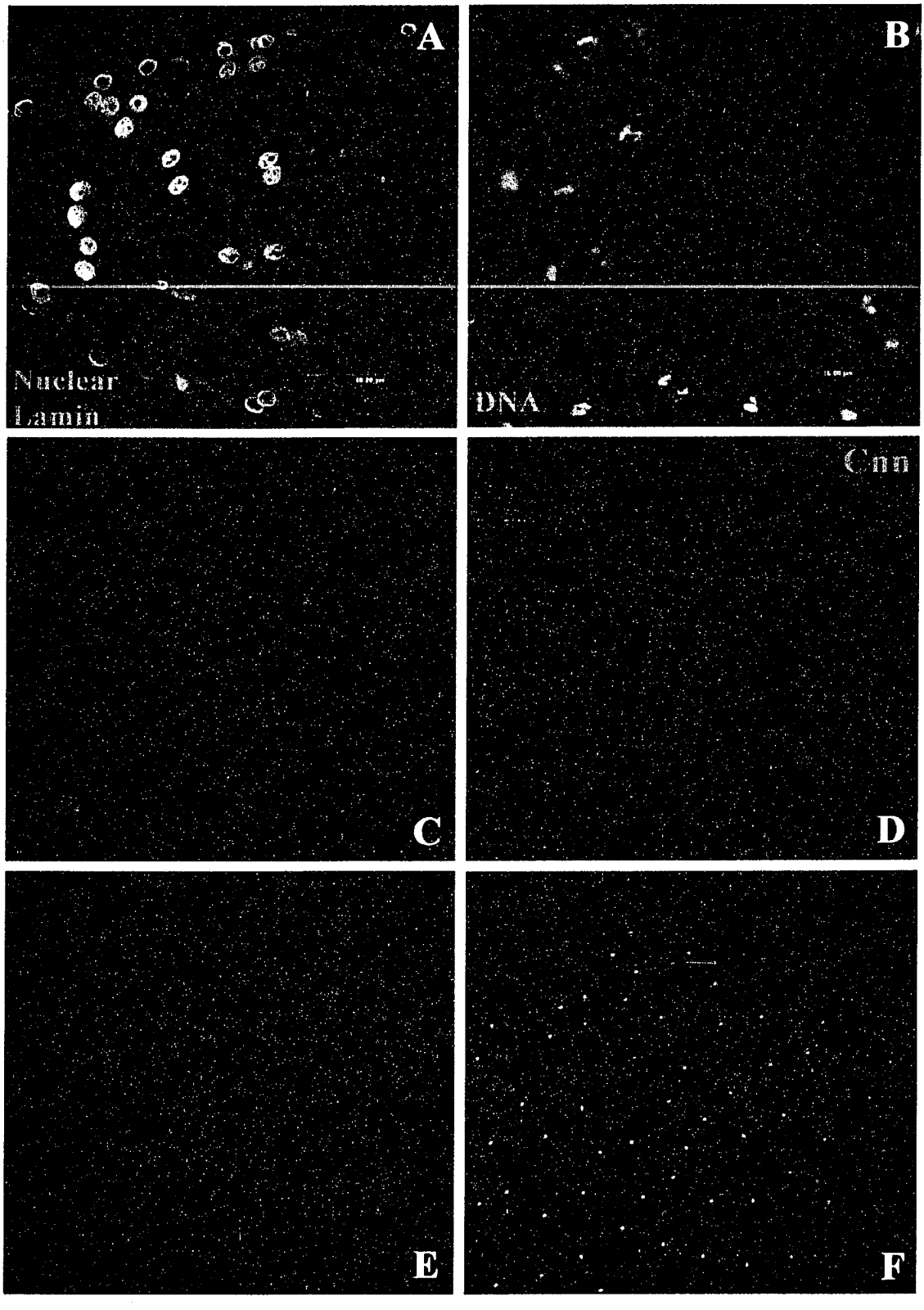

Figure 4-5 Dynamic localization of EGFP-Wee1 during interphase and mitosis in early embryogenesis. (A) A typical nuclear localization of EGFP-Wee1 during interphase. Nuclear Lamin (red) marks the nuclear envelope and GFP is in green. Note that the nuclei appear quite heterogeneous and that EGFP-Wee1 is enriched in unknown "dotted" subnuclear structures in some, but not all nuclei. (B) The hypercondensed DNA morphology observed in nuclei from embryos overexpressing EGFP-Wee1. Note that the DNA only takes up a small fraction of the nuclear space and that EGFP-Wee1 dots are also visible. (C-F) Colocalization of EGFP-Wee1 with mitotic chromosomes during prometaphase. Centrosomin (Cnn, red) marks the centrosomes. Tubulin (blue) marks microtubules. GFP in green. (F) Merged image. This colocalization is very transient (also visualized in life movies), so in fixed embryos only a small percentage (~5%, n=100) of the embryos showed this phenotype.

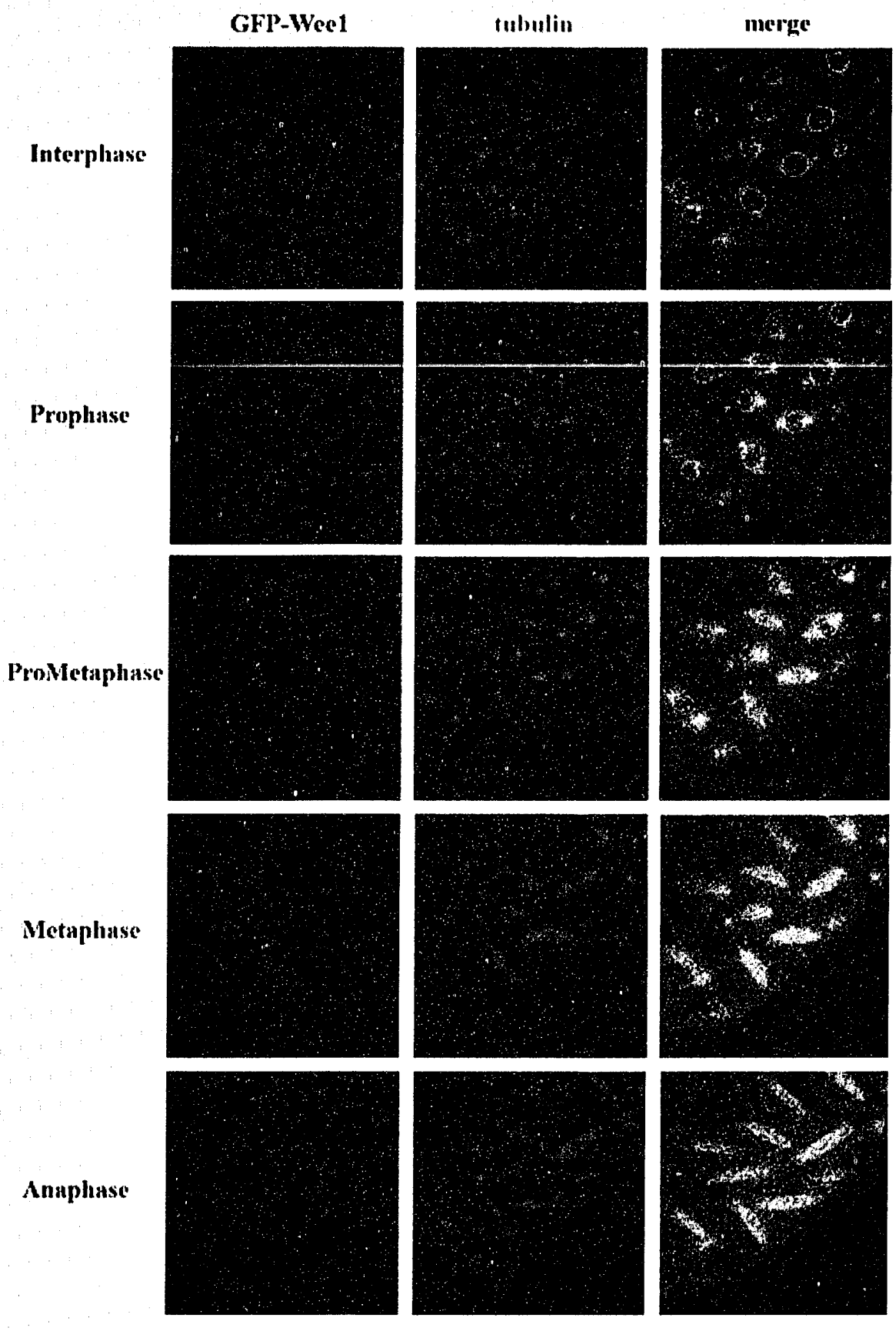

Figure 4-6 Live analysis of the dynamic localization of EGFP-Wee1 during syncytial embryonic cell cycles. Courtesy of E. Homola. All images were taken from a movie made of an EGFP-Wee1 expressing embryo that was injected with Rhodamine-conjugated Tubulin. GFP is shown in green and Tubulin in red. The EGFP-Wee1 protein localizes in a cell cycle dependent manner. During interphase, EGFP-Wee1 shows a nuclear localization. Note that "dotted" subnuclear structures are noticeable at this stage, as also shown in a magnified insert (first figure bottom right). During prophase and prometaphase, EGFP-Wee1 starts to disperse into the cytoplasm and also appears to localize to the spindle poles as well as in the chromosomal region (the exact order of these inferred events has not yet been determined). During metaphase, the majority of EGFP-Wee1 appears dispersed throughout the cytoplasm, with a slight preference for the chromosomal region. In anaphase, EGFP-Wee1 is completely dispersed within the cytoplasm. At the end of telophase and beginning of interphase, EGFP-Wee1 signal reappeared strongly in the nucleus as seen in interphase.

Driver name	Tissue/Cell type/Developmental stage	Localization pattern of tagged Wee1	Figure references
engrailed-GAL4	Late embryogenesis	Within the nucleus	Figure 4-3B
embryonic neuron-GAL4	Late embryogenesis	Stabilized in the axoplasm	Figure 4-3A
follicle cell-GAL4	Follicle cells surrounding the oocyte after stage 12 of oogenesis	In the cytoplasm of the follicle cells	Figure 4-4
maternal tubulin-GAL4	Syncytial embryogenesis:	Within the nucleus in interphase, dispersal in the cytoplasm in mitosis, colocalize with mitotic spindles and chromosomes at the onset of mitosis	Figure 4-5 Figure 4-6

Table 4-4 A summary of localization patterns of tagged Wee1 fusion proteins when overexpressed with different GAL4 drivers.

Because I have already demonstrated that Cdk1 phosphorylation was markedly changed in the presence of EGFP-Wee1 overexpression in biochemical assays, with Cdk1 being one of the key cell cycle regulators, I expected to see various abnormal phenotypes associated with EGFP-Wee1 overexpression. Indeed, embryos overexpressing EGFP-Wee1 had a wide range of defects affecting cell cycle regulated events during the syncytial divisions, which are described in detail below. Most of these embryos could not develop beyond cycle 14, implying that Wee1 expressed at these levels profoundly interferes with early embryonic development and cellularization. For convenience, I divided the defects into two categories: nuclear defects and cytoplasmic defects. Note that during these experiments, both Myc-Wee1 and EGFP-Wee1 overexpression were tested and they showed identical phenotypes. Only the data for EGFP-Wee1 overexpression is discussed in this thesis.

4.3.4.1 Nuclear defects caused by EGFP-Wee1 overexpression:

Consistent with Cdk1 having nuclear functions, for example promoting DNA condensation (Lamb *et al.*, 1990), overexpression of EGFP-Wee1 caused severe nuclear defects. First of all, the DNA morphology was abnormal in embryos overexpressing EGFP-Wee1. Large condensed masses of nuclei were occasionally seen (Figure 4-7B). The interphase nuclei DNA appeared to be abnormally condensed in that the DNA appeared to occupy only a small fraction of the nuclear space (Figure 4-5B). Most embryos contained regions devoid of nuclei (Figure 4-7B). The nuclei were typically seen in clusters instead of being evenly spread out as in control embryos, suggesting a nuclear migration defect or nuclei fallout from the cortex into the interior of the embryo.

In wild type embryos, the syncytial divisions are synchronized such that the nuclei are of similar sizes. In embryos overexpressing EGFP-Wee1, however, the nuclei were of different sizes (Figure 4-7B), suggesting there was a loss of synchrony in cell division with some nuclei divided more times (and thus were smaller) than others. The synchrony of nuclear divisions was also addressed using anti-PH3 antibodies to mark mitotic nuclei. Histone H3 is specifically phosphorylated during mitosis (Hendzel *et al.*, 1997), thus anti-PH3 antibody labels mitotic nuclei. In control mitotic embryos, chromosomes were uniformly PH3 positive as expected, since nuclei are cycling synchronously (Figure 4-7C). In embryos overexpressing EGFP-Wee1 however, various cell cycle stages could be observed in each embryo (Figure 4-7D).

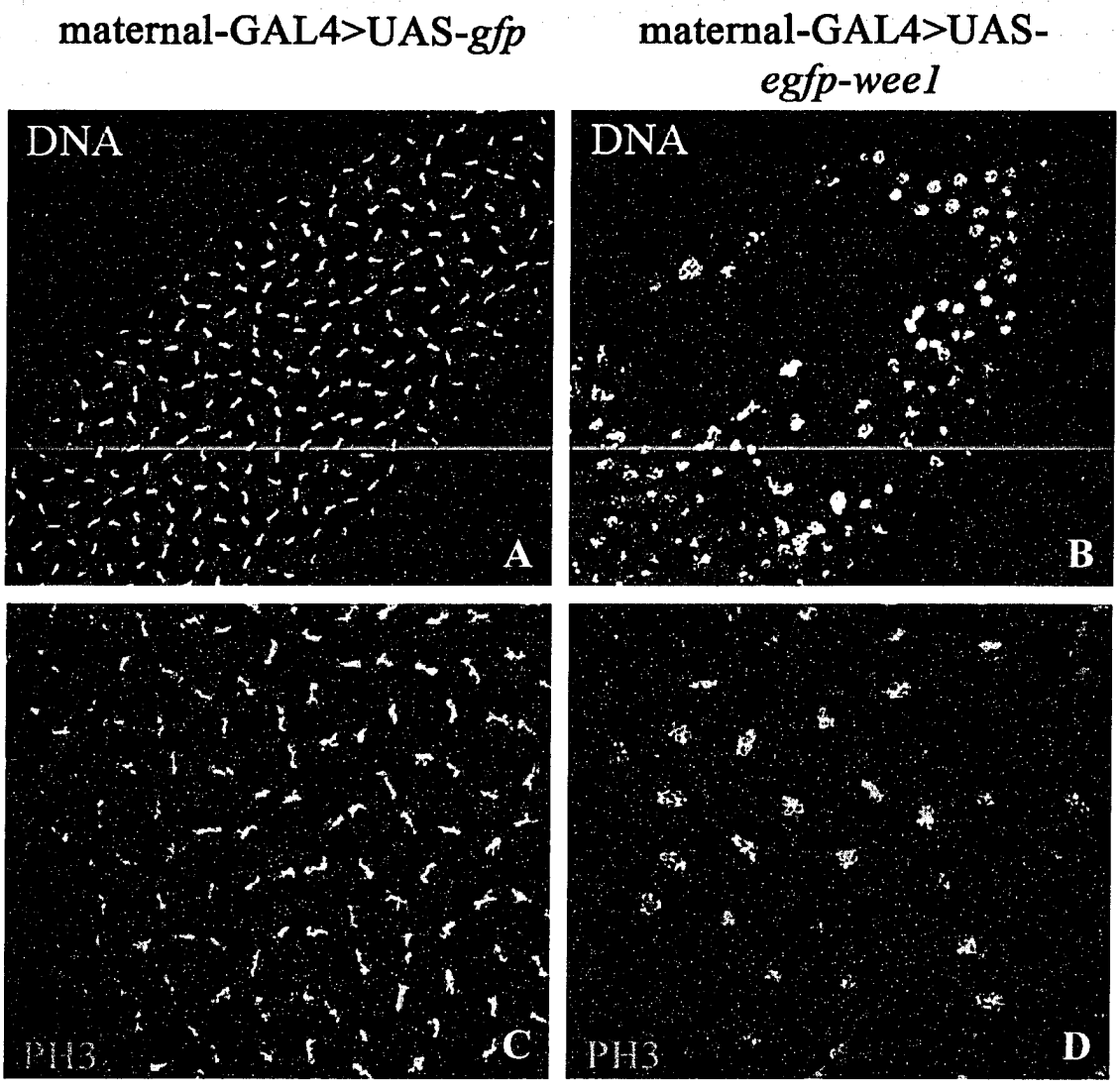

Figure 4-7 Overexpression of EGFP-Wee1 in early embryos resulted in severe nuclear defects. (A) Even distribution of nuclei along the cortex in an embryo overexpressing GFP alone (same as in wild type). (B) Uneven distribution of nuclei in an embryo overexpressing EGFP-Wee1 with a blank region containing no nuclei. The sizes of nuclei and the condensation status of the DNA vary in these embryos. (C) A synchronous dividing embryo overexpressing GFP alone (same as in wild type). PH3 in red and DNA in blue. Note that the chromosomes are uniformly in a metaphase configuration and are all PH3-positive. (D) A loss of synchrony in an embryo overexpressing EGFP-Wee1. Different phases of mitosis can be observed. Also note that the nuclei were not evenly arranged.

