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Functional analysis of *Drosophila* Myt1

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ABSTRACT

Growth, differentiation and morphogenetic movements occur in concert with cell division during animal development. To understand how this coordination is achieved in *Drosophila melanogaster*, a conserved cell cycle regulator called Myt1 was chosen as the subject for this study. Myt1 belongs to a family of Wee1-like kinases, which delay entry into M phase by inhibiting Cdk1/Cyclin B, the master switch for M phase progression. Wee1 and Myt1 are different in sub-cellular localization and phosphorylation site specificity, suggesting that they have redundant as well as distinct functions.

Despite its essential role in regulating syncytial cell cycles during embryogenesis, *wee1* is dispensable throughout zygotic development. In contrast, *myt1* mutants show prominent bristle defects, male sterility, and other zygotic phenotypes. Although *wee1* and *myt1* single mutants are viable, *wee1* and *myt1* double mutants were never recovered as adults, indicating that, besides its unique developmental functions, Myt1 is functionally redundant with Wee1 for viability.

Phenotypic analysis of *myt1* mutants was focused on gametogenesis, a developmental stage that allows evaluation of both cell division and cell differentiation. In addition to its dramatic effects on mitotic cycles of germline cells, lack of *myt1* also disrupts a cell fate switch in a small fraction of secondary spermatogonia. During male meiosis, premature centrosome separation and splitting occurs without chromosome condensation in *myt1* mutants, leading to aberrant meiotic spindles and subsequently aneuploidy in spermatids. Upon reduction of *wee1* copy number, however, premature chromosome condensation does take place, suggesting that nuclear Wee1 maintains the normal timing of this event in *myt1* mutant primary spermatocytes.

These observations, along with other genetic and biochemical evidence, suggest that *myt1* mutant defects are due to mis-regulation of Cdk1 activity. Loss of *myt1* also promotes chromosome non-disjunction at meiosis I in females. Furthermore, some post-mitotic somatic cells divide ectopically in *myt1* mutants, implying a novel role for Myt1 in mediating cell cycle exit in differentiating cells.

Experimental data presented here suggest that Myt1 and Wee1 each provide a mechanism for delaying cell cycle progression in their respective sub-cellular compartments. These mechanisms may be essential for timing specific cellular events during development.

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LIST OF ABBREVIATIONS

aly: *always early*

Aly: the gene product of *always early*, a marker for primary spermatocytes

Axs: the gene product of *aberrant X segregation*

bam: *bag of marbles*

BamC: a cytoplasmic form of *bag of marbles* gene product, a marker for secondary spermatogonia/oogonia

BrdU: 5'-bromo-2'-deoxyuridine

ca: *cannonball*

CAK: Cdk-activating kinase

cdc: *cell cycle division*

Cdks: Cyclin-dependent kinases

Chfr: checkpoint protein with an FHA domain and ring finger

CKIs: Cdk kinase inhibitors

Cnn: Centrosomin, a marker for centrosomes in *Drosophila*

Dap: the gene product of *dacapo*

DSBs: double-strand breaks

DUP: the gene product of *double parked,* as a marker for proliferating cells

EMS: ethyl methane sulfonate

ER: endoplasmic-reticulum

Eya: the gene product of *eyes absent*, a marker for cyst cells

F-actin: filamentous-actin

Fzr: the gene product of *fizzy-related*

G1 phase: the first gap phase

G2 phase: the second gap phase

grp: *grapes*, a *Drosophila* homolog of *chk1*

GRP: the gene product of *grapes*

GSCs: germline stem cells

hs-Cdk1AF: a heat shock-inducible , non-inhibitable transgenic allele of *Cdk1*

Hts: the gene product *hu li tai shao (hts),* an adducin-like protein

IR: ionizing radiation

KLP61F: a kinesin-like motor protein

M phase: mitosis or meiosis

MCM: mini-chromosome maintenance

mia: *meiosis I arrest*

MPF: M-phase or maturation-promoting factor

Myt1: membrane-associated tyrosine and threonine dual specificity protein kinase 1

NCD: the gene product of *non-cleart disjunctional*

NDJ: non-disjunction

NEBD: nuclear envelope breakdown

MPM2: mitotic phosphoproteins monoclonal antibody 2, a marker for M phase cells

ORC: origin recognition complex

PCC: premature chromosome condensation

PCNA: proliferating cell nuclear antigen

PH3: phosphorylated form of histone H3

Rux: the gene product of *roughex*

S phase: DNA synthesis phase

sa: *spermatocytes arrest*

SC: synaptonemal complex

SCF: Skip-Cullin-F-box

SPB: spindle pole bodies

SSCs: somatic stem cells

ß-TrCP: ß-transducin repeat-containing protein

Sub: the gene product of *subito*

T288P-AurA: a phospho-specific T288 Aurora A antibody, as marker for centrioles

Tome-1: a trigger of mitotic entry-1

Vasa: the gene product of *vasa*, a marker for germline cells

Chapter 1 Introduction

This thesis focuses on analyzing the biological functions of Myt1, a member of the Wee1-like kinase family, throughout the development of *Drosophila melanogaster*, with a special emphasis on its role in cell cycle regulation during gametogenesis. Herein, I review our current understanding of the cell cycle and its control by Wee1-like kinases. While *Saccharomyces cerevisiae* is the known exception with only one Wee1-like kinase, most eukaryotic organisms have two or more, suggesting that multiple Wee1-like kinases may regulate different aspects of the cell cycle. Lastly, to familiarize the readers with the experimental system, a discussion of developmental regulation of cell cycles during *Drosophila* gametogenesis is presented.

1.1 An overview of the cell cycle

A fascinating feature of living organisms is their ability to produce offspring. This capability relies on cell division, regardless of whether the organism is unicellular or multicellular. In order to propagate itself, a cell duplicates its DNA content and mass, then divides into two daughter cells with equal amounts of DNA to finish a cell division cycle. Although different cell cycles in various organisms have many variations, most cell cycles can be arbitrarily broken down into four discrete phases, namely: first gap (G1) phase, DNA synthesis (S) phase, second gap (G2) phase and the mitosis/meiosis (M) phase. It has been discovered that in eukaryotic organisms as distantly related as yeast and humans, that the basic cell cycle machinery is well conserved. Multiple Cyclin-dependent kinases (Cdks) and their associated partner Cyclins are the major regulatory factors that promote progression from one phase to the next. Hence, understanding the regulation of the activities of Cyclin-dependent kinases is an important biological problem.

1.1.1 Overview of how Cdk/Cyclin complexes drive cell cycle progression

In multicellular organisms, cells enter the cell cycle in G1 phase when they receive stimulating signals from either growth factors or the extra-cellular matrix (Boonstra, 2003). To prepare the machinery for DNA synthesis at the next phase, cells initially synthesize Cdk4 and Cdk6, each of which forms a functional complex with Cyclin D. With their kinase activity, Cdk4/6-Cyclin D complexes phosphorylate the product of the

retinoblastoma tumor suppressor gene (*pRB*) on some regulatory sites and thereby partially turn on E2F/DP-dependent transcription that is normally suppressed by pRB. Although it is not entirely clear how E2F/DP complexes regulate transcription at G1 phase, some genes have been determined to rely on E2F/DP directed transcriptional activation. Among them, the most important ones seem to be *cdk2* and *cyclin E*, whose gene products promote the G1/S transition by further phosphorylating pRB, thus completely activating the transcription of E2F-responsive genes, such as those encoding Cyclin A, Cdc6, Cdt1, ORC (origin recognition complex), MCM (mini-chromosome maintenance) and PCNA (proliferating cell nuclear antigen) (reviewed in Dyson, 1998; Nevins, 1998).

In G1 phase, a heteromeric hexamer of ORC binds to origins of replication that then recruits Cdc6 and Cdt1 to the origins. Together, these proteins recruit another heteromeric hexamer of MCM to form pre-replication complexes, which are capable of initiating DNA synthesis (reviewed in Kelly and Brown, 2000). Therefore, the assembly of ORC, Ccd6, Cdt1 and MCM proteins is thought to make chromatin competent or "licensed" for DNA replication by providing sites that are competent for assembly of the DNA replication machinery (reviewed in Chevalier and Blow, 1996).

Entry into S-phase is then catalyzed by the concerted action of S-phase promoting factors such as Cdk2/Cyclin E, Cdk2/Cyclin A complexes and the Cdc7-Dbf4 kinases in a step-wise manner: releasing Cdc6 and recruiting Cdc45 protein to the pre-replication complexes. This process promotes loading of DNA polymerase α/primase and single-strand specific DNA-binding protein RPA to make a RNA/DNA primer, allowing the start of leading strand synthesis (reviewed in Maga and Hubscher, 2003). The gradual release of MCM from the pre-replication complexes then suppresses re-initiation at the origins (reviewed in Ritzi and Knippers, 2000). Meanwhile, the loading of PCNA and DNA polymerase δ/ε promotes lagging strand synthesis and coordinates the maturation of Okazaki fragments (reviewed in Maga and Hubscher, 2003).

Once DNA synthesis is finished, cells progress into G2 phase and start accumulating mitotic Cyclins, forming inactive Cdk1/Cyclin A, Cdk1/Cyclin B and Cdk1/Cyclin B3 complexes. Since cell cycle progression is arrested during this period of time, cells can grow in volume, migrate to appropriate positions, or repair DNA damage

when this circumstance arises. Presumably, Wee1 and Myt1 are functioning in G2 phase to keep Cdk1 inactive. During the G2/M transition, activation of Cyclin-associated Cdk1 complexes is triggered by a network of positive regulators that overcome inhibition by the negative regulators, Wee1 and Myt1. Active Cdk1/Cyclin B complexes are thought to serve as the major M-phase kinase, promoting many M-phase events such as chromosome condensation, nuclear envelope breakdown (NEBD), spindle assembly. The other mitotic Cyclin/Cdk1 complexes play poorly understood roles in regulating entry into mitosis, that are at least partially redundant with Cyclin B/Cdk1 in *Drosophila* (Jacobs et al., 1998; Lehner and O'Farrell, 1990).

Therefore, with the sequential action of Cdk4/6-Cyclin D, Cdk2/Cyclin A and Cdk2/Cyclin E, and Cdk1/Cyclin A and Cdk1/Cyclin B in an appropriate order (Edgar and Lehner, 1996; Nasmyth, 1996; O'Farrell et al., 1989), cells undergo transitions from G1 to S and G2 to M in the proper order, finishing cell cycle phase-specific changes before proceeding to the next phase.

1.1.2 Regulation of Cdk/Cyclin activity during the cell cycle

How are the different Cdk/Cyclin complexes activated and inactivated at the appropriate time? Extensive studies have shown that Cdk/Cyclin activity is strictly controlled by at least four different mechanisms to ensure the proper timing of cell cycle events in eukaryotic cells. Normally, protein levels of the different Cdks remain constant throughout the cell cycle and regulation of their kinase activity is imposed at the post-translational level. In metazoans, the binding of each Cyclin partner to a specific Cdk appears to be required for the activation of Cdk/Cyclin complexes, because this triggers a conformational change that exposes the ATP-binding site of each kinase subunit, as required for its catalytic activity (reviewed in Morgan, 1995). Meanwhile, the activation of Cdk/Cyclin complexes also requires the phosphorylation of a conserved theronine residue (T161 in Cdk1 and T160 in Cdk2 of humans), which is carried out by Cdk-activating kinase (CAK), a complex of Cdk7 and Cyclin H (Shiekhattar et al., 1995). As T161 (or T160) phosphorylation seems to be constitutive in most cell types, Cyclin binding is one primary means of regulating Cdk/Cyclin activity. The activity of

Cdk/Cyclin complexes is thereby modulated by Cyclins that are synthesized in a phase-specific manner.

After activation, termination of the activity of specific Cdk/Cyclin complexes at the appropriate time is accomplished through destruction of Cyclins by the ubiquitin/proteasome pathway (reviewed in Ekholm and Reed, 2000). The different Cyclins are recognized by the F-box component of Skip-Cullin-F-box (SCF) complexes that catalyze phosphorylation-dependent ubiquitination. Although many specific proteins that are implicated in the destruction of specific Cyclins remain to be identified, the degradation of Cyclins is a common mechanism for down-regulating Cdk/Cyclin activity.

Cdk/Cyclin complexes can also be inhibited by their association with Cdk kinase inhibitors (CKIs), such as the Cip/Kip family and INK4 family in vertebrates (reviewed in Ekholm and Reed, 2000); and Dacapo (Dap) and Roughex (Rux) in *Drosophila*, respectively (de Nooij et al., 1996; Gonczy et al., 1994; Lane et al., 1996; Thomas et al., 1994). The founding member of the Cip/Kip family is p21, which is believed to inhibit both Cdk2-Cyclin E and Cdk2-CyclinA and Cdk4/6-Cyclin D kinases, whereas INK4 members specifically bind and suppress Cdk4/6-Cyclin D. *Drosophila* Dap functions as a Cip/Kip Cdk2 inhibitor, whereas Rux is required for inhibiting Cdk1/Cyclin A complexes (Foley et al., 1999; Foley and Sprenger, 2001). Depending on the specific context, CKIs are involved in inhibiting Cdk/Cyclin complexes in the DNA damage response, in cell cycle exit and in exit from mitosis (de Nooij et al., 1996; Ekholm and Reed, 2000; Foley et al., 1999; Foley and Sprenger, 2001; Lane et al., 1996).

Of central interest to my thesis, Cdk1 and Cdk2 are also subject to inhibitory phosphorylation by Wee1-like kinases at two distinct sites (T14 and Y15 in human Cdk1 and Cdk2), which are close to the amino-terminus of each protein (Booher et al., 1997; Campbell et al., 1995; Cornwell et al., 2002; Igarashi et al., 1991; Liu et al., 1997; Russell et al., 1989; Wells et al., 1999). T14 and Y15 are available for phosphorylation only after Cyclin binding (Morgan, 1995), indicating that inhibitory phosphorylation targets Cdk/Cyclin complexes rather than Cdk alone. Thus, T14 and/or Y15 phosphorylated Cyclin-Cdk1/2 complexes are kept inactive as a consequence. A family of Cdc25 phosphatases, remove T14 and Y15 phosphates from phosphorylated Cdk1/2-Cyclin complexes to activate them, when necessary.

Throughout the cell cycle, these multiple mechanisms for regulating Cdk/Cyclin complexes are applied in a combinatorial fashion to guarantee critical and accurate transitions from one stage to the next. Because the object of this study, Myt1, is a member of the family of Wee1-like kinases, the functions of these kinases will be discussed further in the next section.

1.2 Wee1-like kinases and their functions

1.2.1 The discovery of Wee1-like kinases

In genetic screens for *cell cycle division* (*cdc*) mutants in the fission yeast *Schizosaccharomyces pombe*, three gene loci: *cdc2* (*S. pombe* homolog of *cdk1*), *wee* and *cdc25*, were determined to be important for timing of cell division with respect to cell size (Fantes, 1981; Nurse, 1975). Based on the genetic interactions of these genes in *S. pombe*, an elegant epistatic pathway for control of mitosis was proposed: that Cdc25 is required for releasing inhibition of Cdc2 by Wee1, upon entry into mitosis (Fantes, 1981).

Cytoplasmic transfer experiments with *Xenopus* oocytes led to the discovery of M-phase or maturation-promoting factor (MPF, first described in Masui and Markert, 1971), which can induce meiosis and mitosis. It was later found that MPF is composed of two subunits: now known as Cdk1 and mitotic Cyclin B (Dunphy et al., 1988; Gautier et al., 1988). Remarkably, the genetic studies of *cdc* mutants in *S. pombe* and the biochemical characterization of MPF converged, when it was subsequently discovered that the Cdk subunit of MPF was the *Xenopus* homolog of the *cdc2* gene product in *S. pombe* (Dunphy et al., 1988; Gautier et al., 1988; Simanis and Nurse, 1986). Later, the revelation of Wee1 as a protein kinase and Cdc25 as a phosphatase in *S. pombe*, explicitly demonstrated that Cdc2 activation is regulated by post-translational phosphorylation (Russell and Nurse, 1986; Russell and Nurse, 1987). Moreover, this mode of Cdc2 activation has proven to be universal, since homologs of Wee1, Cdc25 as well as Cdc2 have been found in various organisms from fission yeast to invertebrates and vertebrates (Edgar and O'Farrell, 1990; Featherstone and Russell, 1991; Gautier et

al., 1991; Igarashi et al., 1991; Kumagai and Dunphy, 1991; Lee et al., 1992; Moreno et al., 1990).

In yeast species, *S. pombe* has two Wee1-like kinases, Wee1 and Mik1 (Lundgren et al., 1991), whereas *S. cerevisiae* only has one: Swe1 (Booher et al., 1993). In metazoans, *Drosophila* is an exception with only one Wee1 (Campbell et al., 1995), while *C. elegans*, *X. laevis* , *M. musculus* and *H. sapiens* all have Wee1 (or Wee1A) and Wee2 (or Wee1B) (Honda et al., 1995; Leise and Mueller, 2002; Mueller et al., 1995a; Nakanishi et al., 2000; Okamoto et al., 2002; Parker et al., 1995; Wilson et al., 1999). The nuclear Wee-like kinases phosphorylate the Y15 residue of Cdk1 (or an analogous site) in all species, and sometimes, can even functionally substitute for one another to a certain extent (Campbell et al., 1995; Igarashi et al., 1991; Russell et al., 1989).

Another type of Wee1-like kinase that has only been found in metazoans is a membrane-associated tyrosine and threonine dual specificity protein kinase called Myt1, that was initially cloned in *Xenopus* and subsequently found in other organisms (Booher et al., 1997; Cornwell et al., 2002; Lamitina and L'Hernault, 2002; Liu et al., 1997; Okumura et al., 2002; Price et al., 2002; Wells et al., 1999). As its name suggests, Myt1 is distinct from other Wee1-like kinases with respect to its cellular localization, being membrane-bound and residing at the Golgi-body and endoplasmic-reticulum (ER) membranes. Moreover, Myt1 phosphorylates both T14 and Y15 residues of Cdk1, unlike nuclear Wee1 kinases that only target the Y15 residue (Mueller et al., 1995b). These localization and Cdk1 target specificity differences suggest that Myt1 might serve specialized functions during the cell cycle in multi-cellular organisms.

1.2.2 Wee1-like kinases and Pre-mitotic Checkpoints that respond to incomplete DNA replication and DNA damage

Ever since *wee1* was found in *S. pombe*, it has been under extensive investigation for its role in regulating the cell cycle. The first clue came from the *wee1* mutant phenotype: where cells initiate mitosis at half the size of wild type, suggesting a faster G2/M transition (Nurse, 1975). However, a mutation in another Wee-like kinase, Mik1, did not show any phenotype, by itself, in this organism (Lundgren et al., 1991). Surprisingly,

functional depletion of both *wee1* and *mik1* resulted in lethality, due to a premature entry into M phase with incomplete DNA synthesis, suggesting that either *wee1* or *mik1* was required for delaying mitosis until S phase was finished (Lundgren et al., 1991). This was the first demonstration that different Wee1-like kinases could serve distinct and also redundant functions in one species. Moreover, this study also provided the experimental evidence for the involvement of Wee1-like kinases in a checkpoint mechanism: that mitosis is dependent on the completion of S phase (Hartwell and Weinert, 1989). At the cellular level, checkpoint mechanisms not only confer interdependency between successive phases of cell cycle, but also allow cells time to deal with the problem that triggers the checkpoint (Elledge, 1996; Hartwell and Weinert, 1989). For example, the DNA damage checkpoint and DNA replication checkpoints provide time for repairing DNA damage and finishing DNA synthesis, respectively, before Cdk1/Cyclin B complexes are activated to promote mitosis.

After the realization that Cdk1 (previously called cdc2)/Cyclin is the main cell cycle kinase for promoting M-phase in eukaryotes, phosphorylation of the Y15 residue of Cdk1 was determined to be the molecular mechanism by which Wee1 kinases regulate Cdk1/Cyclin B complexes (Booher et al., 1993; McGowan and Russell, 1993; Parker and Piwnica-Worms, 1992). In agreement with this notion, fission yeast and mammalian cells arrested in G2 phase after DNA damage or DNA replication inhibition have Y15 phosphorylated Cdk1 (Blasina et al., 1997; Jin et al., 1996; Rhind et al., 1997; Rhind and Russell, 1998), although this is not the case in *S. cerevisiae* (Amon et al., 1992; Sorger and Murray, 1992; Zhou and Elledge, 2000). Many studies have defined Cdc25C as a cell cycle regulator targeted in cellular responses to DNA damage or DNA replication inhibition. When the upstream checkpoint kinases Chk1/Chk2 (or Cds1 in fission yeast) are activated, Cdc25 activity is inhibited by nuclear translocation, preventing Cdc25 from dephosphorylating Cdk1 and thus maintaining Cdk1 inhibition (Blasina et al., 1999; Furnari et al., 1997; Kumagai et al., 1998; Peng et al., 1997; Rhind and Russell, 1998). These regulatory pathways are summarized in Fig.1-2 (page 18). In contrast, the involvement of Wee1 in these checkpoints remained a matter of debate in *S. pombe* (Barbet and Carr, 1993; Boddy et al., 1998; Raleigh and O'Connell, 2000; Rhind and Russell, 2001; Rowley et al., 1992), although it was clear that Mik1 was required for

DNA damage/replication checkpoints at both the establishment and maintenance stages of this response (Rhind and Russell, 2001). In vertebrates, where there are three Wee1-like kinases, a similar conundrum exists, since several studies with mammalian tissue culture cells indicated that Wee1 was either required or not required for a DNA damage checkpoint (Chow et al., 2003; van Vugt et al., 2004; Wang et al., 2004). These results imply that Wee1-like kinases can be functionally redundant with respect to responses to DNA damage and inhibition of DNA replication.

1.2.3 Wee1-like kinases and inhibition of entry into mitosis during embryonic development

Compared to the uncertainties regarding the checkpoint function of Wee1 kinases in mammalian tissue culture cells, it is clear that maternal Wee1 function is absolutely required (Price et al., 2000) during early embryogenesis in *Drosophila*, for a proposed DNA replication checkpoint. Mutant embryos derived from *wee1* females become arrested at cycle 13 during the syncytial stage of development, a phenotype that is reminiscent of *grapes* mutants (*grp* encodes the *Drosophila* ortholog of Chk1) and *mei-41* (the *Drosophila* ortholog of ATR) mutants (Sibon et al., 1999; Sibon et al., 1997). Additionally, genetic interactions between *wee1* and *grp*, *mei-41* placed *wee1* in a *mei-41-grp-wee1* checkpoint pathway functioning in *Drosophila* syncytial stage embryos, presumably triggered by incomplete DNA replication as maternal supplies of replication factors become depleted (Price et al., 2000). Zygotic *wee1* hemizygous mutants did not show discernable mutant phenotypes however, except in being sensitive to treatment with hydroxyurea (HU, a DNA replication inhibitor), which triggers the DNA replication checkpoint. These observations indicate that zygotic *wee1* function is not essential for viability in *Drosophila*, suggesting a possible functional redundancy between Wee1 and Myt1 in this species.

The first cell cycle of *Xenopus* is distinct from mitotic cycles 2-12 in that it has a G2 phase, which is maintained by p42 mitogen-activated protein kinase mediated Wee1 activity (Murakami et al., 1999; Walter et al., 2000). This developmentally regulated G2 phase was proposed to ensure the fusion of pronuclei (Walter et al., 2000). In

gastrulating *Xenopus* embryos, both maternal Wee1 and zygotic Wee2 functions were reported to be involved in coordinating cell division with gastrulation (Leise and Mueller, 2004; Murakami et al., 2004). Morphogenetic processes such as cell movements and/or cell shape changes occur frequently in gastrulating embryos, and these processes are generally incompatible with cell division because they all rely on reorganization of the cytoskeleton. Using the antisense morpholino technique in *Xenopus* embryos, the knockdown of maternal Wee1 disrupted early gastrulation, while the depletion of zygotic Wee2 affected morphogenetic events during mid-late gastrulation. In both cases, Y15 phosphorylation of Cdk1 promoted by Wee1 and Wee2 was responsible for regulating entry into mitosis during gastrulation. In *Drosophila* gastrulating embryos, a negative regulator of Cdc25 called Tribbles, is required to prevent invaginating cells from entering mitosis prematurely, emphasizing the fact that inhibitory phosphorylation of Cdk1 must be in place for down-regulation of Cdc25 to be effective in regulating Cdk1 in this context (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Therefore, the paradigm that affecting the balance of inhibitory phosphorylation of Cdk1 emerged as a general strategy to delay progression through the cell cycle during critical developmental transitions in metazoans.

1.2.4 Functions of Wee1-like kinases during Meiosis

In many species of metazoans, immature oocytes arrest at prophase I of meiosis for a relatively long period of time, and mature oocytes are later arrested at either metaphase of meiosis I (in *Drosophila*) or meiosis II, in *Xenopus* and many other vertebrates (Sagata, 1996). In *A. pectinifera* and *Xenopus*, an upstream kinase inactivates Myt1 kinase by phosphorylation, allowing activation of Cdk1/Cyclin B during the G2/M phase transition that accompanies oocyte maturation (Okumura et al., 2002; Palmer et al., 1998). Therefore, Myt1 was proposed to be the inhibitory kinase that is responsible for prophase I arrest in the oocytes of these animals. A genetic study in *C. elegans* revealed that gain-of-function alleles of *myt1* did not affect the progression of meiosis in oocytes but blocked meiosis entry in spermatocytes instead (Lamitina and L'Hernault, 2002). These observations demonstrated that Myt1 is sufficient for prophase I arrest of meiosis in

different species, but they did not address whether or not Myt1 is necessary for this arrest during meiosis.

In *S. cerevisiae*, if **d**ouble-strand **b**reaks (DSBs) originating from meiotic recombination and chromosome synapsis are not repaired, then a pachytene checkpoint (or a meiotic recombination checkpoint) is activated to prevent entry into meiosis (Lydall et al., 1996). The underlying mechanism for this pachytene checkpoint is similar to that of the DNA replication checkpoint described above. For instance, Mec1 (the ortholog of ATR) functions as an upstream activator kinase in the pachytene checkpoint pathway (Bailis and Roeder, 2000), whereas Swe1 functions downstream to inhibit Cdc28 (budding yeast homolog of Cdk1) via inhibitory phosphorylation (Leu and Roeder, 1999). Deletion of *SWE1* caused resumption of meiosis approximating wild type levels in several mutants that would otherwise be arrested in meiotic prophase. In addition, the activated pachytene checkpoint pathway also limited Cyclin production as a parallel mechanism for suppressing the activity of Cdc28/Cyclin complexes (Leu and Roeder, 1999).

Recently, Mei-41 (ATR), Chk2 and Wee1 were also found to function in the pachytene checkpoint in *Drosophila*, apparently responding to unrepaired DSBs during female meiosis (Abdu et al., 2002). Unlike the situation in *S. cerevisiae*, however, loss of *wee1* function did not provoke premature meiosis in mutant oocytes with DSBs, despite the fact that Wee1 appeared to be activated by Chk2 in those oocytes. Besides the possibility of limiting Cyclin production as in *S. cerevisiae*, this could also be attributed to redundancy between Wee1 and Myt1 in *Drosophila* for the pachytene checkpoint, similar to an observed redundancy between Wee1 and Mik1 in *S. pombe* for the meiotic DNA replication checkpoint (Murakami and Nurse, 1999). When treated with HU to inhibit DNA replication, *S. pombe* cells are blocked at prophase I of meiosis due to an activated DNA replication checkpoint, by the activity of checkpoint kinases orthologous to those used for the pachytene checkpoint in *S. cerevisiae* (Murakami and Nurse, 1999). The *S. pombe* DNA replication checkpoint pathway also operates by inhibiting Cdk1 kinase activity through Y15 phosphorylation, but a single mutation in either *wee1* or *mik1* does not abolish the prophase I arrest triggered by HU treatment. Under similar conditions, although *wee1 mik1* double mutant cells did enter into meiosis, they were

arrested at metaphase I with separated and occasionally extra spindle pole bodies (SPB). These results suggested that Wee1-like kinases could also be functionally redundant for regulating meiosis in metazoans, which have two or three Wee1-like kinases.

A distinctive feature of meiosis is the lack of DNA synthesis between meiosis I and meiosis II. In *Xenopus*, this avoidance of S phase during meiosis is accomplished by down-regulating Wee1 protein levels at the meiosis I to meiosis II transition, presumably via a translational repression mechanism (Iwabuchi et al., 2000; Nakajo et al., 2000). If Wee1 was added exogenously during meiosis I however, oocytes start DNA replication after meiosis I is finished, converting meiosis into mitosis (Nakajo et al., 2000). The absence of Wee1 allows a residual amount of Cdk1/Cyclin B activity, which inhibits S phase during interphase of meiosis II (Iwabuchi et al., 2000). Since Wee1 proteins are also undetectable in *Xenopus* spermatocytes and in *S. pombe* during meiosis (Daya-Makin et al., 1992), Nakajo et al. proposed that lack of Wee1 might be a conserved mechanism for skipping S phase between the two meiotic divisions (Nakajo et al., 2000). This model is attractive but it also raises some interesting questions: Is Wee1 still required for blocking meiosis in the DNA pachytene checkpoint or DNA replication checkpoint in these systems? Can Myt1 functionally substitute for Wee1 at this stage?

1.2.5 The degradation of Wee1-like kinases at the G2/M transition in different species

Although Swe1 mediated phosphorylation of the Y19 residue of Cdc28 (equivalent to Y15 of Cdk1 in *S. pombe*) is not required for the DNA damage and DNA replication checkpoints in *S. cerevisiae*, regulation of phosphorylation of this site is essential for the morphogenesis checkpoint, which refers to a mechanism that triggers a G2 arrest in the event of disrupted septin filament assembly at the bud neck (Lew and Reed, 1993; Lew and Reed, 1995). This was proposed as a special checkpoint mechanism because delay of mitosis before a bud has been formed can prevent the formation of bi-nucleate cells. Once the morphogenesis checkpoint is activated, Swe1 is stabilized at the bud neck and constrains Cdc28-Cyclin activity through inhibitory phosphorylation (Barral et al., 1999; McMillan et al., 1999; Shulewitz et al., 1999).

Genetic analysis in this system has shown that proteins such as Hsl1 (histone synthetic lethal 1) and Hsl7 work coordinately to target and tether Swe1 to the bud neck to promote its degradation upon entry into mitosis, but the mechanism that mediates Swe1 destruction remains to be established (Barral et al., 1999; McMillan et al., 1999; Shulewitz et al., 1999). Recently, Cdc5 (the *S. cerevisiae* homolog of Polo, a protein kinase) and Cla4 (the *S. cerevisiae* homolog of PAK) have been reported to phosphorylate Swe1 at S phase and the G2/M transition, respectively, initiating a phosphorylation-dependent degradation process (Sakchaisri et al., 2004). The degradation of Swe1 also requires an E3 ubiquitin ligase called SCF^{Met30} as well as Cdc28 activity (Kaiser et al., 1998; Sia et al., 1998).

As in *S. cerevisiae*, Polo or Cdk1/Cyclin B dependent phosphorylation of Wee1 is indispensable for Wee1 degradation at the G2/M transition in human tissue culture cells (Watanabe et al., 2004). Specifically, certain F-box components of SCF protein complexes (functioning in ubiquitin mediated proteolysis) such as Tome-1 (**t**rigger **o**f **m**itotic **e**ntry-1) and ß-TrCP (ß-transducin repeat-containing protein) are reported to recruit phosphorylated Wee1 and to tag it with ubiquitin for subsequent proteolysis (Ayad et al., 2003; Watanabe et al., 2004). Consistent with these observations, Polo is also essential for the degradation of Wee1, allowing mammalian cells to re-enter mitosis after DNA damage (van Vugt et al., 2004). As mentioned earlier, Polo is also required for Myt1 degradation in immature oocytes during meiosis (Nakajima et al., 2003). Since degradation of Wee1 kinases at the G2/M transition has been documented in several different organisms, this may be a common mechanism for entering M phase (although not in early *Drosophila* embryos, personal communication with E. Homola).

1.2.6 Wee1-like kinases and Cdk1 activation at the G2/M transition

Compelling evidence in several different model systems has shown that the trigger for progressing from G2 phase to M phase relies on the removal of T14 and/or Y15 inhibitory phosphorylation from Cdk1, but the actual regulatory circuits that accomplish this goal may differ in these systems (Ayad et al., 2003; Hirota et al., 2003; Karaiskou et al., 2004; Lim and Surana, 2003; Okano-Uchida et al., 2003; Okumura et al., 2002; Palmer et al., 1998; Sakchaisri et al., 2004). The different mechanisms that regulate how

Cdk1 activation can be triggered in different eukaryotic organisms are summarized below (also see Figure 1-1, page 16).

Firstly, down-regulation of Myt1 or other Wee1-like kinases activity can be accomplished by the activity of other protein kinases, such as $p90^{RSK}$, Akt1, Polo and Cla4 by phosphorylation, either by inactivating kinase activity or by targeting the protein for ubiquitination-dependent proteolysis (Ayad et al., 2003; Karaiskou et al., 2004; Nakajima et al., 2003; Okano-Uchida et al., 2003; Okumura et al., 2002; Palmer and Nebreda, 2000; Sakchaisri et al., 2004; van Vugt et al., 2004; Watanabe et al., 2004). Secondly, accumulation of Cyclin B by release of translational repression of Cyclin B1 has been documented in *Xenopus* oocytes; or alternatively, by inhibition of Fzr-dependent proteolysis of Cyclin B in *Drosophila* embryos (Cao and Richter, 2002; Dienemann and Sprenger, 2004; Nakahata et al., 2003). Thirdly, the recruitment of positive regulators of Cdk1/Cyclin B complexes such as Ajuba, Aurora A, Polo, Cdc25 and Cdk1/Cyclin B onto centrosomes occurs, which serves as a cytoplasmic organizing center for Cdk1/Cyclin B activation (Grallert and Hagan, 2002; Hirota et al., 2003; Jackman et al., 2003; Kumagai and Dunphy, 1996). Lastly, positive feedback loops that are initiated by a small amount of activated Cdk1/Cyclin B activity can further activate Cdc25 activity and suppress the activity of Wee1-like kinases (Abrieu et al., 1998; Hoffmann et al., 1993; Kumagai and Dunphy, 1995; Mueller et al., 1995b; Patra et al., 1999). Presumably these positive feedback loops are crucial for generating the none-or-all type of switch from G2 into M phase that is normally observed (Ferrell, 1998; Ferrell and Machleder, 1998; O'Farrell, 2001).