4.3.4.2 Cytoplasmic defects associated with EGFP-Wee1 overexpression

a). Centrosomes failed to attach to bipolar spindle poles

Since Cdk1 also regulates cytoplasmic events such as spindle assembly, I also examined the cytoplasmic defects associated with EGFP-Wee1 overexpression. To examine effects on the mitotic apparatus, I performed immunofluorescence with antibodies that recognize Centrosomin, alpha or beta-Tubulin to visualize centrosomes and spindles. Two predominant defects were observed. Firstly, free centrosomes were commonly seen, either singly or in groups (Figure 4-8L and O). This phenotype suggests defects in centrosome attachment to the spindle poles. Judged by fixed embryo staining, centrosomes were apparently detached from the spindle poles suggesting there was indeed a poor affinity between the centrosomes and the spindle poles (Figure 4-8L and O). Centrosomes replicate during interphase and then the two daughter centrosomes migrate to opposite sides of the nucleus at the very onset of mitosis. It has been previously demonstrated that the two centrosomes are associated with the nuclear envelope and migrate along it to assume the opposite positions before mitosis (Robinson *et al.*, 1999). In embryos overexpressing EGFP-Wee1, centrosomes were not tightly associated with the nuclear envelope. Often, one of the centrosomes appeared to be detached from the nuclear envelope (Figure 4-8H). Thus some centrosomes were detached from their correct positions even before mitotic spindles were assembled. None of these abnormal phenotypes was observed in control embryos overexpressing EGFP alone (Figure 4-8C and F).

The other defect observed in EGFP-Wee1 expressing embryos was abnormal spindle configurations. The normally uniform spacing between spindles within the syncytium was lost. Frequently, I noticed that some bipolar spindles had none or more that one centrosome at each pole (Figure 4-8L and O), likely due to detached centrosomes. The spindles with a missing centrosome often showed a tendency to collapse at the end with no centrosome. This type of spindle pole therefore appeared blunt-ended (Figure 4-8L and O). Occasionally, two neighboring spindles were fused partially together at one end (Figure 4-8O). This fusion resulted in the formation of a multipolar spindle structure. Two adjacent spindles sometimes appeared to share a centrosome at one pole, but had their own centrosomes at the other poles (Figure 4-8O). None of these phenotypes were observed in control embryos overexpressing EGFP alone (Figure 4-8 F).

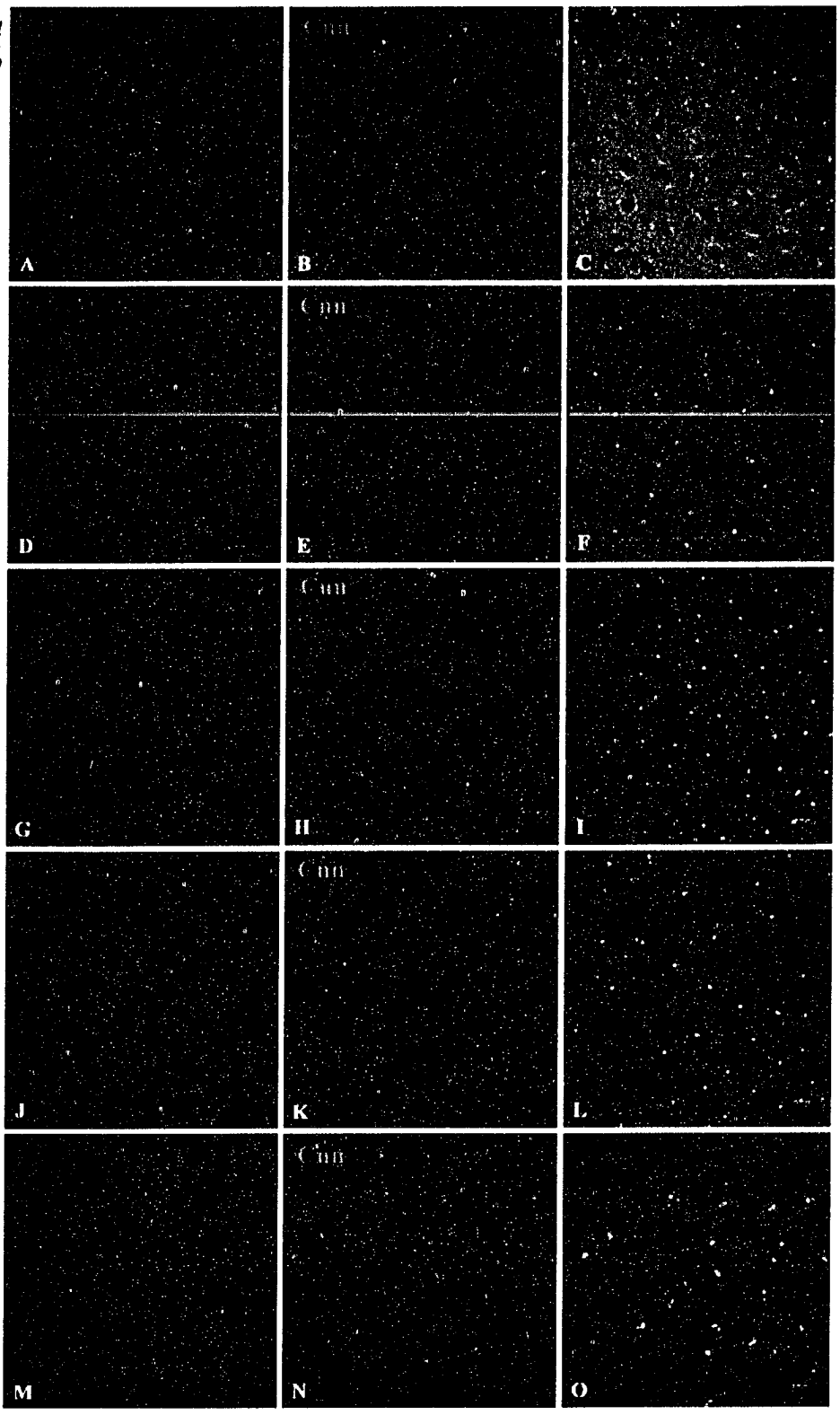

Figure 4-8 Overexpression of EGFP-Wee1 in early embryos resulted in centrosome and spindle defects. Centrosomin (Cnn, red) marks centrosomes. Tubulin (green) labels microtubules. Top two rows show embryos overexpressing EGFP alone. (A) Microtubules in an embryo overexpressing EGFP at late interphase. (B) Centrosomes attaching to the nuclear envelope and well separated. (C) Merged image. (D) Mitotic apparatus in an embryo overexpressing EGFP. Spindles are evenly spread out along the cortex. Spindle morphology is normal. (E) Centrosomes. (F) The overlay of (D) and (E). Note that centrosomes are attached to the bipolar spindle poles. (G) Microtubules in an embryo overexpressing EGFP-Wee1 in interphase. They lost the even distribution. (H) Centrosomes detaching from the nuclear envelope in interphase. The detached centrosomes reside in the cytoplasm. As a result, free centrosomes are often seen. (J and M) Mitotic apparatus in embryos overexpressing EGFP-Wee1. Spindles are unevenly spread out along the cortex. Spindle morphology is abnormal. The following abnormalities were observed: blunt-ended spindle poles, multipolar spindle poles and fused adjacent spindles. (K and N) Centrosomes during mitosis. They are unevenly arranged. Some of them are fused together. (L and O) Overlay of J and K, and M and N. Note that the centrosomes are detached from the spindle poles. Many free centrosomes are in the cytoplasm.

b) Pseudo-cleavage furrow morphology was severely affected

Besides the defects in centrosomes and mitotic spindles, another striking cytoplasmic defect observed involved the pseudo-cleavage furrows. Pseudo-cleavage furrows are actin-rich structures during the late syncytial divisions, which undergo dynamic changes analogous to cytokinesis in a typical cell cycle. During syncytial divisions, the nuclei are not separated by cell membranes (the entire embryo is surrounded by a continuous cell membrane). Although individual cell membranes do not form at the end of mitosis, individual pseudo-cleavage furrows that transiently surround each nucleus are formed, similar to cell membranes exist and cleavage furrows form to mediate cytokinesis. In addition to actin, other proteins have been identified that are associated with pseudo-cleavage furrows, such as Peanut and Annilin (Neufeld and Rubin, 1994; Fares *et al.*, 1995; Field *et al.*, 2005). Previous studies in our lab revealed that pY15-Cdk1 is also localized to the pseudo-cleavage furrows in wild type embryos, however it required tyramide amplification to be observed. This signal becomes much stronger during cycle 14 as cellularization occurs, implying that pY15-Cdk1 also localizes onto the cleavage furrows (E. Homola, personal communication).

In control embryos overexpressing EGFP alone, pseudo-cleavage furrows stained with anti-pY15 Cdk1 were thin-layered structures surrounding each individual nucleus and all the furrows were of similar sizes and evenly spread out along the cortex (Figure 4-9A). In embryos overexpressing EGFP-Wee1, however, furrow staining with the anti-pY15 Cdk1 antibody before cellularization was much stronger than what was seen in the controls and the pseudo-cleavage furrows appeared malformed (Figure 4-9B). These furrows were thickened and many were arranged in small clusters that did not contain nuclei, suggesting that the nuclear division and pseudo-cleavage division cycles were uncoupled in these embryos. These observations are consistent with the expectation that the levels of Y15 phosphorylation would be increased by Wee1 overexpression as well as the biochemical assays shown earlier that increased levels of Y15 phosphorylation were detected in western blotting experiments. The accumulation of the inhibited isoform of Cdk1 on defective pseudocleavage furrows suggests a potential role of Cdk1 in the regulation of the actin cytoskeleton.

Collectively I demonstrated that overexpression of EGFP-Wee1 in syncytial embryos caused a variety of defects in nuclear and cytoplasmic events, consistent with Wee1 being a key regulator of Cdk1, which has many substrates affecting a variety of cell cycle regulated processes.

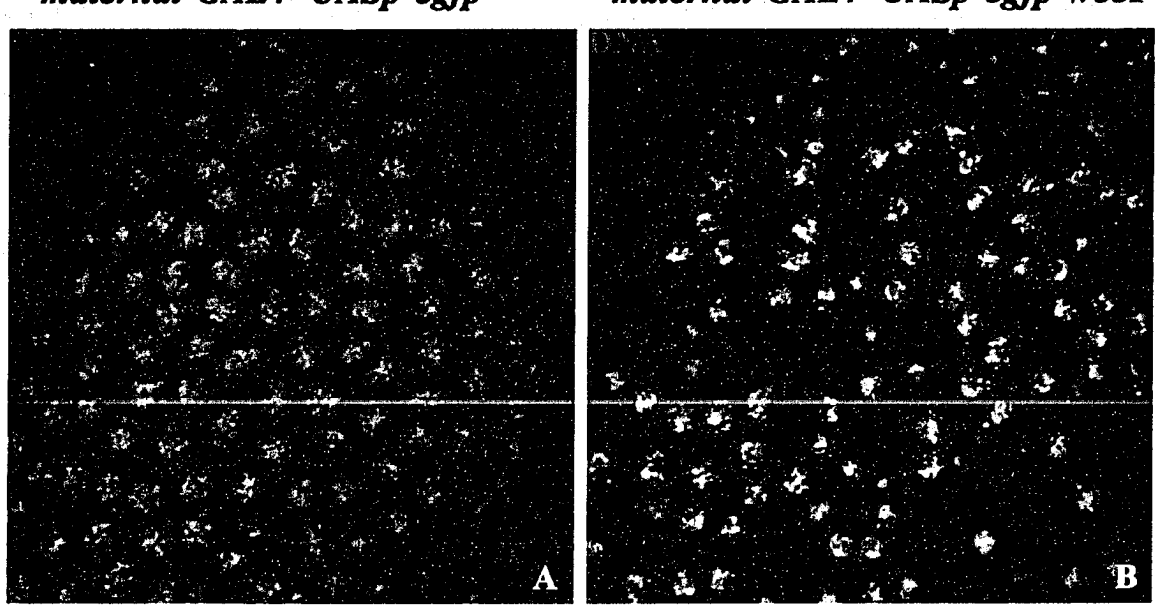

Figure 4-9 Pseudo-cleavage furrow morphology was abnormal in embryos overexpressing EGFP-Wee1. phospho-tyr 15 Cdk1 (green) labels the pseudo-cleavage furrows during late syncytial cycles. DNA in red. (A) Normal pseudo-cleavage furrow pattern in an embryo overexpressing EGFP alone (same as in wild type). The pseudo-cleavage furrow is a thin layer of cytoskeletal network that surrounds each nucleus during cycle 11 to cycle 13. Each furrow has an irregular shape and is contacting other furrows. (B) Abnormal morphology of the pseudo-cleavage furrows in an embryo overexpressing EGFP-Wee1. Note that the furrows are thickened and arranged in clusters leaving gaps between clusters. Nuclei are not residing inside many of the furrows, suggesting a loss of coordination between nuclear division and cytoplasmic division cycles.