Conversely, when the G2/M transition is delayed in response to the above mentioned checkpoint mechanisms or to developmental cues, the common regulators of Cdk1/Cyclin B complexes are targeted again, but in an opposite direction (see Figure 1-2, page 18). For example, Wee1-like kinases can be stabilized and up-regulated via Chk1 and/or Chk2-mediated phosphorylation to maintain a G2 arrest after DNA damage, incomplete DNA replication, activation of the pachytene or morphogenesis checkpoints, or by developmental mechanisms that delay entry into mitosis, in different organisms (Baber-Furnari et al., 2000; Leise and Mueller, 2004; Leu and Roeder, 1999; Michael and Newport, 1998; Murakami et al., 2004; Petrus et al., 2004; Raleigh and O'Connell, 2000;

Sakchaisri et al., 2004; van Vugt et al., 2004). Reciprocally, Cdc25C is excluded from the nucleus after being phosphorylated by Chk1 and/or Chk2, preventing Cdk1/Cyclin B activation in the nucleus after DNA damage or inhibition of DNA replication (Blasina et al., 1999; Boddy et al., 1998; Furnari et al., 1999; Furnari et al., 1997; Lopez-Girona et al., 1999; Peng et al., 1997; Zeng and Piwnica-Worms, 1999). Likewise, Polo, another positive regulator of Cdk1/Cyclin B, is also degraded by a Chfr-dependent (checkpoint protein with an FHA domain and ring finger) mechanism in response to activation of either the DNA damage checkpoint or the DNA replication checkpoint, in mammalian tissue culture cells (Kang et al., 2002). The nuclear exclusion of Cdc25 is of particular importance during checkpoint triggered G2 arrest, since nuclear Wee1 is capable of protecting the nucleus from cytoplasmically activated Cdk1/Cyclin B activity in mammalian cells (Heald et al., 1993).

Cdk1/Cyclin B activation is regulated by a complicated network of interacting proteins, in different eukaryotes that have been studied. One of the major targets of this network is the Wee1-like kinases, whose Cdk1 inhibitory activity has to be either eliminated or stabilized, depending on the circumstances. The specific roles of Wee1 and Myt1 kinases in regulating M-phase entry are still controversial in the literature, partially because mutations affecting these proteins were not available until recently for analyzing their functions *in vivo*, in metazoans. To address this question, our lab set out to evaluate how loss of Wee1 or Myt1 would affect the regulation of Cyclin-associated Cdk1 activity during *Drosophila* development.

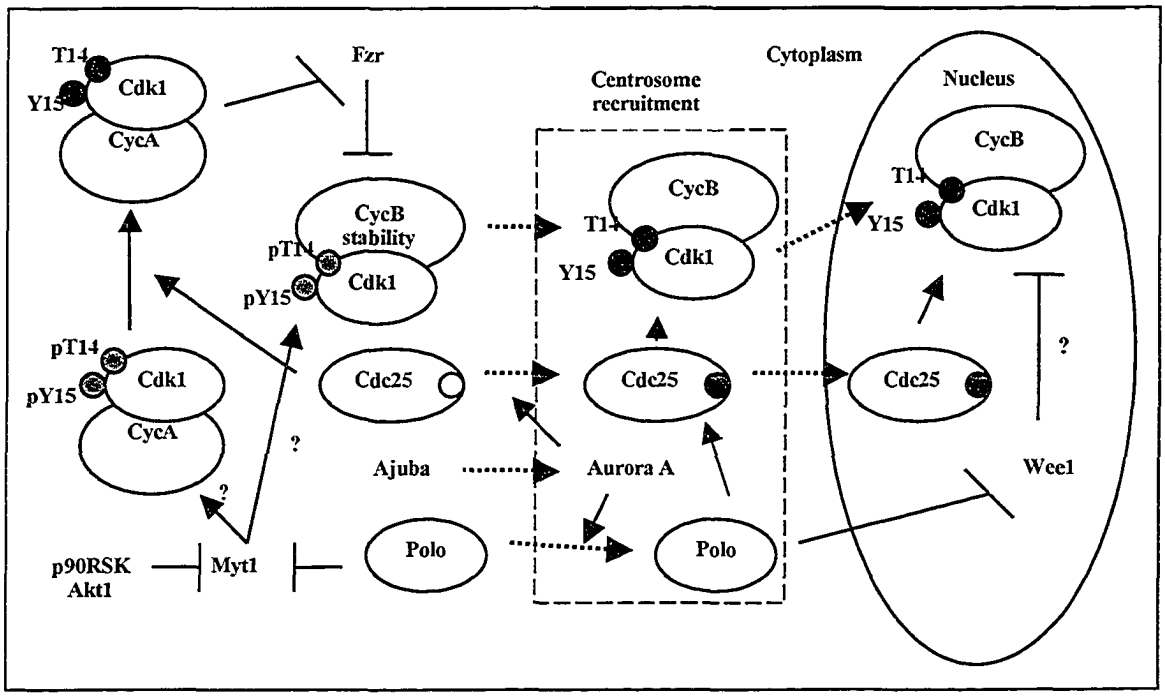


Fig. 1-1 A scheme illustrating established pathways leading to the activation of Cdk1/Cyclin B at the G2/M transition. Myt1/Wee1 kinases keep Cdk1/Cyclin A/B complexes inactive via inhibitory phosphorylation of T14 and Y15 of Cdk1 during G2 phase. Upon entry into mitosis, T14 and Y 15 phosphorylation on Cdk1 is reversed by down-regulating Myt1/Wee1 activity and/or stability and up-regulating Cdc25 activity. Extrinsic or intrinsic cues inactivate Myt1/Wee1 and promote their instability. Cdk1/Cyclin A can inhibit Fzr mediated Cyclin B degradation and thereby facilitate the accumulation of Cdk1/Cyclin B complexes. Centrosomes serve as a center to recruit Cdk1/Cyclin B, and its activators such as Cdc25, Polo for the activation of Cdk1/Cyclin B in the cytoplasm. Aurora A appears to promote Cdk1 activation by enhancing centrosome recruitment of Cdc25 and Polo. Once a small pool of Cdk1/Cyclin B complexes are activated, they further activate Cdc25 and Polo and inactivate Myt1/Wee1, generating positive feedback loops that are required for the none-or-all switch for Cdk1/Cyclin B activation. Due to space limitations, these feedback loops are not indicated in the scheme. An arrow with a solid line and a bar with a solid line indicate positive and negative effect on a target or a process, respectively. An arrow with a dotted blue line means centrosome recruitment, whereas an arrow with a dotted green line shows nuclear translocation. Blue text and dotted blue line box are symbols for centrosome recruitment of certain proteins. Green and red circles denote positive and negative phosphorylation of the target. Question marks indicate a suggested but unproven effect.

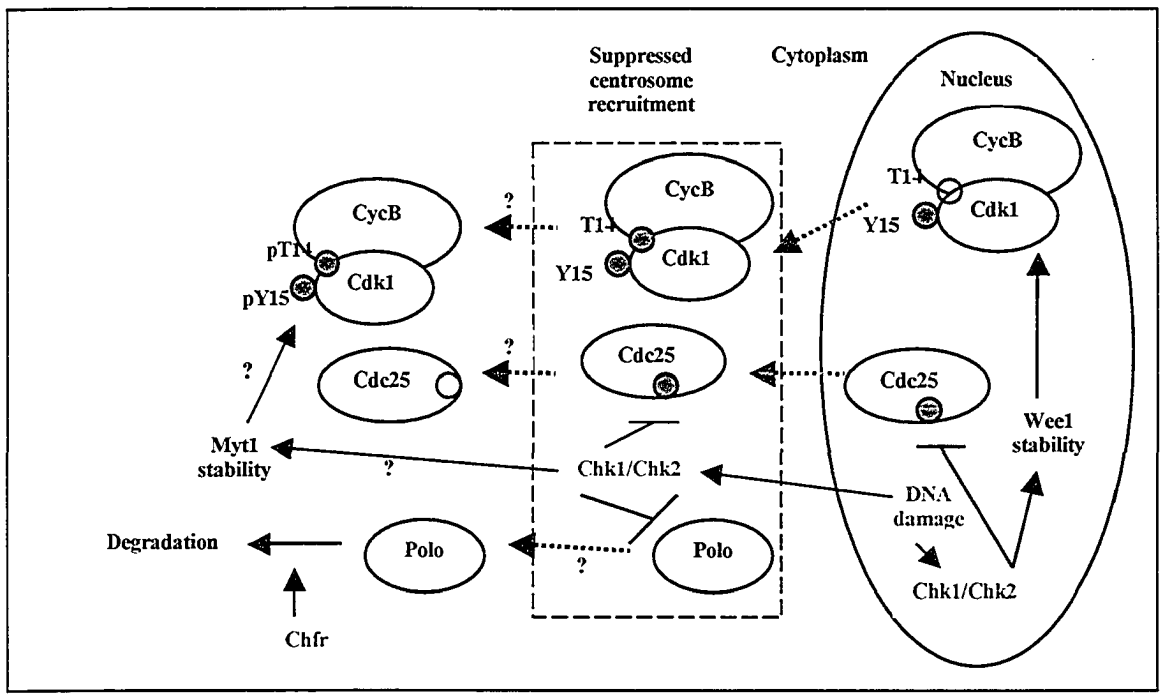


Fig. 1-2 A scheme illustrating the checkpoint pathways that inactivate Cdk1/Cyclin B after DNA damage at the G2/M transition. Once DNA damage is sensed by the cell, a checkpoint mechanism is triggered to activate down stream effectors such as Chk1 and Chk2 in the nucleus or on the centrosome. In the nucleus, Cdc25 is phosphorylated by Chk2 to promote its nuclear export, whereas Y15 of Cdk1 is phosphorylated by Wee1 to suppress its activity. In the cytoplasm, Myt1 may phosphorylate T14 and Y15 of Cdk1 to suppress Cdk1/Cyclin B activation. Both Chk1 and Chk2 can inactivate Cdc25 and/or Polo on the centrosome. Moreover, Chfr functions to promote Polo degradation more efficiently after DNA damage, although we do not know exactly how Chfr is activated in this case. Moreover, Chk1 and/or Chk2 may increase the stability of Myt1/Wee1 after DNA damage. An arrow with a solid line and a bar with a solid line indicate positive and negative effect on a target or a process, respectively. An arrow with a dotted black line means blocked centrosome recruitment, whereas an arrow with a dotted red line shows nuclear export. Red text delineates checkpoint components, whereas red circles denote negative phosphorylation of the checkpoint target. Question marks indicate a suggested but unproven effect.

1.3 Regulation of cell divisions during gametogenesis

During male and female *Drosophila* gametogenesis, a stereotypical developmental sequence of cell cycles takes place, including both mitotic and meiotic cell divisions. Thus, comparative studies can be done between *myt1* mutant testes and ovaries, with regard to similarities and differences in these mitotic and meiotic cycles. According to previous studies in *Xenopus*, Myt1 functions at prophase I to arrest immature oocytes (Palmer and Nebreda, 2000). The activity of Myt1 also needs to be down-regulated at the G2/M transition into meiosis during spermatogenesis in *C. elegans* (Lamitina and L'Hernault, 2002). In *Drosophila*, *myt1* mutants are male sterile but only partially female sterile, suggesting that loss of *myt1* function has different effects during male and female meiosis. To understand these phenotypes in a developmental context, I undertook a phenotypic analysis of *Drosophila myt1* mutants in their reproductive tissues. To familiarize readers with the experimental system, this last section summarizes the general features of gametogenesis in *Drosophila*.

1.3.1 Germline stem cell division and the mitotic cycles of gametogenesis

The term gametogenesis will be used to refer to spermatogenesis and oogenesis in male testes and female ovaries, respectively (Fig.1-3, A, B, page 24). Testes are composed of two curved tubes (Fuller, 1993), whereas ovaries are formed by two sets of 15-17 ovarioles (Spradling, 1993). Each ovariole has a germarium at the tip and a string of egg chambers following the germarium. As in most other animals, spermatogenesis and oogenesis in *Drosophila* begin with a small population of germline stem cells (GSCs) undergoing asymmetric cell division, renewing themselves while generating gonialblasts (or primary spermatogonia) in males and cystoblasts (also known as primary oogonia) in females, as daughter cells. The gonialblasts or cystoblasts then undergo four rounds of incomplete mitoses to give rise to 2, 4, 8, 16-cell inter-connected secondary spermatogonia or oogonia. Up to this point, the cell division cycles are considered to be similar in males and females. For instance, the cytoplasmic form of Bam (*bag of marbles* gene product) is expressed in both secondary spermatogonia in males and secondary oogonia in females (Gonczy et al., 1997; McKearin and Ohlstein, 1995). Moreover,

mutations in *bam* block the differentiation of both spermatogonia and oogonia, leading to a germline tumor phenotype (Gonczy et al., 1997; McKearin and Spradling, 1990).

1.3.2 Regulation of Meiosis in *Drosophila* males and females

In males, secondary spermatogonia switch their developmental program after 4 synchronous mitotic divisions, differentiating into 16-cell primary spermatocytes which are then destined to execute meiosis and spermatid differentiation (reviewed in Fuller, 1998). In *Drosophila*, spermatid differentiation follows immediately after male meiosis, consequently G2 phase of male meiosis is extended to ~90 hr to allow time to synthesize transcripts and proteins needed for spermatid differentiation. The translation of germline-specific $Cdc25^{twine}$ protein, which is required for meiosis in both males and females (Alphey et al., 1992; Courtot et al., 1992), is turned on by one or more transcripts that are synthesized along with those needed for spermatid differentiation, thereby coupling male meiosis to spermatid differentiation (White-Cooper et al., 1998). Genes such as *aly* (*always early*), *mia* (*meiosis I arrest*), *ca* (*cannonball*) and *sa* (*spermatocytes arrest*) are instrumental for this coupling mechanism. Mutations in these genes cause a similar phenotype, characterized by the absence of meiosis and spermatid differentiation in the testes. Meiosis is not a prerequisite for spermatid differentiation however, as the latter takes place when only meiosis is blocked (Courtot et al., 1992; White-Cooper et al., 1993), suggesting that spermatid differentiation can be independent of meiosis (Fuller, 1998).

In females, oocyte differentiation begins simultaneously with commitment to meiosis. Immediately after the completion of 4 synchronous mitotic cycles and pre-meiotic S phase, the formation of a synaptonemal complex can be seen in at least two pre-oocytes among the 16-cell secondary oogonia, arrested at prophase I of meiosis (Manheim and McKim, 2003; Page and Hawley, 2001). Through a poorly understood process, one pre-oocyte is then selected as the oocyte and continues meiotic–specific events, while the other pre-oocyte exits from meiotic prophase I and assumes a nurse cell fate along with the remaining cells in the cyst (reviewed in McKim et al., 2002). After the formation of the synaptonemal complex, DSBs are made in each pair of homologous chromosomes to facilitate meiotic recombination at the pachytene stage of meiosis in the

oocyte. Normally, these DSBs are under the surveillance of a pachytene checkpoint mechanism and will be repaired before the oocyte can develop further. All of these changes in the oocyte are finished in the middle/posterior end of the germarium at the tip of the ovariole, however the resumption of meiotic division I and chromosome segregation does not start until approximately 7 days later in the stage 14 oocyte, indicating that prophase I arrest lasts ~7 days in *Drosophila* female oocytes. In immature *Xenopus* oocytes, a Mos-mediated mitogen-activated protein kinase signaling pathway (Nebreda and Hunt, 1993; Posada et al., 1993; Shibuya and Ruderman, 1993) operates at prophase I to down-regulate Myt1 kinase activity and thus initiate entry into meiosis I (Palmer et al., 1998). However, a recent study of *Drosophila* Mos suggested that there are alternative mechanisms for initiating meiosis, because oocytes could enter meiosis even without Mos activity (Ivanovska et al., 2004).

Although the development of 16-cell secondary spermatogonia and oogonia proceed in very different manners, both male and female meioses have a relatively long "G2-like" prophase I arrest similar to that described in *Xenopus* immature oocytes. These two parallel systems provide a useful model for testing if Myt1 activity is important for "G2-like" prophase I arrest of meiosis in *Drosophila.*

1.3.3 Somatic cell divisions and cell lineage during gametogenesis

In the *Drosophila* testes, developing germline cells (GSCs) are in intimate contact with germline-associated somatic cells. Each GSC is enveloped by two progenitor cyst cells (or somatic stem cells), which divide to renew themselves and produce daughter cyst cells, two of which associate with each gonialblast (see Fig. 1-3, A, page 24). After two cyst cells encompass each gonialblast, these cells no longer divide but differentiate as they remain with the derivative secondary spermatogonia and primary spermatocytes formed as the cyst develops. The descendents of the gonialblasts are referred to as a cyst, containing 2, 4, 8 or 16 cells.

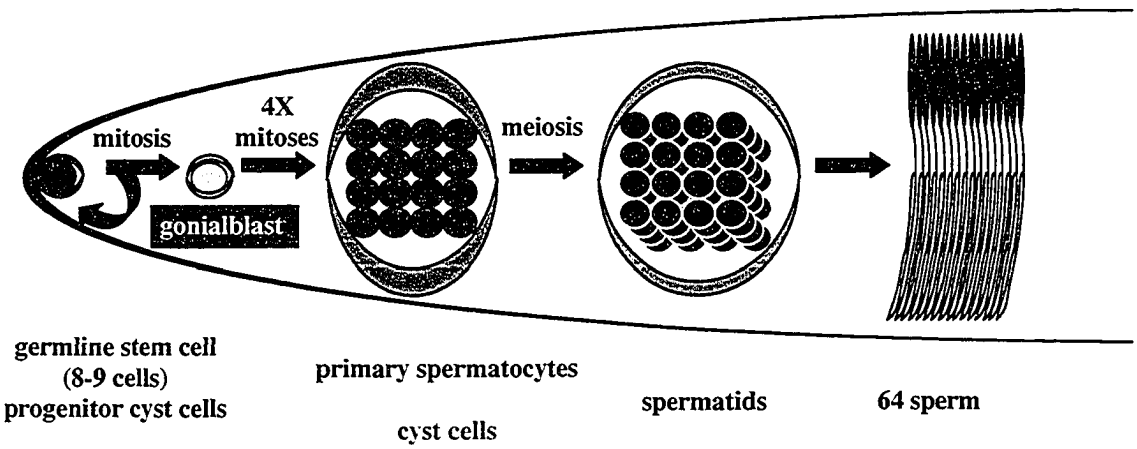
During oogenesis, unlike *Drosophila* spermatogenesis, the GSCs, cystoblasts and 2-8 cell cysts are not encased by somatic stem cells. The 16-cell cysts do become surrounded by pre-follicle cells (also known as follicle cell progenitors) however, which are generated by 2-3 somatic stem cells (SSCs) residing in the middle of the germarium

(see Fig. 1-3, B). Once a 16-cell cyst reaches the end of the germarium together with its overlying pre-follicle cells, it forms a ball-shaped structure known as a stage 1 follicle (or egg chamber). By this time, oocyte and nurse cell fate have been determined in the germline cells and the oocyte has finished meiotic recombination and DSB repair (Jang et al., 2003).

As the egg chamber becomes mature and moves along the ovariole towards the posterior, different developmental programs unfold in the pre-follicle cells and germline cells (Bai and Montell, 2002; Margolis et al., 1995; Tworoger et al., 1999). Specifically, two subtypes of pre-follicle cells, i.e., stalk cells and polar cells, cease cell proliferation after the stage 1 egg chamber budding off from the germarium; whereas the remainder of the pre-follicle cells are now called epithelial follicle cells and continue to divide 3-4 times until the egg chamber reaches stage 6 (see Fig. 1-4, A, page 26). Simultaneously, the 15 nurse cells begin endoreplicating to become polyploid. Thereafter, all of the pre-follicle cells exit the cell cycle and differentiate into three major groups: the anterior group, main body group and posterior group. At the anterior end of a stage 6 or older egg chamber (starting from the differentiated pair of anterior polar cells onward), there are border cells (6-9 cells), stretched cells and centripetal cells (see Fig. 1-4, B, page 26). At the posterior end of these egg chambers, a group of follicle cells are set aside, surrounding the posterior polar cells. Follicle cells that are located between the anterior and posterior groups of follicle cells are referred to as main body follicle cells, which then undergo genome endoreplication and chorion gene amplification during the remainder of oogenesis.

Compared to our more advanced knowledge of the developmental control during *Drosophila* spermatogenesis and oogenesis, less is known regarding regulation of the cell cycle at these stages. Moreover, even less is clear is how the germline and somatic cell divisions in testes and ovaries occur in concert with key developmental transitions. Considering that Wee1-like kinases are involved in developmental regulation of the cell cycle in *Drosophila* and *Xenopus* embryos (Leise and Mueller, 2004; Murakami et al., 2004; Price et al., 2000), phenotypic analysis of *myt1* mutants for defects in gametogenesis may provide insights into the function of this atypical Wee1-like kinase in a different developmental context.

A. Spermatogenesis



B. Oogenesis

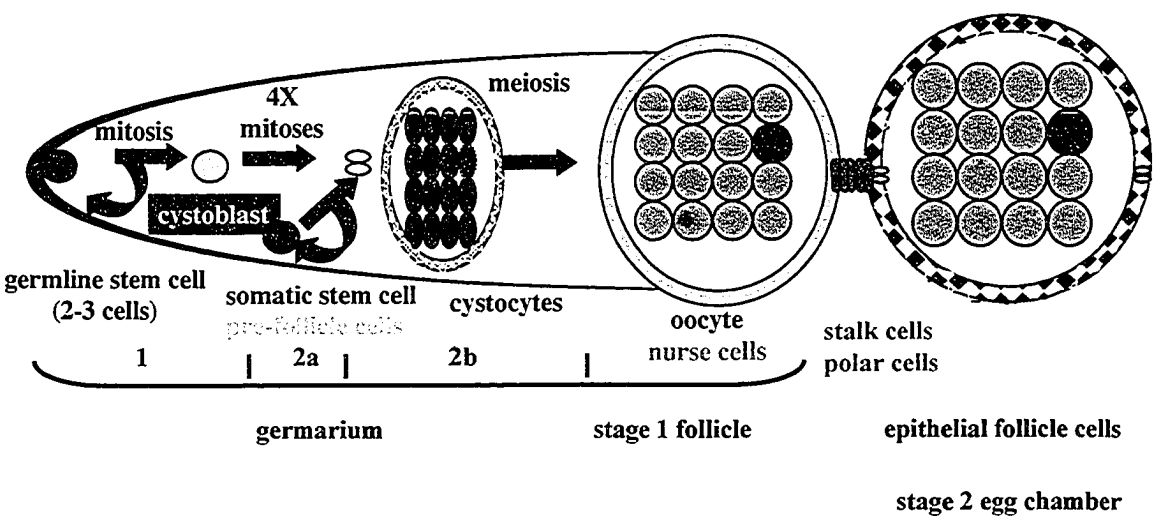
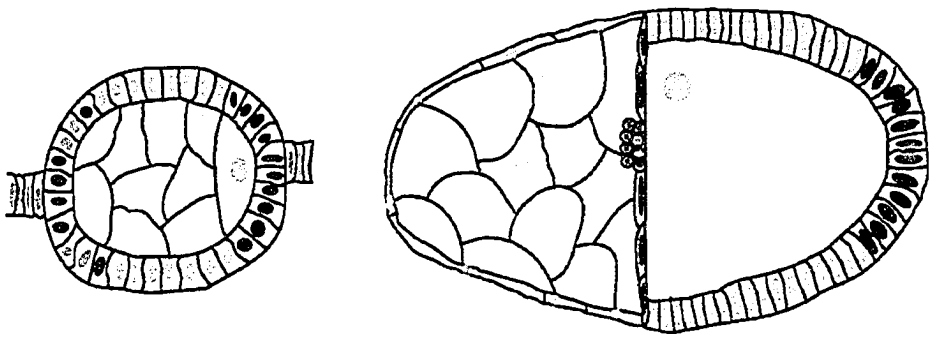


Fig. 1-3. Schematic diagrams showing different cell cycles during spermatogenesis (A) and oogenesis (B) in *Drosophila*. The testis apical end (A) or germarium (B) are arranged with anterior towards the left. Equivalent cell types in testes (A) and ovaries (B) are color coded to match the text boxes as following: germline stem cells (light green), gonialblast/cystoblast (yellow), progenitor cyst cells/somatic stem cells (light red), primary spermatocytes/cystocytes and oocyte (light brown). Male-specific cell types are cyst cells (blue) and spermatids (black). Female-specific cell types are pre-follicle cells (pink), stalk cells (cyan), polar cells (dark red), epithelial follicle cells (dark green) and nurse cells (purple). See text section 1.3 for details.



A. Stage 6 egg chamber **B. Stage 10 egg chamber**

Fig. 1-4. Types of differentiated follicle cells in developing egg chambers. Stage 6 (A) and stage 10 egg chambers (B) with their anterior end towards the left. The arrangement and fates of different cells are indicated by another set of colors of their nuclei: oocyte (dark brown), nurse cells (light brown), main body follicle cells (gray), polar cells (red), stalk cells (light blue), outer border cells (green), stretched cells (pink), centripetal cells (dark blue) and posterior terminal cells (black). (Adapted from Grammont, M. et al. Development 2002;129:5131-5140).

1.4 Concluding remarks

Since *wee1* mutants were initially identified in *S. pombe*, analyses of Wee1-like kinases in several different model organisms have greatly advanced our understanding of these important negative cell cycle regulators. As an atypical Wee1-like kinase, Myt1 is not found in unicellular organisms but coexists with other Wee1-like kinases in metazoans. To date, functional studies of Myt1 protein kinases have been hampered by a lack of mutants in these organisms. With a single Myt1 and Wee1 in its genome and the powerful genetic tools available in this organism, *Drosophila* appears to be a good choice to study the function of Wee1 and Myt1 kinases in metazoans. Previously, *Drosophila wee1* mutants were identified and characterized by a former student in our lab, Don Price. As a complementary project, my goal was to identify and analyze *myt1* mutants in *Drosophila melanogaster*, addressing the question of whether Myt1 has a function that is distinct from Wee1. More importantly, the availability of both *wee1* and *myt1* mutants in the same organism makes it possible to assess to what extent these two Wee1-like kinases are functionally redundant, since many of the ambiguities described above may stem from such functional redundancy. *Drosophila*, with the possibility of unmasking the redundancy of Wee1-like kinases, as well as a wealth of literature about development in this organism, should contribute to our understanding of how Myt1 and Wee1 are involved in developmental control of the cell cycle. Because these kinases are evolutionarily conserved among metazoans, what is learned from *Drosophila* may apply to other metazoans including humans.

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Chapter 2 The Cdk1 inhibitory kinase Myt1 regulates both mitotic and meiotic cell cycles during *Drosophila* gametogenesis

2.1 Introduction

During *Drosophila* embryonic development, the timing of mitosis is both spatially and temporally regulated, to prevent interference with morphogenetic cell movements (Edgar and O'Farrell, 1989; Foe, 1989; Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). As in other eukaryotic organisms, the molecular mechanism controlling mitosis involves a conserved cyclin-dependent kinase called Cdk1 (O'Farrell, 2001). During most of the cell cycle, Cdk1 is inactivated by inhibitory phosphorylations, which prevents premature initiation of mitotic events. Cdk1 activity is triggered at the G2/M transition by the expression of *Cdc25^{stg}* phosphatase, a regulatory mechanism that integrates developmental processes with cell cycle behavior (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). Two Cdk1 inhibitory kinases have been identified in *Drosophila*: dWee1 and dMyt1 (Adams et al., 2000; Campbell et al., 1995). dWee1 is a nuclear kinase required for regulating Cdk1 during the rapid S/M nuclear divisions of early embryogenesis (Price et al., 2000; Stumpff et al., 2004). dWee1 phosphorylates a specific tyrosine residue (Y15) of Cdk1, as do other nuclear Wee1 kinases (Campbell et al., 1995; Heald et al., 1993; Parker et al., 1995). In contrast, dMyt1 is cytoplasmic, being localized to Golgi and endoplasmic reticulum membranes (Z. Jin, unpublished), like *Xenopus* and human Myt1 kinases (Booher et al., 1997; Liu et al., 1997; Mueller et al., 1995). Myt1 kinases have been characterized as dual specificity Cdk1 kinases: phosphorylating a threonine (T14) residue of Cdk1 in addition to the Y15 site that is a shared target with Wee1 kinases (Booher et al., 1997; Liu et al., 1997; Mueller et al., 1995).

These localization and Cdk1 target site differences suggest that Wee1 and Myt1 have evolved distinct regulatory roles; a hypothesis supported by our previous characterization of *wee1* maternal effect lethal mutants (Price et al., 2000). Transgenic over-expression and RNAi experiments also suggested that dMyt1 acts principally during G2 phase of the cell cycle, however direct evidence that dMyt1 has specific Cdk1 regulatory functions *in vivo* has been lacking (Cornwell et al., 2002; Price et al., 2002). The functional analysis of dMyt1 reported here suggests that this Cdk1 inhibitory kinase serves several distinct cell cycle regulatory functions required for both mitotic and meiotic cell cycles during male and female gametogenesis, implicating dMyt1 as a key

male and female gametogenesis, implicating dMyt1 as a key component of the molecular mechanism that coordinates cell cycle behavior with critical developmental events.

2.2 Materials and methods

2.2.1 Generation and identification of *myt1* mutants

The *myt1* locus is at region 64F of the 3rd chromosome, corresponding to predicted gene *CG10569* (Adams et al., 2000). I used a chromosomal deletion called *Df(3L)64D-F* for a genetic screen, that was predicted to uncover the *myt1* locus and ~25-30 other genes (Garcia-Bellido et al., 1994). I confirmed that this deletion removed the *myt1* locus by complementation tests with lethal *P*-element alleles of genes flanking the gene (Figure 2-1B) before undertaking an F2 genetic screen (more details of mutant screen can be found in appendix) for mutants uncovered by *Df(3L)64D-F* that could be rescued by a transgenic *myt1* rescue construct: *P{myt1+}*, described below. Males from an isogenized p^p e^l stock were fed a 10 mM solution containing the mutagen ethyl methane sulfonate (EMS), then mated with females carrying a third chromosome balancer. The heterozygous F1 male progeny were then crossed to females carrying *Df(3L)64D-F* and the hemizygous non-balancer F2 progeny were scored for morphological defects, lethality and female sterility. To make the *P{myt1+}* rescue construct, a 3.6Kb SacI-EcoRI fragment, including the entire *myt1* gene and approximately 1 kb of upstream and downstream non-coding DNA, was sub-cloned from a P1 clone (DS04757) into the *pCaspeR* transposon vector (Pirrotta, 1988). Transgenic lines carrying the integrated construct were then generated by standard procedures (Spradling, 1986).

2.2.2 Molecular analysis of the *myt1* mutant alleles

To sequence genomic DNA, single flies of the appropriate genotype were homogenized in a tube with 50μl buffer (100 mM Tris-Cl pH 7.6, 100 mM EDTA, 100 mM NaCl and 0.5% SDS). Proteinase K was added to a final concentration of 100 μg/ml, and the samples were incubated at 37°C for 30 minutes. 1μl of genomic DNA isolated by this procedure was then used as a template for DNA sequencing (Amersham), using oligonucleotide primers positioned at 300-400 bp intervals on both template strands. The

sequencing reactions were repeated three times with genomic DNA isolated from different flies of the same genotype, for confirmation.

2.2.3 Phenotypic analysis of the *myt1* mutants and *hs-Cdk1AF* expression experiments

I used *myt1*^{1}*/Df(3L)64D-F* hemizygotes as the representative *myt1* mutants for the phenotypic analysis and their *myt1*^{1}*/TM3,Sb* heterozygous siblings, as controls. For testes preparations, 1 or 2-day old male flies were dissected in 1X PBS or in testis buffer: 183 mM KCl, 47 mM NaCl, 10 mM EDTA (Casal et al., 1990a) plus 1 mM PMSF, then rinsed twice with the same buffer. Established protocols were followed for the testes and ovariole immuno-fluorescent localization experiments (Bonaccorsi et al., 2000; Mattheis et al., 2000). The primary antibodies and concentrations used were: rabbit anti-PH3 (1/2000; Upstate), rabbit anti-anillin at 1/300, (Field and Alberts, 1995), mouse anti-beta-tubulin (1/100; Sigma), mouse anti-BrdU (1/20; Jackson Labs), mouse anti-Hts, clone C17.9C6, obtained from the Developmental Studies Hybridoma Bank (DSHB) at 1/10 (Zaccai and Lipshitz, 1996), rat anti-BamC at 1/2000 (McKearin and Ohlstein, 1995), rabbit anti-Aly at 1/2000(White-Cooper et al., 2000), rabbit (used at 1/500) and rat (used at 1/1000) anti-Vasa (Lasko and Ashburner, 1990), mouse anti-spectrin obtained from DSHB at 1/200 (Dubreuil et al., 1989), mouse anti-Eya obtained from DSHB at 1/100 (Bonini et al., 1993), rabbit anti-Cnn at 1/500 (Heuer et al., 1995), mouse anti-MPM2 (1/200, Cell Signaling) and mouse anti-FasIII obtained from DSHB at 1/5; (Patel et al., 1987). Alexa-488 and Alexa-568 conjugated secondary antibodies (used at 1/1000) were obtained from Molecular Probes, as was rhodamine-conjugated phalloidin. BrdU (5-bromo-2'-deoxyuridine) incorporation was assayed in 1 or 2-day old dissected testes, using published protocols (Wolff, 2000). For the *Cdk1-AF* experiments, 1-2 day old *y w*; *hs-Cdk1AF* transgenic flies and *y w* controls received 1 hour, 37°C heat shocks twice a day for three consecutive days, before examining the testes.

2.2.4 Genetic tests for segregation defects during female meiosis

Since female myt1 mutants are fertile, a genetic cross (cross #1) was set up between *myt1*^{1}*/myt1*^{2} virgins (with *myt1*^{1}*/TM3, Sb* virgins used in a control cross) and *C (1;Y), v, f,*

B ; C(4) RM, ci, ey [R] males to detect non-disjunction (NDJ) events during meiosis. In this cross, non-disjunction of homologous chromosomes was recognized by scoring for exceptional progeny: X chromosome NDJ was represented by females with normal eyes or *v,f, B* males; whereas 4th chromosome NDJ was scored as adults expressing the recessive *ci, ey[R]* phenotype. The formula for the calculation of NDJ frequency is: 2x exceptional progeny/ (2x exceptional progeny + regular progeny) for each chromosome.

To determine at which meiotic division(s) the X chromosome NDJ occurred in *myt1* mutant females, *FM7, y, B/y; myt1^{1}/myt1^{2}* females were crossed to *y+* males (cross #2). NDJ at meiosis I would produce *FM7,y, B/y* or nullo-X eggs, whereas NDJ at meiosis II would result in either *FM7,y,B/FM7,y,B, y/y* or nullo-X eggs. X-chromosome NDJ events resulting in viable progeny were scored as adult females with Bar eyes and yellow bodies, or double-Bar eye (two copies of the *B* allele), yellow females or females with normal eyes and yellow bodies. The first class is meiosis I NDJ-specific, whereas the latter two classes are meiosis II NDJ-specific progeny.

2.3 Results

2.3.1 Molecular and genetic characterization of *Drosophila myt1* mutants

Drosophila myt1 is predicted to encode a 61 kDa protein (533 amino acids). Within the kinase domain, dMyt1 shares 49% and 47% amino acid sequence identity with *X. laevis* and *H. sapiens* Myt1 homologs (Fig. 2-1A, purple bar), and 31% sequence identity with dWee1 in the same region (Campbell et al., 1995). dMyt1 also shares two conserved domains with other Myt1 kinases, that are not present in nuclear Wee1 kinases (Lamitina and L'Hernault, 2002; Liu et al., 1997; Wells et al., 1999): a potential trans-membrane domain (Fig. 2-1A, green bar) and a C-terminal putative Cyclin B interaction motif (Fig. 2-1A, brown bar).

I isolated two *myt1* alleles (*myt1^{1}* and *myt1^{2}*) in a genetic screen for hemizygous mutants with phenotypic defects that could be rescued by a *P{myt1^{+}}* transgene (Fig. 2-1B, Materials and Methods). The original *myt1^{1}* and *myt1^{2}* alleles had markedly different viability as hemizygotes. This difference was removed by out-crossing and was thus due to secondary lesions. Genomic sequencing of *myt1^{1}* and *myt1^{2}* identified identical mutations in both alleles, a single nucleotide deletion at position 514 (amino acid 173).

This mutation is predicted to cause a frame-shift alteration in the protein sequence, followed by a premature stop codon at nucleotide 689 (amino acid 232). This would truncate the protein near the beginning of the kinase domain and delete the conserved sequence motifs near the C-terminus of the protein (Fig. 2-1C), suggesting that these are likely null alleles. *myt1* mutants (either $myt1^{1}/Df(3L)64D\text{-}F$ or $myt1^{1}/myt1^{2}$) are viable but exhibit bristle defects affecting the dorsal thorax, head and eye (see chapter 4, Fig. 4-8) and are male sterile, also implying by genetic criteria that these are null alleles (Muller, 1932). The phenotype of *myt1* mutant females is less clear-cut: although *myt1* females are fertile, I observed variable maternal effect lethal embryonic phenotypes in their progeny. Since male sterility is the most consistent mutant phenotype, I initially focused on analyzing spermatogenesis in *myt1* mutants.

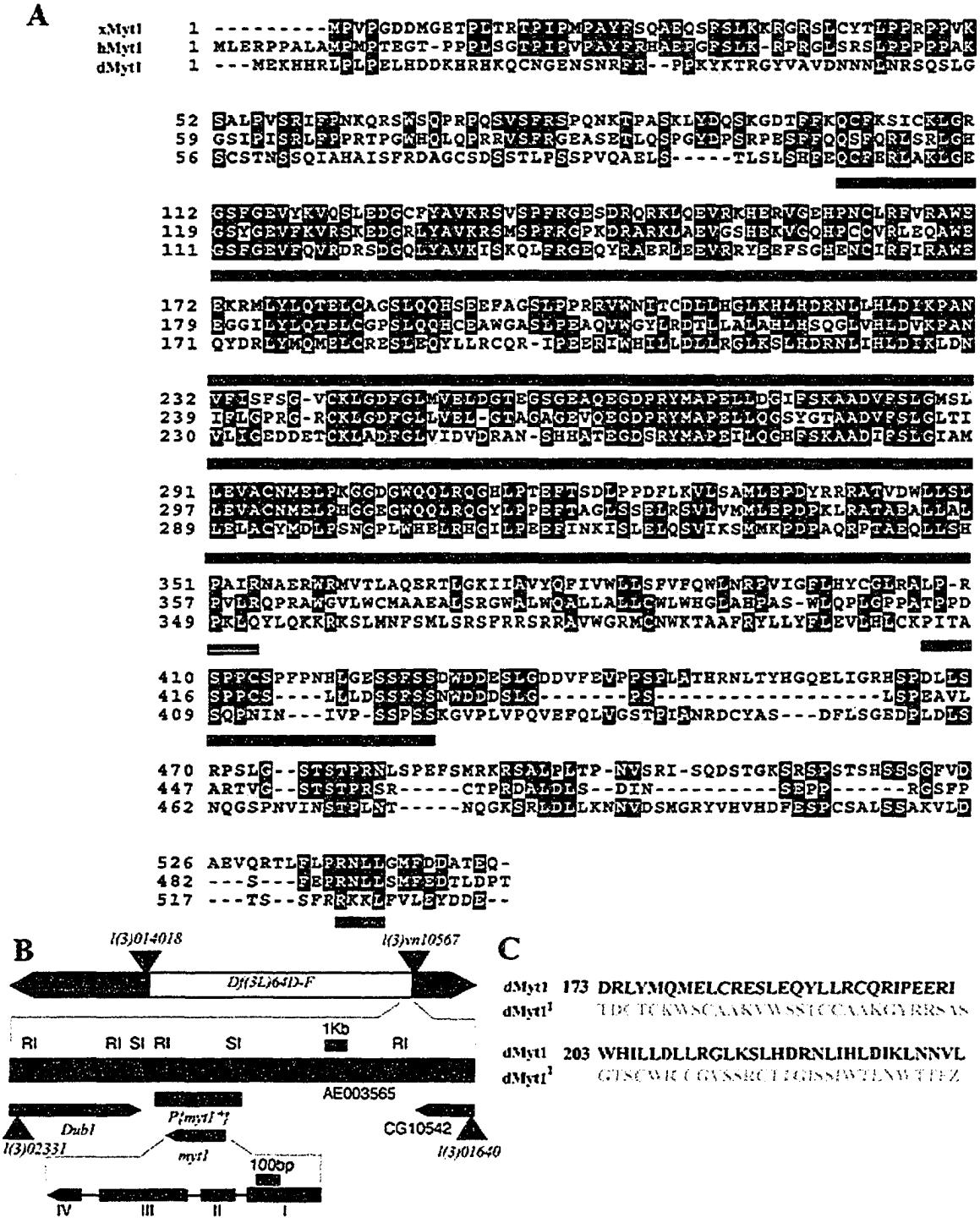

Fig. 2-1 Genomic and molecular analysis of *myt1*. (*A*) An alignment of Myt1 sequences from *Xenopus laevis*, *Homo sapiens* and *Drosophila melanogaster*. Black shading indicates identical amino acids and gray shading highlights similar amino acids. The dMyt1 protein sequence contains a kinase domain (purple bar), a potential trans-membrane domain (green bar) and a predicted C-terminal Cyclin B interaction motif (brown bar). (*B*) Physical and genetic map of *myt1* and flanking genes uncovered by *Df(3L)64D-F* (white box). Black triangles indicate the position of the nearest lethal *P*-element insertions that complement *Df(3L)64D-F*. The black bar showing restriction enzyme sites (RI: *EcoRI*; SI: *SalI*) represents a 24 kB DNA sequence (coordinates 240,000 to 264,000) in AE003565, a genomic BAC clone. The narrow black bar represents the DNA fragment that was sub-cloned to generate $P\{myt1^{+}\}$. Black arrow bars represent transcripts of $myt1^{+}$ and the nearest flanking genes. Roman numerals indicate exons I to IV of the *myt1* gene. (*C*) A single base deletion mutation in $myt1^{1}$ (and $myt1^{2}$) causes a frame-shift, producing an altered amino acid sequence (red letters) beginning at amino acid position 173 and ending with a premature stop codon at position 232.

2.3.2 Mitotic cell proliferation defects during *myt1* mutant spermatogenesis

Male germline development begins with stem cell divisions that generate gonialblasts, which then undergo four synchronous mitotic divisions to produce cysts of 16 cells (Fuller, 1993). After completion of these mitotic cell divisions, the germline cells differentiate as primary spermatocytes. These cells remain arrested in G2 phase for ~90 hr before undergoing meiotic divisions to produce cysts containing 64 syncytial spermatids that differentiate into mature sperm. To analyze how loss of dMyt1 function affects these cell divisions, I used an antibody that recognizes a phosphorylated form of histone H3 (PH3) as a marker for mitotic or meiotic cells (Kiger et al., 2000). In control testes, small numbers of mitotic cells were usually seen near the tip of the testis (Fig. 2-2A, white arrow). More distally along a control testis, one often observes a single PH3-positive meiotic cyst (Fig. 2-2A, blue arrow). I observed a striking increase in the numbers of mitotic cells near the tip of the testes in *myt1* mutants (Fig. 2-2B, white arrow), as well as cysts apparently undergoing meiosis (Fig. 2-2B, cyan arrows). I also observed isolated PH3-positive cells along the length of *myt1* mutant testes (Fig. 2-2B, green arrows), as well as PH3-positive cells at the distal end of the testes (yellow arrow), that were never seen in controls (Fig. 2-2A). These cell proliferation defects were suppressed, male fertility was restored and the adult bristle phenotype was rescued when a *P{myt1$^+$}* transgene was introduced into the *myt1* mutant background, confirming that these mutant phenotypes were all due to a loss of dMyt1 activity.

To determine if there were other proliferation defects observable in the *myt1* mutants, I used BrdU incorporation to assay for DNA replication in short-term (30 min) cultures of dissected testes. BrdU incorporation was only seen in the cells near the tip of the testes in the controls, implying that pre-meiotic S phase was essentially complete by the time the spermatocyte cysts move away from the tip (Fig. 2-3A). I observed a marked increase in BrdU-incorporating cells near the apical tip of the testes in the *myt1* mutants, relative to controls (Fig. 2-3A, B). These observations and the cell division data could be explained if cells in the *myt1* mutants undergo faster cell cycles or if they continue to cycle instead of undergoing developmental cell cycle arrest. Alternatively, delays during S phase and mitosis could be responsible.

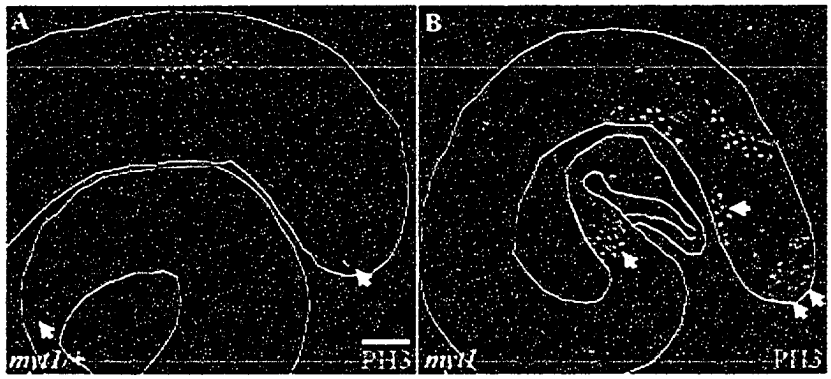

Fig. 2-2 Cell proliferation defects observed in *myt1* mutant testes. (A) Phospho-histone H3 (PH3, red) antibody staining of *myt1*/+ controls, shows mitotic cells at the apical tip (white arrow) and a cyst of meiotic cells (cyan arrow) farther along the testis. No additional cells were labeled at the distal end (yellow arrow). (B) PH3 staining of mitotic cells (white arrows) at the apical tip of the testes in *myt1* mutants and, in this example, 2 cysts of meiotic cells (cyan arrows). Isolated mitotic cells (green arrows) were also found along the testis as well as a large cluster of labeled cells at the distal end (yellow arrow). Bar: 20 μm.

2.3.3 Loss of *myt1* activity affects the developmental switch from mitotic to meiotic cell cycles

Previously, male-sterile mutants were described with similar overproliferation defects that were caused by problems with germline stem cells (GSC) or spermatogonia failing to differentiate and continuing to undergo mitotic cell cycles (Gonczy et al., 1997; Kiger et al., 2000; Matunis et al., 1997; Tulina and Matunis, 2001). To determine if the proliferation defects observed in *myt1* mutants might be attributable to similar defects, I used established cell fate markers to examine germline stem cells, spermatogonia and spermatocytes. GSCs in testes can be identified by positive-Vasa (the gene product of *vasa*) staining and direct contact with somatic hub cells, which are marked by FasIII staining (Gonczy and DiNardo, 1996; Lasko and Ashburner, 1990; Patel et al., 1987). The *myt1* mutants have normal numbers of germline stem cells (Fig. 3B, E). There was, however, a significant increase in secondary spermatogonial cells marked by antibodies against BamC (a cytoplasmic form of *bag of marbles* gene product) in the *myt1* mutants (Fig. 2-3C, F), relative to controls (Gonczy et al., 1997; Ohlstein and McKearin, 1997). This could occur if the secondary spermatogonia undergo extra round(s) of cell division(s), in which case I would expect PH3-positive spermatogonial cysts with 16 or more cells.

As one test of this possibility, I performed BamC and PH3 co-localization experiments. In controls (Fig. 2-4A) I never observed cysts containing more than 8 cells that were both BamC and PH3-positive, consistent with spermatogonia only undergoing 4 mitotic divisions. In contrast, approximately 30% of the *myt1* mutant testes examined (N= 20) contained at least one 16-cell cyst that was both BamC and PH3-positive (arrow, Fig. 2-4B), implying that spermatogonia can undergo an extra round of cell division. Since BamC is weakly expressed in primary spermatocytes, I also used antibodies against Aly (the gene product of *always early*) as a complementary marker for identifying primary spermatocytes (White-Cooper et al., 2000). I was unable to do Aly/PH3 co-localization with the available antibodies for technical reasons, so I used monoclonal antibodies against MPM2 (mitotic phospho-proteins monoclonal antibody 2) as an alternative marker for mitotic cells (Westendorf et al., 1994). MPM2-positive and Aly-negative cysts with 8 cells or less could be seen in controls (Fig. 2-4C, C', C"). In ~30%

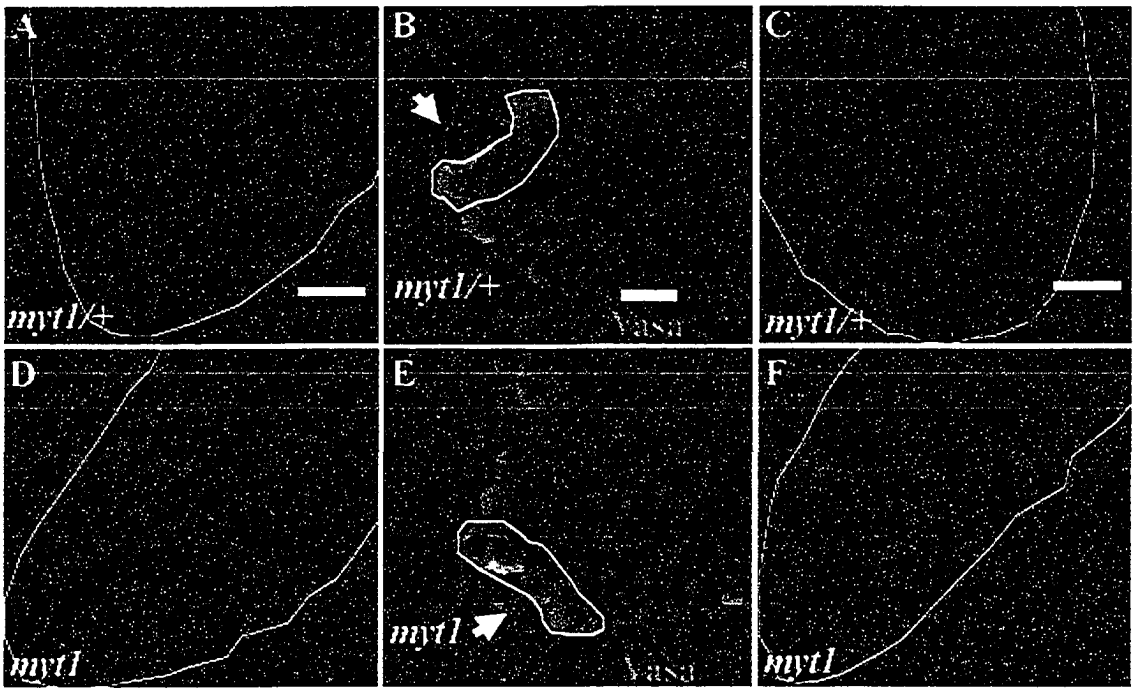

Fig. 2-3 Increased numbers of *myt1* mutant secondary spermatogonia. (A, D) BrdU-incorporation labels S phase cells (green), showing that more cells are labeled in *myt1* mutant testes (D) than in controls (A). (B, E) FasIII labeling of hub cells (green, arrow) and Vasa germline-specific staining indicates the position of germline stem cells (outlined), showing that there are similar numbers of stem cells in *myt1* mutants (E) and controls (B). (C, F) BamC (green) staining of secondary spermatogonia shows more spermatogonia in *myt1* mutants (F) than in controls (C). Size bars for (A, C, D, F): 16 μm, bar for (B, E): 8 μm.

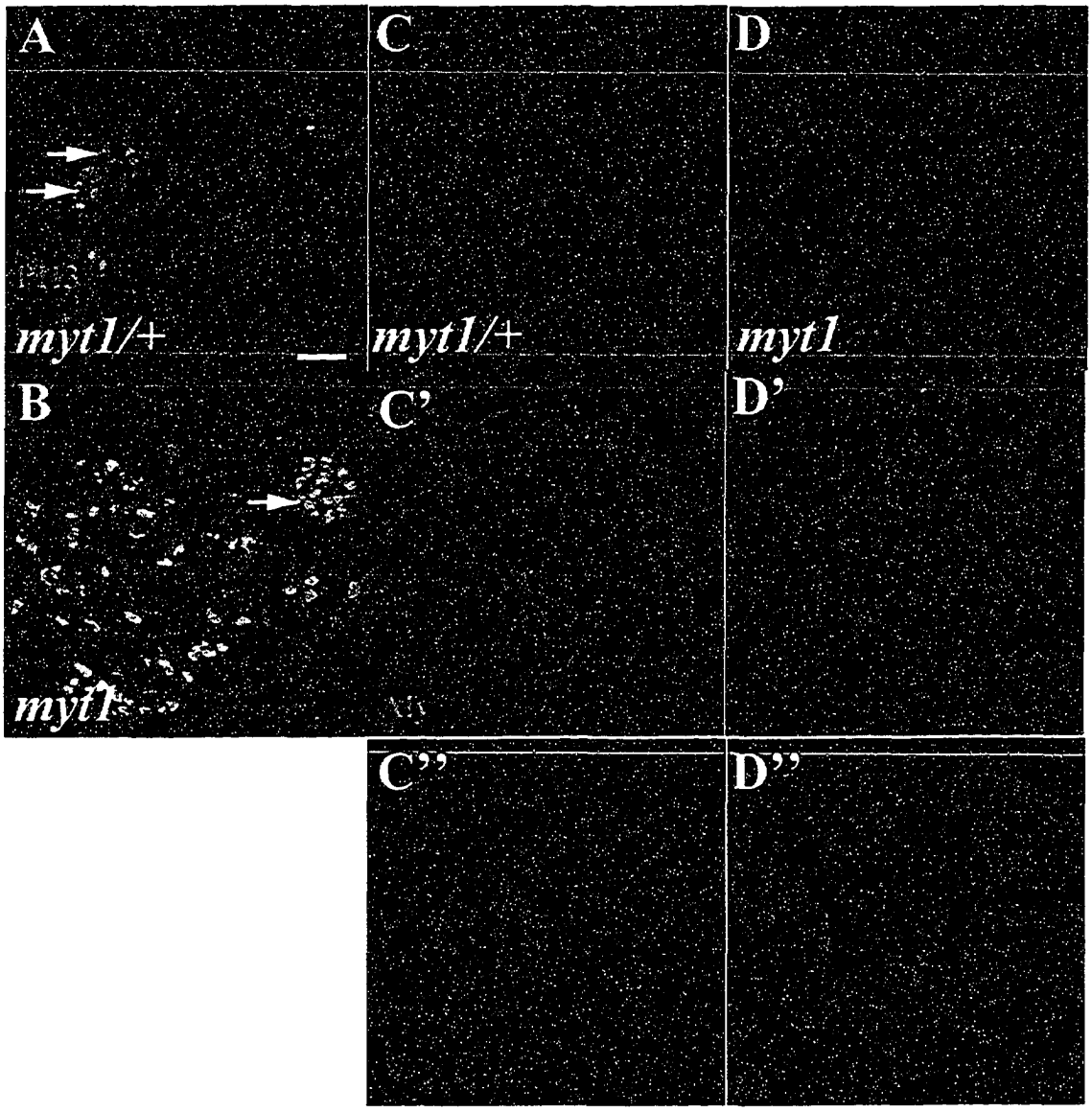

Fig. 2-4. Secondary spermatogonia can undergo an extra round of mitosis in *myt1* mutants. (A, B) Testes stained with anti-BamC (green) and anti-PH3 (red) antibodies. In the controls, cysts of dividing secondary spermatogonia do not contain more than 8 cells (A, white arrows), whereas the *myt1* mutant also contains a 16-cell cyst (B, white arrow), indicating an extra cell division. There were also many scattered PH3-positive cells not co-stained with BamC but intermingled with BamC-positive cells, in the *myt1* mutants. These were probably ectopically dividing somatic cells (see Figure 5, for explanation). (C, C', C'') and (D, D', D'') Controls and *myt1* mutant testes, respectively, showing DNA staining in blue (C, D), Aly staining in red (C', D') and MPM2 staining in green (C'', D''). The green outline delineates MPM2-positive and Aly-negative regions in the testes. In the *myt1* mutants, a 16-cell cyst can be seen (compare C" and D"), indicating ectopically dividing spermatogonial cells. Bar: 20 μm.

of *myt1* mutant testes however (N= 20), one or more MPM2-positive and Aly-negative 16-cell cysts were seen (region outlined in green, Fig. 2-4D, D', D''), confirming that loss of dMyt1 allows some secondary spermatogonia to undergo an extra round of cell division before they differentiate into spermatocytes. The majority of Aly-positive cysts in the *myt1* mutant testes contained only 16 cells however, indicating that most secondary spermatogonia in *myt1* mutants differentiate into primary spermatocytes after the normal developmental program of 4 mitotic divisions.

2.3.4 Germline-associated somatic cells also divide ectopically in *myt1* mutant testes

I next examined whether defects in the behavior of somatic cells might contribute to the *myt1* over-proliferation phenotype. The apical tip of the testes contains somatic stem cells that divide to generate cyst cells whose fate is intimately coupled with male germline development (Gonczy and DiNardo, 1996; Matunis et al., 1997). Two cyst cells associate with each gonialblast and remain associated with its descendants for the remainder of spermatogenesis. These somatic cyst cells do not divide again, implying that they exit the cell cycle as they differentiate. I used antibodies against Eya (the gene product of *eyes absent*) to mark the cyst cells (Bonini et al., 1993; Fabrizio et al., 2003) and observed a striking increase in the numbers of cyst cells in the *myt1* mutants, relative to controls (Fig. 2-5A, E). Because the cyst cells are quiescent, they are PH3-negative in the controls (Fig. 2-5A). In *myt1* mutants however, cyst cells can be double-labeled with antibodies to PH3 and Eya (Fig. 2-5F, white arrows). Cells produced by these ectopic divisions remained associated with germline cysts during meiosis, since Aly-positive cysts with more than two Eya-positive cyst cells were never observed in controls (Fig. 2-5B), but were often seen in the mutants (Fig. 2-5F, arrows). The terminal epithelial cells located at the distal end of the testes also appear to divide ectopically in the *myt1* mutants; since these are also PH3-positive, unlike controls (Fig. 2-2A, B, yellow arrow). These observations further distinguish *myt1* mutants from other types of male-sterile over-proliferation mutants and implicate dMyt1 in a molecular mechanism that promotes cell cycle exit during terminal differentiation.

2.3.5 Meiosis and spermatid differentiation in *myt1* mutant testes

Entry into meiosis is also regulated by inhibitory phosphorylation of Cdk1 in *Drosophila* (Alphey et al., 1992; Courtot et al., 1992). To examine how loss of dMyt1 function affected meiosis, I analyzed primary spermatocytes and their meiotic products by phase contrast microscopy and by immunofluorescence. Using Aly antibodies to label spermatocytes, I confirmed that *myt1* mutants enter meiosis (Fig. 2-5B, F). Several obvious defects were observable in *myt1* spermatocytes, however. Unlike normal spermatocytes (Fig. 2-5C), multi-lobed nuclei with aberrant and in some cases apparently missing nucleoli were seen in *myt1* mutant pre-meiotic spermatocytes (Fig. 2-5G, inset). I observed meiotic cell divisions in both the controls and *myt1* mutants (Fig. 2-5D, H); the examples shown having a morphology characteristic of meiotic telophase (Cenci et al., 1994). Spermatids were also observed in the *myt1* mutant spermatocytes that had unevenly sized nuclei and Nebenkern (compare Fig. 2-5I, M, arrows). These results suggest that *myt1* mutants experience meiotic segregation defects, as seen in other mutants with individualization defects (Casal et al., 1990b; Fabrizio et al., 1998).

Once meiosis is completed, a filamentous-actin (F-actin) based structure called the individualization complex (IC) forms around each spermatid (Fig. 2-5J), separating them into 64 individual sperm (Fabrizio et al., 1998; Noguchi and Miller, 2003; Tokuyasu et al., 1972). I also observed problems in *myt1* mutants during spermatid individualization using phalloidin staining to detect F-actin (Fabrizio et al., 1998) and antibodies to Anillin (Field and Alberts, 1995), as well as DAPI to detect DNA (Fig. 5N). Spermatid nuclei appear scattered in the *myt1* mutants at this stage (Fig. 2-5N, O), rather than tightly bundled as in controls (Fig. 2-5J, K). Although F-actin was clearly associated with spermatid nuclei in the *myt1* mutants, there were usually fewer than 10 Anillin-positive ICs (rather than 64) which appeared to translocate along the spermatid cysts in a disorganized fashion. After individualization, mature sperm translocated into the seminal vesicle in controls and could be visualized by staining for DNA (Fig. 2-5L). In *myt1* mutants, the seminal vesicle appeared to be empty (Fig. 2-5P). Collectively, these observations suggest that dMyt1 is required for normal male meiosis and perhaps also for subsequent spermatid differentiation, in addition to regulating mitotic cell cycles.

2.3.6 *myt1* mutant defects can be phenocopied by expression of non-inhibitable Cdk1

Given the established enzymatic activity of Myt1 kinases, I expected the phenotypic defects of *myt1* mutants to reflect a failure in Cdk1 inhibitory phosphorylation in mutant cells (Mueller et al., 1995). I used a heat shock-inducible, non-inhibitable transgenic allele of Cdk1 (*hs-Cdk1AF*) that can by-pass a developmentally regulated G2 arrest in embryonic germline cells, to test this idea (Su et al., 1998). Expression of *Cdk1AF* caused germline over-proliferation defects similar to those seen in *myt1* mutants (Fig. 2-2B), notably increased PH3-positive cells at the tip of the testes (Fig. 2-6D, E) as well as close to the tip (arrows), relative to heat-shocked controls (Fig. 2-6A, B). I confirmed that some of these PH3-positive cells in the *Cdk1AF*-expressing testes were secondary spermatogonia undergoing an extra round of mitosis (Fig. 2-6E, arrow), by co-staining with BamC antibody. Induction of *hs-Cdk1AF* also phenocopied the ectopic cell division phenotype of somatic cyst cells labeled with Eya (compare Fig. 2-6C, F), similar to what was seen in *myt1* mutants (Fig. 2-5E). These results indicate that dMyt1 regulates proliferation behavior of these cells by inhibitory phosphorylation of Cdk1.

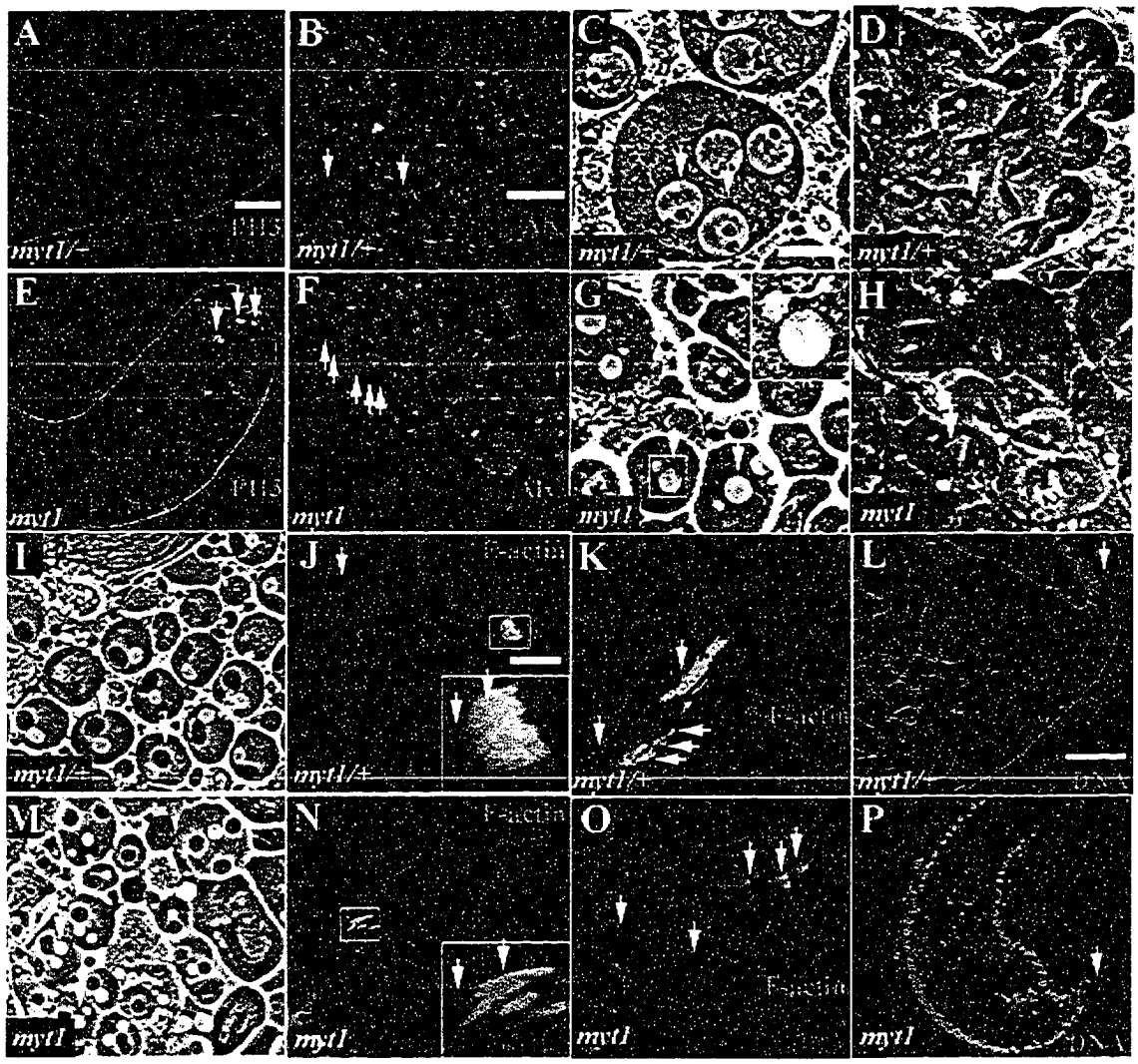

Fig. 2-5 Germline-associated somatic cells divide ectopically in *myt1* mutant testes. (A, E) Testes stained with anti-Eya (green) and anti-PH3 (red) antibodies. Increased numbers of cyst cells (E, green) were seen in the *myt1* mutants relative to controls (A) and ectopically dividing cyst cells were also detected in the mutants (E, arrows). (B, F) cysts of primary spermatocytes stained with anti-Eya (green) and anti-Aly (red) antibodies. Extra cyst cells (green) associated with Aly-positive spermatocytes (red) were seen in *myt1* mutants (F, arrows), but were not seen in controls (B). (C, D, G, H, I, M) Spermatocytes and spermatids analyzed by phase contrast microscopy. (C, G) shows defects in nuclear and nucleolar morphology in *myt1* mutants (G) not seen in controls (C). Note the presence of *myt1* mutant nuclei containing two lobes (arrows, G, inset), not seen in controls (C). The nucleolus in *myt1* mutant cells seems to be aberrantly located between these two nuclear lobes and thus only appears to be absent. (D, H) Dividing telophase nuclei were seen in both controls (D) and *myt1* mutants (H). (I, M) Shows aberrant spermatids in *myt1* mutants (M) that were not seen in controls (I). Examples of multiple small nuclei (pale circles) accompanying a single mitochondrial derivative (dark circle) in *myt1* mutants (arrows, M), are compared with controls (I) (J, K, N, O) Individualizing spermatids labeled for F-actin (red), Anillin (green) and DNA (blue), showing *myt1* mutant spermatid nuclei (N, O, white arrows) in small, disorganized clusters that form abnormal investment cones (yellow arrow, compare insets, N), unlike controls (J, K). (L, P) Optical sections through seminal vesicles are shown, that were stained to visualize DNA (blue, arrow shows proximal end of vesicle). Sperm were missing in the *myt1* mutant seminal vesicle (P), in contrast to controls that were full of mature sperm (L). Bars for (A, E): 40 μm; bars for (C, D, G, H, I, M): 16 μm; bars for (J, K, N, O): 20 μm; bars for (B, F, L, P): 16 μm.