4.4 Discussion

4.4.1. The EGFP tagged Wee1 fusion proteins are functional *in vivo*

Genetic rescue experiments showed that mild overexpression of EGFP-Wee1 by maternal heat shock could partially rescue the *wee1* mutant maternal embryonic lethal phenotype. This result is consistent with a previously published result where overexpression of an untagged Wee1 transgene with heat shock also partially rescued the *wee1* mutant phenotype (Price *et al.*, 2000). Supporting this conclusion from the rescue experiments, biochemical assays demonstrated that Y15 phosphorylation of Cdk1 was elevated as a result of EGFP-Wee1 overexpression, as expected. Taken together, these results demonstrated that the EGFP-Wee1 fusion protein is functional *in vivo* and suggest that subsequent localization studies and phenotypic analysis associated with EGFP-Wee1 overexpression are meaningful in that they are associated with abnormal regulation of Cdk1.

4.4.2. Dynamics of tagged Wee1 localization

Wee1 proteins exhibit nuclear localization during interphase in many organisms, including *S. pombe*, *Xenopus* and humans (Heald *et al.*, 1993; Mitra and Schultz, 1996; Wu *et al.*, 1996; Nakanishi *et al.*, 2000). In *S. cerevisiae* and humans, Wee1 was also detected on cytoskeletal structures during mitosis: the budding neck (in *S. cerevisiae,* Sakchaisri *et al.*, 2004) and the cleavage furrows (in humans; Baldin and Ducommun, 1995). In *Drosophila*, antibodies directed against Wee1 show that Wee1 has a nuclear localization during interphase, consistent with observations in other systems (E. Homola, personal communication). Due to the low reactivity of the antibody to the endogenous Wee1 protein (or otherwise low endogenous Wee1 protein levels), however, it was technically challenging to resolve the details of Wee1 localization in various cell types at different cell cycle stages. Using epitope tagged Wee1 fusion proteins greatly facilitated the visualization of Wee1 proteins and has revealed that indeed, EGFP-Wee1 showed a dynamic localization pattern in different cells.

Overexpression under the control of *engrailed-GAL4* showed that tagged Wee1 localized within the nucleus of interphase cells as expected, during late embryogenesis when the body segments are forming. However, *embryonic neuron-GAL4* controlled overexpression of EGFP-Wee1 resulted in accumulation of the protein in the central commissure of the embryonic nervous system, implying that the protein is cytoplasmic in these cells. We are not sure yet why EGFP-Wee1 was apparently stabilized in the axons or whether this effect may be merely an artifact of overexpression. If EGFP-Wee1 protein is stabilized in the axoplasm, this subcellular localization may be connected to a possible neural function for Wee1, as discussed in Chapter 2. Defects in locomotor activity in older *wee1* mutants suggested a role for Wee1 in the nervous system that may or may not involve Cdk1. One alternative candidate substrate for Wee1 would be Cdk5, which was also discussed in Chapter 2. Given that overexpression of Wee1 led to many defects in cytoskeletal structures in embryos, it is also possible that endogenous Wee1 could be involved in regulating the cytoskeleton in neurons. I did not examine whether abnormal phenotypes were associated with overexpression of EGFP-Wee1 in the embryonic neurons. The possible neural function of Wee1 is worth pursuing in future studies. I recognize the possibility that this neural pattern may just be an overexpression artifact

and endogenous Wee1 may not have the same expression pattern. This can be examined if better Wee1 antibodies are obtained in the future.

maternal tubulin-GAL4 overexpression of EGFP-Wee1 in syncytial embryos was associated with strong EGFP-Wee1 signals in the interphase nuclei, consistent with previous reports of Wee1 in other systems (Heald *et al.*, 1993; Mitra and Schultz, 1996; Wu *et al.*, 1996; Nakanishi *et al.*, 2000), and supporting the idea that the fusion proteins were localizing properly. The fact that EGFP-Wee1 signal reappeared at the end of mitosis suggested that the majority of EGFP-Wee1 was not degraded during mitosis, in contrast to previous reports in *Xenopus* and human cells, which suggest that Wee1 is degraded during mitosis (Mueller *et al.*, 1995; Ayad *et al.*, 2003). Because it takes newly synthesized EGFP approximately an hour to show fluorescence and mitosis during syncytial stages is roughly 5 minutes long (Foe and Alberts, 1983; Tsien, 1998), the EGFP-Wee1 signal I observed could not be due to newly synthesized proteins. Syncytial cycles are so rapid that there may not be enough time to synthesize key cell cycle regulators during each cycle. Instead, syncytial embryos may utilize other mechanisms to regulate the activities of those proteins, such as protein phosphorylation and protein translocation. It is reasonable to speculate that the dispersal of EGFP-Wee1 upon entry into mitosis observed in live analysis might be an effective way to downregulate Wee1 activities, thus decreasing its chance to phosphorylate and thus inactivate Cdk1 during mitosis. Given that overexpression may cause artifacts, I recognize the caveat to this idea: there was such a large amount of EGFP-Wee1 proteins that not all of them could be degraded in each cycle. However, preliminary results suggest that some endogenous Wee1 proteins are indeed localized in the cytoplasm during mitosis as detected by Wee1 antibody staining (E. Homola, personal communication). To answer the question whether Wee1 is degraded during syncytial embryogenesis, we can look at Wee1 protein levels during interphase and mitosis by western blots when we have a better antibody. An alternative solution is to establish a system to visualize the endogenous Wee1 protein. For example, construct an *egfp-wee1* transgene with endogenous *wee1* promoter, as it would allow EGFP-Wee1 protein levels to be kept closer to the endogenous levels.

Live fluorescent microscopy analysis also revealed an apparent transient colocalization of EGFP-Wee1 with mitotic spindles and chromosomes during mitosis (E. Homola, personal communication). Wee1 has been demonstrated to localize to cytoskeletal structures during mitosis, such as the budding neck in *S. cerevisiae* (Sakchaisri *et al.*, 2004) and the cleavage furrows in humans (Baldin and Ducommun, 1995). However, evidence for colocalization with mitotic spindles was not reported in those systems. EGFP-Wee1 may localize to the mitotic spindles to regulate local Cdk1 activity, consistent with recent reports that Cdk1 regulates spindle assembly (Jaspersen *et al.*, 2004; Mishima *et al.*, 2004). E. Homola also found that Cdk1 Y15 levels are much lower, but still detectable, in mitotic embryos (E. Homola, personal communication). Collectively, these results suggest the possibility that a pool of Cdk1 on the spindles could be inhibited during mitosis, even when the majority of Cdk1 in the embryo is active.

EGFP-Wee1 localization in ovaries was very different from that observed in embryos. *follicle cell-GAL4* driven overexpression of EGFP-Wee1 resulted in accumulation in the cytoplasm of the endoreplicating follicle cells after stage 12 of oogenesis. Endocycles consist of alternating S phases and G phases, in the absence of

mitosis. Previous evidence in human cells and *Drosophila* indicated that endocycles occur concurrently with the downregulation of Cdk1 activity, either by Cyclin B degradation or by inhibitory phosphorylation on Cdk1 (Garcia and Cales, 1996; Vidwans *et al.*, 2002; Kramer *et al.*, 2004). If endogenous Wee1 behaves similarly, the cytoplasmic localization of EGFP-Wee1 seems to be incompatible with inhibition of nuclear Cdk1, unless Cdk1 cycles between the nucleus and cytoplasm. Down regulation of Cdk1 in follicle cells may normally be carried out by Cyclin B degradation or degrading Cdc25, since follicle cell defects are not normally seen in *wee1* mutant embryos.

To summarize the above, different drivers drove overexpression of tagged Wee1 in different subcellular localizations in different cell types, suggesting that the fusion proteins localize in a context-specific manner. The dynamic localization of EGFP-Wee1 in syncytial embryos implies that localization of Wee1 is a cell cycle dependent function. Another interesting result of these studies is that the apparent colocalization of EGFP-Wee1 with mitotic spindles and chromosomes indicates a possible role for Wee1 in specifically inhibiting localized Cdk1 activities during mitosis.

4.4.3. EGFP-Wee1 overexpression was associated with severe cell cycle defects in syncytial embryos

Because Cdk1 has been implicated in many different aspects of cell cycle regulation, including nuclear envelope breakdown, chromosome condensation and spindle assembly (Lehner and O'Farrell, 1990), I expected a wide range of defects associated with misregulated Cdk1 as a result of EGFP-Wee1 overexpression. In fact, overexpression of EGFP-Wee1 did cause major defects in DNA condensation and spindle assembly, in addition to defects in other nuclear and cytoplasmic processes.

a) EGFP-Wee1 overexpression led to DNA hypercondensation in interphase and a loss of synchrony in syncytial cycles

In the presence of excessive EGFP-Wee1, interphase DNA appeared to be abnormally condensed. This phenotype is difficult to explain based on established roles of Wee1 and Cdk1. Active Cdk1 has been shown to promote DNA condensation at the onset of mitosis (Lehner and O'Farrell, 1990), therefore inhibition of Cdk1 activity by overexpression of EGFP-Wee1 would be expected to delay or prevent DNA condensation. One possible interpretation of this result is that if the nuclei exit mitosis prematurely with condensed DNA due to the presence of inhibited Cdk1, in the next interphase the DNA will still be condensed. Other abnormal DNA morphology defects were also observed. For example, variable sized nuclei seem to suggest that the cell cycles in these embryos are so severely disrupted that such DNA defects may well be a result of several interacting factors.

In wild type embryos, the syncytial cycles are strictly regulated and synchronized. The communication between nuclei is likely critical in establishing the synchrony of these cell cycles. Misregulation of Cdk1 could disrupt this communication, leading to a loss of cell cycle synchrony and explaining the loss of synchrony observed in embryos overexpressing EGFP-Wee1.

b) Cdk1 may regulate pseudo-cleavage furrow function

Pseudo-cleavage furrows are cytoskeletal structures that play an important role during the syncytial divisions, in a process analogous to cytokinesis. In embryos overexpressing EGFP-Wee1, pseudo-cleavage furrow morphology was strikingly abnormal. Staining of pY15-Cdk1 showed that phospho-tyrosine 15-Cdk1 strongly localized to the furrows during syncytial cycles in embryos overexpressing EGFP-Wee1. In controls, the same staining with anti-pY15-Cdk1 antibody only showed a weak signal on the furrows at the same stage. This localization of pY15-Cdk1 suggests that a pool of inhibited Cdk1 resides on the pseudo-cleavage furrows, which may be activated later to regulate the furrow function or to perform other roles during the cell cycle. It is already known that Cdk1 is involved in the regulation of cytokinesis in different systems (Litvak *et al.*, 2004; Wolfe and Gould, 2005). However, the exact mechanism is not well understood. There have not been any previous reports of Cdk1 regulating the pseudo-cleavage furrows in *Drosophila*, therefore my results are novel and implicate Cdk1 in serving a conserved role in regulating the actin cytoskeleton in fly embryos.

Another phenotype is that pseudo-cleavage furrows were apparently disassociated from nuclei in embryos overexpressing EGFP-Wee1, suggesting that nuclear divisions and furrow divisions were uncoupled. Under normal conditions, nuclear division and cytoplasmic division are tightly regulated, so that they occur in proper sequence. Regulation of these two processes is at least partially carried out by Cdk1, because loss of Cdk1 has been demonstrated to lead to defects in both (Satterwhite *et al.*, 1992; Ohi and Gould, 1999; Litvak *et al.*, 2004; Morris and Jalinot, 2005). There may be other players as well. For example, APC (anaphase promoting factor) complexes promote both the segregation of chromosomes and cytokinesis (Irniger, 2002). The fact that these two processes can be uncoupled demonstrated that they each have their own unique regulatory mechanisms, in addition to shared pathways. These considerations may explain why overexpression of Wee1 and misregulated Cdk1 could give rise to defects in both nuclear division and cytoplasmic division.

c) Defects in nuclear migration, centrosome attachment to the spindle poles and spindle morphology caused by EGFP-Wee1 overexpression

Other cytoplasmic defects caused by EGFP-Wee1 overexpression included abnormal nuclear migration, centrosomes detaching from mitotic spindles and abnormal spindle morphology. In wild type embryos before syncytial cycle 7, the nuclei are still embedded inside the embryo. At cycle 7 to 9, they migrate to the cortex of the embryo, a process mediated by microtubule-dependent motor proteins (Foe *et al.*, 1993). In embryos overexpressing EGFP-Wee1, the nuclei lost their normal uniform spacing. Instead, they tended to cluster, leaving areas on the cortex free of nuclei. Live analysis also revealed abnormal movement of the nuclei. These phenotypes suggested that there is a nuclear migration defect associated with EGFP-Wee1 overexpression. Excessive EGFP-Wee1 could disrupt the signaling pathway that triggers nuclear migration. Alternatively, it might affect the microtubules and motor proteins involved in coordinating the migration process. Misregulated Cdk1 could well affect microtubule-dependent motor proteins involved in this process, since Cdk1 has been previously reported to regulate the dynein intermediate chain (Addinall *et al.*, 2001), a component of a microtubule-dependent motor.

Overexpression of EGFP-Wee1 also caused centrosome and spindle defects. Centrosomes lost their proper positioning at the spindle poles, as well as along the nuclear envelop. Mitotic spindle morphology was also disrupted, perhaps as a result of the defects in centrosome attachment. The spindle poles with missing centrosomes tended to collapse. Occasionally two bipolar spindles from two adjacent nuclei appeared to be fused together, generating multipolar spindles. This range of phenotypes strongly mimics the phenotypes of mutants for some microtubule-dependent motor proteins, such as *Dhc64C* (dynein heavy chain) mutants (Robinson *et al.*, 1999). Dynein heavy chain is a minus-end microtubule directed motor protein and a component of the Dynein-Dynactin complexes that are involved in diverse intracellular transport processes (Robinson *et al.*, 1999). Loss of *Dhc64c* affects centrosome migration along the nuclear envelope during interphase and centrosome attachment to the spindle poles during mitosis (Robinson *et al.*, 1999).

It is thought that Dynein as well as other motor proteins like KLP61F (kinesin-like protein) and Ncd (non-claret disjunctional, another kinesin protein) regulate centrosome positioning (Endow *et al.*, 1994; Nigg *et al.*, 1996; Sharp *et al.*, 1999 and 2000; Debec *et al.*, 2001). KLP61F is a plus-end directed microtubule directed motor; whereas Ncd and Dynein are minus-end microtubule directed motors. They exert opposite forces to keep centrosomes at the right position, when the forces are in balance. During late interphase and early prophase, one of two centrosomes migrates along the nuclear envelope to assume opposite positions. The migration is achieved through Dynein exerting a force that pulls the centrosome along the nuclear actin network. Dynactin is the regulatory unit associating Dynein possibly with the nuclear actin network, instead of cytoplasmic actin network, because there is no individual cell membrane in syncytial cycles. Ncd exerts an opposite force to balance the force generated by Dynein (Figure 4-10A). During prometaphase/metaphase, centrosomes need to reposition themselves because spindles are being assembled in between two centrosomes. In this process, Dynein and KLP61F exert outward forces, whereas Ncd exerts an inward force. It is the balance of these forces that determines the correct positioning of the centrosomes (Figure 4-10B; Sharp *et al.*, 2000).

Centrosome attachment to the spindle poles is also important for spindle morphogenesis. Without centrosomes attached, spindles collapse or adjacent spindles sometimes fuse. Infrequently, multipolar spindles form, presumably due to presence of free centrosomes in the cytoplasm, which are capable of initiating spindle assembly.