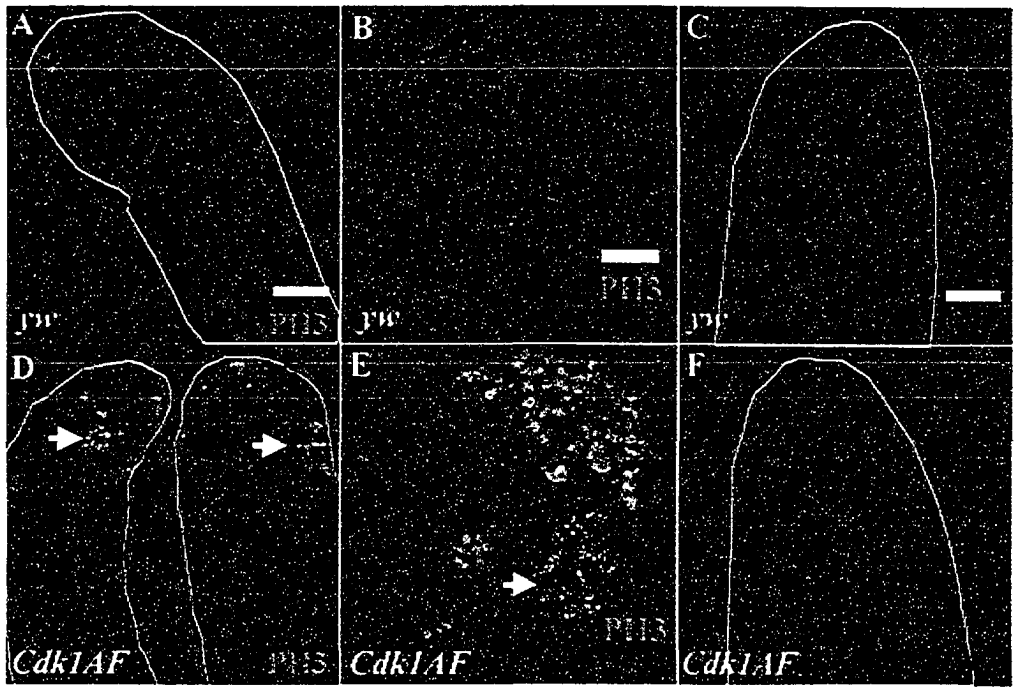

Fig. 2-6 The *myt1* mutant over-proliferation defects are due to a failure of Cdk1 inhibitory phosphorylation. (A, D) PH3 (red) staining shows that heat-shocked *Cdk1AF* testes (D) have extra dividing cells, compared with controls (A). (B, E) BamC (green) and PH3 (red) staining identifies dividing secondary spermatogonia. In the heat-shocked *Cdk1AF* testes a 16-cell cyst undergoing ectopic mitosis (E, white arrow) is shown, which was never seen in the controls (B). (C, F) Eya (green) staining to label somatic cyst cells shows that heat-shocked *Cdk1AF* testes have more cyst cells (F), than controls (C). Bar for (A, D): 40 μm; bar for (B, E): 8 μm; (C, F): 20 μm.

2.3.7 dMyt1 also regulates germline cell proliferation during oogenesis

Female *myt1* mutants are fertile, however a high incidence of early lethality in maternally affected mutant embryos (not shown) suggested that dMyt1 might also function during oogenesis. Oogenesis begins with stem cell divisions, producing cystoblasts that undergo 4 synchronous mitotic divisions to generate 16 cell cysts (Spradling, 1993). A single cell in each cyst then differentiates into an oocyte that progresses into prophase of meiosis I, whereas the remaining cells differentiate as nurse cells. Two or three germline stem cells (GSCs) are located at the tip of the germarium, each containing a ball-shaped fusome-related structure called a spectrosome (Lin and Spradling, 1995). Using the position of GSCs and antibodies against Hts (the gene product of *hu li tai shao*) to label spectrosomes (Lin et al., 1994; Zaccai and Lipshitz, 1996), I determined that the number of GSCs was comparable in *myt1* mutants and controls (Fig. 2-7A, B inset a1, b1, green arrows). I also counted the numbers of dividing GSCs, cystoblasts and cystocytes and calculated a mitotic index for each cell type (Table 2-1), using antibodies against Cnn and Hts to mark spindle poles and spectrosomes (or fusomes, in cystocytes) respectively, as well as the DNA-labeling dye Hoechst 33258 to mark condensed mitotic chromosomes (Fig. 2-7A-D, outlined areas). Female GSCs typically undergo one cell cycle per day, consequently mitotic GSCs are rarely observed and the mitotic index is very low (Lin and Spradling, 1993). I found only 2 mitotic GSCs in 130 germaria from control ovarioles (Table 2-1). In contrast, I found 15 mitotic GSCs among 50 *myt1* mutant germaria, a 20-fold increase in the mitotic index. The mutant cystoblasts and their cystocyte descendants also had a significantly higher mitotic index than normal (Table 2-1), so it was not uncommon to find a metaphase stem cell (green arrows) and a metaphase cystoblast or cystocyte (yellow arrows) in a single *myt1* mutant germarium (Fig. 2-7D). In the controls, a single dividing cyst at most was seen in each germarium (Fig. 2-7A, C). Thus, loss of *myt1* activity causes germline cell proliferation defects in females, as well as males. Egg chambers with greater than 16 cells were never observed in *myt1* mutants however, indicating that ectopic cell division did not contribute to this phenotype in females.

Oocyte and nurse cell differentiation appears normal in the *myt1* mutants, with respect to expression of Orb and Gurken in oocytes (Schupbach and Roth, 1994) and DNA morphology of the nurse cells (not shown). When I examined *myt1* mutant germaria with antibodies against the Vasa germline marker, I noted a significant increase in the number of cysts (2-3 fold, N=20), relative to controls (Fig. 2-7E, F). Although *myt1* mutant cystoblasts and cystocytes were similar in size to controls, the GSCs appeared slightly smaller than controls (compare Fig. 2-7E, F, green arrows). These data suggest that GSCs are cycling more rapidly than normal and were therefore producing more germline cysts. I noted similar increases in BamC-positive cysts in *myt1* mutant germaria, relative to controls (not shown). Since these differences were not as extreme as the GSC mitotic index measurements would predict, these results imply that compensatory delays likely occur during these mitotic cell cycles, that can account for this discrepancy in the *myt1* mutants.

To investigate whether there were effects on homologous chromosome segregation during female meiosis in *myt1* mutant females, standard genetic tests were undertaken to identify non-disjunction (NDJ) events, as described in the Materials and Methods (Giunta et al., 2002). In cross #1, the NDJ frequency for *myt1* mutants was much (100-fold) higher than controls for the X chromosome and the 4^{th} chromosome (Table 2-2), indicating that loss of dMyt1activity compromises the fidelity of female meiosis. All of the exceptional progeny derived from *myt1* females in cross #2 had Bar eyes and yellow bodies and were therefore derived exclusively from *FM7, y, B/y* eggs, indicating that achiasmate NDJ occurs primarily at meiosis I in the mutants (Table 2-2).

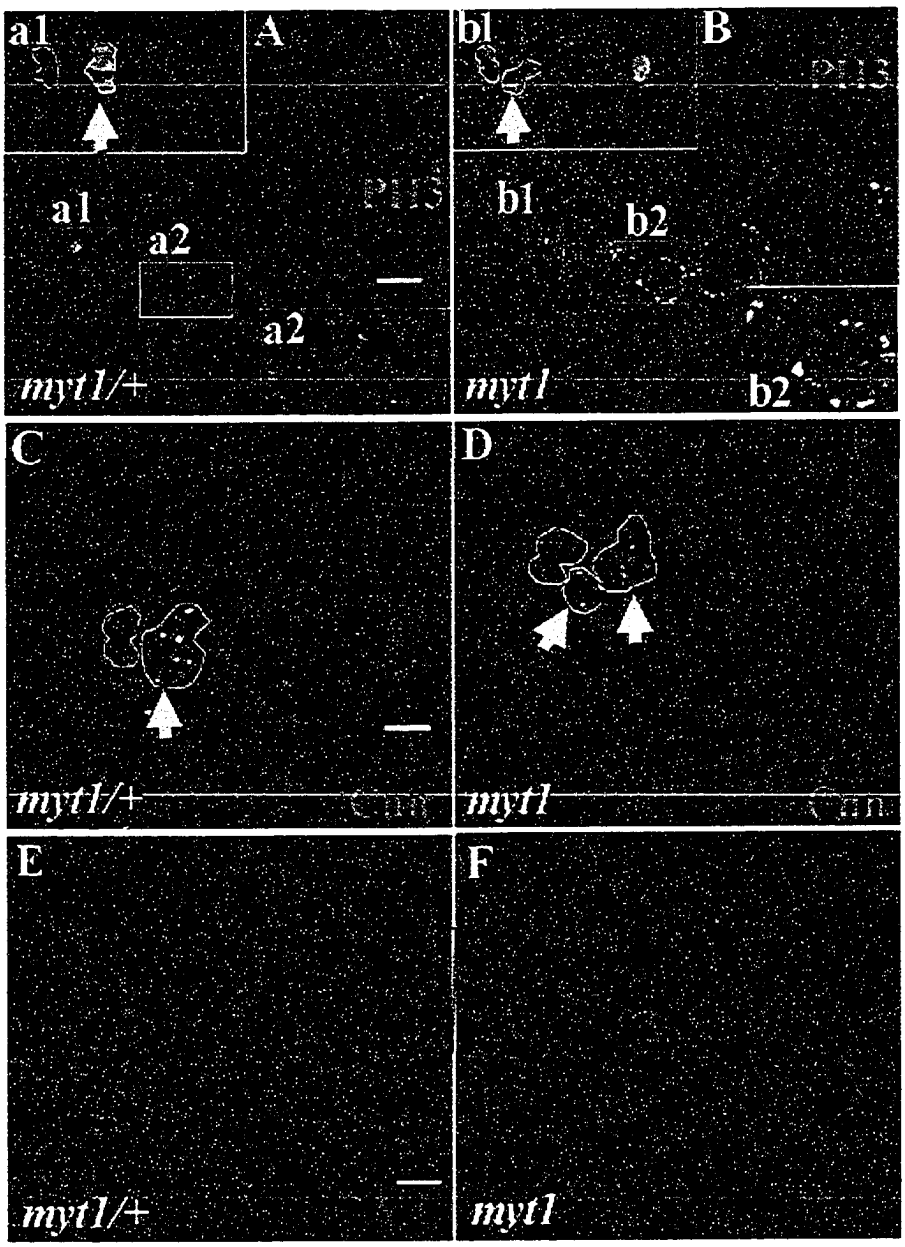

Fig. 2-7 Mitotic over-proliferation defects seen in *myt1* mutant ovaries. (A, B) Ovarioles labeled by antibodies against PH3 (red) to mark mitotic cells, Hts (green) to mark spectrosomes and stained with Hoescht 33258, marking DNA (blue). In *myt1* mutants (B), germaria often contained both dividing stem cells (green arrow) and dividing cystocytes (yellow arrow), which was never seen in controls (compare insets: a1, b1). The *myt1* mutants also had more PH3-positive somatic follicle cells surrounding the egg chambers, than controls (compare insets: a2, b2). (C, D) Centrosomin (Cnn) staining (red) for centrosomes, Hts (green) staining for fusomes and DNA (blue) staining showed significantly more metaphase stem cells (green arrow) and cystocytes (yellow arrows) in *myt1* mutants (D) compared with controls (C). (E, F) Stacked confocal images of a Vasa-stained germarium, showing that *myt1* mutant germaria (F) contain more cysts than controls (E). The *myt1* mutant germline stem cells (F, green arrows) were also slightly smaller than controls (E). Bar for (A, B): 40 μm; bars for (C-F): 8 μm.

Table 2-1. Quantification of the mitotic index associated with different cell types in *myt1* mutant ovaries

Genotype	Mitotic index				
	Stem cell	cystoblast	2-cell cystocyte	4-cell cystocyte	8-cell cystocyte
myt1/+	0.015	0.000	0.032	0.048	0.016
	(N=130)	(N=62)	(N=62)	(N=62)	(N=62)
myt1	0.300	0.160	0.160	0.160	0.180
	(N=50)	(N=50)	(N=50)	(N=50)	(N=50)

The mitotic index was calculated as the number of PH3-positive cells divided by the total number of cells, for each indicated cell type (scored by criteria that are illustrated in Fig. 6). The number of germaria counted for each sample is indicated in brackets.

2.3.8 Germline-associated follicle cells also undergo ectopic cell division in *myt1* mutants

Female germline cells are associated with somatic follicle cells derived from stem cell precursors (Margolis and Spradling, 1995). When each 16-cell germline cyst buds from the germarium as an egg chamber, a layer of undifferentiated follicle cells surrounds it. These follicle cells then differentiate into functionally distinct sub-classes. The stalk cells (located between each egg chamber) and the polar cells (located at each end of the chamber), cease dividing immediately after the egg chamber forms, whereas the remaining follicle cells proliferate asynchronously until stage 6 of oogenesis (Bai and Montell, 2002). Accordingly, early egg chambers have only small numbers of PH3-positive follicle cells, in the controls (inset a2, Fig. 2-7A, Fig. 2-8A, red). In *myt1* mutant egg chambers, there was a marked increase in PH3-positive follicle cells before stage 6 (Fig. 2-7C, inset c2, Fig. 2-8D), as well as ectopic PH3-positive follicle cells after stage 6 (compare Fig. 2-8B, E, arrows). Curiously, these ectopic PH3-positive follicle cells primarily appeared at the anterior and posterior ends of each egg chamber (Fig. 2-8E).

Each of the four major types of follicle cells can be distinguished by their cell shape and by expression of distinct molecular markers. Stalk cells have a unique disc-like shape and inter-egg chamber location (Fig. 2-8A, arrow); polar cells are located at the end of each egg chamber and express FasIII before stage 9 (Fig. 2-8C, green); border cells maintain Fas III expression and migrate towards the posterior after stage 9 (Fig. 2-8G); and stretched cells extend over the 15 nurse cells and express Eya (Fig. 2-8H). By these criteria, the different follicle cell types all appeared to be represented in *myt1* mutants, however unlike the controls, many of these cells were PH3-positive, suggesting that they were undergoing ectopic cell divisions (Fig. 2-8D-F, arrows, J, K). Consistent with this interpretation, there were significantly more Eya-expressing cells in the mutants than in controls by stage 9 (compare Fig. 2-8H, K), indicating that some of these cells were able to complete cell division. The typical "stretched" morphology characteristic of this cell type was disrupted however, presumably as a result of cytoskeleton re-organization accompanying mitosis (Fig. 2-8H, K, arrows). I also observed ectopic PH3-positive main body follicle cells in mutant egg chambers after stage 9, long after these

cells normally cease dividing (Fig. 2-8I, L). Thus, germline-associated somatic cells lacking dMyt1 function undergo ectopic cell divisions in both males and females.

Table 2-2. Non-disjunction at meiosis I in *myt1* mutant females

Female genotypes	Regular progeny	Regular progeny	Diplo-X progeny	Nullo-X progeny	% of X-NDJ	4^4	% of 4-NDJ	Total progeny
Cross #1	X/O	XX^Y	XX	X^Y				
myt1^{1}/*myt1*^{2}	135	144	5	13	11.40	1	0.70	315
myt1^{1}/*TM3**	459	340	0	1	0.10	0	0	801
Cross #2	y^{+} females	*y* males	*FM7,y,B/y* females	y^{+} males	% of X-NDJ	*FM7,y,B/FM7,y,B* OR *y/y* females		Total progeny
FM7,y,B/y;myt^{1}/*myt1*^{2}	300	279	51	63	28.25	0		807
FM7,y,B/y;myt1^{1}/*TM3**	347	360	1	4	1.39	0		717

Note: Females were crossed to *C (1;Y), v, f, B ; C(4) RM, ci, ey [R]* males in corss #1 and *y+* males in corss #2 (see materials and methods for more details). While regular progeny received one X and one fourth maternal chromosome, the diplo-X progeny (genotype as XX^Y) received two maternal X chromosome, nullo-X progeny (genotype as X^Y) received no maternal X chromosome, and nullo-4 progeny (genotype as 4^4) received no maternal fourth chromosome. The percentage of NDJ of X chromosome is higher in cross #2 than in cross #1 indicated that loss of myt1 *primarily* affects achiasmate chromosome segregation. * The introduction of TM3 balancer in control crosses may interfere with the NDJ rate of X chromosome and thus should be substituted with a + 3rd chromosome instead.

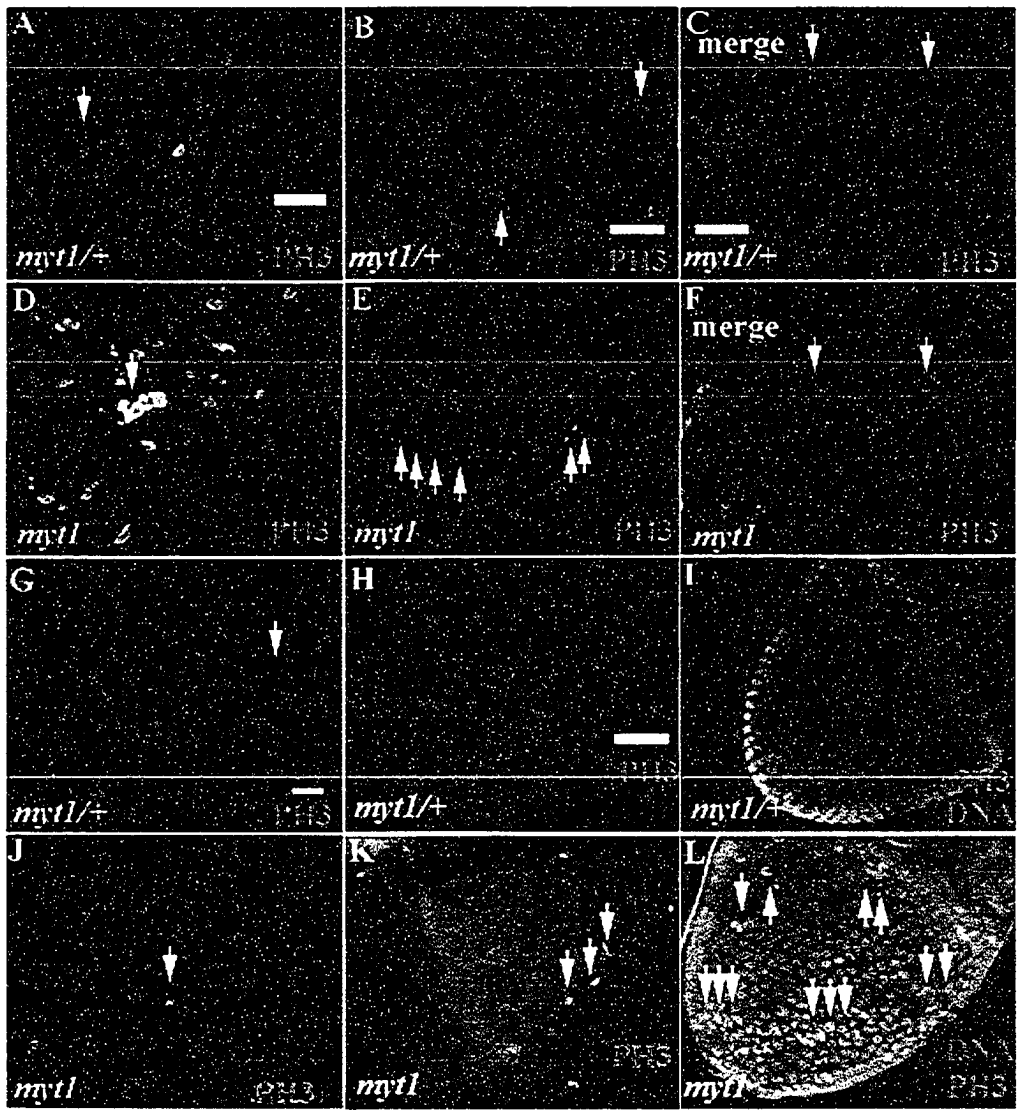

Fig. 2-8 Follicle cell defects observed in *myt1* mutant ovarioles at different stages of development. (A, D) Stage 2 egg chambers stained for Hts (green) and PH3 (red). Stalk cells (arrow) are PH3-positive in ~90% of *myt1* mutants at this stage (D), but are PH3-negative in controls (A). (B, E) Stage 7 egg chambers stained for PH3 (red). Note that follicle cells at each end of the egg chamber (arrows) are PH3-positive in *myt1* mutants (E), compared with controls (B). (C, F) Stage 7 egg chambers stained for FasIII (green) and PH3 (red). Arrow indicates a PH3-positive polar cell (seen in 100% of *myt1* mutants, F), never seen in controls (C). (G, J) Stage 10A egg chambers stained for Fas III (green) and PH3 (red). Arrow in (J) indicates a PH3-positive border cell (seen in ~20% of *myt1* mutant ovarioles), never seen in controls (G). (H, K) Stage 10A egg chambers stained for Eya (green) and PH3 (red). Arrows indicate PH3-positive, Eya-positive stretched cells (seen in ~16% of *myt1* mutants, K), never seen in controls (H). (I, L) Stage 10B egg chambers stained for DNA (cyan) and PH3 (red). Arrows indicate PH3-positive main body follicle cells (seen in ~6% of *myt1* mutants, L), never seen at this stage, in controls (I). Bar for (A, D): 8 μm; bars for (B, C, E, F, G, J): 40 μm, bar for (H, I, K, L): 20 μm.

2.4 Discussion

Phenotypic analysis of *Drosophila myt1* mutants has revealed distinct and unexpected roles for dMyt1 in regulating both mitotic and meiotic cell cycles. Previously, Myt1 inhibitory phosphorylation of Cdk1 was implicated in the prolonged "G2-like" growth state of immature oocytes in *Xenopus* (Furuno et al., 2003; Karaiskou et al., 2004; Nakajo et al., 2000; Palmer et al., 1998; Peter et al., 2002) and the starfish *A. pectinifera* (Okano-Uchida et al., 2003; Okumura et al., 2002). Although female *myt1* mutants are fertile they exhibit an elevated rate of homologous chromosome non-disjunction during meiosis I, indicating that dMyt1 function is required for faithful chromosome segregation during *Drosophila* female meiosis. I observed more severe pre-meiotic and meiotic defects in male *myt1* mutants, suggesting that dMyt1 has a more critical role in regulating male meiosis. An essential role in male meiosis was also proposed for a *C. elegans* Myt1 homolog, *wee1.3* (Lamitina and L'Hernault, 2002). A developmental feature shared by *Xenopus* immature oocytes and *Drosophila* spermatocytes is that they arrest in a G2-like state for relatively long periods of time while synthesizing cellular components required for subsequent development. During *Drosophila* oogenesis, the endoreplicating nurse cells (and not the oocyte) are primarily responsible for synthesizing components required for later development. These similarities and differences suggest that Myt1 kinases serve specialized functions in cells undergoing developmentally regulated G2 or G2-like growth phases and provide a plausible rationale for why Myt1 activity is not as important for regulating female meiosis in *Drosophila*, as it is in *Xenopus* and *A. pectinifera*.

My analysis also provides evidence that dMyt1 activity is important for regulating mitotic cell proliferation during gametogenesis, in both sexes. I have identified several distinct defects that apparently contribute to the mitotic over-proliferation phenotype. In male *myt1* mutants, spermatogonial cysts occasionally underwent an extra round of cell division before they differentiated into spermatocytes, suggesting that dMyt1 activity contributes to the fidelity or timing of this developmentally regulated cell fate decision. Unlike previously described male-sterile over-proliferation mutants with cell fate defects (Gonczy et al., 1997; Kiger et al., 2000; Matunis et al., 1997; Tulina and Matunis, 2001), the majority of *myt1* mutant cysts did not undergo ectopic cell divisions, however.

Germline-associated somatic cells also underwent ectopic cell divisions in the mutants, suggesting that dMyt1 activity is required for these cells to exit from the cell cycle as they differentiate. Mitotic index measurements in female *myt1* mutants and observations of increased numbers of germline cysts also suggested that cells were progressing through the cell cycle at a faster rate than normal. Delays in mitosis probably contribute to the phenotype also, since observed increased numbers of germline cysts in *myt1* mutant germaria were not proportional to the mitotic index elevation. These data provide the first evidence that dMyt1 serves a critical role in coordinating mitotic cell cycle behavior with developmental cues. Precisely how dMyt1 accomplishes these diverse functions is not yet clear; however, my results support the hypothesis that Myt1 kinases are required for spatially and temporally regulating Cdk1 activity within the cytoplasm.

Is there some unified basis for the diverse effects on mitotic cell proliferation observed in the *myt1* mutants? Previously, ectopic expression studies of dMyt1 suggested that its major effect was to lengthen G2 phase (Cornwell et al., 2002; Price et al., 2002). Cell cycle compensatory mechanisms have been described that can adjust to experimentally induced reductions or increases in the length of G1 or G2 phases (Neufeld et al., 1998; Reis and Edgar, 2004). In principle, then, Myt1-regulated prolongation of G2 phase would not necessarily have an effect on overall cell cycle length. An appealing hypothesis that could account the mitotic phenotypes and for the observed specificity of meiotic *myt1* mutant phenotypes is that dMyt1 activity is required in cells that are undergoing developmentally delayed G2 phases; a function permissive for cell growth or for the assembly of structures required for subsequent stages of development. Premature exposure to activated Cdk1 could interfere with these developmentally regulated biosynthetic processes in the *myt1* mutants, thereby compromising the fidelity of meiotic cell fate transitions and uncoupling somatic cell differentiation from cell cycle exit.

Other than gametogenesis, most aspects of morphological development appeared normal in *myt1* mutants except for adult sensory bristle development (not shown). Perdurance of maternal Myt1 activity could be responsible for this result, however another possibility is that the other Cdk1 inhibitory kinase dWee1 is functionally redundant with dMyt1 at these stages of development, in addition to its unique function

during early embryogenesis (Price et al., 2000). Unlike other model metazoans, which have more than two Wee1-like kinases (Lamitina and L'Hernault, 2002; Leise and Mueller, 2002; Murakami et al., 2004; Nakanishi et al., 2000; Okamoto et al., 2002; Wilson et al., 1999), *Drosophila* has only one Wee1 and one Myt1 kinase. This circumstance makes *Drosophila* a particularly favorable system for identifying and characterizing both unique and functionally redundant regulatory mechanisms involving these two Cdk1 inhibitory kinases. Results reported here suggest the testable hypothesis that dMyt1 serves unique regulatory functions linking prolonged G2 growth phases with specific developmental transitions. It will be interesting to determine whether Myt1 kinases serve similar developmental functions in other organisms, besides their established role in regulating meiosis.

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Chapter 3 *Drosophila* Myt1 and Wee1 dictate the timing of distinct meiotic events in primary spermatocytes

3.1 Introduction

During G2 phase and early prophase of mitosis, Cdk1/Cyclin B complexes required for coordinating mitotic events are phosphorylated by Wee1-like kinases and thus remain inactive (Russell and Nurse, 1987). Activity of Cdc25C phosphatase reverses this inhibitory phosphorylation of Cdk1, allowing cells to enter mitosis (Russell and Nurse, 1986). In HeLa cells, the earliest sign of commitment to mitosis occurs at late G2 phase, when a LIM protein called Ajuba becomes concentrated at centrosomes, where it activates Aurora A that in turn stimulates Cdc25C to activate Cdk1/Cyclin B *in situ* (Hirota et al., 2003). Through a mechanism that remains to be characterized, the active form of Cdk1/Cyclin B then translocates into the nucleus to bring about nuclear Cdk1/Cyclin B activation (Ferrell, 1998; O'Farrell, 2001), when negative signals such as damaged DNA and incomplete DNA replication are not encountered. Because of this dynamic chain of events, coordinated activation of Cdk1/Cyclin B complexes in different cellular compartments may not only contribute to the synchrony of chromosome condensation, nuclear envelope breakdown and spindle assembly at the G2/M transition, but also provide a molecular switch to shut down inappropriate mitotic entry when DNA is not ready for division and thus maintain genome stability. Presumably, this model would also apply to meiosis; a specialized cell division that also involves coordinated changes in the cytoplasm and the nucleus.

Meiosis is different from mitosis in that it includes two successive nuclear divisions with only one round of pre-meiotic S phase. Studies using *Xenopus* oocyte extracts discovered that the absence of Wee1 protein kinase ensures that S phase does not occur between meiosis I to meiosis II, since residual Cdk1 activity is required to inhibit S phase re-entry at this point. Provision of ectopic Wee1 activity allows an ectopic S phase to occur after MI, converting meiosis into mitosis (Iwabuchi et al., 2000; Nakajo et al., 2000). The other Wee1-like kinase, Myt1, has been proposed to suppress Cdk1 activity during prophase I of meiosis. Myt1 is present in immature oocytes from *X. larvis* and *A. pectinifera* and is inactivated at the beginning of meiosis I (Okumura et al., 2002; Palmer et al., 1998), supporting the idea that Myt1 is the only Cdk1 inhibitory kinase necessary for female meiosis I. *S. cerevisiae* Swe1, which is the only Wee1-like kinase in this

organism, is required during meiosis for a "pachytene checkpoint" function that responds to double strand breaks triggered by meiotic DNA recombination (Leu and Roeder, 1999). This response may be regulated similarly in very early immature *Xenopus* oocytes, since a small amount of Wee1 protein can also be detected around the pachytene stage, implying that Wee1 is also required for this stage before disappearing as meiosis I initiates (Iwabuchi et al., 2000). These data imply that Myt1 and Wee1 serve different functions during meiosis.

To test this possibility, I examined the Wee1-like kinases in *Drosophila melanogaster*, where mutants affecting specific genes can be synthesized by positional genetic screens. Unlike vertebrates, which have three Wee1 kinases: Wee1A, Wee1B and Myt1 (Booher et al., 1997; Leise and Mueller, 2002; Liu et al., 1997; Mueller et al., 1995; Okamoto et al., 2002), *Drosophila* only has Wee1 and Myt1 (Campbell et al., 1995; Cornwell et al., 2002). Like their vertebrate counterparts, the nuclear dWee1 kinase phosphorylates the Y15 residue of Cdk1, whereas the membrane-bound, Golgi body and endoplasmic reticulum (ER) resident dMyt1 kinase phosphorylates both T14 and Y15 residues of Cdk1 (Booher et al., 1997; Lee et al., 2001; Liu et al., 1997; Mueller et al., 1995 and our unpublished data). Maternal *Drosophila wee1* is essential for completing the syncytial embryonic cell divisions in embryos laid by *wee1* hemizygotes (Price et al., 2000). The Schupbach lab had also implicated *wee1* in the pachytene checkpoint during female meiosis, although their evidence was indirect (Abdu et al., 2002).

My project has involved isolating and characterizing *myt1* mutants, which are also zygotic viable but have phenotypes that are distinct from *wee1* mutants, including: male sterility, partial female sterility and bristle defects. The initial phenotypic analysis was focused on germline cells and germline-associated somatic cells in *myt1* mutant testes and ovaries, where over-proliferation defects were observed that involved the developmentally regulated mitotic cell cycles (Chapter 2, manuscript submitted). In contrast, primary spermatocytes in *myt1* mutants underwent abnormal meiosis, suggesting that dMyt1 has an essential role in regulating male meiosis.

In *Drosophila* testes, germline cells destined for meiosis called primary spermatocytes, remain in a prolonged G2 phase for up to 90 hr (Fuller, 1998). I

speculated that dMyt1 might be important for this G2 phase arrest, implying that lack of *myt1* would disrupt male meiosis and cause male sterility. In support of this hypothesis, my studies of primary spermatocytes showed that loss of *myt1* function triggers specific defects in the cytoplasm that result in aneuploidy during male meiosis. Moreover, chromosome condensation occurred prematurely in *myt1* primary spermatocytes, when nuclear dWee1 level was reduced, emphasizing the importance of dWee1 for protecting the nucleus from premature Cdk1activity.

These results provide the first direct evidence that dMyt1 and dWee1 each negatively regulate Cdk1 in their respective cellular compartments. dMyt1 is required for maintaining the normal timing of centrosome separation and subsequent splitting in the cytoplasm (as well as an undefined role in establishing or maintaining normal fusome structure), whereas dWee1 is required for regulating chromosome condensation in the nucleus during male meiosis.

3.2 Materials and Methods

3.2.1 Fly stocks

myt1^{1}/*myt1*^{2} trans-heterozygotes were used as the representative of *myt1* mutants and their *myt1*^{1}/*TM3,Sb* heterozygous siblings served as controls for the phenotypic analysis of male meiosis. A null allele of *wee1* (*wee*^{ES1}), a hypomorphic allele of *wee1* (*wee1*^{DS1}) and a balancer chromosome with a dominant marker *CyO* were used in genetic crosses to generate mutants flies of *wee1*^{ES1} or *wee1*^{DS1}/*CyO*; *myt1*^{1}/*myt1*^{2} genotype. For the induction of *Cdk1AF* experiments, 1-2 day old *y w*; *hs-Cdk1AF* transgenic flies and *y w*, *hs-Cdk1* controls received 2X 1 hr heat shocks at 37°C for three consecutive days, prior to the testes being examined.

3.2.2 Immunofluorescence

For testes preparations, 1 or 2 day old male flies were dissected in 1X PBS (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.2) or in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM EDTA, pH 7.2), then rinsed twice with the same buffer. Established protocols were followed for the testes immunofluorescent localization experiments. (Bonaccorsi et al., 2000). The primary antibodies and concentrations used were: rabbit anti-PH3 (1/2000; Upstate), mouse anti-beta-tubulin (1/100; Sigma), rabbit anti-Cnn at 1/500 (Heuer et al., 1995), rabbit anti-T288P-Aurora A (1/200, Cell Signaling) and rat anti-KLP61F (1/1000, Barton et al., 1995). Alexa-488, Alexa-568 conjugated secondary antibodies (used at 1/1000) were purchased from Molecular Probes. DNA was visualized by Hoechst 33258 at 1 μg/ml. Images were initially acquired with a Leica TCS-SP2 Multiphoton Confocal Laser Scanning Microscope (TCS-MP) and then were enhanced with Photoshop software.

3.3.3 Western blotting

Twenty pairs of testes of desired genotypes were dissected from 1-2 day old adult males in EB buffer (10 mM Tris, pH 7.5, 80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 2 mM Na3VO4, 1 mM benzamidine, 0.2 mM PMSF, 1 μg/ml leupepsin, pepstatin A and aprotinin), and then rinsed twice before they were transferred to an Eppendorf tube with 20μl EB buffer. Each sample was kept in an ice bucket for 1 hr.

20μl of 2XSDS-PAGE sample buffer was added in each tube and immediately boiled for 5 min before it was stored in -20°C. 25 μl of each sample was run on 10% SDS-PAGE gel for western blotting. Blots were blocked with 5% BSA and then probed with anti-T14-Cdk1 (1 μg/ml, pers. comm. with E. Homola) first, followed by HRP-conjugated secondary antibody (1/10000, Amersham). ECL mediated detection was performed as the manufacturer instructed. After stripping the previous antibodies, the same blot was used again with anti-Y15-Cdk1 (1/1000, Cell signaling) and then anti-PSTAIR Cdk1 (1/1000, Santa Cruz Biotechnology) as primary antibodies.

3.3 Results

3.3.1 Abnormal centrosome behavior in *myt1* primary spermatocytes

Drosophila myt1 mutants are male sterile, a phenotype that cannot be readily explained simply by defects in the mitotic cycles of germline cells, as I reported previously (Chapter 2, manuscript submitted). My hypothesis is that *myt1* is required for primary spermatocytes to remain arrested in the pre-meiotic G2 phase. The biological significance of this 90 hr long G2 arrest is that it allows primary spermatocytes to grow roughly 25-fold in size and express gene products required for post-meiotic differentiation (Fuller, 1998). This developmentally regulated G2 phase arrest provided us with an opportunity to examine the timing of several well-characterized landmark meiotic events that occur at the G2/M transition, in *myt1* mutants. Using γ-tubulin (data not shown) and Centrosomin (Cnn) antibodies (Li et al., 1998), I observed that centrosome separation occurs much earlier in *myt1* mutant primary spermatocytes (Fig. 3-1D, white circle) than it does in normal spermatocytes (Fig. 3-1A, B, white circles), soon after their appearance and well before chromosome condensation and microtubule spindle formation mark the onset of meiosis. Occasionally, control G2 phase spermatocytes also appeared to have separated centrosomes (Fig 3-1B, right of the white circle), but this was probably due to mechanical forces caused by the testes squash protocol.

Strikingly, the separated centrosomes that were consistently seen in the *myt1* mutants then appeared to "split", often forming four distinct "poles" (Fig. 3-1E, white circle). One possible cause of these multiple Cnn-positive foci would be an extra

duplication of centrosomes, in which case each separate pole in a *myt1* mutant primary spermatocyte would be composed of a pair of centrioles. To examine this possibility, I used a phospho-specific T288 Aurora A antibody (T288P-AurA, described in Hirota et al., 2003), which recognizes centrosomes in control primary spermatocytes as either four individual rod-like centrioles in a juxtaposed position during G2 phase (Fig. 3-1G, inset), or two rod-like centrioles at each pole at prometaphase I (Fig. 3-1H, inset). In the *myt1* mutants, the centrosomes in most primary spermatocytes were seen as four individual centrioles, either as 2 pairs of centrioles or as 4 separated centrioles, presumably representing a temporal transition from G2 phase (Fig. 3-1I, inset) into meiosis I (Fig. 3-1I, white circle). The observation that loss of *myt1* is associated with centrosomal defects suggests that dMyt1 is necessary to regulate the timing of centrosome separation and splitting in primary spermatocytes. Similar experiments were also performed with *wee1* mutant testes, however no abnormalities were observed in that case (data not shown).

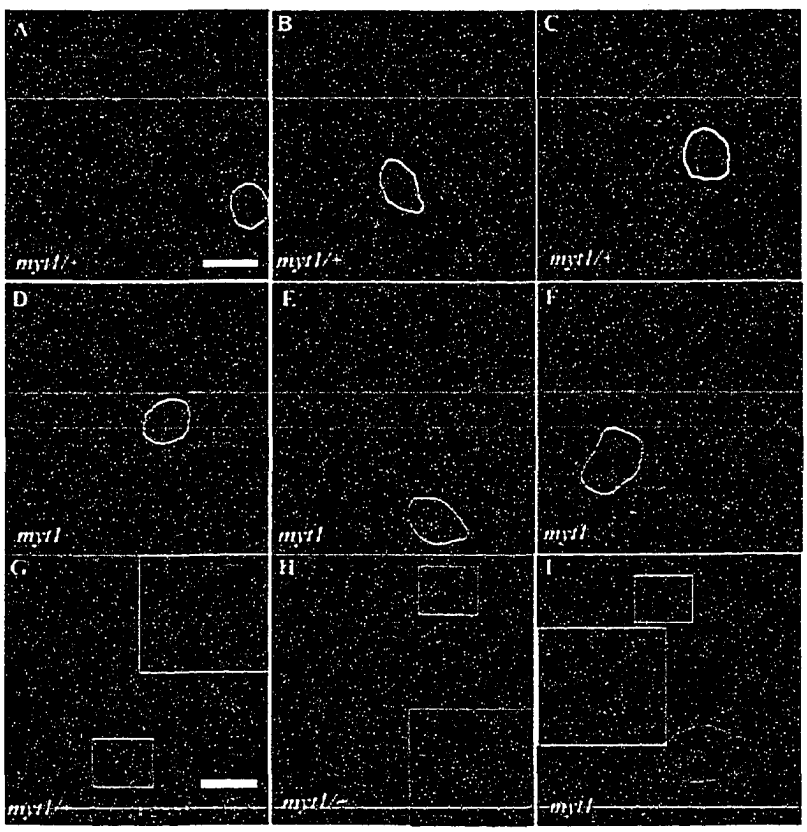

Fig. 3-1 Centrosomal defects in *myt1*mutant primary spermatocytes. A-C, G and H were controls, whereas D-F and I were *myt1*mutants. Centrosomes were marked with either Cnn (red) in A-F or T288P-AurA (red) in G-I. DNA was visualized with Hoechst 33258 dye. White circles highlight individual primary spermatocytes. (A, D) Stage S2 primary spermatocytes. (B, E) Stage S5 primary spermatocytes. (C, F) Metaphase I stage primary spermatocytes. Note that in *myt1* mutants, the centrosomes (D, two red dots in a white circle) were separated already at the S2 stage and then split into four Cnn-positive structures (E and F, four red dots in a white circle) as the primary spermatocytes mature. Centrosomes did not separate to two poles in the controls until metaphase I (compare juxtaposed red dots in A, B to separated red dots in C). (G-I) Primary spermatocytes representing successive developmental stages. Boxed area in each panel is shown in the magnified inset: each cell only had four rod-like centrioles, however in the controls (insets in G, H), pairs of centrioles remained juxtaposed, whereas in *myt1* mutants (inset in I) centrosome splitting had occurred and isolated centrioles could be observed. Bar for A-F: 20 μm; bar for G-I: 16 μm.

3.3.2 Disruption of fusome structure in the cytoplasm of *myt1* spermatocytes

To investigate whether other cytoplasmic organelles might also be disrupted in *myt1* mutant primary spermatocytes, I next examined a germline specific structure called the fusome. Fusomes have a branch-shaped structure containing many protein components, which are believed to help transport other proteins that are important for cell division and differentiation in each cyst of 16 cells (de Cuevas and Spradling, 1998; Hime et al., 1996). Fusomes become more extensive as primary spermatocytes grow, keeping the cells in each cyst inter-connected during pre-meiotic G2 phase. When meiosis I begins, the fusome disassembles and many of its components relocate to the cytoplasm (Hime et al., 1996). I monitored the behavior of three proteins that are established components of fusomes: α-Spectrin, an adducin-like protein (the *Hts* gene product) and KLP61F (a kinesin-like motor protein) in *myt1* mutant males (Lin et al., 1994; Wilson, 1999). In *myt1* mutant primary spermatocytes, KLP61F was scattered in the cytoplasm, rather than localized to a well-defined fusome structure (Fig. 3-2D, E, F). In control testes however, KLP61F showed the typical fusome localization as a branch-shaped structure in G2 phase spermatocytes (Fig. 3-2A, B, C). α-Spectrin and Hts also behaved similarly to KLP61F in *myt1* mutant primary spermatocytes (data not shown). From these observations, I conclude that dMyt1 is required for establishing or maintaining fusome-specific protein structures during pre-meiotic G2 phase of spermatogenesis.

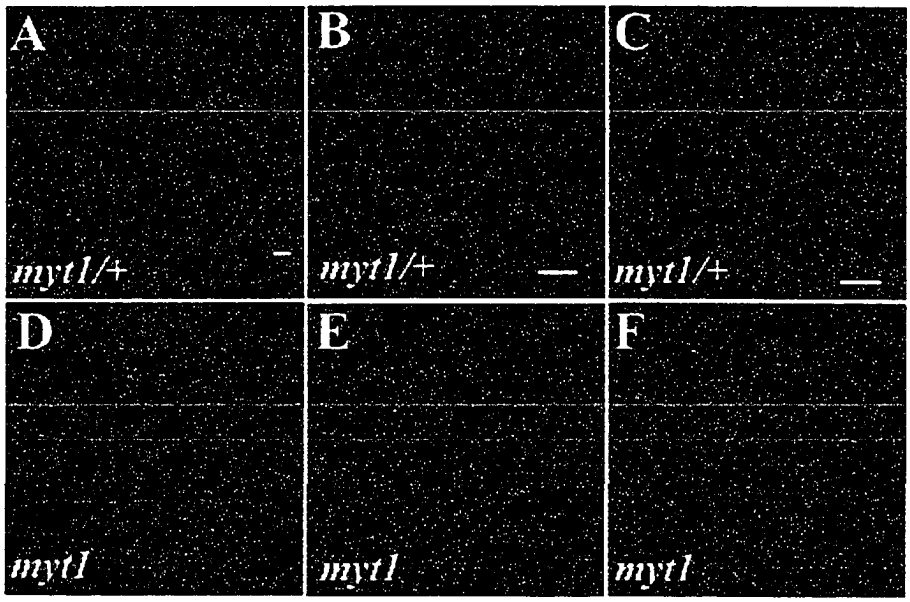

Fig. 3-2 Fragmentation of fusomes, as revealed by KLP61F staining in primary spermatocytes of *myt1* mutants. A-C and D-F are controls and *myt1* mutants, respectively. KLP61F was labeled in green and DNA was stained in blue, in all panels. (A, B, D, E) Apical tips of testes: (C, F) Close-up micrographs of primary spermatocytes. Compare the fragmented fusome staining in *myt1* mutants (D-F, green) to the typical branch-shaped fusome structure in controls (A-C, green). Bar for A, D: 40 μm; bar for B, C: 20 μm; bar for C and F: 8 μm.

3.3.3 Formation of multipolar spindles causes unequal chromosome segregation during Meiosis in *mytl* male mutants

To evaluate the consequences of premature centrosome separation and splitting in the *mytl* mutant spermatocytes, I analyzed meiosis by following the behavior of microtubules, centrosomes (or centrioles) and chromosome. Although the behavioral changes of centrosomes and fusomes suggest that certain meiotic events were occurring prematurely in the mutants, chromosome condensation appeared similar to the controls (Fig. 3-3A, B) and the mutants (Fig. 3-3E, F). Similarly, the timing of nuclear envelope breakdown (NEBD), as judged by nuclear exclusion of β-Tubulin staining and spindle assembly apparently occurs with similar timing in the controls (Fig. 3-3A, B) and in the mutant primary spermatocytes (Fig. 3-3E, F).

Not until pro-metaphase did I start seeing a significant degree of chromosome condensation and spindle assembly in both controls (Fig. 3-3C, D) and mutant cells (Fig. 3-3G, H). While the nuclear envelope disappears at metaphase I in control cells (Fig. 3-3I, J) and the mutant cells (Fig. 3-3M, N), most mutant spermatocytes appear to arrange their four poles (Fig. 3-3N, red) in such a manner that the spindle looks much like a bipolar spindle (Fig. 3-3M), with the chromosomes spreading along the length of the spindle (Fig. 3-3N, blue) rather than lining up at the metaphase plate, as seen in controls (Fig. 3-3J, blue). Due to premature centrosome splitting in the *mytl* mutants, cells in meiosis I always have multi-polar spindles (Fig. 3-3M, N). In the controls, both chromosome and centrioles segregate normally (Fig. 3-3K). In *mytl* mutants, whereas chromosome seems to be segregated into two masses at the end of meiosis I (Fig. 3-3O, Q, U, blue), the centrioles appear to segregate randomly, resulting in 1, 2 or 3 centriole(s) in each resulting daughter cell (Fig. 3-3P, R, V, red, arrows). When examining meiosis II in controls (Fig. 3-3S, T) and in *mytl* primary spermatocytes (Fig. 3-3W), apparent examples of chromosome segregation defects were frequently seen in the mutants; presumably due to problems encountered when these cells attempt to divide with multipolar spindles , as was previously described for mitotic cells with multi-polar spindles (Hut et al., 2003). Consistent with these results from immuno-fluorescent

staining, phase contrast microscopy also revealed apparent defects in chromosome segregation in the *myt1* mutants, but not in controls (compare Fig. 3-3T and Fig. 3-3X).

The unfaithful segregation of chromosome during male meiosis in *myt1* mutants probably originates from an inability to maintain the proper timing of centrosome separation and splitting, a process that occurs prematurely with respect to other meiotic events in the absence of dMyt1 function. Despite these premature cytoplasmic events occurring in *myt1* mutant primary spermatocytes during pre-meiotic G2 phase, the timing of chromosome condensation, spindle assembly and NEBD seem to be unaffected. These observations suggest that lack of Myt1 activity uncouples these normally coordinated these events.

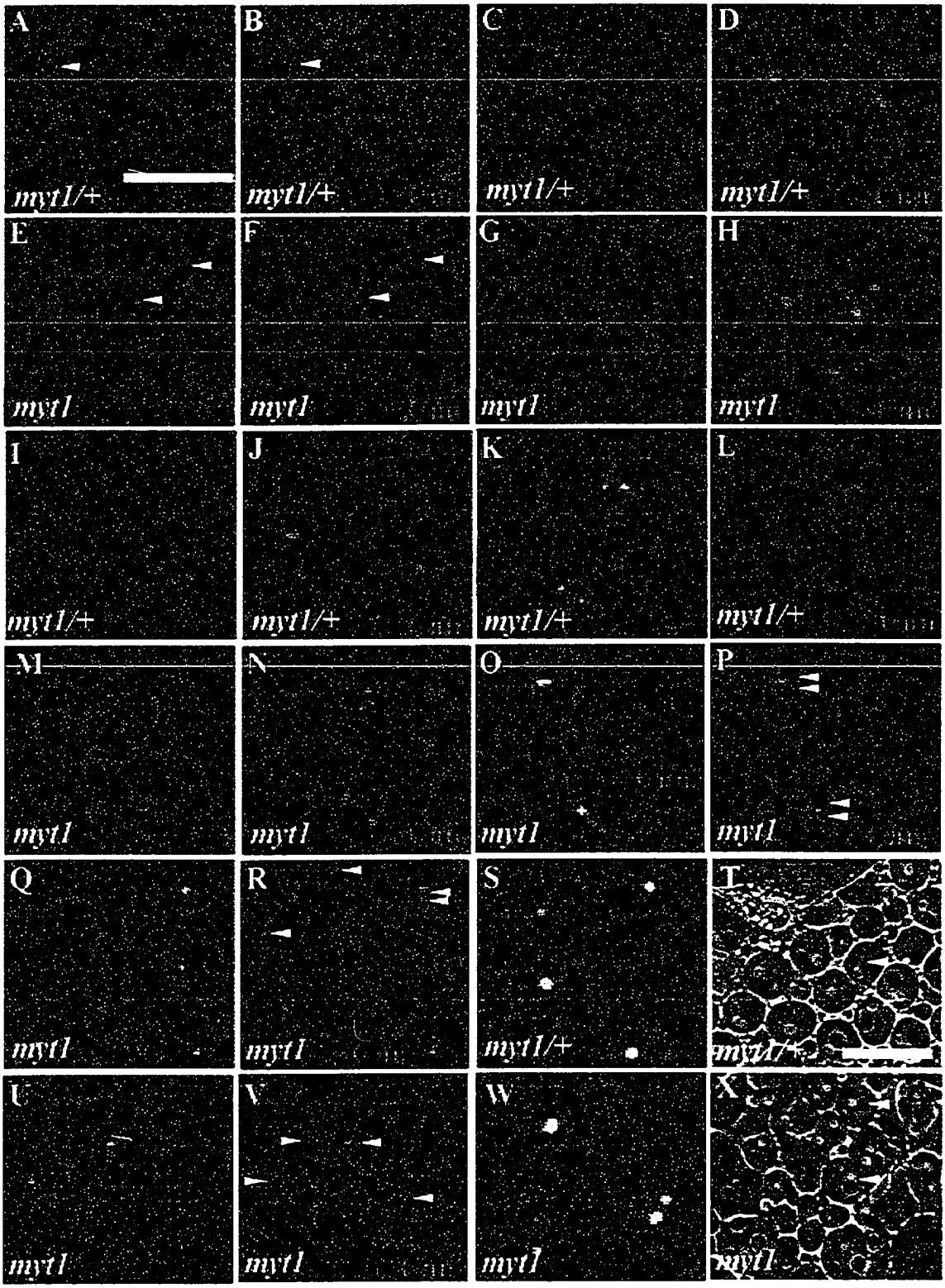

Figure. 3-3 High incidence of abnormal multipolar cells and unevenly segregated chromosome in *myt1* mutants, during male meiosis. Fluorescence micrographs of primary spermatocytes from controls (A-D, I-L, S) or *myt1* mutants (E-H, M-P, Q, R, U-W) stained for Cnn (red), Tubulin (green) or DNA (blue), with (K, O, Q, U, S, W) showing merged images. (A, B, E, F) Late prophase I spermatocytes. Only faint astral staining (arrows) can be seen in wild type (A) and mutants (E), although *myt1* mutants (F) are different from wild type (B) in that centrosomes and centrioles (red, arrows) are already separated. Note that chromosome condensation is not complete yet. (C, D, G, H) Prometaphase I spermatocytes. The *myt1* mutant cells form four asters (G, green) rather than two asters (C, green), which correlate with four centrioles (H, red). (I, J, M, N) Metaphase I spermatocytes. The *myt1* mutant cells apparently form a bipolar spindle (M) albeit with four poles (N, red), whereas a bipolar spindle (I) with two poles (J, red) form in the control. (K, L, O, P, Q, R, U, V) Telophase I spermatocytes. In *myt1* mutants, chromosome segregates into two cells with two poles (O, P), three poles (Q, R) or four poles (U, V). Note that in (R, green arrows) centrosome segregation is also unequal between two daughter cells. (R, S, V, W) Metaphase-telophase II spermatocytes. The *myt1* mutant cells often have chromosome lagging behind during telophase and results in unequal segregation of chromosome. (T, X) Phase-contrast micrographs of spermatids from controls and mutants, respectively. Both DNA (phase bright) and the mitochondrial derivatives called Nerbenken (phase dark) show unequal segregation in *myt1* mutant spermatids (X, arrows). Bar for A-S, U-W: 20 μm; bar for T, X: 40 μm.

3.3.4 Inappropriate Cdk1 activity in the cytoplasm is responsible for centrosomal defects

To explain the meiotic defects in *myt1* mutant males, I hypothesized that the loss of *myt1* activity caused inappropriate cytoplasmic Cdk1 activity, which in turn could trigger premature centrosomal events in the mutant primary spermatocytes. In order to demonstrate that Cdk1 phosphorylation status is altered in *myt1* mutants, I performed Western blotting experiments with phospho-specific Cdk1 antibodies, on proteins extracted from control and *myt1* mutant testes (Fig 3-4A). As predicted, the T14 phospho-isoform of Cdk1 (expected to be a Myt1-specific phosphorylation event), was virtually abolished in *myt1* mutant testes. The low levels of antibody staining remaining in the mutants may be due to small amounts of antibodies recognizing the unmodified Cdk1 protein, that were not completely removed during the affinity purification of these antibodies (E. Homola, pers. comm.). Surprisingly, levels of the Y15 Cdk1 phospho-isoform appeared to be higher in the *myt1* mutants, implying that Wee1 activity might be up-regulated as a compensatory response, when Myt1 is absent.

As another means of testing the hypothesis that defects seen in the *myt1* mutants were a consequence of failure to regulate Cdk1 properly, I utilized a non-inhibitable form of Cdk1, Cdk1AF, which is resistant to inhibitory phosphorylation by Myt1 and Wee1 kinases. A transgene carrying this allele can be induced in flies by heat shock treatment, whereas under normal temperature conditions the transgenic flies do not express Cdk1AF. When I performed this experiment, I observed that many primary spermatocytes from the heat-shocked animals (Fig. 3-4B, white circle) showed premature centrosome separation and splitting phenotypes, similar to those seen in *myt1* mutants (78%, N=50). Similar changes were also occasionally seen in heat-shocked control animals (12%, N=50) carrying an inducible inhibitable form of Cdk1 (Fig. 3-4C, white circle), suggesting that under these conditions the levels of dMyt1 activity become limiting and are thus insufficient to completely inhibit all of the ectopic Cdk1. In both cases, KLP61F staining of fusomes appeared diffuse, suggesting that the heat-shock protocol and/or the presence of active Cdk1 can disrupt the fusome structure. These results allowed me to conclude that inhibitory phosphorylation of Cdk1, especially on the T14 residue, is important for

regulating the timing of centrosome separation and splitting during male meiosis in *Drosophila*.

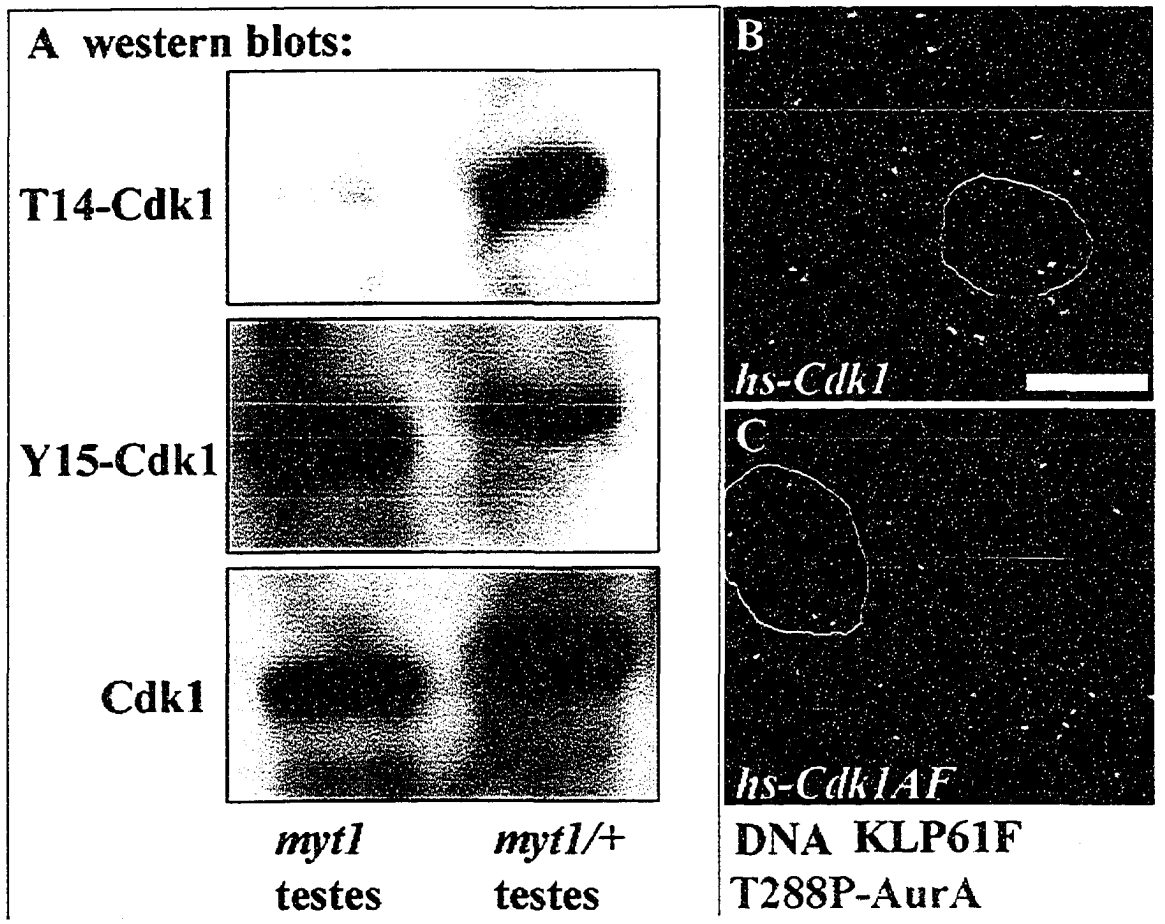

Fig. 3-4 Loss of inhibitory phosphorylation of Cdk1 causes centrosomal defects in *myt1* mutant primary spermatocytes. (A) Phosphorylation status of Cdk1 in proteins extracted from testes. Myt1-mediated T14 phosphorylation of Cdk1 was virtually undetectable in *myt1*mutant testes (left lane) when compared to control testes (right lane). However, Y15 Cdk1 phosphorylation seemed to be higher in the *myt1* mutants (left lane) than in controls (right lane). Non-phospho-specific Cdk1 antibody staining served as a loading control for both lanes. The bands in the right lane appeared to be slightly up-shifted, suggesting they represent the doubly phosphorylated (T14Y15) form of Cdk1 which is expected to be present in the controls and not in the mutants. (B, C) Primary spermatocytes stained for fusome (KLP61F in green), centrioles (T288p-AurA in red) and DNA (Hoechst 33258 in blue), with white circles outlining individual cells. Approximately 78% (N=50) of the primary spermatocytes from heat-shock treated Cdk1AF animals showed separated and/or split centrosomes (C, white circle). This phenotype was infrequent (12%, N=50) in controls with heat-shocked inhibitable Cdk1 (B). Bar for (B, C): 8 μm.

3.3.5 Reducing *wee1* results in a "pulverized" chromosome defect, apparently due to premature chromosome condensation (PCC) during male meiosis of *myt1* mutants

Results discussed above indicated that inappropriate cytoplasmic activation of Cdk1 complexes is likely responsible for the premature centrosomal separation and splitting defects, as well as for the fusome defects observed in *myt1* mutant primary spermatocytes. The regulatory mechanism that prevents other meiotic events from also occurring prematurely is not accounted for by this explanation, however, since the prevailing model describing G2/M transitions predicts that once a small amount of active Cdk1 becomes available, a positive auto-amplification mechanism rapidly converts the remainder to this form (Abrieu et al., 1998; Hoffmann et al., 1993; Kumagai and Dunphy, 1995; Mueller et al., 1995; Patra et al., 1999). Because metazoans have both Myt1 and Wee1 as Cdk1 inhibitory protein kinases, I considered the possibility that nuclear Wee1 might compensate for the absence of Myt1 to maintain the timing of nuclear meiotic events in *myt1* mutant males. Indeed, a previous study showed that Wee1 was sufficient for normal timing of mitosis, as long as Cdk1 was only active in the cytoplasm (Heald et al., 1993). It is clear that dWee1 has no essential role in regulating male or female meiosis, since zygotic wee1 mutants are viable and fertile (Price et al., 2000). To investigate whether Wee1 might serve an essential role during male meiosis in a sensitized genetic background, I removed one gene copy of *wee1* in a *myt1* mutant background by genetic manipulation, to examine whether Wee1 serves a detectable function in this background.

Double mutants, *wee1; myt1,* were never recovered as adults, suggesting a synthetic lethality phenotype. Strikingly, *wee1* is haplo-insufficient in a *myt1* mutant background, as indicated by a significant decrease in adult viability and enhancement of the adult bristle phenotypes detectable in microchaetae. As these results concern other aspects of redundancy between Myt1 and Wee1, they will be described in more detail elsewhere (in Chapter 4). In this chapter, I only focus on analyzing male meiotic defects in flies of $wee1^{DSI}$/+; *myt1* genotype. The hypomorphic allele $wee1^{DSI}$ was chosen for this analysis because it caused meiotic defects that were similar to those seen with the

null allele ($wee1^{ES1}$) in *myt1* mutant primary spermatocytes, but permitted the recovery of more viable adults for experiments.

In these experiments, primary spermatocytes from $wee1^{DS1}$/+; *myt1* flies exhibited aberrant chromosome morphology: chromosome is pulverized into more than 10 pieces (Fig. 3-5C, C', inset), in contrast to the four dense clouds of chromosome seen in *myt1* mutants (Fig. 3-5B, B', inset) or wild type (a $wee1^{DS1}$/+ control was the same as wild type, data not shown) controls (Fig. 3-5A, A', inset). At pro-metaphase I, spermatocytes from $wee1^{DS1}$/+; *myt1* flies form multipolar spindles (Fig. 3-6C, β-Tubulin in green), similar to those seen in *myt1* mutants alone (Fig. 3-6B, Tubulin in green), however the chromosome appears more condensed and fragmented (Fig. 3-6B, DNA in blue) than was seen in *myt1* mutants alone (Fig. 3-6C, DNA in blue). It was also noticeable that chromosome condensation in *myt1* primary spermatocytes (Fig. 3-6B, DNA in blue) seems to be lagging behind, compared to controls (Fig. 3-6A, DNA in blue) or $wee1^{DS1}$/+; *myt1* flies (Fig. 3-6C, DNA in blue). In sharp contrast to the normally condensed and aggregated chromosomes seen in controls at metaphase I (Fig. 3-7A, PH3 in red) and in *myt1* mutants (Fig. 3-7B, PH3 in red), the $wee1^{DS1}$/+; *myt1* mutants exhibited fragmented and dispersed chromosomes in primary spermatocytes (Fig. 3-7C, PH3 in red). These $wee1^{DS1}$/+; *myt1* mutant testes produced spermatids with hyper-condensed nuclei of uneven sizes and strong PH3 staining (Fig. 3-7C, PH3 in red, arrow), which were never seen in either controls or *myt1* mutants alone (data not shown).

In conclusion, removing one copy of *wee1* from *myt1* mutants has an extraordinary effect on the timing of chromosome condensation during male meiosis. This result provides the first experimental evidence that Wee1 has a meiotic function that is distinct from that of Myt1, during *Drosophila* spermatogenesis.

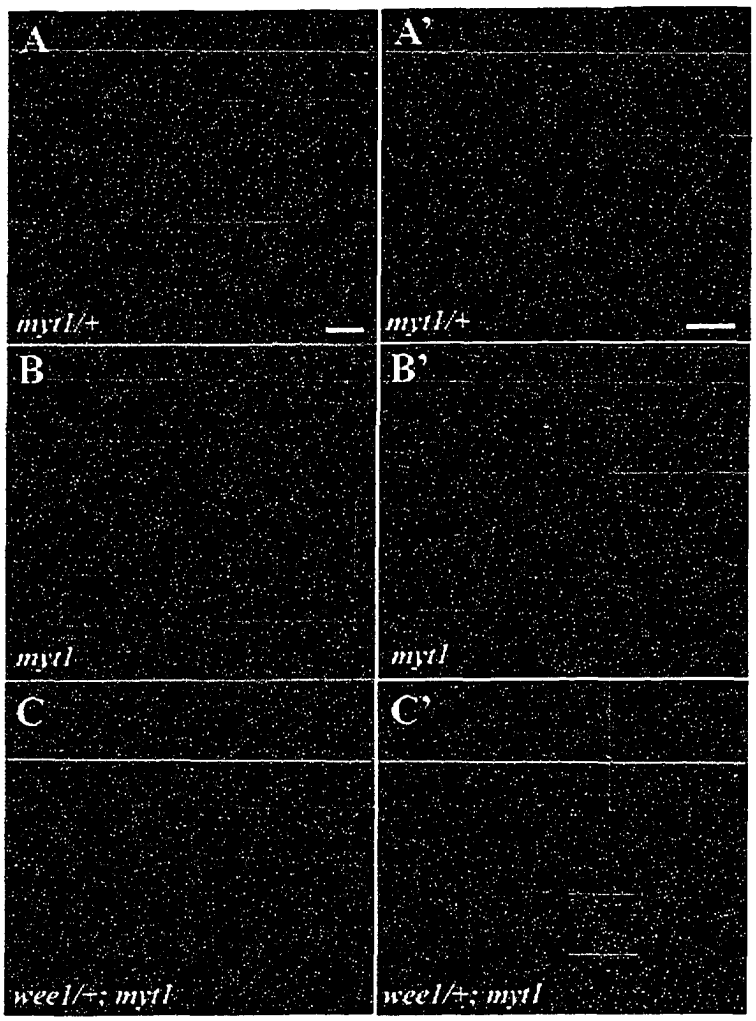

Fig. 3-5 Reduction of *wee1* in a *myt1* mutant background leads to the appearance of "pulverized" chromosomes, presumably reflecting premature chromosome condensation in stage 4 primary spermatocytes. DNA stained with Hoechst 33258 in blue. (A, B, C) The apical tips of testes from *myt1/+*, *myt1* and $wee1^{DSI}$/+; *myt1* animals, respectively. Boxed areas are shown in A', B', C'. (A', B', C') higher magnification of boxed areas in A, B, C. Insets are close-ups for boxes in A', B, C'. Note that chromosome condenses into 3-4 normal-looking clumps in primary spermatocytes from *myt1/+* (A') and *myt1* (B') males, but not in $wee1^{DSI}$/+; *myt1* (C') males, where chromosome appears "pulverized". Bar in A-C: 40 μm; bar in A'-C': 20 μm.