The fact that overexpression of tagged Wee1 phenocopies the loss of *dynein heavy chain* suggests Wee1 might also be involved in regulating centrosome positioning. The overexpression phenotype is consistent with a role for Wee1 as either as a negative regulator of the force pulling centrosomes towards the spindle poles or as an enhancer of the forces pushing centrosomes away. Overexpression of Wee1 may cause a loss of balance in the forces exerted by antagonizing motor proteins, resulting in the forces pushing centrosomes outward being greater, with enhanced Dynein function or KLP61F function. This could conceivably cause centrosomes to detach from the spindle poles. Such a function of Wee1 could be mediated through the regulation of Cdk1, since Cdk1 regulates motor protein dynein intermediate chain (Addinall *et al.*, 2001).

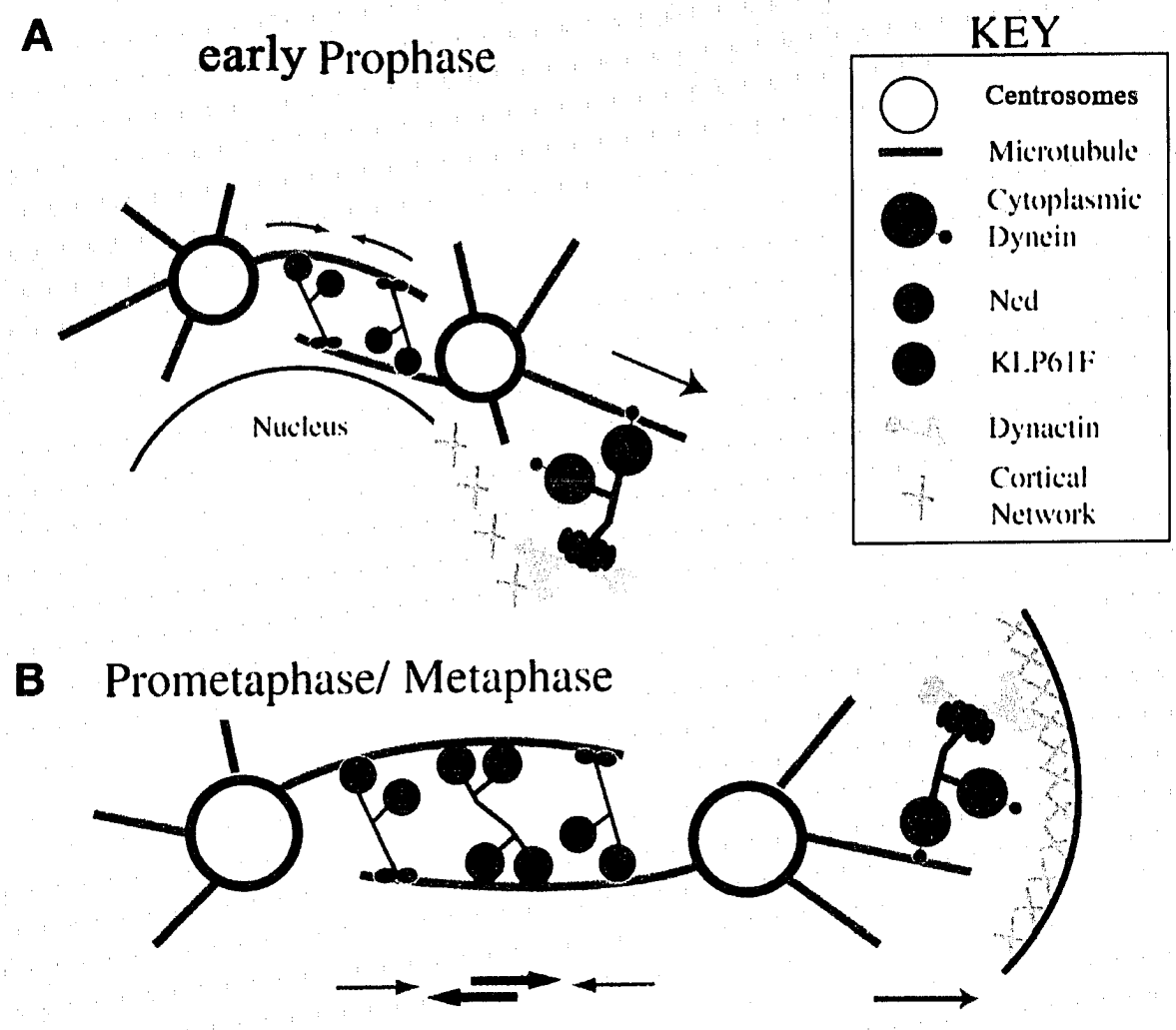

Figure 4-10 Roles of microtubule-dependent motor proteins in the regulation of centrosome positioning during syncytial divisions. Adapted from Sharp *et al.*, 2000. The color code is at the right upper corner of the figure. (A) During late interphase and early prophase, one of two centrosomes migrates along the nuclear envelope to assume opposite positions. The migration is achieved through Dynein exerting forces that pull the centrosome along the nuclear actin network. Dynactin is the regulatory unit associating Dynein possibly with the nuclear actin network instead of cytoplasmic actin network, because there is no individual cell membrane in syncytial cycles. Ncd exerts opposite forces to balance the forces generated by Dynein. (B) During prometaphase/metaphase, centrosomes need to reposition themselves because spindles are being assembled in between two centrosomes. In this process, Dynein and KLP61F exert outward forces, whereas Ncd exerts an inward force. It is the balance of these forces that determines the correct positioning of the centrosomes.

d) Cdk1 T14 phosphorylation levels were modified by overexpression of EGFP-Wee1

Western blots demonstrated that tyrosine 15 and threonine 14 phosphorylation levels were increased in embryos overexpressing EGFP-Wee1. The increase in Y15 phosphorylation levels was expected, but the increase in threonine 14 phosphorylation levels was not. Wee1 in higher eukaryotes has long been thought to phosphorylate the tyrosine 15 residue only, whereas Myt1 can phosphorylate both residues (Igarashi *et al.*, 1991; Honda *et al.*, 1995; Mueller *et al.*, 1995; Booher *et al.*, 1997; Nakanishi *et al.*, 2000; Okamoto *et al.*, 2002; Leise and Mueller, 2002). The current hypothesis is that Myt1 phosphorylates Cdk1 primarily on the T14 residue in the cytoplasm, but also some on the Y15 residue. Then, Cdk1 is translocated into the nucleus to be phosphorylated by Wee1 on the Y15 residue, leading to a further reduction in Cdk1 activity. My western results questioned this model, as it does not predict that T14 phosphorylation levels would be affected by EGFP-Wee1 overexpression. My western results were consistent, however, with independent results indicating that threonine 14 phosphorylation levels decreased in *wee1* mutants (E. Homola, personal communication).

One explanation for these results would be that *Drosophila* Wee1 could phosphorylate the T14 residue on Cdk1 in embryos. It was previously demonstrated in our lab that in a *myt1* mutant testis, Cdk1 threonine 14 phosphorylation is completely lost, implying that in this tissue Myt1 is solely responsible for T14 phosphorylation (Jin, 2005). If Myt1 is also solely responsible for T14 phosphorylation in embryos, then an alternative explanation for these results would be that Wee1 affects the phosphorylation of the Cdk1 threonine 14 residue indirectly. It is unlikely that Wee1 would do this by directly regulating Myt1, given the different subcellular localizations of the two proteins. More likely, these results may reflect a preferential order of phosphorylation events. For example, Cdk1-Cyclin B complexes may be transported into the nucleus and be phosphorylated on Y15 by Wee1 first. This event could result in Cdk1 translocating into the cytoplasm where it would be further phosphorylated on T14 (and perhaps also on some Y15) by Myt1 (Figure 4-11). According to this hypothesis, Cdk1 tyrosine 15 phosphorylation by Wee1 could increase the accessibility of Cdk1 complexes to Myt1. For example, the tyrosine 15 phosphorylation may alter the ability of Cdk1 to interact with chaperones needed for transport out of the nucleus. Alternatively, Wee1 phosphorylation of Cdk1 may induce a conformational change that facilitates Myt1 phosphorylation of Cdk1 on the T14 residue. A third possibility is that phospho-T14-Cdk1 is not stable by itself, without the Y15 phosphorylated. All these possibilities can explain why the T14 phosphorylation levels are altered when altering the amount of Wee1 in the embryos, despite of the assumption that Wee1 does not phosphorylate T14. Such mechanisms would provide a means for generating Cdk1 with different activity levels, which could be important for regulating specific cell cycle events.

To summarize the above discussion, the EGFP-Wee1 fusion proteins constructed are functional by two criteria: rescue of a *wee1* mutant phenotype and Cdk1 inhibitory kinase activity. Furthermore, the fusion proteins showed different subcellular localizations when overexpressed in different cell types. A particularly dynamic localization pattern was observed in early embryogenesis, consistent with the observed dynamic localization of Wee1 described for other organisms (Baldin and Ducommun, 1995; Lee *et al.*, 2001; Sakchaisri *et al.*, 2004). Transient colocalization of EGFP-Wee1

with mitotic spindles and chromosomes during the syncytial mitoses also suggests that Wee1 could serve novel roles in regulating specific subcellular Cdk1 pools. Misregulation of Cdk1 by EGFP-Wee1 overexpression affected multiple aspects during syncytial cycles, such as DNA condensation, synchrony in divisions, nuclear migration, centrosome attachment, spindle morphogenesis and pseudo-cleavage furrow function. The observed defects in pseudo-cleavage furrows are consistent with the idea that spatial regulation of Cdk1 might be important for regulating actin cytoskeleton.

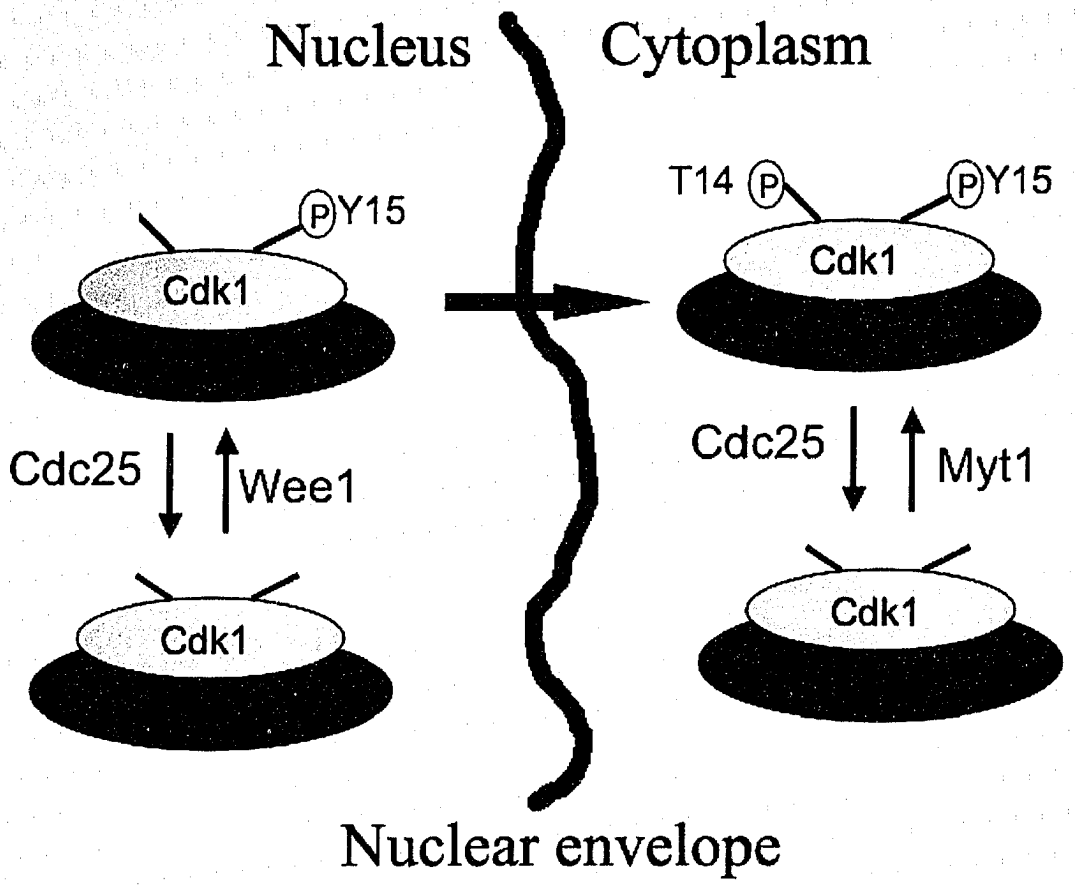

Figure 4-11 An alternative hypothesis on the phosphorylation regulation of Cdk1 by Wee1-like kinases. After Cdk1-Cyclin B complexes form in the cytoplasm, they may be transported into the nucleus and be phosphorylated on Y15 by Wee1 first. This event could result in Cdk1 translocating into the cytoplasm where it would be further phosphorylated on T14 (and perhaps also on some Y15) by Myt1. According to this hypothesis, Cdk1 tyrosine 15 phosphorylation by Wee1 could increase the accessibility of Cdk1 complexes to Myt1. For example, the tyrosine 15 phosphorylation may alter the ability of Cdk1 to interact with chaperones needed for transport out of the nucleus. Alternatively, Wee1 phosphorylation of Cdk1 may induce a conformational change that facilitates Myt1 phosphorylation of Cdk1 on the T14 residue.

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Chapter 5
Overexpression analysis of Wee1 and Myt1 during oogenesis

5.1 Introduction

Each female *Drosophila* has two ovaries that consist of a number of ovarioles. One ovariole is made of a string of orderly connected egg chambers, with the germarium at the anterior end. New egg chambers bud off the germarium as they mature, giving rise to a chain of egg chambers, with the more mature ones always being located at the posterior end of the ovariole. Each egg chamber is comprised of 15 nurse cells and one oocyte, all of which originated from a cystoblast derived from a germline stem cell. These cells are eclosed by a layer of somatic follicle cells. Nurse cells are the germline cells that provide nutrients to the oocytes. The oocytes are always located at the posterior end of each egg chamber (Figure 5-1; Spradling, 1993).

During *Drosophila* oogenesis, cells undergo a remarkable sequence of different types of cell cycles, in a temporally controlled manner. First of all, a germline stem cell divides asymmetrically to generate a cell called a cystoblast and regenerate a stem cell. The cystoblast then undergoes four rounds of mitoses to generate 16 cystocytes. All of the cystocyte divisions during this process are synchronous. Initially, several of the 16 cystocytes initiate early meiotic events, such as the formation of Synaptonemal Complex. However, only one of these cells becomes the oocyte and completes meiosis, whereas the remaining 15 cells in the cyst become nurse cells. Once their cell fate has been determined, nurse cells initiate endoreplication and begin producing mRNAs that will be supplied to nourish the oocyte. All the cell cycles discussed above occur in the germarium, a structure that is at the anterior tip of an ovariole. The germarium can be divided into three regions, with regard to cell cycle progression. Region one is located at the very anterior end of the germarium and consists of successive stages of cysts (cysts after the first, second, third and fourth divisions). Region two and region three are located at the posterior end of the germarium and occupied by the sixteen-cystocyte cysts (Figure 5-1). Meiosis initiates in region two, but by region three the oocyte/nurse cell fate is determined and nurse cells are undergoing the transition from mitotic cell cycles to endoreplication cycles. The oocyte has progressed to meiosis I prophase by the time it reaches this region, where it remains arrested until the end of oogenesis (Spradling, 1993).

There are several unique features about the germline divisions during *Drosophila* oogenesis. First of all, cystoblasts undergo exactly four rounds of mitoses to generate 16 cystocytes. Cystocytes divide synchronously in order to make sure that the final number of cells corresponds to 2^4 (16). These cycles are relatively rapid and not accompanied by cell growth, so they result in reduction of cell volume. In addition, the cystocytes divide with incomplete cytokinesis, so that the cells remain interconnected by cytoskeletal structures called ring canals. Ring canals are thought to be involved in oocyte fate determination. It has been hypothesized that oocyte determinants are transferred from nurse cells into the future oocyte through the ring canals. In a 16-cystocyte cyst, there are two cells with four ring canals, two cells with three ring canals, four cells with two ring canals and eight cells with a single ring canal (Figure 5-2A). It is always one of the two cells with four ring canals that becomes the oocyte (one of the two red cells in Figure 5-2A; Spradling, 1993).

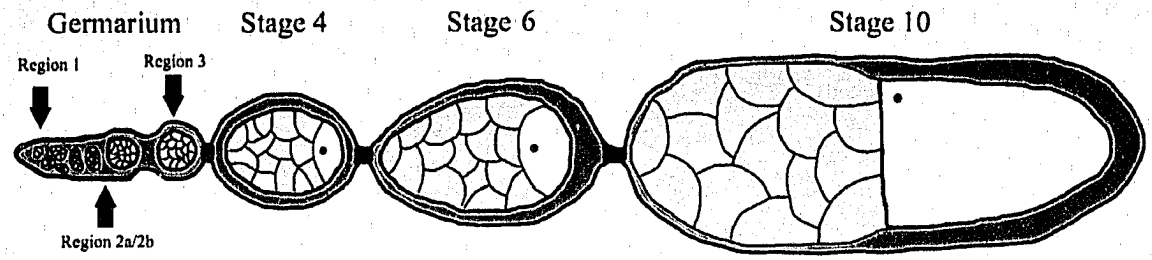

Figure 5-1 A simple schematic representation of an ovariole. A typical ovary is made of ~16 ovarioles. Adapted from Pedersen, 2005. Anterior is oriented to the left and posterior to the right. Nurse cells are shown in yellow, somatic follicle cells in blue, the oocyte in white and the oocyte nucleus in red. The germarium is always located at the very anterior tip of an ovariole, followed by successive stages of egg chambers. A germline stem cell derived cystoblast undergoes four rounds of mitoses within the germarium. Region 1 in the germarium have cysts of different number of cystocytes (2, 4, 8 and 16). Region 2a/2b and 3 only have 16-cystocyte cysts. By region 3, oocyte/nurse cell fate has been determined (15 nurse cells and 1 oocyte) and nurse cells are switching to endoreplication. As the cysts mature, somatic follicle cells form a coat around them and bud off the germarium forming egg chambers. Egg chambers mature as they proceed to the posterior end of an ovariole. The oocyte always locates at the posterior end of an egg chamber. The increasing size of the oocyte represents a nutrient transfer from the nurse cells to the oocyte.

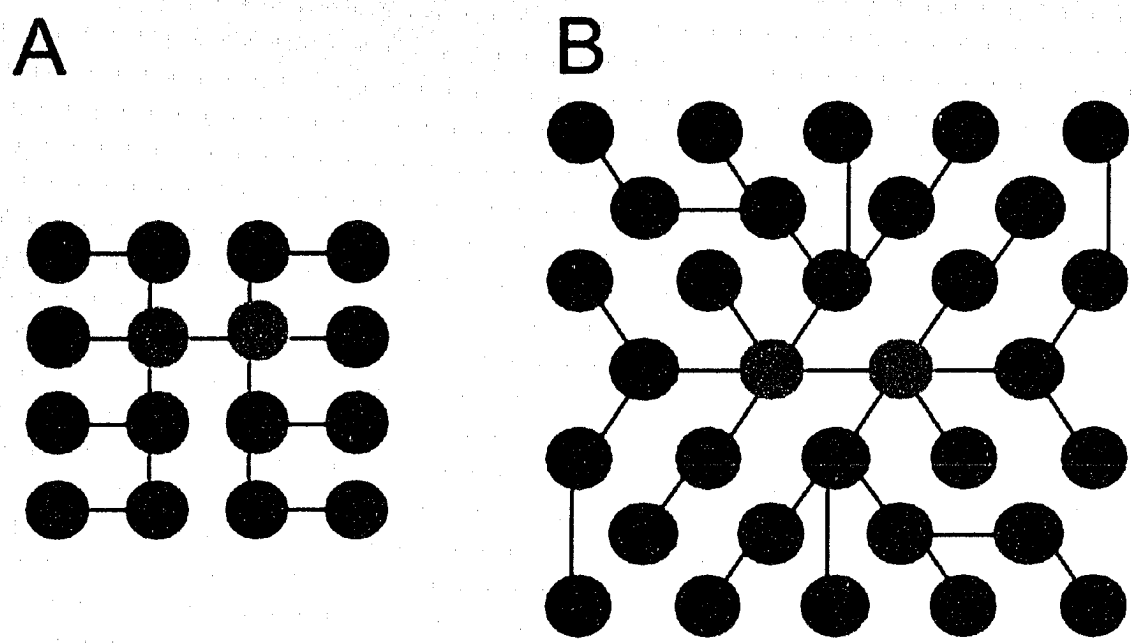

Figure 5-2 A. The formation of 15 ring canals between 16 (2^4) cystocytes in a wild type germarium. The two red circles represent the two oocyte candidates with the most number of ring canals (4 ring canals).

B. The formation of 31 ring canals between 32 (2^5) cystocytes in a germarium with one extra round of cystocyte mitosis. The two red circles represent the two oocyte candidates with the most number of ring canals (5 ring canals).

Another important feature of oogenesis is that nurse cells start to undergo endocycles after the cystocytes have completed four rounds of cell divisions. The pro-nurse cells have finished their mitotic program and initiated endoreplication cycles by region three in the germarium. During this period, the nurse cells replicate genes necessary for oocyte maturation and early embryogenesis. They will later transfer these mRNAs and proteins to the oocyte through the ring canals, after which they undergo apoptosis at stage 11 (Spradling, 1993).

The cell cycle regulators that regulate germline divisions are similar to those regulating cell cycles in other developmental stages. Modifying the dosage of critical regulators led to a disruption in the number of cystocyte divisions. For example, overexpression of Cyclin A or Cyclin B in the germlines causes one extra round of division resulting in 32 (2^5) cystocytes (Lilly *et al.*, 2000). It was thought that excessive amount of Cyclin A or Cyclin B shortened G2 phase and drove one extra round of division during a given period of developmental time. Overexpression of Cyclin E however, did not show such a phenotype, perhaps because Cyclin E is a G1 cyclin (Lilly *et al.*, 1996). Loss of *cyclin E* did lead to one less round of mitosis, however, presumably due to loss of the ability to promote the G1/S transition. Overexpression of Cdc25, a mitotic activator, surprisingly, also led to one less round of cystocyte mitosis (Mata *et al.*, 2000; Johnston, 2000). Consistent with the Cdc25 overexpression results, loss of *tribbles*, which is a gene encoding for a protein that promotes Cdc25 protein degradation, led to an 8-cystocyte phenotype. Furhtermore overexpression of Tribbles caused a 32-cystocyte phenotype (Mata *et al.*, 2000; Johnston, 2000; Seher and Leptin, 2000). These results suggested that *tribbles* is involved in regulating the number of cystocyte divisions. Mutations in *encore*, which encodes for a protein involved in SCF-Ubiquitin-proteasome-dependent proteolysis result in similar phenotypes (Hawkins *et al.*, 1996; Van Buskirk *et al.*, 2000; Ohlmeyer and Schupbach, 2003). Loss of *encore* led to one extra round of mitosis in the germline, likely due to a less efficient proteolysis of certain mitotic cyclins like Cyclin A (Ohlmeyer and Schupbach, 2003). The phenotypes described above have an interesting common feature: there is either exactly one extra or one less mitotic cell cycle associated with different expression levels of those genes. Odd-numbered cysts were not observed in these situations, which implies that the synchrony of cystocyte divisions was not disrupted. Oocyte fate determination was also unaffected in the above scenarios (except in *cyclin E* mutants), suggesting that the germline division counting mechanism and oocyte determination are differentially regulated.

Oocyte determination is a strictly regulated process. The cell that becomes an oocyte is always one of the two cystocytes with four ring canals in wild type ovaries (Spradling, 1993). This process occurs within the germarium, implying that the fate determination is relatively rapid and efficient. Several genes that regulate cystocyte divisions during oogenesis are also involved in oocyte determination. For example, *cyclin E* mutants sometimes had two or three oocytes (Lilly *et al.*, 2000). On the other hand, *ovarian tumor*, *morula*, *bag of marbles*, and *small ovaries* mutants occasionally had no oocyte at all, but either too many or too few nurse cells (Storto and King, 1988; Steinhauer and Kalfayan, 1992; Wayne *et al.*, 1995; Reed and Orr-Weaver, 1997; Parisi *et al.*, 2001; Riparbelli *et al.*, 2004). These results suggest that some regulatory factors are shared between the two processes of germline divisions and oocyte determination. Once oocyte fate is determined, the oocyte will express many oocyte-specific markers,

such Orb, Vasa and Gurken. These proteins are involved in anterior-posterior and dorsal-ventral patterning in oocytes (Christerson and McKearin, 1994; Styhler *et al.*, 1998). These proteins often have very specific localization patterns in the oocyte; therefore can be used as oocyte markers in developing egg chambers. I used anti-Orb antibodies in my experiments.

As mentioned earlier, most of the genes shown to affect the mitotic cell cycle counting mechanism during oogenesis are conserved cell cycle regulators. However, *wee1* mutants show no obvious defects during oogenesis. Therefore I decided to examine whether overexpression of EGFP-Wee1 during oogenesis would generate defects in germline mitotic cycles. This experiment also gave me an opportunity to examine how Wee1 protein localized in a different tissue than embryos and to compare its effects with those of the related Cdk1 inhibitory kinase, Myt1, using available reagents. Strikingly, germline overexpression of EGFP-Wee1 caused one extra round of mitosis in cystocyte cycles, but did not disrupt oocyte determination. This study also demonstrates that the overexpression patterns of EGFP-Wee1 and EGFP-Myt1 under the same GAL4 line were different from each other, indicating that there are differences in post-transcriptional regulation of the two proteins during oogenesis.

5.2 Materials and Methods

5.2.1. Fly stocks

The strains w^{1118}; *P{GAL4::VP16-nos.UTR}MVD1* (Van Doren *et al.*, 1998) and *yw*; *P{UASp-egfp-wee1}* flies were crossed to drive overexpression of EGFP tagged Wee1 in germline cells. w^{1118}; *P{GAL4::VP16-nos.UTR}MVD1* and *w; P{UASp-egfp}* were crossed to drive overexpression of EGFP alone in germline cells as a control. w^{1118}; *P{GAL4::VP16-nos.UTR}MVD1* and *w; P{UASp-wee1}*; w^{1118}; *P{GAL4::VP16-nos.UTR}MVD1* and *w*; *P{UASp-egfp-myt1}* were also crossed to drive overexpression of Wee1 only and EGFP-Myt1 in germline cells to serve as a further control and comparison. For all crosses, F1 generation female ovaries were examined.

5.2.2. Immunofluorescence

Newly eclosed F1 females from the appropriate crosses were transferred into vials with fresh food containing yeast and sibling males for mating. After three to five days, the females were dissected and ovaries were teased apart using tungsten needles (to facilitate access of the antibodies to the tissue). The ovaries were fixed in standard fixative (1 part [PBS+0.05%NP-40+3.7% formaldehyde]: 3 parts heptane) for 20 minutes, washed in PBST (PBS + 0.1%TritonX-100) three times for 5 minutes each time, and then blocked in PBST+10%NGS (normal goat serum) for an hour at room temperature. Samples were then incubated with primary antibodies at 4°C overnight with agitation. Primary antibodies were diluted in PBST at the following concentrations: 1:500 for rabbit anti-GFP (BD Science), 1:200 for mouse anti-Orb (Developmental Studies Hybridoma Bank), 1:100 for rabbit anti-pY15 Cdk1 (Cell Signaling). The next day, samples were washed in PBST four times for 15 minutes each time, and then incubated with secondary antibodies for one hour at room temperature. The dilutions used for secondary antibodies were: 1:1,000 for anti-rabbit Alexa Fluor 488 (green signal) or 568 (red signal), 1:1,000 for anti-mouse Alexa Fluor 488 or 568 (all secondary antibodies used were from Molecular Probes). After completion of the secondary antibody

incubation, samples were washed in PBST four times for 15 minutes each time, and then stained with Hoechst 33258 (1:100 in PBST, final working concentration was 5 μg/ml) for 5 minutes. A final wash was performed to remove unbound Hoechst dye and then the samples were mounted in anti-fade mounting media (9 parts glycerol+1 part 1XPBS containing 10 mg/ml 1, 4-phenylenediamine).

5.2.3. Confocal microscopy

All samples were examined under Leica confocal microscope (Model: TCS SP2). Data were collected and compiled using Leica confocal software and Adobe Photoshop CS software.

5.3 Results

In order to test whether overexpression of EGFP-Wee1 proteins would have an effect on the germline mitosis and to compare it with EGFP-Myt1 overexpression, I used the UAS-GAL4 system to drive overexpression of the two fusion proteins in the germline cells. Nanos is a protein that is only expressed in the germline, where it is involved in pattern formation (Forbes and Lehmann, 1998). *nanos-GAL4* is a strong GAL4 line used to drive overexpression of genes of interest only in germline cells. When I examined the overexpression pattern of EGFP-Wee1 using the *nanos-GAL4* line, I found that approximately 50% of the egg chambers showed a cystocyte overproliferation phenotype. After counting the number of nurse cells in each egg chamber, I found that cystocytes underwent one extra mitotic cycle and generated 32 cystocytes, with 31 nurse cells and a single oocyte (determined by staining with anti-Orb antibodies). This observation indicated that elevated levels of Wee1 affected the number of germline divisions. In contrast, overexpression of EGFP-Myt1 did not cause such a defect.

Using the same overexpression system, I also examined the difference in localization patterns between EGFP-Wee1 and EGFP-Myt1 driven by the same *nanos-GAL4*. Surprisingly, overexpression of EGFP-Wee1 and EGFP-Myt1 showed very different expression and localization patterns in the oocyte. EGFP-Wee1 was not detected in the oocyte after stage 6, whereas EGFP-Myt1 was strongly detected in the cytoplasm of the oocyte at the same stage (Figure 5-5). This result suggests that these two proteins, although overexpressed by the same driver, were differentially regulated in the oocyte.

5.3.1. Localization patterns of EGFP-Wee1 and EGFP-Myt1 during oogenesis

To study the localization of EGFP-Wee1 in the ovaries and compare it to that of EGFP-Myt1, the *nanos-GAL4* strain was used to drive overexpression of EGFP-Wee1 and EGFP-Myt1 in the female germlines. Anti-GFP antibody staining of controls overexpressing EGFP alone detected signals in cystocytes in germarium region one and two, indicating that the driver can promote expression very early during oogenesis (Figure 5-3A). From germarium region three to oogenesis stage 5, the EGFP signal was very weak (Figure 5-3A and Figure 5-4 top panels). After stage 6, the EGFP signal reappeared very strongly in the egg chambers, both in the nurse cells and in the oocyte (Figure 5-5 top panels), consistent with previously established expression patterns for Nanos (Forbes and Lehmann, 1998). The EGFP signal could be seen in the nuclei as well as in the cytoplasm, with slightly higher levels in the nuclei (Figure 5-5 top panels, arrow

marks the oocyte nucleus, determined by its unique karyosome morphology at this stage of development).