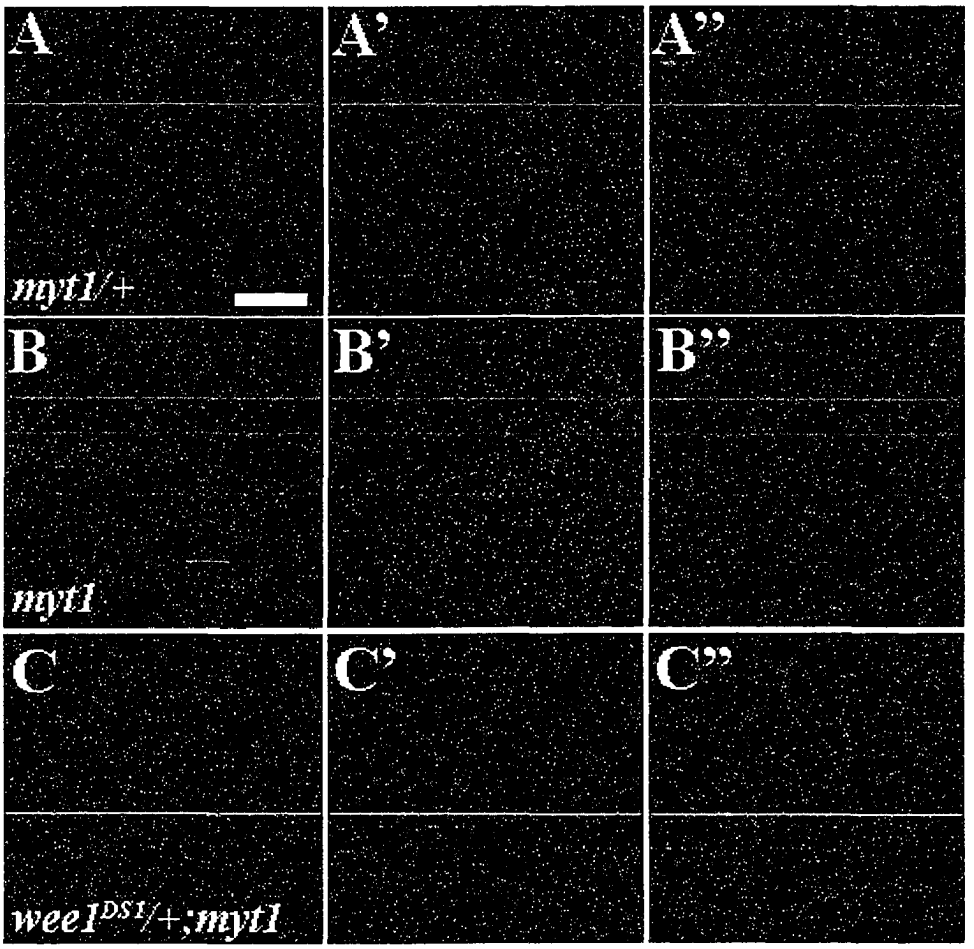

Fig. 3-6. Meiosis proceeds with fragmented nuclei in primary spermatocytes from *wee1*^{DSI}/+; *myt1* males. Pro-metaphase I primary spermatocytes: (A) *myt1*/+; (B) *myt1* and (C) *wee1*^{DSI}/+; *myt1* males that were stained for _-Tubulin (green in A, B, C and A'', B'', C'') and DNA (blue in A', B', C' and A'', B'', C''). The formation of prominent asters was used to score the beginning of pro-metaphase I in primary spermatocytes of all genotypes (A-C, green). When comparing their chromosome morphology (A'-C'), the spermatocytes from *wee1*^{DSI}/+; *myt1* mutants (C'') revealed apparently fragmented nuclei relative to controls and *myt1* mutants alone due to premature chromosome condensation in early prophase I. Bar for all panels: 8 μm.

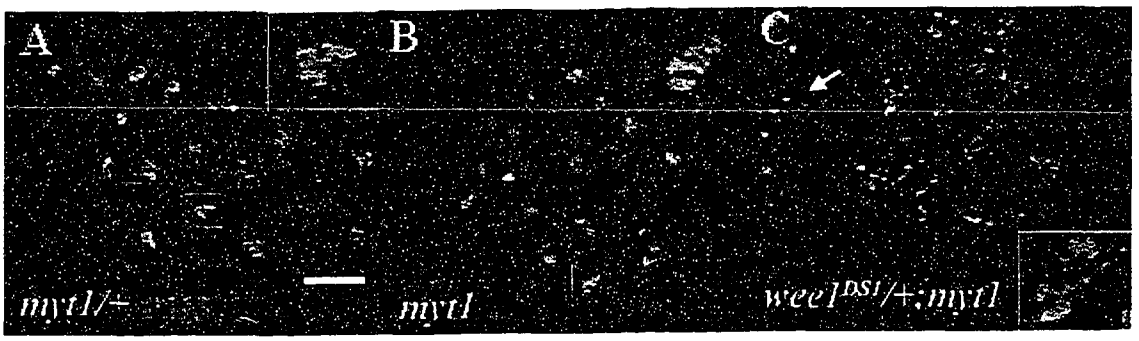

Fig. 3-7 Aberrant meiosis in *wee1*^{DSI}*/+; myt1* males, apparently due to premature chromosome condensation. A cyst of metaphase I primary spermatocytes from (A) *myt1/+*; (B) *myt1* and (C) *wee1*^{DSI}*/+; myt1* males, respectively, stained for _-Tubulin (green) and PH3 (red). Boxed area in A, B and C are shown in corresponding insets. The chromosome morphology of metaphase I primary spermatocytes is almost the same in *myt1/+* as it is *myt1* mutants (A, B, insets), but very different from that seen in *wee1*^{DSI}*/+, myt1* mutants, where the chromosome appears spread out and fragmented (inset in C). The meiotic spindle morphology (green in A, B and C) also appears more aberrant in *wee1*^{DSI}*/+, myt1* mutants than in controls or *myt1* mutants alone (data not shown). Bar for (A-C): 20 μm.

3.4 Discussion

Our analyses of the meiotic behavior of primary spermatocytes in *myt1* mutants and in *wee1/+, myt1* mutants suggest that Myt1 and Wee1 are responsible for the timing of distinct meiotic events. Although it has previously been proposed that Myt1 and Wee1 serve distinct roles in regulating Cdk1 activity in the cytoplasm and nucleus, respectively (O'Farrell, 2001; Takizawa and Morgan, 2000), the results present here provide the first direct evidence for this model in a developmental context. It is, therefore, of special interest to discuss whether or not these findings may apply to meiosis and mitosis in other systems.

3.4.1 Are the *myt1* mutant centrosomal defects specific to male meiosis?

I presented evidence for an early defect: premature centrosome separation, in *myt1* mutant primary spermatocytes, prompting a search for a similar defect in mitotic cells. However, neither germline stem cells nor spermatogonial (or oogonial) cells in mutant testes (or ovaries) showed any obvious centrosome separation defect, nor was one observed in somatic cells in these tissues (data not shown). Moreover, dividing cells from 3rd instar wing imaginal discs and cellularized, gastrulating embryos did not display this defect (data not shown). In the animal kingdom, oocytes in most species do not contain centrosomes, although they are required in some (Cullen and Ohkura, 2001; Theurkauf and Hawley, 1992; Waters and Salmon, 1997). Since I was only able to detect a premature centrosome separation phenotype in *myt1* mutant primary spermatocytes, my results suggest that Myt1 is specifically required for normal timing of centrosome separation in these cells during the ~90 hour long G2 phase arrest. Real time recording of cell division events in *myt1* mutant flies in multiple tissues might help to determine whether timing events are disrupted, as a means of testing this hypothesis.

Another peculiar centrosomal defect seen in *myt1* mutant primary spermatocytes was centrosome splitting – the separation of two centrioles, which normally takes place at the beginning of meiosis II in secondary spermatocytes. One possible explanation for this defect is that Myt1 somehow protects the cohesion of mother-daughter centrioles at meiosis I indirectly by inhibiting Cdk1/cylin B activity, a possibility supported by data

showing that *cdk1AF* expression can also produce a similar phenotype. Also possibly relevant to this issue was the observation that centrosomes split into centrioles when mammalian cells enter mitosis with damaged DNA or with unfinished DNA replication due to drug inhibition (Hut et al., 2003). Similarly, using other drug treatments to bypass checkpoint mechanisms and apoptosis, some mammalian cell lines can form multipolar spindles, presumably caused by a similar "centrosome splitting" (Castedo et al., 2004). This form of centrosome splitting has also been considered to be part of a "centrosome inactivation" mechanism to terminate defective mitotic cells, because such consequent chromosome segregation defects would typically result in aneuploidy and cell death (Hut et al., 2003). Another relevant case was observed in *S. pombe*, where conditional double deletion of *wee1* and *mik1* (the second *S. pombe wee1* homolog), caused a meiotic metaphase I arrest with separated spindle pole bodies – which are equivalent to centrosomes (Murakami and Nurse, 1999). Between these possibilities, I believe the data favor the first possibility as being most likely correct (ie. that Myt1 activity is required for normal centrosome cohesion). This is because primary spermatocytes in *myt1* mutants with split centrosomes did not appear to display two prominent features seen in mammalian cells (, that were forced into mitosis with DNA damage or incomplete DNA replication) : being blocked at metaphase and segregating their chromosome in to 3-4 cells at a later stage (Castedo et al., 2004; Hut et al., 2003).

Although I postulate that Myt1 normally functions to suppress premature Cdk1/Cyclin activation in primary spermatocytes and thereby prevent premature centrosome separation during a prolonged G2 phase, it remains unclear whether Myt1 regulation of centrosome and centriole cohesion would be direct (due to inhibition of centrosome-localized Cdk1 complexes) or indirect (inhibition of Cdk1 elsewhere in the cytoplasm, preventing active Cdk1 from gaining access to centrosomes). One possibility that could specifically account for the centriole splitting phenotype is that a small pool of Myt1 may associate with two centrioles and remain active prior to the beginning of meiosis II. It is possible to test this idea by determining if Myt1 is down-regulated during meiosis I and when this occurs, if entry into meiosis is regulated by mechanisms similar to those documented in *Xenopus* oocytes (Karaiskou et al., 2004; Palmer et al., 1998).

3.4.2 How is centrosome separation uncoupled from other meiotic events in *myt1* mutants?

Cdk1 forms complexes with 3 different mitotic Cyclins (A, B, B3-type), each of which can promote mitotic events (Jacobs et al., 1998; Lehner and O'Farrell, 1990). However, Cdk1/Cyclin B complexes appear to play the major role in catalyzing mitotic events in most systems examined. In HeLa cells, activation of Aurora A (marked by T288 phosphorylation of Aurora A: T288P-Aurora A) at centrosomes occurs first in late G2 phase (Hirota et al., 2003), which then mediates Cdk1/Cyclin B activation, subsequently catalyzing the phosphorylation of many target proteins at the onset of mitosis. One of the many targets of Aurora A and Cdk1/Cyclin B is the motor protein KLP61F (Blangy et al., 1995; Giet et al., 1999), which is thought to play an important role in partitioning centrosomes, by pushing them apart from each other to two poles at the onset of mitosis (Blangy et al., 1995; Giet et al., 1999; Heck et al., 1993). In addition to centrosome proteins, activated Cdk1/Cyclin B complexes act on many other target proteins to promote different mitotic events: including chromosome condensation, NEBD, and spindle assembly. Accordingly, the cytoplasmic activation of centrosome-localized Cdk1/Cyclin B complexes has been proposed to serve as the key factor in coupling centrosome separation with other mitotic events (Nigg et al., 1996). Since a similar coupling of M phase events also exists during *Drosophila* male meiosis, conserved regulatory mechanisms for Cdk1/Cyclin B activation may also be used in this process.

In very young control primary spermatocytes (approximately 90 hours before meiosis I) T288P-Aurora A immunolocalization appears as four closely juxtaposed centrioles, however the two centrosomes do not normally separate until prometaphase. A polyclonal antibody against *Drosophila* Aurora A has also described (Berdnik and Knoblich, 2002) that only stains two centrosomes, suggesting that (the monoclonal antibody against human) T288P-Aurora A specifically cross-reacts with a subset of *Drosophila* Aurora A proteins. In *myt1* mutant primary spermatocytes, T288P-Aurora A also localizes to the centrosomes as seen in controls, however, the two centrosomes separated prematurely and then split into four centrioles well before prometaphase, as described earlier. Since *cdk1AF* mutants could phenocopy the premature centrosome

separation/splitting defects of *myt1* mutants in primary spermatocytes, these results suggest that these centrosomal defects were due to inappropriate Cdk1/Cyclin B activation. The biochemical data presented earlier indicated that Myt1 mediated T14 phosphorylation of Cdk1 was largely absent in *myt1* mutant testes, although Y15 phosphorylation was still prominent, suggesting that the T14-specific phospho-isoform might have a distinct behavior (with respect to Cdk1 activity, localization or substrate recognition behavior), that is relevant to understanding the behavior of the mutants. Despite premature centrosome separation behavior in *myt1* mutants, other meiotic events do not seem to be advanced, implying that centrosome separation and splitting are uncoupled from these other M phase events. Therefore, my results support the hypothesis that dMyt1 regulates the timing of centrosome separation and splitting during male meiosis, by inhibiting cytoplasmic Cdk1/Cyclin B activity.

More than ten years ago, Heald and colleagues demonstrated in HeLa cells that nuclear Wee1 kinase can protect the nucleus from cytoplasmically activated Cdk1/Cyclin B complexes (Heald et al., 1993). These authors did not report any centrosomal defects in their experiments, however, raising an important question regarding the generality of my model. Perhaps the centrosomal defect occurred but was too subtle to be seen with immuno-staining. Alternatively, endogenous Myt1 may have still been able to protect the centrosomes from artificially induced Cdk1/Cyclin B activity in those experiments. Regardless, the present results in *Drosophila myt1* mutant primary spermatocytes have disclosed several novel features regarding Myt1 function that are important for understanding how meiosis is normally initiated. Since activated Cdk1/Cyclin B in the cytoplasm of *myt1* primary spermatocytes was insufficient to induce nuclear meiotic events such as chromosome condensation and NEBD (at least in *Drosophila* primary spermatocytes), the strong implication of these studies was that nuclear and cytoplasmic Cdk1/Cyclin B can be activated separately.

3.4.3 Wee1 dictates the timing of chromosome condensation in primary spermatocytes

Unlike the situation in *myt1* mutants alone, $wee1^{DSI}$/+; *myt1* flies displayed "pulverized" **p**remature **c**ondensed **c**hromosomes (PCC) as early as G2 phase, suggesting that Wee1

serves a conserved role in protecting the nuclei of primary spermatocytes so that they are unaffected by inappropriate Cdk1 activity in the *myt1* mutants. Presumably, activated Cdk1/Cyclin B complexes can translocate from the cytoplasm into the nucleus and initiate chromosome condensation prematurely, when dWee1 levels are limiting (ie. in $wee1^{DS1}$/+; *myt1* mutant primary spermatocytes). In this situation, the capacity for protecting nuclear structures from prematurely active Cdk1 would be diminished, apparently resulting in chromosome fragmentation. As meiosis I proceeds, chromosomes in primary spermatocytes from $wee1^{DS1}$/+; *myt1* mutants appeared fragmented and to segregate improperly. This was a strong enhancement of the *myt1* phenotype, because in *myt1* mutants alone chromosome condensation was not premature and the chromosome segregation problem was not obvious until meiosis II.

One might wonder why lowering the *wee1* gene dosage causes "pulverized" looking chromosome only in primary spermatocytes? Early reports of a similar phenotype were first described as premature chromosome condensation (PCC) in a study of mammalian cells forced to prematurely enter into M phase prior to the completion of DNA replication by caffeine treatment (Johnson and Rao, 1970). Without enough nuclear Wee1 protein, "pulverized" PCC could form as a consequence of aberrant chromosome condensation prior to completion of pre-meiotic DNA replication in the primary spermatocytes of $wee1^{DS1}$/+; *myt1* mutants. Further experiments are required to examine the possible explanations for this phenotype.

Unlike the chromosome condensation defects described above, NEBD and spindle assembly are not obviously premature in the primary spermatocytes of $wee1^{DS1}$/+; *myt1* animals. Since these animals still have a wild copy of $wee1^{+}$ in addition to a partially functional hypomorphic allele of *wee1*, different requirements for Cdk1 regulation of these structures may explain why no defects were observed in NEBD and spindle assembly. That possibility was not tested, because complete removal of both *wee1* and *myt1* was not compatible with adult viability. It will be interesting to determine if such double mutants die from premature timing of all cytoplasmic and nuclear mitotic events, as our model predicts. Other factors such as translation control of $Cdc25^{twine}$ or the accumulation or stability of Cyclin B may also contribute to the timing of NEBD and

spindle assembly during prophase I of male meiosis; ideas that could also be examined in the future.

Although I have provided evidence that *myt1* and *wee1* are required for regulating distinct cytoplasmic and nuclear events during male meiosis, these genes were not apparently required for similar events during female meiosis. *Drosophila* female meiotic spindles are acentrosomal (Matthies et al., 1996), so centrosomal defects are an irrelevant issue in *myt1* mutant oocytes. Genetic data (presented in Chapter 2) did indicate, however, that *myt1* mutations caused elevated X chromosome non-disjunction (NDJ) to a frequency of ~11% during female meiosis. Currently, I am investigating the possibility that loss of Myt1 disrupts meiotic spindle assembly in females. In a drastic contrast to the "pulverized" appearance of chromosome in primary spermatocytes of $wee1^{DS1}$/+; *myt1* flies, chromosome condensation appeared normal in oocytes of female siblings with this genotype (data not shown). Interestingly, ~5% (N=150) of the egg chambers from $wee1^{DS1}$/+; *myt1* females contain only 8 germline cells (7 nurse cells and 1 oocyte) rather than 16 germline (15 nurse cells and 1 oocyte), suggesting that reducing Wee1 in a *myt1* mutant background shortens the typical four mitotic cycles to three cycles in some egg chambers (*myt1* alone females do not contain any 8-cell egg chambers). Collectively, my results show that Myt1 and Wee1 exhibit striking sexual dimorphism with respect to regulation of meiosis in *Drosophila* males and females, suggesting another layer of mechanistic complexity between these two seemingly parallel biological processes. Future studies focused on the relationship of Myt1 and Wee1 in regulating *Drosophila* female meiosis should further advance our knowledge of Wee1-like kinases in metazoans.

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Chapter 4 Other results

4.1 Initial characterization of embryos derived from *myt1* females

Fertilized *Drosophila* embryos undergo rapid nuclear division with alternating S/M cycles but no detectable G2 phase for 13 cycles (reviewed in Foe, 1993). A G2 phase is first introduced at cycle 14, as Cdk1 is inactivated by inhibitory phosphorylation on its T14 and Y15 residues. Entry into mitosis is regulated by the activity of $Cdc25^{stg}$ phosphatase, which removes inhibitory phosphates from Cdk1 and thereby promotes its activation (Edgar and O'Farrell, 1990). Since Myt1 was reported to phosphorylate Cdk1 at T14 and Y15 sites (Booher et al., 1997; Fattaey and Booher, 1997; Liu et al., 1997; Mueller et al., 1995), it is relevant to ask whether *myt1* is required for maintaining this or other G2 phases during embryonic development. Embryos derived from *myt1* mutant females (referred to here as *myt1* mutant embryos) can be grouped into the following categories, according to their developmental stages and/or appearance: (early) arrested embryos, "chromosome bridge" and "sinking nuclei" syncytial embryos, a small number of embryos were found with "extra division" during cycle 14 (which was not documented in this study), deformed gastrulating embryos, which may arise as a consequence of cycle 14 defect and "normal" gastrulating embryos.

When stained for DNA, some embryos (age 7-19 hr) derived from *myt1* mothers showed very little DNA staining and thus were apparently arrested at an early stage of embryonic development. To analyze this early arrest phenotype, a shorter collection (3-5 hr) was made for immunofluorescence staining. The youngest embryos in this collection (3 hr) should have completed the syncytial divisions and be in cycle 14, under normal developmental conditions. In mutant embryos from this collection, 56.4% were "early arrest" (Table 4-1), containing only 1-3 masses of PH3-positive staining (red), which marks condensed mitotic chromosomes (Fig. 4-1, A-I, red). α-Tubulin staining of these embryos showed that most contained abnormal looking spindle-like structures (Fig. 4-1, A-I), having a spindle morphology distinct from that of embryos from the control collection, an example of which is shown in Fig. 4-3G.

The chromosome (Fig. 4-1A, B, D, E, I) and microtubule morphology of *myt1* mutant embryos resembles that of polar body nuclei in unfertilized eggs (Endow and Komma, 1997; Foe, 1993; Shamanski and Orr-Weaver, 1991), suggesting that these mutant embryos are arrested at metaphase, either during meiosis or the first mitotic cell

division. The proportion of mutant embryos with this "early arrest" phenotype apparently decreased to 29% after 7 hr as shown in Table 4-1. This may be due to a variable penetrance of this phenotype, be related to maternal effects or, conceivably, some of the embryos scored as "early arrest" may be able to proceed further in development if given more time. Alternatively, this result may simply reflect experimental variation.

Considering the similarity between *myt1* mutant embryos and unfertilized eggs, as described previously (Endow and Komma, 1997; Shamanski and Orr-Weaver, 1991), one possibility is that *myt1* may be involved in the transition from meiosis to mitosis during early embryogenesis. For example, fertilized *Xenopus* eggs are maintained in a G2 phase by up-regulated Wee1 activity at the first embryonic cell cycle, presumably allowing sufficient time for the fusion of pronuclei from the egg and the sperm (Murakami et al., 1999; Walter et al., 2000). Therefore, it is tantalizing to speculate that Myt1 may serve a similar function in *Drosophila.* Future experiments should focus on female meiosis to determine how it is affected in *myt1* mutants and to explore the relationship between meiosis and the first cell cycle during early development. Alternatively, the anterior polar cells, which are required for establishing a functional conduit for sperm called a micropyle (Spradling, 1993), frequently undergo ectopic mitosis in *myt1* mutants (as described in chapter 2 and chapter 4), meaning that loss of *myt1* activity in females may hamper sperm entry and result in a higher frequency of unfertilized eggs. Experiments with *Gal4*-driven germline-specific and polar cell-specific expression of *UAS-myt1* should help to distinguish these two possibilities.

Another phenotype seen in embryos lacking maternal *myt1* appears around embryonic cycle 11. During mitosis, chromosome bridges (DNA in blue) between segregating anaphase nuclei may be observed (Fig. 4-2B, arrows and inset), although insufficient embryos were analyzed to be sure that this was a meaningful observation. Another maternal *myt1* mutant phenotype seen during embryogenesis was the occurrence of "orphan" centrosomes, as if the nuclei were detaching from the centrosomes and sinking inwards (Fig. 4-2B, C, E, F, red dots in white circles). Curiously, the number of "orphan" centrosomes appears to increase progressively during later embryonic cell

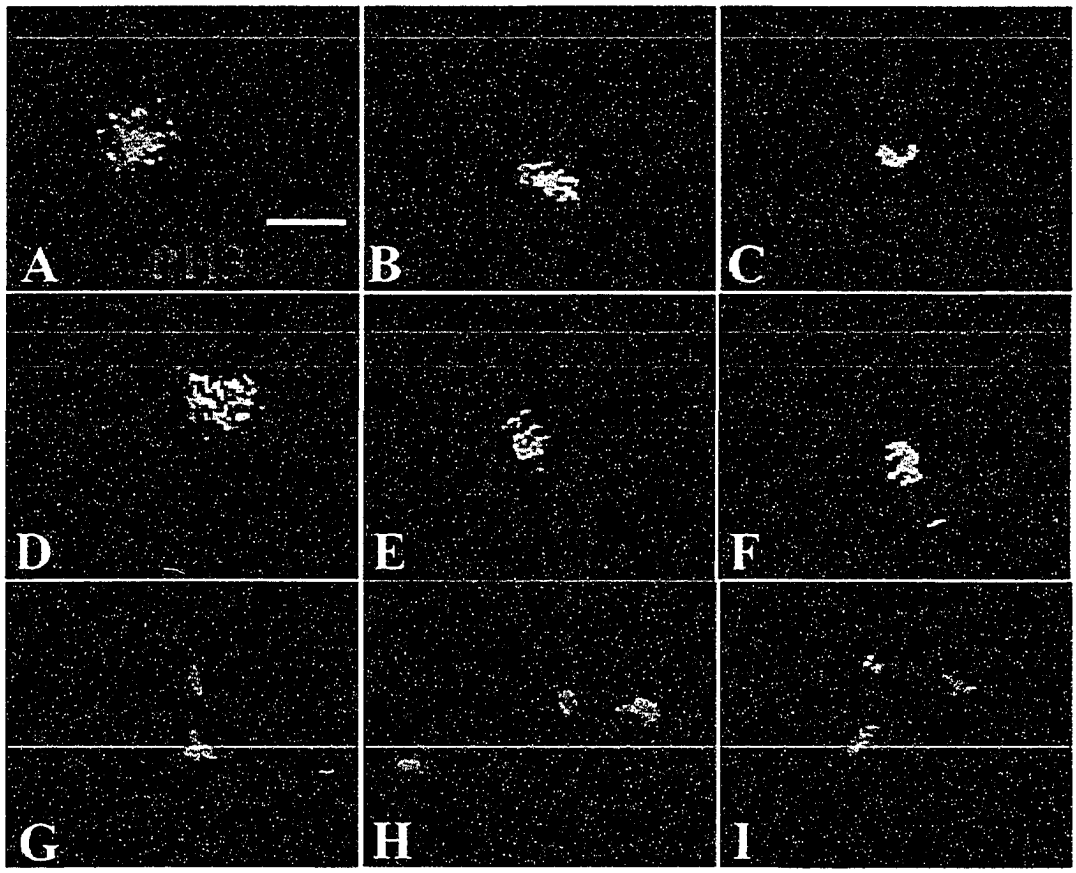

Fig. 4-1 Examples of "early arrest" embryos derived from *myt1* mutant females. Age 3-5 hr old embryos (grown at 25°C) were stained for α-Tubulin (green) and PH3 (red) to visualize mitotic spindles and chromosomes, respectively. (A, D, G-I) are images taken from different embryos, whereas (B, C) and (E, F) represent two different fields of two individual embryos. These arrested *myt1* mutant embryos contain up to 3 nuclei and therefore do not appear to have undergone any zygotic cell divisions. Bar: 20 μm.

Table 4-1.

Quantification of different phenotypes in embryos derived from *myt1* females

female genotype	age	arrested	deformed	"normal"	total	hatch rate
$myt1^1/myt1^2$	3-5 hr	56.4%	12.4%	31%	250	NC
yw	3-5 hr	3.4%	0.9%	95.7%	210	NC
$myt1^1/myt1^2$	7-19 hr	29%	40%	31%	210	NC
yw	7-19 hr	0.9%	0.9%	98%	210	NC
$myt1^1/myt1^2$	48-72 hr	NC	NC	NC	600	28%
yw	48-72 hr	NC	NC	NC	600	98%

Note: The categorization of "chromosome bridge" and "sinking nuclei" syncytial embryos was not collected in these experiments. NC indicates data not collected.

cycles in *myt1* mutant embryos (compare red dots in white circles in Fig. 4-2B, C, E and F).

In *Drosophila* embryos, DNA damage and inhibition of DNA replication can also lead to the appearance of "orphan" centrosomes, thought to be a result of "centrosome inactivation", a process mediated by Chk2 for removing damaged nuclei from the cortex (Takada et al., 2003). The presence of "orphan" centrosomes in *myt1* mutant embryos thus suggests that maternal *myt1* function could be required for DNA damage and/or DNA replication checkpoint responses; consequently embryos lacking *myt1* would be expected to trigger Chk2-mediated centrosome inactivation (Takada et al., 2003). In order to investigate the potential connection of Myt1 with these established checkpoint functions, further experiments are needed to examine whether Chk2 protein kinase is actually activated in *myt1* mutant embryos and if Chk2 interacts with Myt1, either genetically or by other criteria, under these conditions.

Another phenotype observed in 12.4% embryos (referring to the 3-5 hr collection, Table 4-1) laid by *myt1* females involves severe disruption of gastrulation, with the following range of defects: 1) "giant nuclei" without centrosomes (Fig. 4-3B, inset) or with multiple (more than two) centrosomes (Fig. 4-3C) as revealed by Cnn (red) and DNA (blue) staining, 2) an overall disorganization of cells and disrupted mitotic pattern as shown by α-Tubulin (green) and PH3 (red) double staining (Fig. 4-3 E, F, green, 3) metaphase chromosomes arranged in a circular form with no bipolar spindle (Fig. 4-3E, inset). Caution needs to be taken in interpreting this latter defect however, as cells dividing perpendicular to the embryo cortex could also show a similar circular arrangement of chromosomes. All of these phenotypes are consistent with the idea that a Chk2-mediated centrosome inactivation response is operating in the "deformed" class of gastrulating *myt1* mutant embryos. To investigate this possibility, immuno-staining of Chk2 was performed. Normally, no obvious cytoplasmic Chk2 staining was seen in wild type embryos at cycle 14 (Fig. 4-3G, inset). However *myt1* mutant cycle 14 embryos (Fig. 4-3H, I, insets) exhibit many cells with Chk2-positive foci in the cytoplasm, often involving apparent "giant" nuclei that may represent cell fusion events. Moreover, the deformed embryos from *myt1* mothers show progressively more cytoplasmic Chk2 staining as the disruption of gastulation becomes more severe (compare red dots in insets

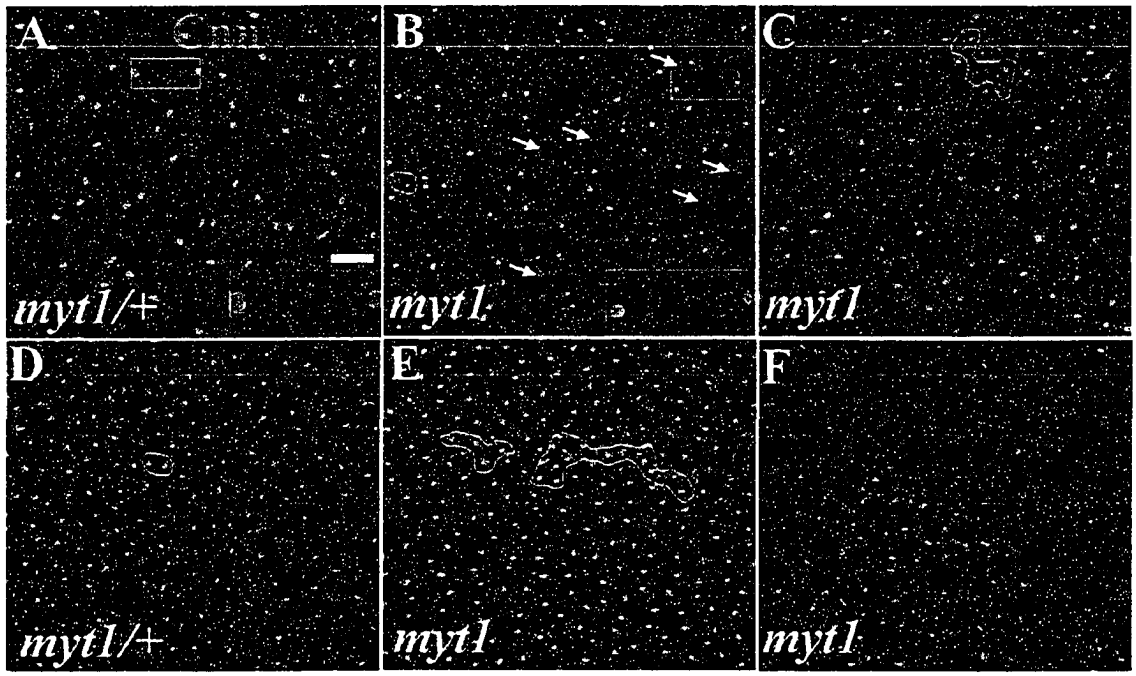

Fig. 4-2 Chromosome bridges and "orphan" centrosomes (or inactivated centrosomes) in embryos derived from *myt1* females. Control (A, D) and *myt1* mutant embryos (B, C, E, F) were stained for Cnn and Hoechst 33258 to label centrosomes (red) and DNA (blue), respectively. Boxed areas in (A, B) are shown magnified in insets. White circles mark "orphan" centrosomes. (A, B, C) Cycle 10 embryo (A) and cycle 11 embryos in (B, C), showing telophase nuclei (blue) and centrosomes (red). Arrows in B) are pointing to nuclei linked by a chromosome bridge. Note the chromosome bridge and 2 "orphan" centrosomes seen in *myt1* mutant embryos (B, inset and isolated, red dots that have been circled) but not in a control embryo (A). A late telophase cycle 11 embryo in (C) contained more "orphan" centrosomes (C, isolated, red dots are indicated by white line). (D-E) Cycle 13 embryos with interphase centrosomes (red) and nuclei (blue). *myt1* mutant embryos (E) possess many more isolated centrosomes, than control embryos (D), which occasionally also show a small number of isolated centrosomes (D, red dots in a white circle). (F) A cycle 14 *myt1* mutant embryo with many interphase nuclei (blue) losing associated centrosomes (red). Bar for all panels: 20 μm.