When EGFP-Wee1 was expressed with the same *nanos-GAL4* driver, the fluorescent signal was localized in the cystocytes of germarium region one and two, then disappeared in region three (Figure 5-3B and Figure 5-4 middle panels). EGFP-Wee1 was not localized to the nucleus in cystocytes but could be detected throughout the cell. EGFP-Wee1 protein levels were below detection levels in germarium region three and stage 1 to stage 5 egg chambers (Figure 5-3B). Beginning in stage 6, EGFP-Wee1 fluorescence reappeared strongly in the nurse cells, both in the nucleus and in the cytoplasm, but was not observed in the oocyte (Figure 5-5 middle panels, arrow marks the site of the oocyte nucleus). The EGFP-Wee1 signal was much stronger in the nurse cell nuclei than in the cytoplasm and appeared to colocalize with chromosomes in the nurse cell nuclei (Figure 5-6C and D). The nurse cell DNA morphology appeared abnormal after stage 10, compared to that of the controls expressing EGFP alone. DNA in these nuclei seemed to be hypercondensed with a "wheel"-like structure (Figure 5-6C).

A different pattern of subcellular localization was observed when EGFP-Myt1 was expressed with the same GAL4 driver. Rather than being evenly distributed, EGFP-Myt1 was localized in a punctate cytoplasmic pattern (Figure 5-3C), presumably reflecting the association of Myt1 with Golgi bodies and the ER in the cytoplasm as was previously described for Myt1 in *Drosophila* and human cells (Booher *et al.*, 1997; Jin, 2005). As in the EGFP alone controls, EGFP-Myt1 was visible in germarium region one and two, but not region three (Figure 5-3C and Figure 5-4 bottom panels). EGFP-Myt1 was also not detectable from stage 1 to stage 5 (Figure 5-3C). By stage 6, EGFP-Myt1 fluorescence levels appeared high in the cytoplasm, both in the nurse cells and in the oocyte (Figure 5-5 bottom panels, arrow marks the oocyte nucleus).

The major significant difference between the overexpression patterns of these two Cdk1 inhibitory kinases was that EGFP-Wee1 was not detected in oocytes after stage 6, even though EGFP-Myt1 expressed using same GAL4 line was highly enriched in oocytes.

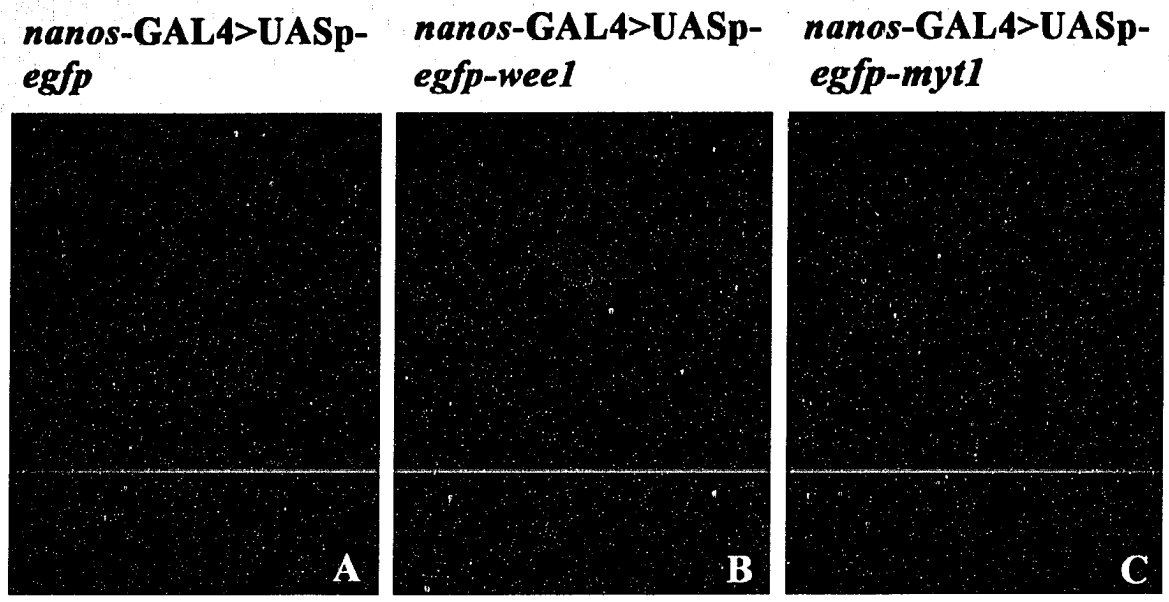

Figure 5-3: Overexpression patterns for EGFP-Wee1 and EGFP-Myt1 in ovarioles under the *nanos-GAL4* driver. DNA in blue and GFP in green. *nanos-GAL4* drove overexpression of EGFP, EGFP-Myt1 or EGFP-Wee1 in the germarium and in egg chambers after stage 6. (A) EGFP is localized both in the cytoplasm and in the nucleus. (B) EGFP-Wee1 is localized in the nucleus, but also it is detectable in the cytoplasm. (C) EGFP-Myt1 is exclusively in the cytoplasm.

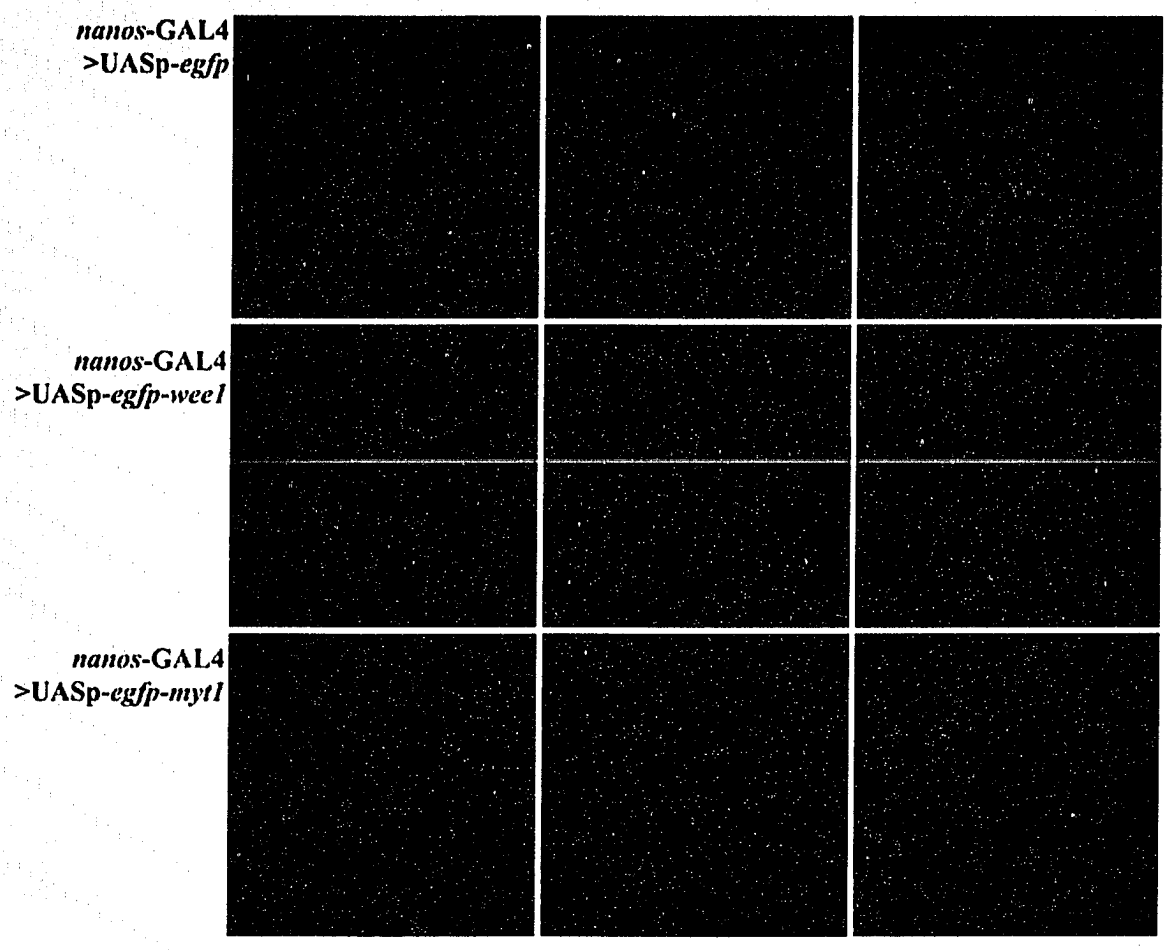

Figure 5-4: Overexpression patterns of EGFP-Wee1 and EGFP-Myt1 in the germarium under the *nanos-GAL4* driver. DNA in blue and GFP in green. The third panels in each row show the merged images of the other two panels. EGFP, EGFP-Wee1 and EGFP-Myt1 are expressed in germarium region one and two, but not region three. EGFP-Myt1 shows a cytoplasmic pattern, whereas both EGFP alone and EGFP-Wee1 show expressions in the cytoplasm and in the nucleus.

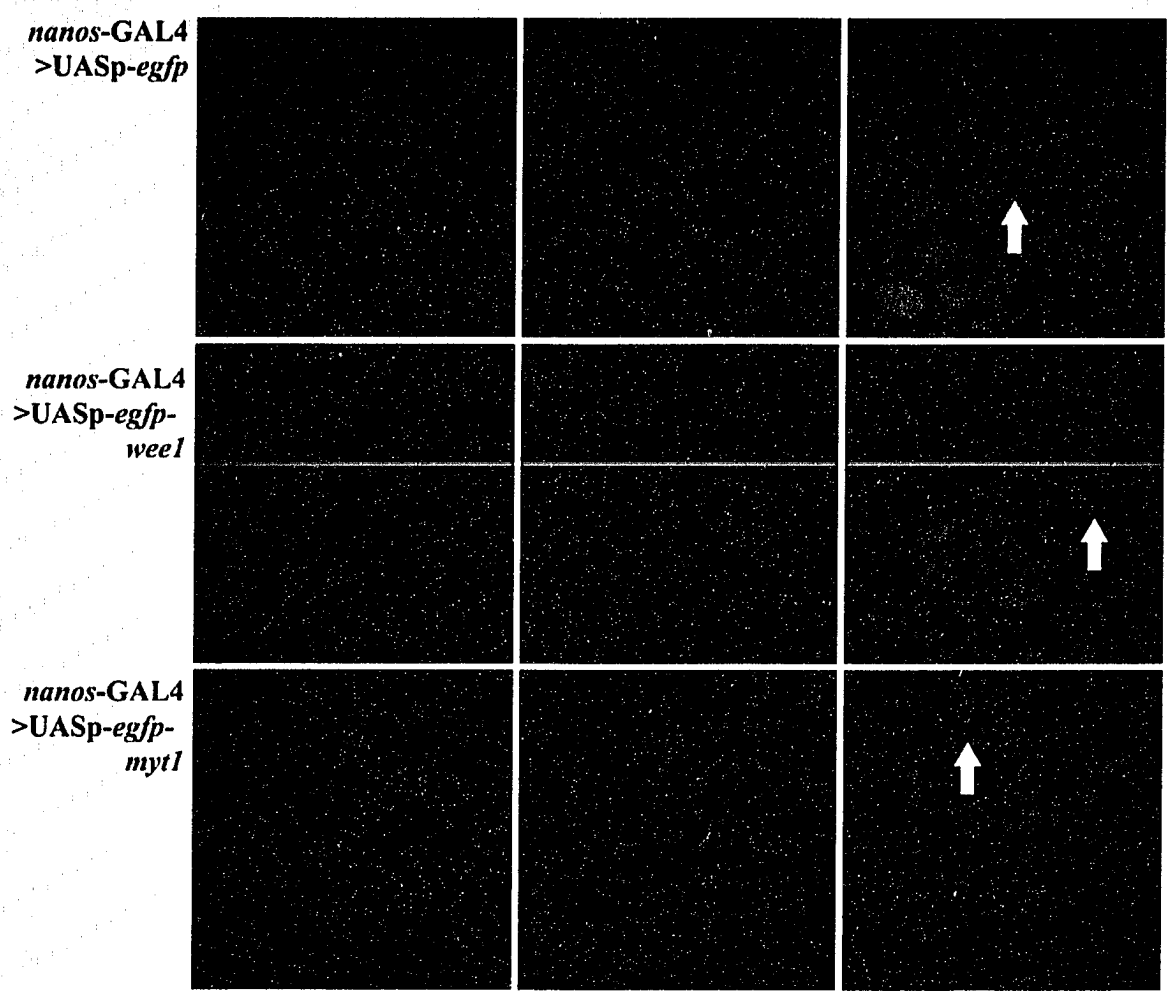

Figure 5-5: Overexpression patterns of EGFP-Wee1 and EGFP-Myt1 in the egg chambers under the *nanos-GAL4* driver at stage 6. DNA in blue and GFP in green. The third panels in each row show the merged images of the other two panels. In the top panels, EGFP is localized both in the cytoplasm and in the nucleus, with a slight preference for the nucleus. The EGFP alone signal is particularly enriched in the oocyte nucleus (arrow). In the middle panels, EGFP-Wee1 is localized to the nurse cell nuclei, and it is faintly detectable in the nurse cell cytoplasm. In the oocyte, there is no EGFP-Wee1 signal either in the nucleus or the cytoplasm (the arrow points to the oocyte nucleus). Note that the morphology of nurse cell nuclei is abnormal compared to the controls. In the bottom panels, EGFP-Myt1 is localized exclusively in the cytoplasm and strongly enriched in the oocyte cytoplasm (the arrow marks the oocyte nucleus that lacks EGFP-Myt1).

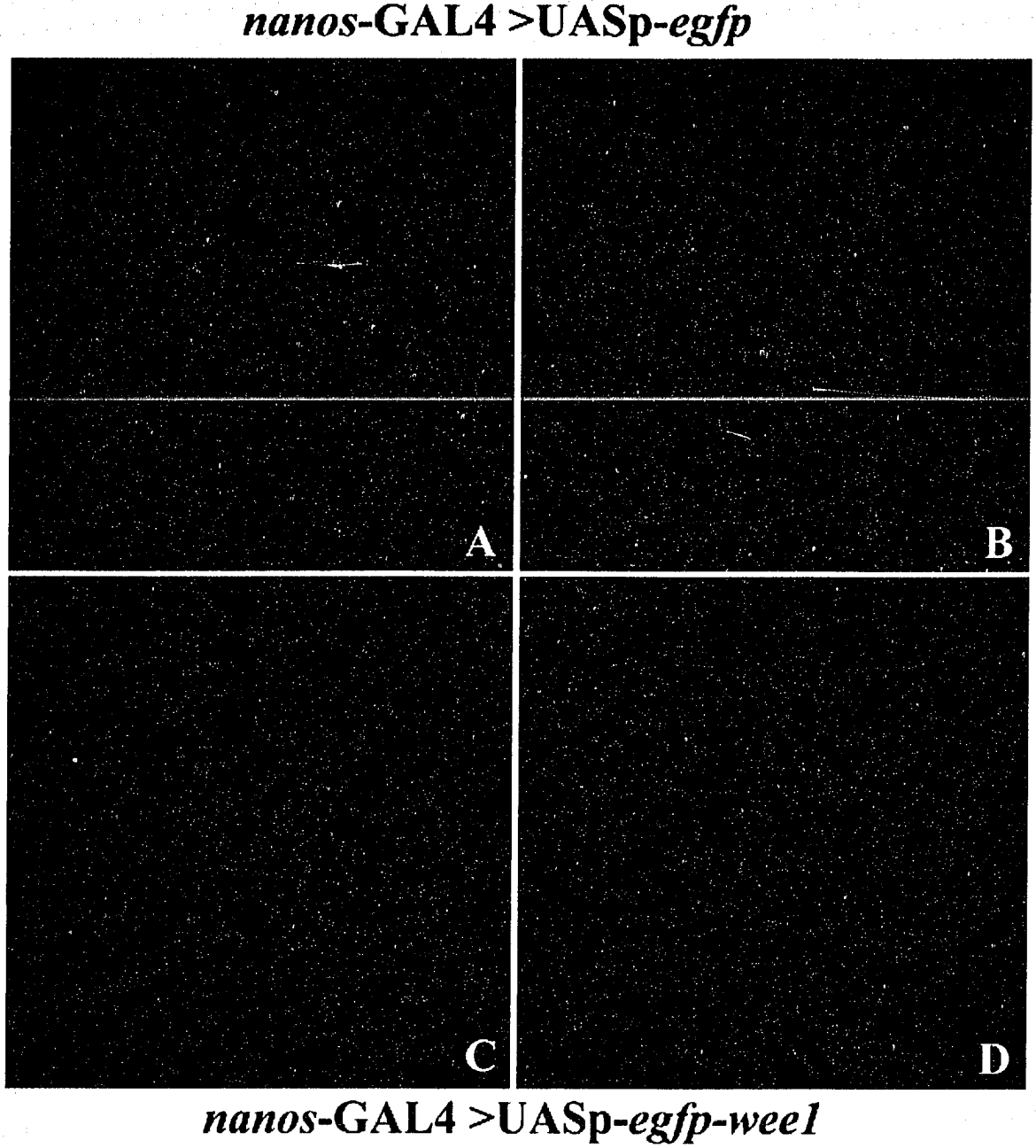

Figure 5-6 Abnormal DNA morphology of nurse cell nuclei after stage 10 caused by EGFP-Wee1 overexpression. DNA in blue and GFP in green. (A) Normal morphology of chromosomes in nurse cells in an egg chamber overexpressing EGFP alone. (B) The localization of EGFP in the nurse cells. (C) Chromosomes assume a condensed "wheel"-like morphology in an egg chamber overexpressing EGFP-Wee1. This defect can sometimes be observed in earlier egg chambers, as shown in Figure 5-5. (D) Colocalization of EGFP-Wee1 with chromosomes.

5.3.2. Overexpression of EGFP-Wee1 caused cystocyte mitosis defects, however overexpression of EGFP-Myt1 did not.

Previous studies have demonstrated that overexpression or mutation of cell cycle regulators, such as Cdc25, Cyclin A and Tribbles could affect the number of cystocyte divisions and subsequent number of germline cells in egg chambers. In order to test whether increasing the amount of Wee1 in the germline cells would also affect cystocyte divisions, Wee1 or EGFP-Wee1 was ectopically expressed using the *nanos-GAL4* driver. Excessive amounts of EGFP-Wee1 or Wee1 alone (data not shown) caused a "31+1 cell" cyst phenotype, in approximately 50% of the egg chambers (n>100, Figure 5-7B). The fact that 32-cell cysts were observed suggests that the germline stem cell derived cystoblast underwent five rounds instead of four rounds of divisions, giving rise to 32 cystocytes instead of 16. Antibody staining for the oocyte marker Orb showed that there was only one oocyte in the egg chambers containing 31 nurse cells (Figure 5-8B). In egg chambers with additional cells, the 31 cells are clearly nurse cells that are endoreplicating their DNA, as the DNA content increased while egg chambers aged. The overexpression of EGFP alone or EGFP-Myt1 (data not shown) did not result in such an overproliferation phenotype (Figure 5-7A).

Cdk1 is the only known target of Wee1, so examining the activity of Cdk1 under the condition of Wee1 overexpression should provide the information whether the abnormal phenotype observed was likely caused by improper regulation of Cdk1. Antibodies directed against phospho-tyrosine 15-Cdk1 were used to detect how levels of phospho-inhibited Cdk1 were affected by Wee1 overexpression. The hypothesis was that there should be more pY15-Cdk1 associated with excessive amounts of Wee1. Using this antibody, I was able to examine the amount of phospho-inhibited Cdk1 in different genetic backgrounds. Surprisingly, the antibody staining showed ring canal localization of pY15-Cdk1 during all stages of oogenesis (data not shown). In EGFP only controls, the levels of pY15-Cdk1 staining were fairly low, however ring canal staining was still visible (Figure 5-9A). There was also a faint signal in the cytoplasm in these controls. The levels of the ring canal staining increased dramatically when either EGFP-Wee1 or EGFP-Myt1 was overexpressed (Figure 5-9B, C; EGFP-Myt1 has been previously shown to be functional; Jin, 2005). In egg chambers overexpressing EGFP-Wee1 that had 31 nurse cells, the oocyte had five ring canals (Figure 5-9B arrows) and there were 31 ring canals in total (data not shown). In egg chambers overexpressing EGFP alone or EGFP-Myt1, there was no overproliferation. Hence, oocytes only had four ring canals as in wild type (Figure 5-9A and C arrows) and there were 15 ring canals all together (data not shown). The numbers of ring canals observed in egg chambers of different genetic backgrounds confirmed that there was indeed one extra round of mitosis in a significant fraction of the egg chambers overexpressing EGFP-Wee1 (predicted in Figure 5-2B), but not in the presence of EGFP-Myt1 overexpression.

To summarize the above results, I found that EGFP-Wee1 and EGFP-Myt1 had different expression patterns in the oocyte after stage 6. EGFP-Wee1 was not detected in the oocyte, where EGFP-Myt1 was strongly detected in the cytoplasmic compartment of the oocyte. Besides these localization differences, EGFP-Wee1 overexpression affected the number of cystocyte divisions in a large proportion of egg chambers by promoting one extra round of division, whereas EGFP-Myt1 overexpression did not.

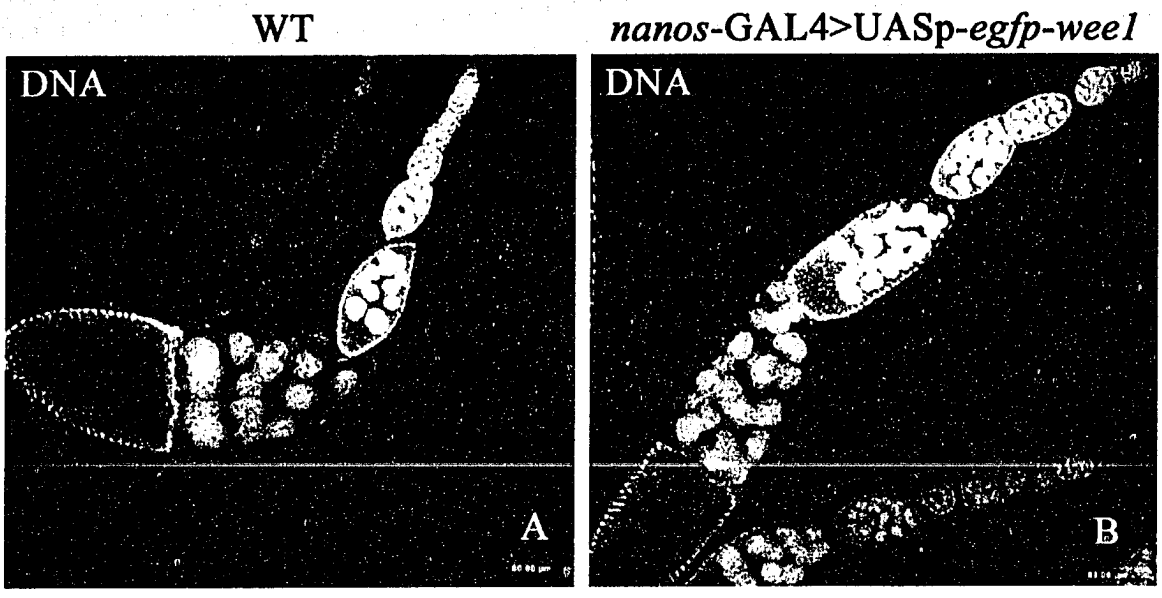

Figure 5-7: Overexpression of EGFP-Wee1 caused a cystocyte overproliferation phenotype. In wild type egg chambers (A), there are usually 15 nurse cells. But in egg chambers overexpressing EGFP-Wee1 (B), there are 31 nurse cells as shown by Hoechst staining of DNA. 32 (2^5) germline cells in (B) suggest that there may be one extra round of cystocyte mitosis (normally 2^4 cells).

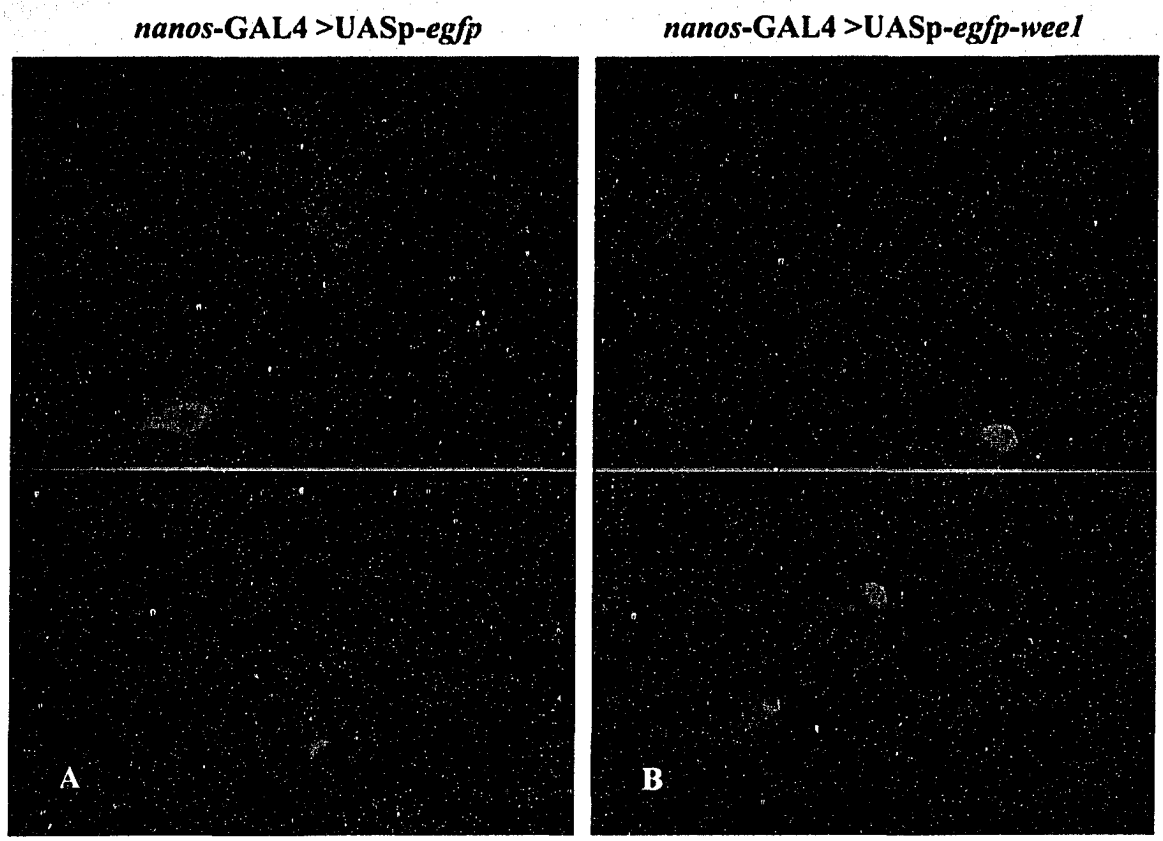

Figure 5-8: Overexpression of EGFP-Wee1 did not affect the number of oocyte per egg chamber. Orb is an oocyte marker. There was only one oocyte per egg chamber in both the controls overexpressing EGFP alone and in egg chambers overexpressing EGFP-Wee1.

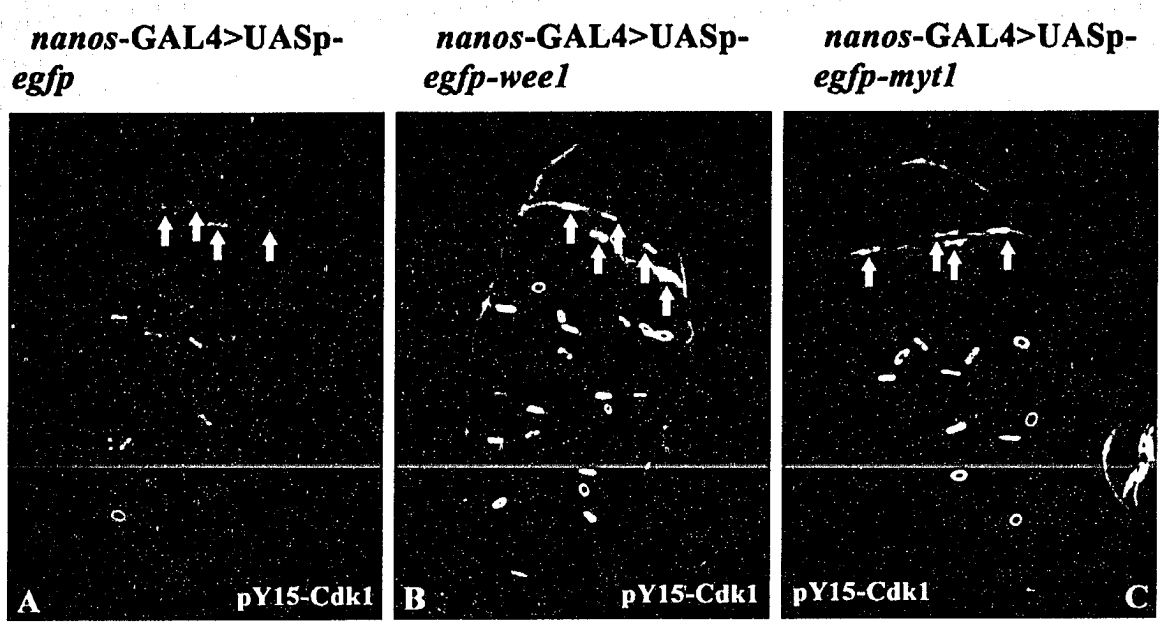

Figure 5-9 Overexpression of EGFP-Wee1 caused one extra round of cystocyte mitosis in the germline. Rings canals were labeled by anti-pY15-Cdk1 staining. In both overexpression of EGFP alone (A) and overexpression of EGFP-Myt1 (C) egg chambers, each oocyte has only four ring canals (arrows), correlating with four rounds of cystocyte mitoses. In egg chambers overexpressing EGFP-Wee1 (B), the oocyte had five ring canals (arrows), indicating there had been five rounds of cystocyte mitoses. The levels of pY15-Cdk1 in (A) are much lower than those in (B) and (C), presumably because EGFP-Wee1 and EGFP-Myt1 overexpression increased the phosphorylation leveles on the Tyr 15 residue (Jin, 2005).