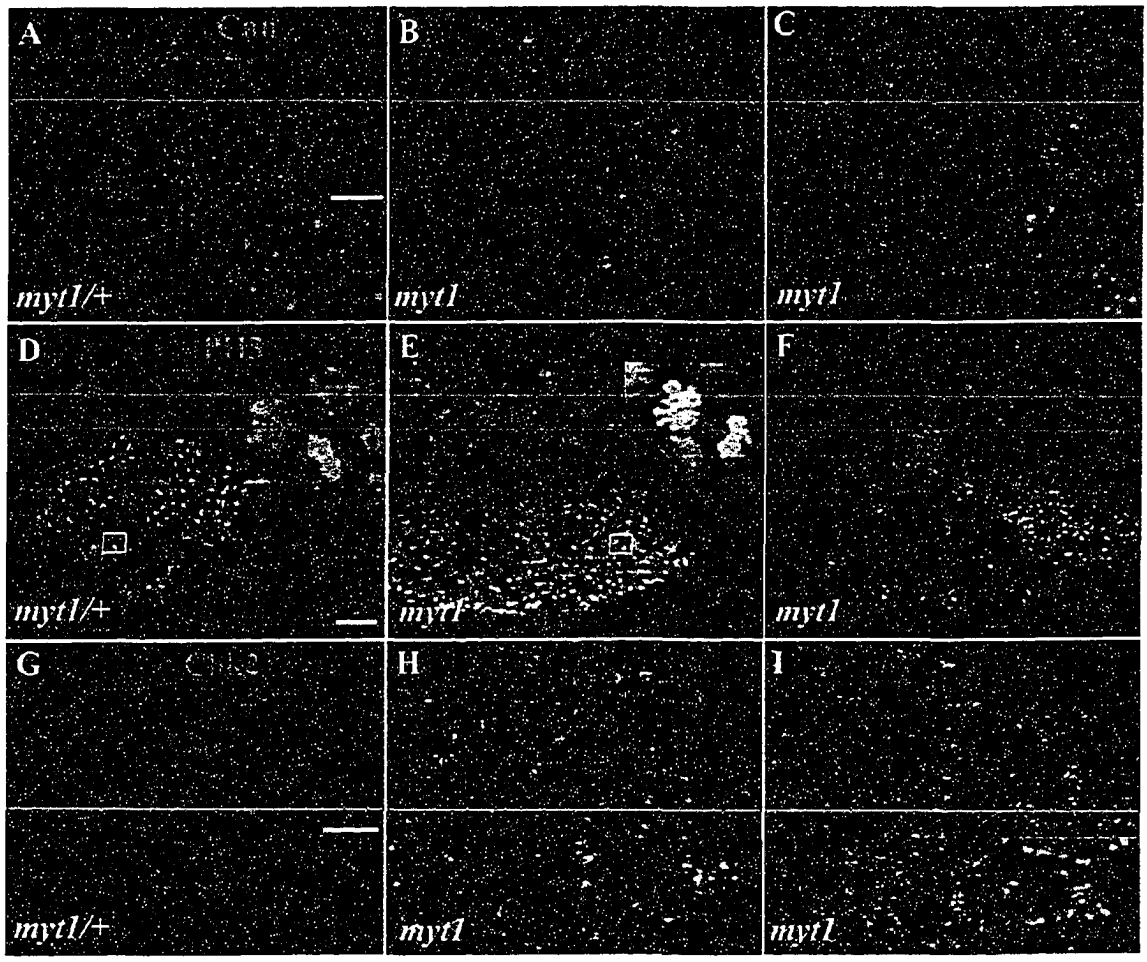

Fig. 4-3 Deformed gastrulating *myt1* mutant embryos (3-5 hr collection) contain giant nuclei. Embryos stained for Cnn (red) and DNA (blue) in (A-C), PH3 (red) and α-Tubulin (green) in (D-F) or Chk2 (red), α-Tubulin (green) and DNA (blue) in (G-I). A, D and G are control embryos, whereas B, C, E, F, G, and H are *myt1* mutant embryos. Boxed areas are shown in insets. (A-C) The control embryos show either interphase nuclei or metaphase chromosomes with two centrosomes (A, inset). *myt1* mutant embryos exhibit many giant nuclei that are not associated with any centrosomes (B, C, inset B), however occasionally some giant nuclei are observed that are associated with multiple centrosomes (B, C, inset C). (D-F) While control embryos (D) reveal the typical PH3-positive pattern of mitotic domains (Foe, 1989), deformed gastrulating *myt1* mutant embryos were observed displaying aberrant mitotic patterns (E,F). Note that in one cell (E, inset), metaphase chromosomes appear to be arranged in a circular array without a bipolar spindle. See the text for an alternative explanation. (G-I) Cytoplasmic foci of Chk2 staining were not seen in control embryos (G). The giant nuclei in *myt1* mutant embryos were often surrounded by such Chk2 foci (H and I, insets), however. Most cells in the apparently "later" gastrulating *myt1* mutant embryo shown in (I) have lost tubulin staining and appear to have arrested with partially condensed chromosomes, many of which are associated with Chk2-positive foci. Bar for (A-C); 20 μm; bar for (D-F); 40 μm; bar for (G-I); 20 μm.

of Fig. 4-3H and I). The formation of deformed *myt1* embryos may stem from abnormal cell division patterns (Fig. 4-3E, red) from cycle 14 onwards, producing gastrulating embryos with giant nuclei and isolated centrosomes, shown by Chk2 staining (Fig. 4-3H, I, insets) and Cnn staining (Fig. 4-3B, C, inset). Since these deformed embryos with giant nuclei are only seen from cycle 14 onwards, these data suggest that dMyt1 contributes to this stage of embryonic development, perhaps for maintaining (the first) G2 phase at cycle 14 during the early stages of gastrulation. Since many of the cells in an apparently "later" gastrulating *myt1* mutant embryo (Fig. 4-3I) with partially condensed chromosomes are associated with multiple Chk2-positive foci, these cells may be undergoing a centrosome inactivation analogous what was seen during syncytial stage, but in this case the affected nuclei cannot be removed by sinking into the interior.

Because the characterization of *myt1* mutant embryo defects is still preliminary, it is premature to claim any particular function(s) for Myt1 during *Drosophila* embryogenesis. Although the underlying mechanisms remain unclear, the defects described above suggest that maternal loss of *myt1* activity is problematic during at least two stages during embryogenesis: the first being the meiosis to mitosis transition, the second being gastrulation. As summarized in Table 1, only 28% of *myt1* mutant embryos (presumably comprising the "normal" class of gastrulating embryos) hatch, emphasizing that maternal dMyt1 function is important, although not absolutely essential, for *Drosophila* embryonic development.

4.2 *myt1* mutants may be defective for the DNA damage checkpoint

Upon DNA damage or inhibition of DNA replication, the Chk1 and Chk2 checkpoint kinases are activated to recruit components that are responsible for repairing DNA damage (Blasina et al., 1997; Rhind et al., 1997; Rhind and Russell, 1998), and at the same time to delay cell cycle progression by inhibiting Cdk1/Cyclin B activation; a process that involves the activity of Wee1 kinases as a potential target (Jin et al., 1996; Price et al., 2000; Raleigh and O'Connell, 2000; Rhind and Russell, 2001). To assess whether *myt1* is involved in these checkpoint responses to DNA damage, *myt1* mutant larvae were treated with 40G of ionizing radiation (IR) and their wing imaginal discs

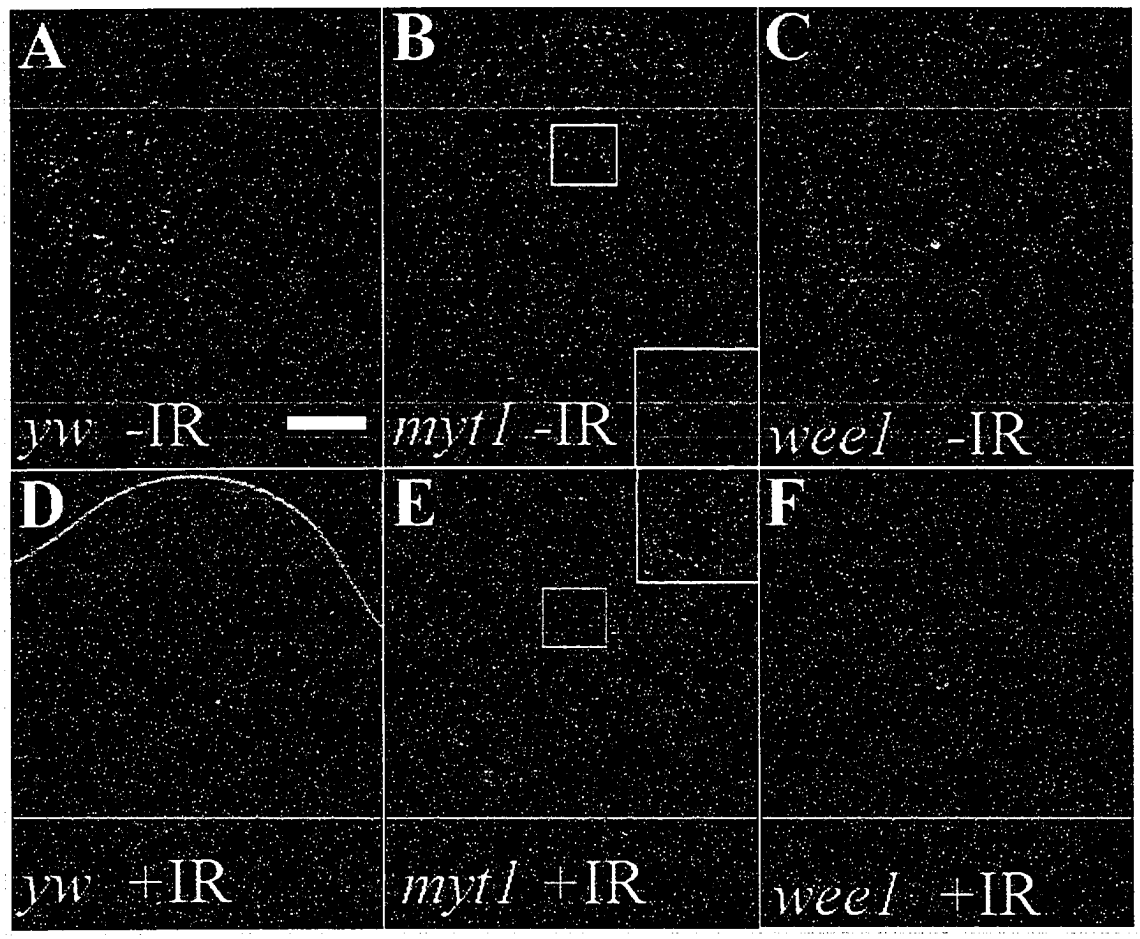

Fig. 4-4 Wing discs from *myt1* mutants contain PH3-positive cells with dispersed PH3-positive staining 30 min after IR treatment. Wandering 3rd instar wing discs from *y w* control larvae (A, D), *myt1* larvae (B, E) and *wee1* larvae (C, F) stained for PH3 (red) before IR (A-C) and after IR (D-F). Boxed areas are shown in insets. (A-C) Many PH3-positive proliferating cells can be found in control (A), *myt1* (B) and *wee1* (C) mutant discs prior to IR. (D-F) After IR exposure, the vast majority of PH3-positive cells disappear in control discs and *wee1* mutant discs, whereas many fragmented PH3-positive staining cells (compare insets in B and E, red) persist in *myt1* mutant discs. Bar for all panels: 60 μm.

were stained 30 min later for PH3 to monitor mitotic cells (a method described in Xu et al., 2001). Before IR treatment, *myt1* mutant discs appeared to contain similar number of PH3-positive cells as the discs from controls and *wee1* mutants (Fig. 4-4A, B, C). Thirty min after IR treatment, wild type discs show very little PH3 staining (meaning there are no dividing cells), an indication that the DNA damage checkpoint has been activated (Fig 4-4D). *wee1* mutant discs exhibited only a few PH3-positive cells after IR, suggesting that the DNA damage checkpoint is also intact in these *wee1* mutant cells (Fig. 4-4F). There were many PH3-positive cells present in *myt1* mutant discs after IR however, and the staining appeared to be extremely dispersed suggesting that the chromosome is fragmented, compared to the unirradiated controls (Fig. 4-4B and E, insets). This defect resembles the fragmented chromosome seen in mammalian cells undergoing mitotic catastrophe, owing to a failure of the DNA damage checkpoint (Castedo et al., 2004). These results indicate that dMyt1 and not dWee1 is responsible for the checkpoint response to DNA damage in *Drosophila.*

An early stage of the "centrosome inactivation" response to DNA damage in *Drosophila* embryos is Chk2 localization to centrosomes, followed by the removal of damaged nuclei (Takada et al., 2003). To test whether wing disc cells exhibit similar behavior after encountering DNA damage, I looked for loss of Cnn (a centrosomal protein) staining as a sign of centrosome inactivation after IR treatment. It was clear that centrosome inactivation can occur in *Drosophila* wing discs cells, since no Cnn staining was seen 30 min after IR in the controls (Fig. 4-5D). Unlike my prediction however, Cnn staining in *chk2* mutants was similar to the controls after irradiation (Fig. 4-5E), suggesting that either the centrosome inactivation response is mediated by a Chk2-independent mechanism at this stage of development, or that loss of Cnn staining is not a marker for this response. Interestingly, *grapes* (*grp*, the *Drosophila* homolog of *chk1*) mutants are compromised with respect to centrosome inactivation, exhibiting similar levels of Cnn staining in both unirradiated and irradiated wing discs (Fig. 4-5F). Most wing discs from *myt1* mutants showed centrosome inactivation response, however (Fig. 4-5H). Only ~20 percent (N= 40) of the *myt1* mutant wing discs were defective in centrosome inactivation (Fig. 4-5I), a much less severe phenotype than seen in *grp* mutants (compare Fig. 4-5F and I). These results suggest that dMyt1 activity is required

for the DNA damage checkpoint, although one aspect of this response: centrosome inactivation, may remain intact. These results may imply that dMyt1 functions at a later stage of the DNA damage checkpoint response than Grp/Chk1 and would thus be compatible with Grp/Chk1 being an upstream regulator of dMyt1. My data also showed that nuclear dWee1 was not essential for the DNA damage checkpoint response under these experimental conditions, although *wee1* is obviously important for a Grp/Chk1-regulated apparent DNA replication checkpoint required during embryogenesis (Price et al., 2000).

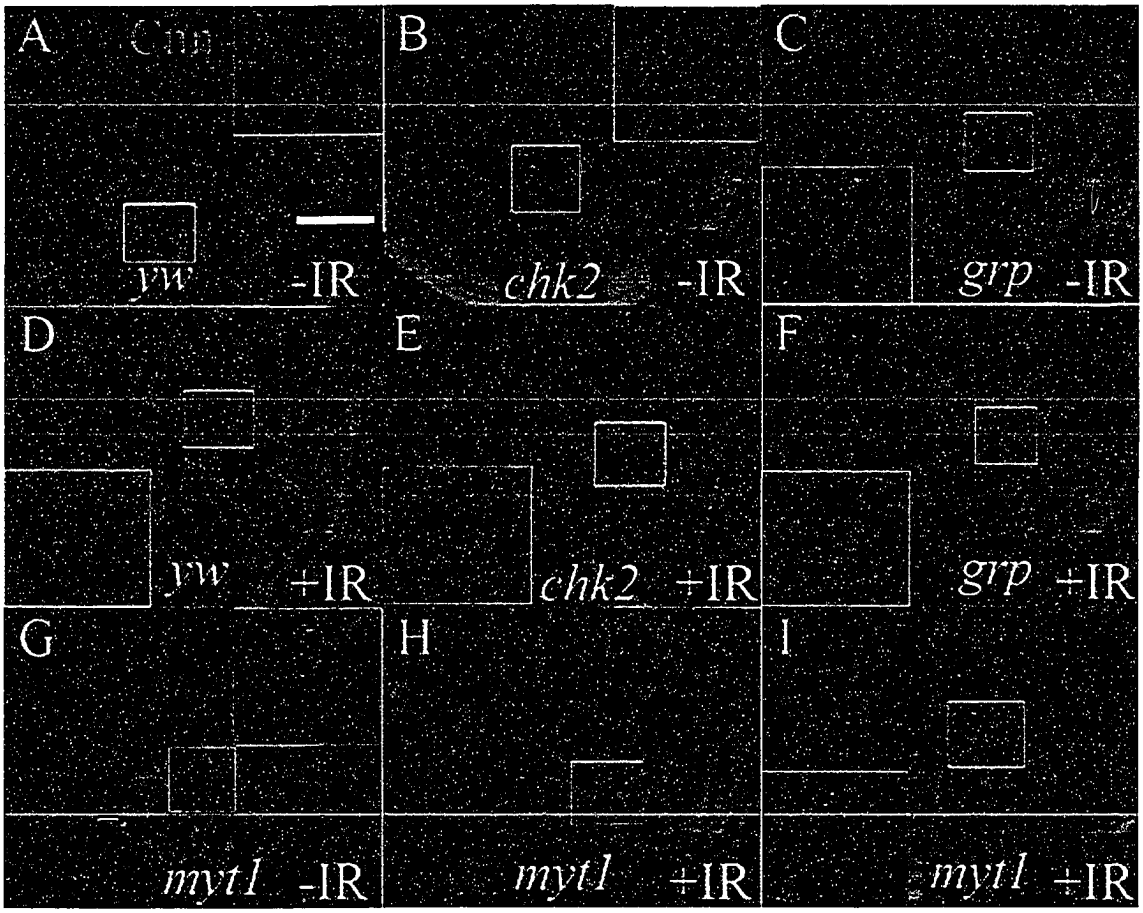

Fig. 4-5 Centrosome inactivation is partially impaired in *myt1* mutant wing discs. Wandering 3rd instar wing discs from *y w* control larvae (A, D), *chk2* larvae (B, E), *grp* larvae (C, F) and *myt1* larvae G-I) stained for Cnn (red) before IR (A-C, G) and after IR (D-F, H and I). Boxed areas are shown in insets. Control discs (D) and *chk2* (E) discs show inactivation of centrosomes after IR treatment. However, *grp* mutant discs show bright metaphase staining before (C) and after IR treatment (F). 80% of *myt1* discs still display apparent centrosome inactivation after IR (H), but 20% of mutant discs (I) maintain metaphase Cnn staining. Bar for all panels: 40 μm.

Table 4-2. Quantitative data for genetic interactions between *myt1*, *grp* and *chk2*

Genotypes	Percentage in progeny		p value
	obtained	expected	
CyO or Sp/+; myt1^2/TM6B	100.00 (337)	100.00	
CyO or Sp/+; myt1^1/TM6B	67.66 (228)	100.00	
Sp/+; myt1^1/myt1^2	28.49 (96)	50.00	
CyO/+; myt1^1/myt1^2	27.60 (93)	50.00	
grp/CyO; myt1^1/TM6B	100.00 (302)	100.00	
grp; myt1^1 or myt1^2/TM6B	50.66 (153)	50.00	
grp/CyO; myt1^1/myt1^2	25.50 (77)	27.60	
grp; myt1^1/myt1^2	2.98 (9)	13.98	p<0.001
chk2/CyO; myt1^1/TM6B	99.80 (519)	100	
chk2;myt1^1 or myt1^2/TM6B	47.69 (248)	47.69	
chk2/CyO; myt1^1/myt1^2	5.19 (27)	27.60	p<0.001
chk2; myt1^1/myt1^2	1.16 (6)	13.16	p<0.001

Note: Data were collected from these crosses: (1) *CyO/Sp; myt1^1/TM6B* X *myt1^2/TM6B*; (2) *CyO/grp; myt1^1/TM6B* X *CyO/chk1; myt1^2/TM6B*; (3) *CyO/chk2; myt1^1/TM6B* X *CyO/chk2; myt1^2/TM6B*. *CyO/+; myt1^1/myt1^2* mutants have lower viability (55.19%) when compared to their siblings *CyO/+; myt1^1/TM6B* (67.66%) or *CyO/+; myt1^2/TM6B* (100%), but they have similar viability as *Sp/+; myt1^1/myt1^2* (56.97%) siblings. To account for this variation, I used the viability value obtained from cross (1) to normalize the expected ratio for corresponding genotypes in cross (2) and cross (3). Therefore, the expected Mendelian ratio for two genes on two different chromosomes was different from 4:2:2:1 in cross (2) and cross (3). Chi-square tests were performed to confirm the significance of differences seen between the expected and obtained numbers for certain genotypes. Numbers in parentheses refer to the numbers of progeny recovered for each genotype.

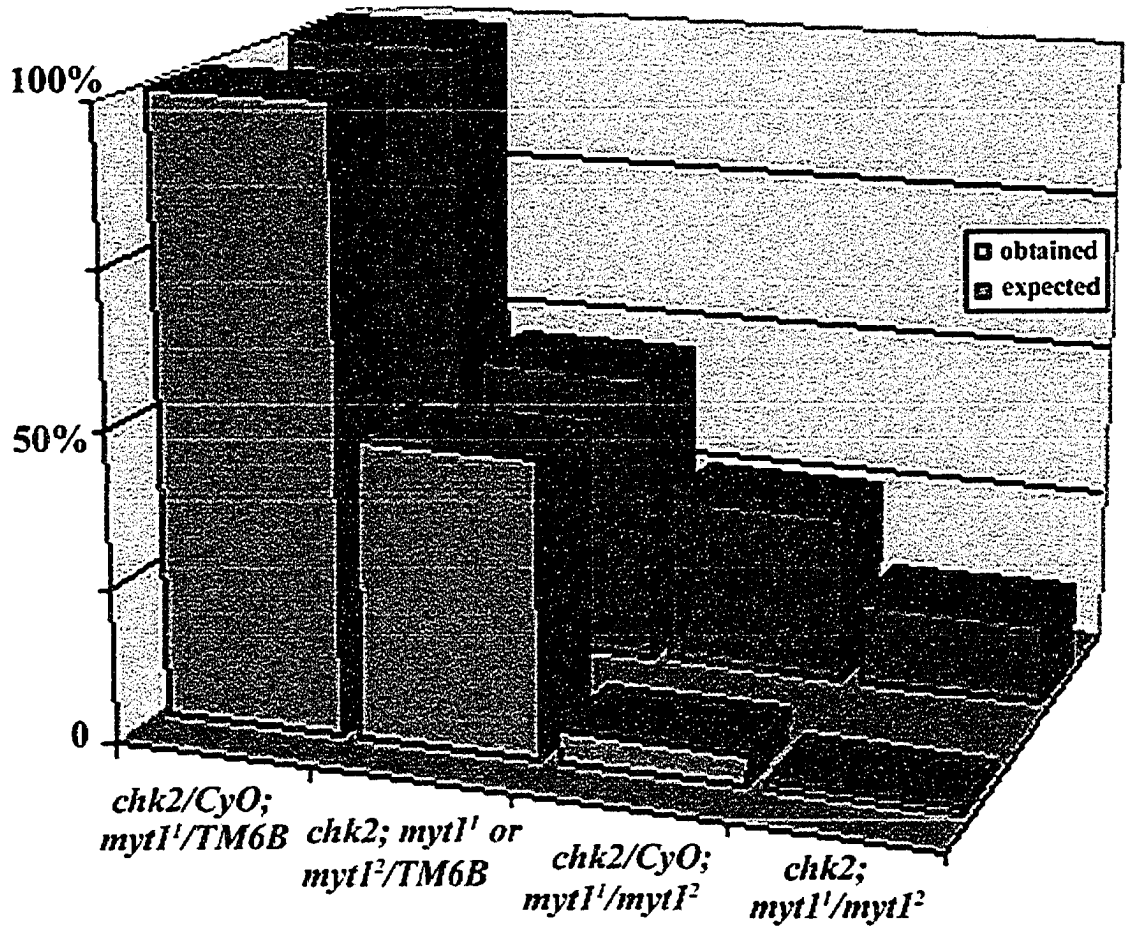

Fig. 4-6 Graph showing the number of progeny recovered from a cross between *chk2/CyO*; *myt1*^{1}*/TM6B* and *chk2/CyO*; *myt1*^{2}*/TM6B* parents. The genotypes of the progeny are listed on the X-axis. The Y-axis indicates the percentage of each genotype present in the whole population. The purple bars represent the expected number of progeny for each genotype, whereas the blue bars stand for the obtained number of progeny for each genotype. Note that the predicted Mendelian 4:2:2:1 ratio was adjusted to take into account the actual survival rate for each genotype, independently (see Table 4-2 for details). *chk2* appears to be haplo-insufficient in *myt1* mutants, for normal adult viability.

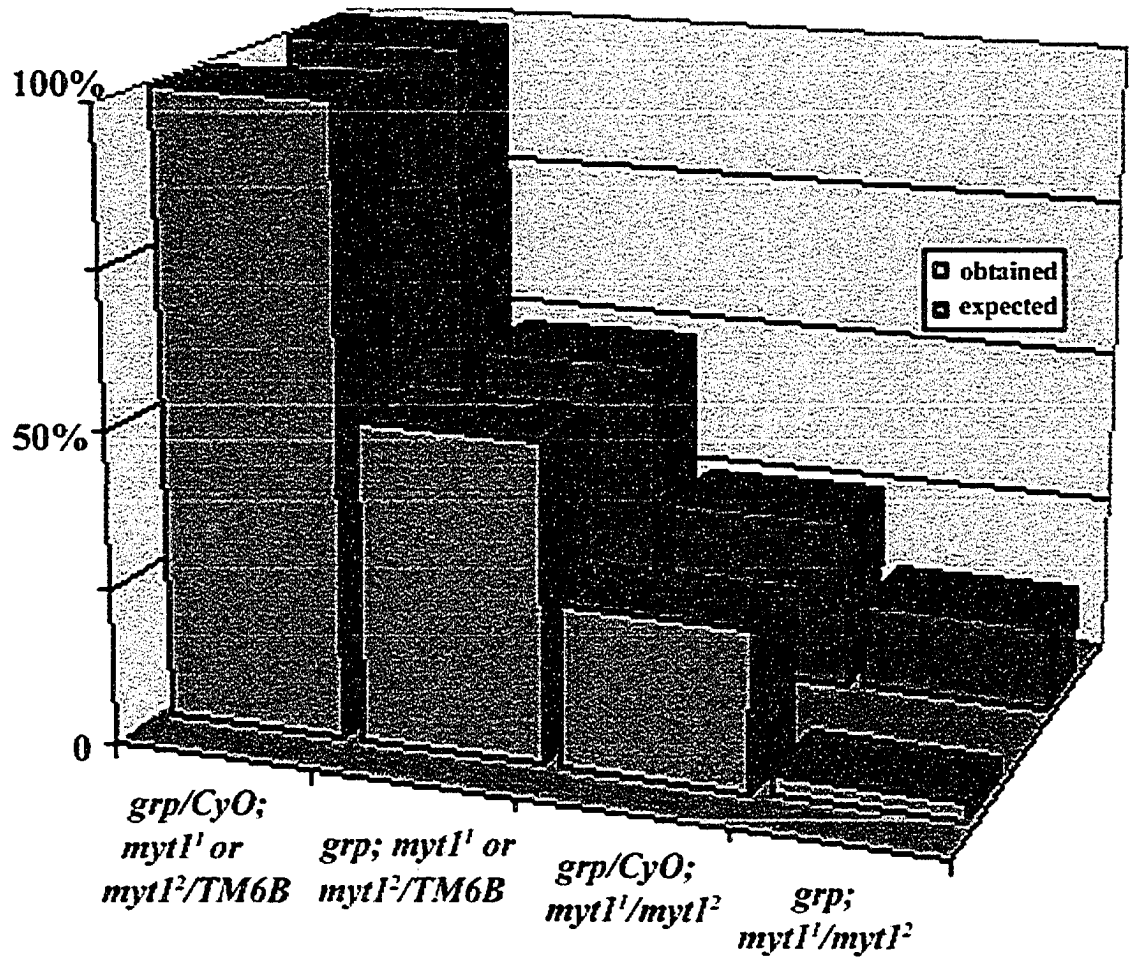

Fig. 4-7 Graph showing the number of progeny recovered from a cross between *grp/CyO*; *myt1*^{1}*/TM6B* and *grp/CyO*; *myt1*^{2}*/TM6B* parents. The genotypes of the progeny are listed on the X-axis. The Y-axis indicates the percentage of each genotype presents in the whole population. The purple bars represent the expected number of progeny for each genotype, whereas the blue bars stand for the obtained number of progeny for each genotype. Note that the predicted Mendelian 4:2:2:1 ratio was adjusted to take into account the actual survival rate for each genotype, independently (see Table 4-2 for details). The adult viability of *myt1* mutants was significantly lowered only when both functional *grp* alleles were removed.

Grp, but not Chk2, is apparently required for centrosome inactivation in *Drosophila* imaginal discs, despite the facts that *chk2* is partially required for cell cycle arrest after irradiation and Chk2 triggers centrosome inactivation in syncytial embryos (Takada et al., 2003; Xu et al., 2001). These observations pose some interesting questions for us regarding the function of Myt1 as part of the DNA damage checkpoint pathway, and regarding the interactions between Myt1, Grp and Chk2 in this pathway. They also suggest that the molecular mechanisms underlying centrosome inactivation may be regulated differently at different stages of development.

4.3 Interactions of *myt1* mutations with *grp* and *chk2* mutations

Consistent with data suggesting a potential role of Myt1 in a Grp/Chk2 mediated DNA damage checkpoint, *myt1* also interacted genetically with *grp* and *chk2* mutations (Table. 4-2). In a *myt1* mutant background, removing one copy of *chk2* resulted in a significant decrease in viability of approximately 5-fold (Fig. 4-6), whereas removing one copy of *grp* had hardly any effect on viability (Fig. 4-7). Removing both copies of *grp* reduced adult viability of the double mutant by ~5-fold (Fig. 4-7), however, whereas loss of both *chk2* and *myt1* lowered the viability approximately 10-fold (Fig. 4-6 and Fig. 4-7). These interactions suggest that *myt1* mutants have different requirements for *grp* and *chk2*, with *chk2* being haplo-insufficient for viability. The strong genetic interaction observed between *myt1* and *chk2* is also in accordance with a previous observation that Chk2 mediated centrosome inactivation is apparently operating in deformed gastrulating embryos that are compromised for dMyt1 function (see Fig. 4-3H and I, red). It will be interesting to test if reduction of *chk2* copy number has an impact on the phenotype of gastrulating *myt1* mutant embryos.

4.4 Characterization of the *myt1* mutant adult bristle phenotype

Drosophila bristles are specialized external sensory organs that are typically composed of four cells: a shaft, a socket, a neuron, and a sheath cell (Hartenstein and Posakony, 1989). Large bristles (macrochaetae) develop at specific sites on the head, thorax and wings, and

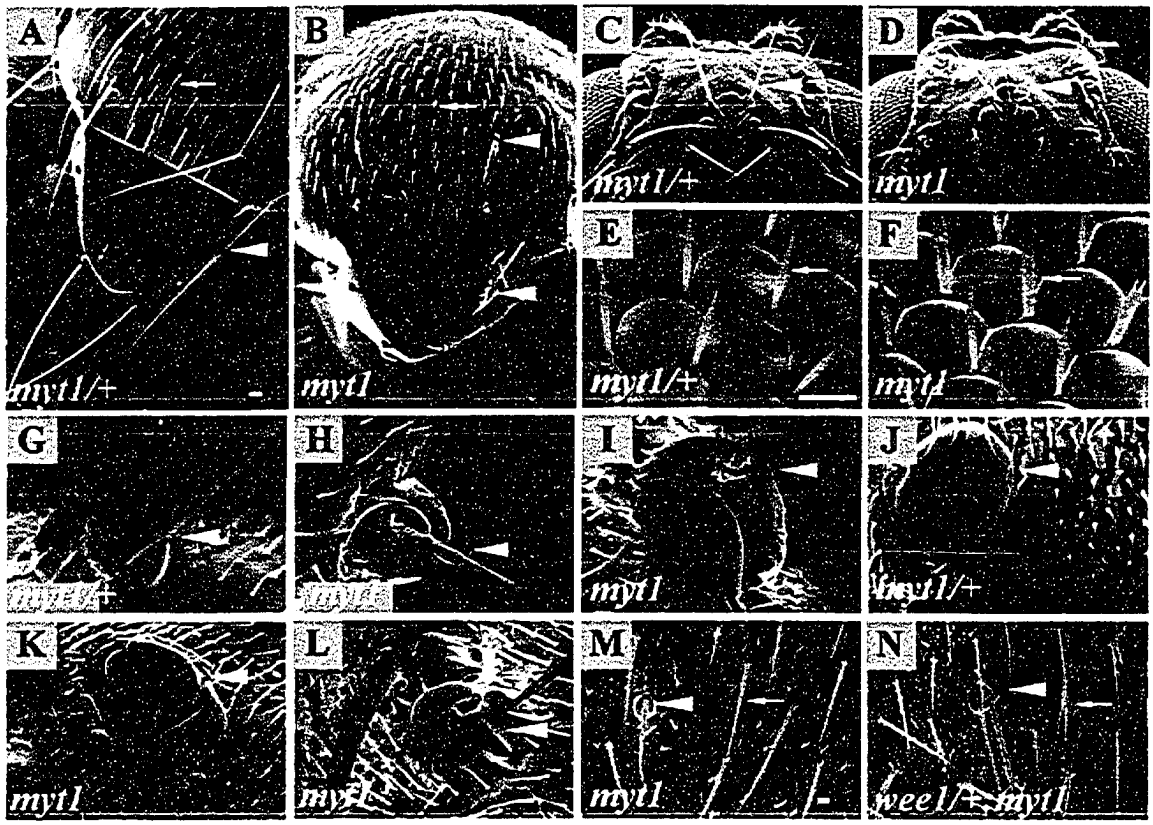

Fig. 4-8 *myt1* mutants have bristle defects affecting the notum, head and compound eye. Scanning electron micrographs of (A, C, E, G, J) wild type, (B, D, F, H, I, K-M) *myt1* or (N) *wee1*/+; *myt1* mutant flies. (A, B, G-N) Arrowheads denote macrochaetae and arrows denote microchaetae. (A, B) Notum. (C, D) Head bristles. (E, F) Interommatidial bristles (arrows) in the compound eye. Note duplicated inter-ommatidial bristles in the *myt1* mutant. (G-I) Posterior scutellar bristles. *myt1* mutants show shortened shaft (H) and/or multiple socket cells (I). (J-L) Head bristles. *myt1* mutants have defects similar to those seen in the notum. (M, N) A higher magnification view of the notum. (N) Removing one copy of *wee1* significantly increases duplication of microchaetae (white arrow). Bar for (A-D): 40 μm; bar for (E-L): 10 μm and bar for (M, N): 20 μm.

small bristles (microchaetae) are spread throughout the surface of the adult epidermis (Hartenstein and Posakony, 1989). Macrochaetae in the *myt1* hemizygous flies exhibit the following defects with variable expressivity: shortened and thinner shafts (Fig. 4-8B, D, H), duplication of bristles (Fig. 4-8B), multiple socket cells (Fig. 4-8I, K, L) and complete loss of bristles (data not shown). The microchaetae were rarely affected, exceptions being occasional microchaetae duplications on the notum (Fig. 4-8B). The macrochaetae bristle shafts in *myt1* hemizygotes were usually shorter than wild type, ranging from approximately 1/10 to 1/2 the length of a normal shaft, and the diameter was approximately 1/2 normal size (Fig. 4-8B, H). Occasionally 2-3 shafts surrounded by 2-3 sockets could be seen growing out from the position where a single macrochaeta would normally be located (Fig. 4-8B). In the compound eye, *myt1* mutants also had many ommatidia with duplicated bristles (Fig. 4-8F).

4.5 *myt1* and *wee1* show strong genetic interactions

There are two Wee1-like kinases in *S. pombe*, Wee1 and Mik1 which are functionally redundant for cell viability, with *mik1* apparently being dedicated to DNA damage and replication checkpoints and *wee1* having a unique role in regulating the G2/M cell size control checkpoint (Lundgren et al., 1991; Rhind and Russell, 2001). Similar functional redundancy may also exist in metazoans, where multiple Wee1-like kinases co-exist. Since *wee1* (Price et al., 2000) and *myt1* mutants are both viable for zygotically regulated development, I could test their redundancy in *Drosophila* by removing both functional alleles through genetic manipulation (see Fig. 4-9).