5.4 Discussion

5.4.1. Wee1 overexpression caused one extra round of cystocyte mitosis, but Myt1 overexpression did not.

Judged by the number of nurse cells in egg chambers overexpressing EGFP-Wee1 as well as the number of ring canals in egg chambers and oocytes, it was concluded that there was one more round of mitosis in germline divisions. This is because only five rounds of incomplete cystocyte mitoses could give rise to 31 ring canals in an egg chamber and five ring canals in the oocyte (Figure 5-2B). It was rather surprising that overexpression of EGFP-Wee1 caused one extra round of mitosis, considering that Wee1 is a mitotic inhibitor. Although I cannot explain my results, as the current understanding of germline division is still very limited, my results are consistent with the Cdc25 overexpression or loss of *tribbles* results (Mata *et al.*, 2000; Johnston, 2000; Seher and Leptin, 2000).

Each egg chamber overexpressing EGFP-Wee1 had only one oocyte, as labeled by anti-Orb antibodies. This suggests that oocyte determination was intact in the presence of excessive EGFP-Wee1, which was further confirmed by the number of ring canals in the oocytes. The oocytes had five ring canals, representing the largest possible number of ring canals out of five rounds of mitoses. Furthermore the mature egg could be fertilized (but not develop beyond embryogenesis, data not shown). These reuslts strongly argue that different mechanisms control oocyte determination and cystocyte divisions.

Since Cdk1 is the only known target of Wee1, I evaluated the pool of tyrosine 15 phosphorylated Cdk1 in ovaries overexpressing EGFP-Wee1. The levels of staining in the overexpression EGFP-Wee1 or EGFP-Myt1 were much higher than those in the controls. Interestingly, pY15-Cdk1 was localized to the ring canals, actin-rich structures that connect cystocytes together as a result of incomplete cytokinesis. This suggests that Cdk1 may have a role in regulating ring canal function. Alternatively, the ring canals are simply a docking area for inhibited Cdk1 in those cells. One relevant example is that in syncytial embryos, pY15-Cdk1 localizes to the pseudo-cleavage furrows (refer to Chapter 4), which are also composed of actin. It will be interesting to test whether Cdk1 regulates the actin network.

5.4.2. Different overexpression patterns suggest differential regulation of Wee1 and Myt1 translation or protein stability.

It was rather surprising that EGFP-Wee1 and EGFP-Myt1 showed such different expression patterns when expressed under control of the same GAL4 driver. EGFP-Myt1 was highly expressed in the oocyte after stage 6, whereas EGFP-Wee1 was not. Because the EGFP alone overexpression controls also resulted in detectable expression of EGFP in the oocyte after stage 6, we can infer that EGFP-Wee1 should at least be transcribed in the oocyte. The fact that EGFP-Wee1 was not detectable in the oocyte at those stages suggests that EGFP-Wee1 was downregulated in oocytes. There are two possibilities for this regulation: downregulation of protein translation or efficient protein degradation. EGFP-Wee1 (or endogenous Wee1) might be actively downregulated in the oocyte after stage 6 perhaps because Wee1 does not have a role in the oocyte at those stages. Myt1, on the other hand, may have a developmental role in the oocyte after stage 6 during oogenesis, which could explain why it can be stably expressed.

Consistent with my results, Wee1 and Myt1 protein levels are strictly regulated during oocyte maturation in *Xenopus* (Charlesworth *et al.*, 2000 and 2004; Furuno *et al.*, 2003). Wee1 protein levels are downregulated during early meiosis and remain low until the first mitotic cycle in embryogenesis. In contrast, Myt1 protein levels and activity are maintained during oocyte maturation until hormonal signals trigger signaling pathways that downregulate it (Karaisko *et al.*, 2004). The regulation of Myt1 and Wee1 protein levels are thought to be achieved by regulating the translation of these two proteins, because the mRNA levels of both remain constant (Furuno *et al.*, 2003). Recent studies have suggested that Wee1 protein translation in *Xenopus* is controlled by a CPE (cytoplasmic polyadenylation element) within the 3'UTR of *wee1* (Charlesworth *et al.*, 2000). The binding of CPEB (cytoplasmic polyadenylation element binding protein) to this CPE inhibits Wee1 protein translation. In *Drosophila*, evidence for translational regulation of *wee1* or *myt1* is lacking because this issue has not been addressed. By analogy to the *Xenopus* story, it seems plausible that the different expression patterns observed in egg chambers are due to different translational controls of the two genes, via sequences in their 3'UTRs. No recognizable consensus CPE binding sites are present in *wee1* or *myt1* sequences (S. Campbell, personal communication), suggesting that such a mechanism would involve regulation by novel factors. The UASp-*egfp-wee1* and UASp-*egfp-myt1* transgenes were constructed by fusing the 5' end of a *myt1* or *wee1* cDNA clone to the *egfp* sequence. The 3'UTRs were not removed from each cDNA during the sub-cloning steps, however. These 3'UTRs in the *egfp-myt1* and *egfp-wee1* constructs could therefore mediate the different protein expression patterns that I observed. If the 3'UTRs of *wee1* and *myt1* are in fact involved in regulating protein translation of these genes during oogenesis, this regulation may be important for different functions of the two genes in the oocytes.

The effects of the 3'UTRs could be tested by swapping the 3'UTRs of *wee1* and *myt1* in future studies. A hybrid containing the *wee1* coding sequence and a *myt1* 3'UTR could give rise to detectable expression of Wee1 in the oocyte after stage 6. Conversely, a hybrid of *myt1* coding sequence and *wee1* 3'UTR would be expected to result in loss of expression of Myt1 in the oocyte. Such proteins encoded by hybrid transgenes would still be expected to exhibit the correct nuclear or cytoplasmic localization, which is controlled by protein coding sequences. Moreover, since the expression patterns of Wee1 and Myt1 might be important for the different functions of these two proteins, it is also possible that expression of these modified proteins might be associated with abnormal phenotypes in the oocyte. Because the downregulation of Wee1 was hypothesized to be necessary for normal meiosis, ectopic expression of Wee1 in the oocyte might interfere with meiosis. If the reciprocal hybrid resulted in absence of Myt1 from the oocyte, expression of this construct in a *myt1* mutant background might compromise meiosis progression. If we see such phenotypes, these observations would be consistent with the idea that translation of *wee1* and *myt1* is differentially regulated by their 3'UTRs in *Drosophila* as in *Xenopus*, and that their functions in meiosis are modulated by this mechanism. Further research should focus on identifying the factors involved in regulating 3'UTR mediated translational control, such as the relevant sequences within the 3'UTRs and binding protein(s) or RNA(s) involved in this regulation.

To summarize the above discussion, overexpression assays revealed evidence for differential regulation of Wee1 and Myt1 protein levels in the oocyte after stage 6 during

oogenesis. Future research should add to our knowledge on the exact molecular mechanisms for regulating Wee1 and Myt1 in the oocytes, and the specific roles of each Cdk1 inhibitory kinase during this developmental stage. My results also raise the challenging question of why excessive amount of Wee1, a mitotic inhibitor, led to one extra round of mitotic cycle in cystocytes. Although contradictory to what we would naively predict, my data along with complementary results reported for overexpression of Cdc25 provides further evidence for a novel regulatory mechanism controlling the number of germline cell divisions.

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Chapter 6
General Discussion and Conclusions

Wee1-like kinases are highly conserved cell cycle regulators, which negatively regulate cell cycle progression by inhibiting Cdk1 through phosphorylation (Campbell *et al.*, 1995). In my thesis, I studied the function the *Drosophila* Wee1. Firstly, I characterized a novel *wee1* mutant phenotype in adult flies. Secondly, I made an attempt to dissect out the role of Wee1 in a DNA replication checkpoint responding to hydroxyurea. Lastly, I examined the localization and function of Wee1 during early embryogenesis and oogenesis using tagged transgenes. In this chapter, the most important conclusions of different aspects of my thesis will be discussed.

6.1 Locomotor defects in *wee1* mutants suggest a neural function of Wee1

In an attempt to identify a novel *wee1* mutant phenotype, I found that *wee1* mutants exhibited a progressive locomotor defect in adulthood. Such a phenotype suggests that Wee1 is required to maintain normal locomotor activity in *Drosophila*. Because many neurons and muscles are involved in the regulation of locomotor activities, this phenotype implies a possible neural function of Wee1. Alternatively, Wee1 might be involved in regulating muscle function. EGFP-Wee1 fusion proteins are stabilized in the axoplasm of the axons, which also suggests a possible neural function of Wee1. As an important regulator of Cdk1, Wee1 could be involved in the formation of functional neurons, or in the maintenance of neural cell fate. Preliminary examination of newly eclosed *wee1* mutant brains by our collaborator did not reveal any obvious defects in the central nervous system (M. Feany, Harvard Medical School, personal communication), suggesting the possibility of Wee1 maintaining neuronal cell fate is more favorable. These results correlate well with previous results in humans, where Wee1 activity was downregulated (accompanied by abnormal Cdk1 activity) in Alzheimer's disease-affected neurons (Tomashevski *et al.*, 2001). The affected neurons then underwent apoptosis or necrosis. If Wee1 has a similar role in the fly nervous system, loss of *wee1* may also lead to ectopic apoptosis of certain neurons, resulting in a loss of locomotor activity.

Another possible target of Wee1 in the nervous system is Cdk5. Cdk5 has important neural functions, such as regulating axonal guidance and patterning (Connel-Crowley *et al.*, 2000). My preliminary results demonstrated an interaction between *wee1* and *Cdk5* in that Wee1 overexpression suppressed the rough eye phenotype caused by Cdk5-p35 overexpression. The misregulation of Cdk5 associated with the loss of wee1 may disrupt key neural developmental processes such as axonal guidance and patterning, leading to a loss of locomotor control.

This study suggested two candidate targets of Wee1 in the nervous system (Cdk1 and Cdk5), both of which are supported by attractive models discussed above. Future research should focus on the identification of the target(s) of Wee1 in the nervous system. One could examine defects in processes regulated by Cdk5 in *wee1* mutants. If such an experiment shows a positive result, then Cdk5 will be a novel target of Wee1.

6.2 Wee1 is not essential in a DNA replication checkpoint in larval wing discs.

A second goal of my thesis was to investigate the role of Wee1 in a DNA replication checkpoint response to hydroxyurea (HU) and to determine if the sensitivity of *wee1* mutant to HU was due to a defective checkpoint. Surprisingly, Wee1 was found to be non-essential in a DNA replication checkpoint in response to HU in larval wing

discs. Considering the functional redundancy between Wee1 and Myt1, it can be hypothesized that Myt1 may activate such a checkpoint in a *wee1* mutant background. This is further supported by a previous result obtained in our lab that *wee1* mutant embryos were able to generate a reduced response to another DNA replication inhibitor, aphidicolin (E. Homola, personal communication). Collectively, these results demonstrate that Myt1 and Wee1 are redundantly required for the DNA replication checkpoint response in *Drosophila.*

However, the ability to generate a functional checkpoint cannot explain why *wee1* mutants are sensitive to HU. The fact that *wee1* mutants exhibit ectopic apoptosis when exposed to HU, offers an explanation for this phenotype. It's possible that current protocols are not suitable for identifying subtle S phase defects that might exist in *wee1* mutants, because contrary to the expectation there was no change detected in the amount of BrdU labeled replicating cells after HU treatment. Also the decreased rate of proliferation in the mutants may account in part for the lethality. In the future, it will be necessary to discover better reagents so that the sublte defects in a DNA replication checkpoint can be easily conducted.

6.3 The dynamic localization of Wee1 is cell type and cell cycle specific

In order to better visualize the localization of Wee1, epitope tagged fusion proteins were constructed under the UAS-GAL4 control. After confirming the functionality of the transgenes by genetic rescue and *in vivo* kinase activity assays, the localization patterns of the tagged Wee1 proteins were examined. The tagged Wee1 showed a highly dynamic localization, as reported in other systems (Baldin and Ducommun, 1995; Lee *et al.*, 2001; Sakchaisri *et al.*, 2004). Furhtermore, the localization was cell type specific, implying that Wee1 may play different roles in different cells and that Cdk1 may be differentially regulated in those cells. In particular, EGFP-Wee1 colocalized transiently with mitotic spindles and chromosomes, suggesting a possible novel regulation of local Cdk1 pools. Cdk1 in these structures may be inhibited by phosphorylation during mitosis, when the majority of Cdk1 is active. This colocalization was also observed in wild type embryos stained with antibodies against Wee1 (E. Homola, personal communication).

The different overexpression patterns of EGFP-Wee1 and EGFP-Myt1 under the same *nanos-GAL4* driver control argue that these two proteins are differentially regulated in the oocyte. The next goal will be to evaluate whether this regulation is at the translational level or at the protein stability level. This can be addressed in this overexpression system. If Wee1/Myt1 translation is regulated through their 3'UTRs as in *Xenopus* oogenesis (Charlesworth *et al.*, 2000 and 2004; Furuno *et al.*, 2003), switching 3'UTRs of the two transgenes will switch the translational patterns of the two genes. This system can also be used to screen for regulators of Wee1 and Myt1 in the oocyte and it will help gain knowledge on the context in which of Wee1 and Myt1 function.

6.4 Overexpression of Wee1 is associated with various defects during early embryogenesis and oogenesis

Overexpression of EGFP-Wee1 led to an increase in the amount of inhibited Cdk1 as shown by western blots and immunofluorescence. This misregulation of Cdk1 caused severe nuclear and cytoplasmic defects in syncytial embryos, consistent with Cdk1 being

a master cell cycle regulator. Three most intriguing phenotypes are ectopic pseudo-cleavage furrows, centrosome detachment from the spindle poles, and an increase in the T14 phosphorylation on Cdk1. The observation that pY15-Cdk1 localized to abnormal pseudo-cleavage furrows implicates Cdk1 in the regulation of actin cytoskeleton. On the other hand, the defect in centrosomal attachment to the spindle poles suggests a potential novel involvement of Wee1 in regulating the microtubule-dependent motor proteins. The increase in T14 phosphorylation on Cdk1 in EGFP-Wee1 overexpressing embryos is consistent with a decrease in T14 phosphorylation on Cdk1 in *wee1* mutants (E. Homola, personal communication). However, both results argue against the current consensus that T14 phosphorylation is independent of Wee1. This study calls for future research on the regulation of Cdk1 inhibitory phosphorylation.

During oogenesis, overexpression of Wee1 promoted one extra round of germline derived cystocyte division, opposite to what one would expect. However, this result is consistent with previous results of Cdc25 overexpression (Mata *et al.*, 2000; Johnston, 2000). These unexpected observations argue against the understanding that Wee1 is solely a mitotic inhibitor, suggesting that the germline division counting mechanism is regulated by a novel process. Solving this apparent paradox in the future will greatly advance our knowledge of cell cycle regulation during this stage of development.

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