Strikingly, *wee1; myt1* double mutants were never recovered in these experiments, using either of two different alleles of *wee1* (see Fig. 4-10, Fig. 4-11 and Table 4-3); indicating that simultaneous loss of *wee1* and *myt1* results in lethality. *wee1* is also functionally haplo-insufficient in a *myt1* mutant background. I documented allele–specific differences in *wee1* haplo-insufficiency: a hypomorphic allele ($wee1^{DS1}$), causes a decrease in viability of ~3-fold, whereas a null allele ($wee1^{ES1}$, or the deficiency chromosome, $Df(2L)wee1^{W05}$, that uncovers *wee1* and its neighboring genes) causes a viability decrease of ~10-fold, (see Table 4-4, compare Fig. 4-10 and Fig. 4-11). Furthermore, occasional survivors of $wee1^{DS1}$or $wee1^{ES1}/CyO$; $myt1^{1}/myt1^{2}$ displayed

many more microchaetae duplications on the notum (Fig. 4-8N), in addition to the macrochaetae phenotypes typically observed in *myt1* mutants with two functional alleles of *wee1* (Fig. 4-8M). Likewise, $wee1^{DS1}/CyO$; $myt1^{1}/myt1^{2}$ or $wee1^{ES1}/CyO$; $myt1^{1}/myt1^{2}$ flies had more enhanced meiotic defects relative to *myt1* mutants alone, apparently caused by a premature chromosome condensation phenotype (discussed in chapter 3). I did not see reciprocal effects in *wee1* mutants when one copy of *myt1* was removed, however. Premature chromosome condensation was not observed in oocytes from $myt1^{1}/myt1^{2}$ or $wee1^{ES1}/CyO$; $myt1^{1}/myt1^{2}$ females. Instead, egg chambers produced by these females were occasionally (~5%) seen with only 8 germline cells, an indication of 3 (rather than 4) rounds of mitotic divisions at the oogonial stage (data not shown). Taken together, these results indicate that either *wee1* or *myt1* is required for organismal viability, although the two genes are differentially required for specific events during development. Determining the nature of this functional redundancy will be an important goal for the future.

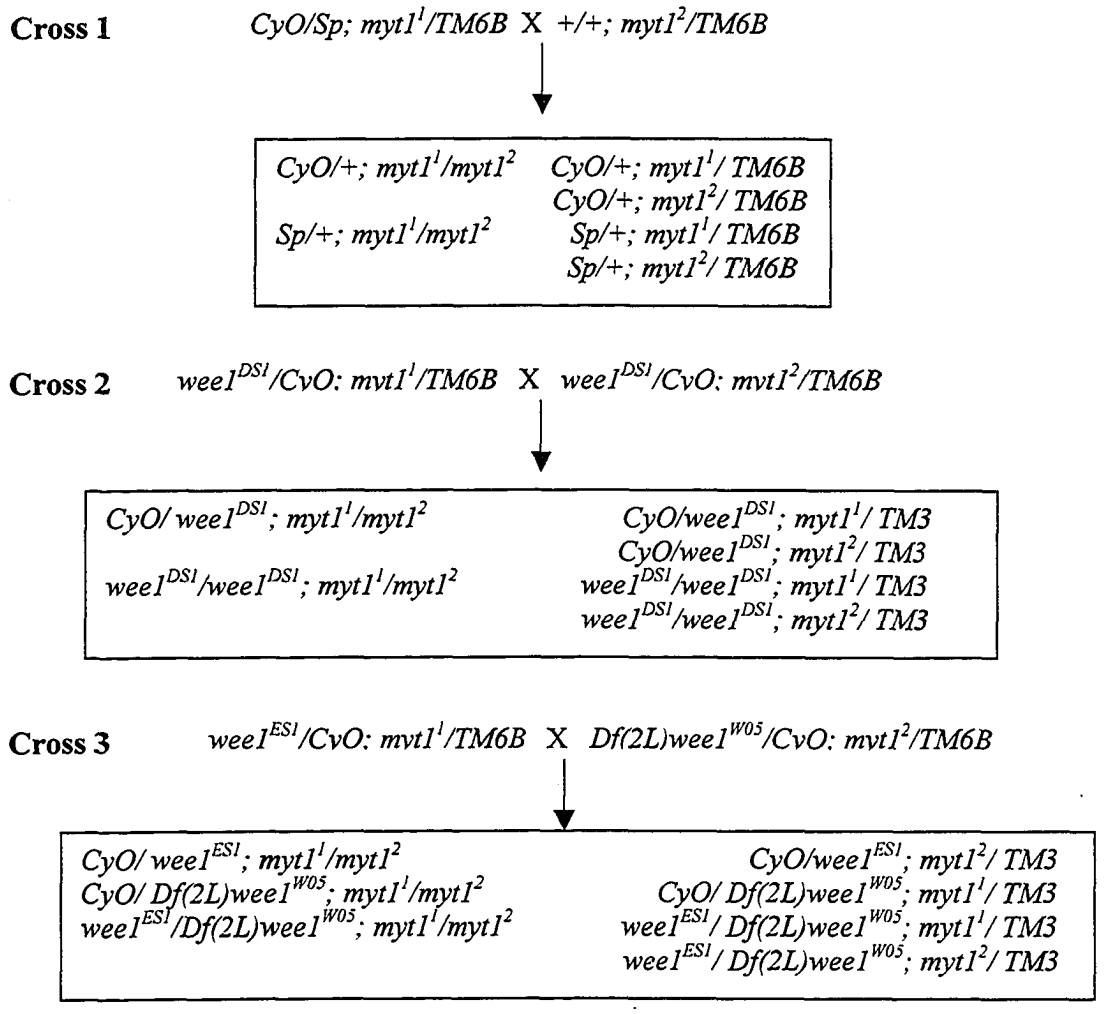

Fig. 4-9 Crosses for testing genetic interaction between *myt1* and *wee1*. For each cross, the predicted genotypes of viable progeny are listed in the box; not listed are the homozygotes for balancer chromosomes such as *CyO*, *TM6B* and TM3, which are not viable. Therefore, the predicted Mendelian ratio predicted for independent assortment is changed from 9:3:3:1 to 4:2:2:1. The purpose of cross 1 was as a control to establish that the balancer chromosome *CyO* does not affect the viability of $myt1^1/myt1^2$ flies.

Table 4-3. Quantitative data for *wee1* and *myt1* genetic interactions

Genotypes	Percentage in progeny obtained	expected	p value
CyO or Sp/+; myt1^{2}/TM6B	100.00 (337)	100.00	
CyO or Sp/+; myt1^{1}/TM6B	67.66 (228)	100.00	
Sp/+; myt1^{1}/myt1^{2}	28.49 (96)	50.00	
CyO/+; myt1^{1}/myt1^{2}	27.60 (93)	50.00	
wee1^{DS1}/CyO; myt1^{1}or myt1^{2}/TM6B	99.74 (375)	100.00	
wee1^{DS1}; myt1^{1} or myt1^{2}/TM6B	49.73 (187)	50.00	
wee1^{DS1}/CyO; myt1^{1}/myt1^{2}	7.98 (30)	27.60	p<0.001
wee1^{DS1}; myt1^{1}/ myt1^{2}	0.00 (0)	13.73	p<0.001
wee1^{ES1}/CyO; myt1^{1}or myt1^{2}/Tm6B	98.04 (549)	100.00	
wee1^{ES1}/ Df(2L)wee1^{W05}; myt1^{1}or myt1^{2}/TM6B	49.22 (251)	50.00	
wee1^{ES1}or Df(2L)wee1^{W05} /CyO; myt1^{1}/myt1^{2}	2.32 (13)	27.60	p<0.001
wee1^{ES1}/ Df(2L)wee1^{W05}; myt1^{1}/myt1^{2}	0.00 (0)	13.58	p<0.001

Note: Data were collected from these crosses: (1) *CyO/Sp; myt1^{1}/TM6B* X *+/+; myt1^{2}/TM6B*; (2) *wee1^{DS1}/CyO; myt1^{1}/TM6B* X *wee1^{DS1}/CyO; myt1^{2}/TM6B*; (3) *wee1^{ES1}/CyO; myt1^{1}/TM3* X *Df(2L)wee1^{W05}/CyO; myt1^{2}/TM3*. *CyO/+; myt1^{1}/myt1^{2}* mutants have a lower viability (55.19%) when compared to their siblings *CyO/+; myt1^{1}/TM6B* (67.66%) or *CyO/+; myt1^{2}/TM6B* (100%), but they have similar viability to their *Sp/+; myt1^{1}/myt1^{2}* (56.97%) siblings. To account for this variation in progeny survival, I used viability data obtained from cross (1) to adjust the expected ratio for corresponding genotypes in cross (2) and cross (3). Therefore, the expected ratio for two genes on two different chromosomes was different from 4:2:2:1 in cross (2) and cross (3). Chi-square testes were then performed to confirm that the differences seen between the expected and obtained numbers for certain genotypes of interest were statistically significant. Numbers in parentheses refer to the numbers of progeny recovered for the indicated genotype.

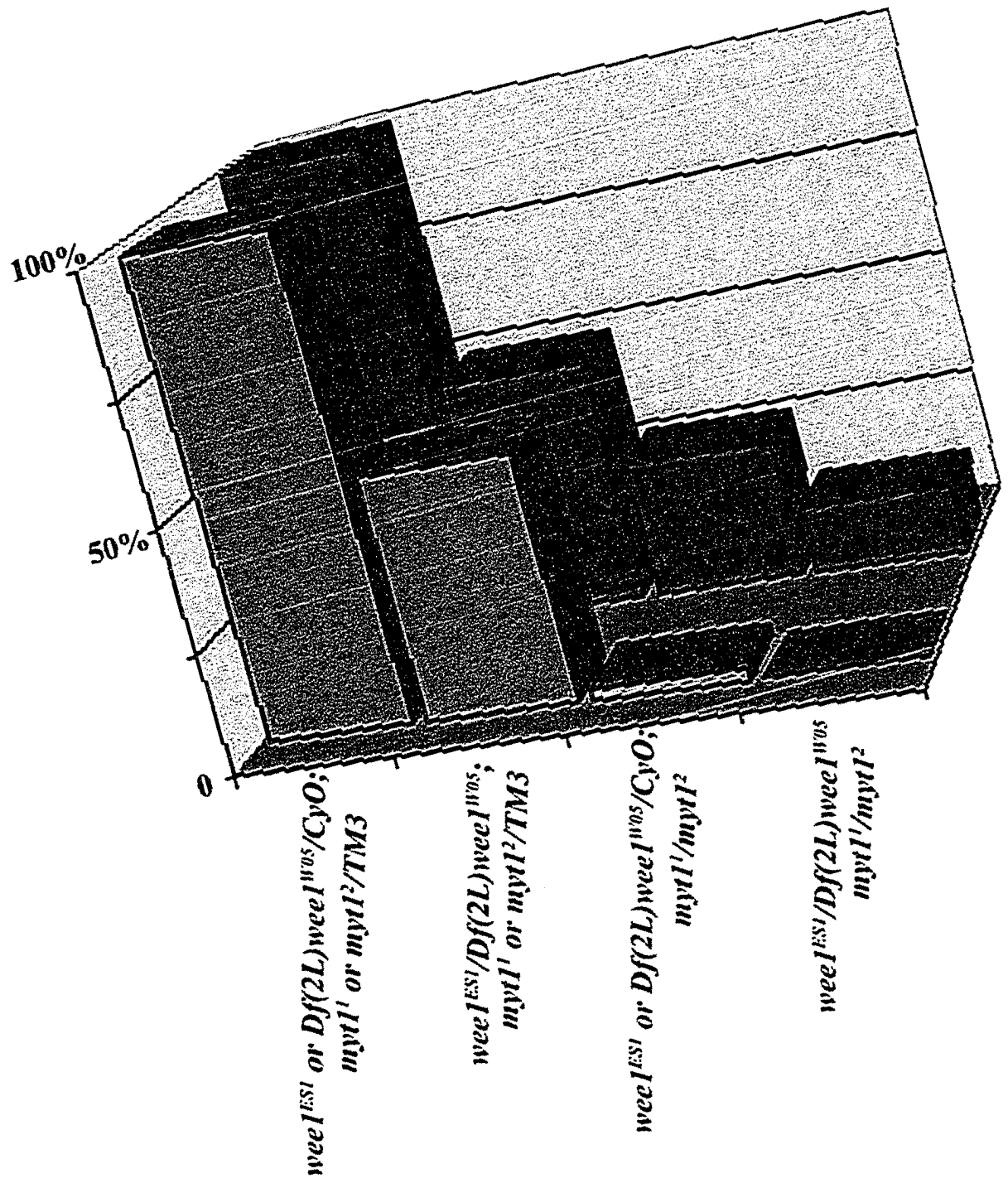

Fig. 4-10 Graph showing the number of progeny recovered from crosses between *wee1*^{ES1}/*CyO*; *myt1*^{1}/*TM3* and *Df(2L)wee1*^{W05} /*CyO*; *myt1*^{2}/*TM3* parents. The genotypes of the progeny are listed on the X-axis. The Y-axis indicates the percentage of each genotype present in the whole population. The purple bars represent the expected number of progeny for each genotype, whereas the blue bars stand for the obtained number of progeny for that genotype. Note that the predicted Mendelian 4:2:2:1 ratio was modified as discussed above to calculate the expected frequency for each progeny type, to take into account the actual survival rate for each genotype (see Table 4-4 for details). The null allele, *wee1*^{ES1}, was haplo-insufficient for viability in a *myt1* mutant background; as there were few *wee1*^{ES1}/*CyO*; *myt1*^{1}/*myt1*^{2} survivors. No double mutants were recovered.

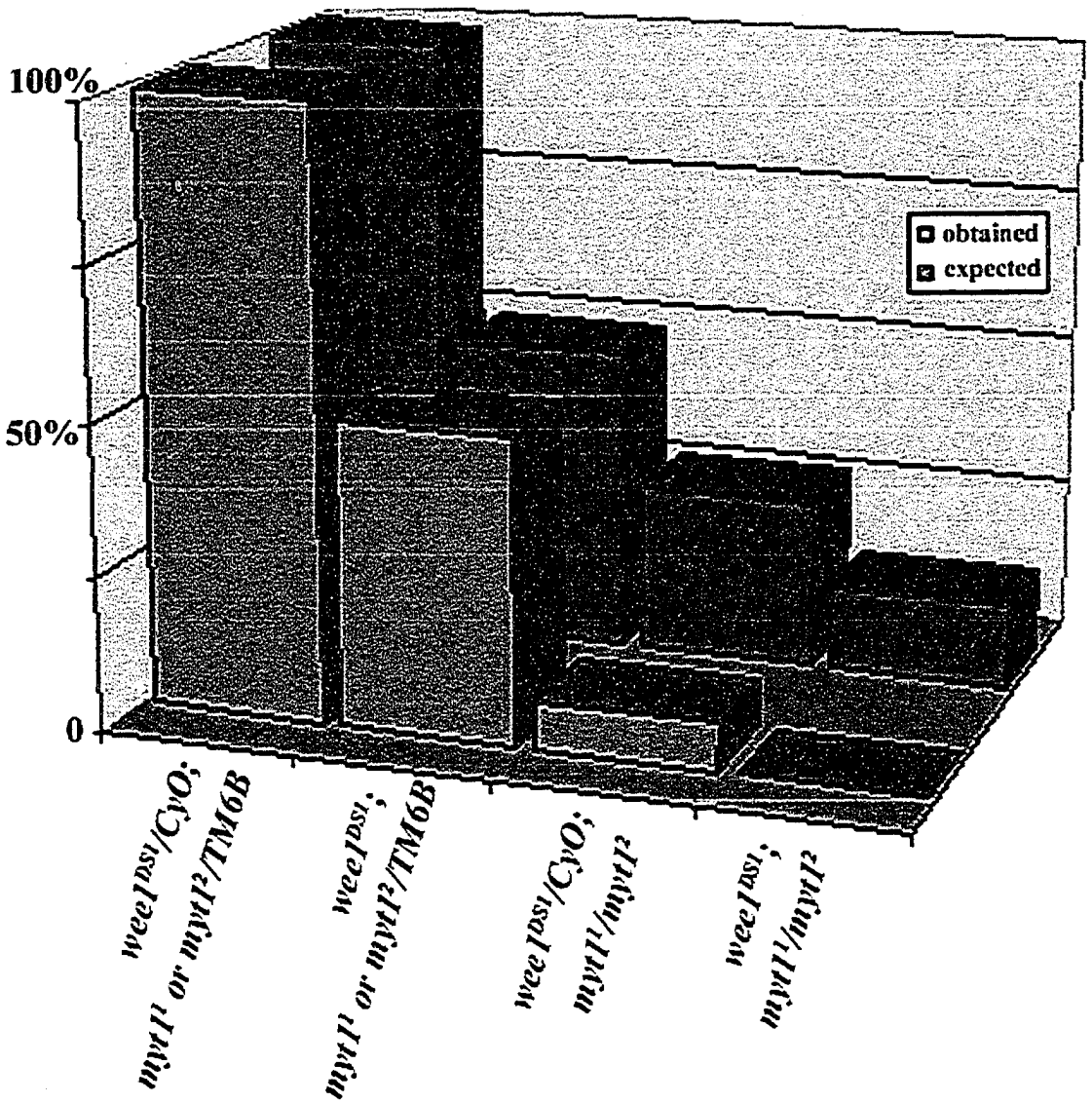

Fig. 4-11 Graph showing the number of progeny recovered from a cross between *wee1*^{DSI}/*CyO*; *myt1*^{1}/*TM6B* and *wee1*^{DSI}/*CyO*; *myt1*^{2}/*TM6B* parents. The genotypes of the progeny are listed on the X-axis. The Y-axis indicates the percentage of each genotype present in the total population. The purple bars represent the expected number of progeny for each genotype, whereas the blue bars stand for the obtained number of progeny for each genotype. Again, the predicted Mendelian 4:2:2:1 ratio was modified to calculate the expected frequency for each progeny type, taking into account the actual survival rate for each genotype (see Table 4-4 for details). The hypomorphic allele *wee1*^{DSI} also shows significant haplo-insufficiency for viability in *myt1* mutants, but with a higher survival rate than *wee1*^{ESI}, consistent with previous characterization of these allele (Price et al., 2000). No double mutants were recovered.

4.6 Additional data showing over-proliferation of follicle cells in *myt1* mutants

Previously in chapter 2, I described changes in cell cycle behavior of somatic cells in *myt1* mutants, including ectopic dividing cyst cells and epithelial terminal cells in testes and follicle cells in ovaries such as main body follicle cells, stalk cells, polar cells, border cells, centripetal cells and stretched cells. Since the development of follicle cells in ovaries is well studied, and each subtype of them is readily recognizable by its distinct morphological and/or molecular marker, I further characterized the effect of the *myt1* mutation on these cells.

First emerging as undifferentiated "pre-follicle cells", all follicle cells are produced by 2-3 somatic stem cells (SSCs) residing at the 2a/2b region of the germarium (refer to Fig. 1-3, page 24). As pre-follicle cells envelop the 16-cell germline cell cyst, they bud off from the germarium to form the first "egg chamber". At this point, the stalk cells and polar cells are the first sub-types that withdraw from the cell cycle, whereas the epithelial follicle cells divide 3 more times (Bai and Montell, 2002; Margolis and Spradling, 1995; Tworoger et al., 1999). At stage 6, the border cells, stretched cells and centripetal cells exit the cell cycle (refer to Fig. 1-4); whereas the rest of epithelial follicle cells cease mitosis and switch to an endo-replication program (reviewed in Spralding, 1993). My initial analysis suggests that *myt1* mutation does not affect cell fate determination of follicle cells, but rather their cell cycle behavior.

PH3-positive pre-follicle cells are found in 50% *myt1* mutant germaria, and only 10% control germaria contain PH3-positive cells (Fig. 4-12A, B). Consistent with this, *myt1* mutants also exhibit more PH3-positive epithelial follicle cells than controls from stage 2 to stage 6 egg chambers. In addition, some stalk cells (Fig. 4-12D, arrow) and polar cells (Fig. 4-12, arrowhead) remain PH3-positive in egg chambers beyond stage 2. After stage 6, border cells, stretched cells, centripetal cells and occasionally some main body follicle cells are also PH3-positive in some *myt1* mutant egg chambers, as documented in chapter 2 (refer to Fig. 2-8 in chapter 2). To rule out the possibility that PH3 staining might not accurately reflect mitotic cells in the *myt1* mutant context but rather reflect a histone modification that might occur when the cell cycle program is disrupted; I also used

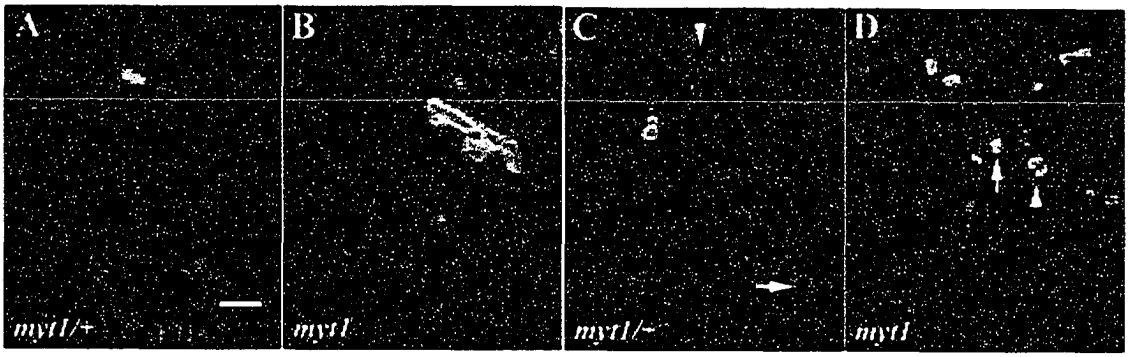

Fig. 4-12 Over-proliferation of follicle cells in *myt1* mutant ovaries. Control (A, C) or *myt1* mutant ovarioles (B, D) stained for PH3 (red), Hts (green) and DNA (blue). Hts (green) is a membrane-bound protein in pre-follicle cells, in addition to its spectrosome/fusome specific localization in the germline cells. (A, B) Control and *myt1* mutant germaria; whereas (C, D) show stage 3 or 4 egg chambers. Note that *myt1* mutants (B, D) have more PH3-positive pre-follicle cells than controls (A, C). Differentiated stalk cells (arrows in C, D) and polar cells (arrowheads in C, D) are always PH3-negative in control ovarioles (C) but are PH3-positive in some mutant ovarioles (D). Bar for all panels: 8μm.

antibodies against MPMII and Cnn as alternative means of marking mitotic cells. Immuno-staining with these antibodies was consistent with the extra PH3-positive cells actually being in mitosis (data not shown). These observations suggest that the *myt1* mutation causes a general over-proliferation defect in somatic follicle cells, including SSCs and many sub-types of follicle cells, particularly stalk cells and polar cells. Although a comprised G2/M transition in the follicle cells of *myt1* mutants would be a straightforward explanation for these observations, other possibilities must also be considered that could account for or contribute to this over-proliferation phenotype.

4.7 Loss of *myt1* activity affects follicle cell division in a cell autonomous manner

Given that I have presented data showing that the *myt1* mutation affects both female germline cells and their overlying follicle cells, the possibility that these two different groups of cells might affect each other's cell cycle behavior as a response to loss of *myt1* was raised. To address this question, I generated *myt1* mutant clones of cells in egg chambers, by FRT-mediated mitotic recombination (Xu and Rubin, 1993). A number of different types of clones could be generated, for example: egg chambers from a *myt1/+* female could have *myt1/+* germline cells, but the overlying follicle cells could be a mosaic of *myt1/myt1* and *myt1/+* cells, and *vice versa.* In my crosses a nuclear–GFP marker was placed on the "*myt1*$^{+}$" chromosome, so that the mutant clones could be easily identified by loss of GFP fluorescence. Similar to what I saw in *myt1* mutant ovaries, *myt1* mutant follicle cell clones from mosaic egg chambers also contained more PH3-positive epithelial cells, as well as PH3-positive stalk cells and polar cells, even if the underlying germline cells were *myt1/+* (arrows in Fig. 4-13A', A''), arguing that loss of *myt1* function acts in a cell-autonomous manner to promote entry into mitosis. Cells from *myt1* mutant somatic clones were essentially the same size as those *myt1* heterozygous clones (Fig. 4-13B''), indicating that the balance between cell division and cell growth is maintained in *myt1* mutant cells, consistent with observations in imaginal cells made by manipulating other cell cycle regulators (Neufeld et al., 1998; Reis and Edgar, 2004).

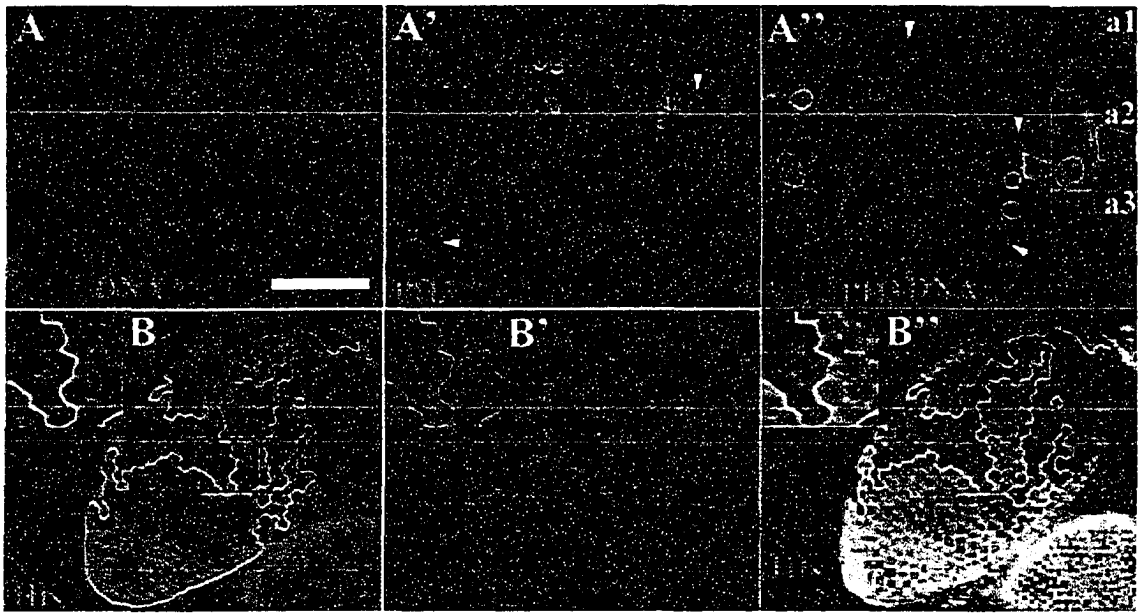

Fig. 4-13 *myt1* is required cell autonomously in follicle cells. *myt1* mutant clones were generated by heat shock-induced mitotic recombination, a technique described in the Materials and Methods. *myt1* mutant clones were labeled by loss of a nuclear-GFP marker, whereas non-recombinant *myt1*/+ cells and recombinant +/+ cells retain nuclear-GFP and were outlined in white. (A) Egg chambers stained for nuclear-GFP (green) and DNA (cyan); (A', A") egg chambers stained for nuclear-GFP (green) and PH3 (red), box in (A') is shown in (A"). Box in (A") is also shown in (a1, a2, a3). Arrowheads indicate PH3-positive follicle cells in *myt1* mutant clones, including polar cells and stalk cells in A' and A''. (B, B') egg chambers stained for Hts (red), nuclear-GFP (green) or both as in (B''). Inset shows boxed area in each panel. Note that cells in the *myt1* mutant and control clones were of similar cell size. Bar: 40 μm.

To examine whether different follicle cell subtypes cells influence each other's behavior, a transgene carrying UASp-*EGFP-myt1* (see appendix for construction of this plasmid), was specifically expressed in the polar cells of *myt1* mutants using a *Neu-Gal4* driver line (Besse and Pret, 2003). *Neu-Gal4* expression restored polar cells to their normal PH3-negative staining pattern, but did not rescue this defect in germline cells, stalk cells or epithelial follicle cells, where the transgene was not expressed (Fig. 4-14D, E, F). Therefore, I conclude that *myt1* is required cell autonomously for regulating normal cell cycle behavior of different follicle cell subtypes, at least in the ovaries. The question remains, however, as to why differentiated follicle cells such as stalk cells and polar cells divide ectopically in *myt1* mutants?

4.8 Loss of *myt1* leads to ectopic expression of S phase and G2 phase markers in polar and stalk cells

One possible explanation for why follicle cells undergo ectopic cell division in *myt1* mutants is that these cells normally arrest in G2 phase because of dMyt1 activity, at the time they apparently withdraw from the cell cycle. A testable prediction of this hypothesis, is that these cells should accumulate G2 Cyclins, such as Cyclin A and Cyclin B. When I tested this idea, to my surprise, certain stalk cells and polar cells contained Cyclin A and Cyclin B in *myt1* mutants, but not in the controls (polar cells in Fig. 4-15D, stalk cells in Fig. 4-15B, Fig. 4-15B). These observations suggest that some stalk cells and polar cells are in G2 phase in the *myt1* mutants, a completely opposite result to what was expected from my original hypothesis: that these cells arrest in G2 phase under normal conditions. An alternative hypothesis that can account for these results is that the differentiated stalk cells and polar cells normally arrest in a quiescent G0 or G1 stage in ovaries and thus do not accumulate G2 Cyclins. Since stalk cells and polar cells in *myt1* mutants do not apparently exit the cell cycle, they can be expected to accumulate G2 Cyclins as they pass through G2 phase. I also noticed that many more normally follicle cells were accumulating G2 Cyclins in the *myt1* mutant egg chambers (Fig. 4-15D, Fig. 4-16B) relative to controls (Fig. 4-15B, Fig. 4-16A), an observation that is also consistent with the higher mitotic index seen in *myt1* mutant epithelial follicle cells.

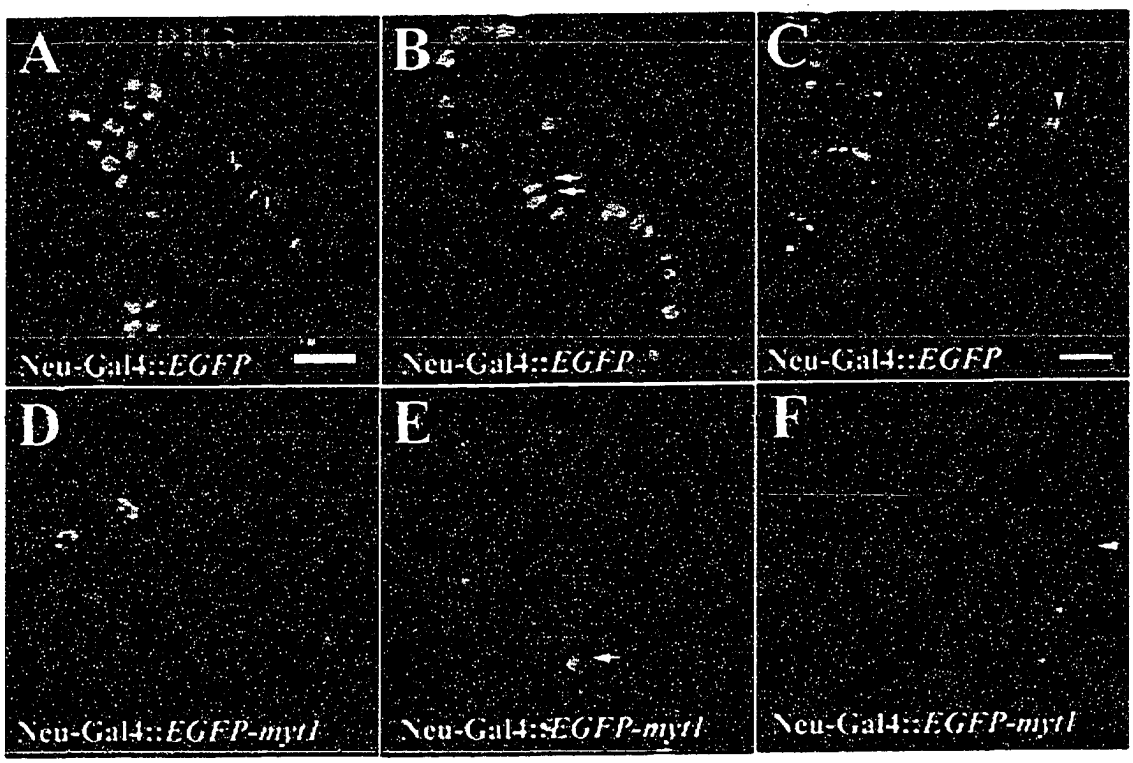

Fig. 4-14 Polar cell-specific expression of *EGFP-myt1* specifically rescues the over-proliferation defect of polar cells in *myt1* mutants. A *Neu-Gal4* driver was used to direct polar cell-specific expression of either *EGFP* as a control (A-C) or *EGFP-myt1* (D-F), in *myt1* mutant ovarioles. Mitotic cells and nuclei were labeled by PH3 (red) and DNA (blue) staining, in all panels. Arrows point to stalk cells, whereas arrowheads indicate polar cells. *myt1* mutant ovarioles contain many mitotic cells in the germline (A), epithelial follicle cells (B and C, red staining in egg chambers), stalk cells (B, arrows) and polar cells (C, arrowheads). Polar cell-specific expression of *EGFP-myt1* completely suppressed the ectopic mitosis usually seen in polar cells in *myt1* mutants (F, arrowhead) but not in other cell lineages such as germline cells (D), epithelial follicle cells (E, F) or stalk cells (E, arrow). Bar for (A, B, D, E): 8 μm, bar for (C, F): 20 μm.

During exit from the cell cycle, quiescent cells normally down-regulate Cdc6, MCM and Cdt1 proteins, which are required for DNA synthesis (Stoeber et al., 2001; Xouri et al., 2004). In control ovaries, neither stalk cells nor polar cells were ever observed that were positive for DUP antibody staining (Dup is the gene product of *double parked*, which is the *Drosophila* homolog of Cdt1). In contrast, some of those cells were DUP-positive, whereas others were not, in *myt1* mutant ovaries (Fig. 4-15E, F, arrows). These observations suggest that in *myt1* mutant ovaries, some stalk cells and polar cells do not down-regulate DUP but continue proliferating. The results also indicate that stalk cells and polar cells are at different phases of the cell cycle. Strikingly, DUP staining was also prominent in Cycling follicle cells and even endo-replicating nurse cells in *myt1* mutants, indicating that loss of *myt1* alters DUP accumulation in these cells as well. With these results, I conclude that normally quiescent stalk cells and polar cells do not exit the cell cycle in *myt1* mutants, since these cells express markers specific for S phase, G2 phase and M phase. Similarly, the staining pattern for DUP and the G2 Cyclins was also altered in cycling follicle cells (and sometimes nurse cells, for DUP staining) of *myt1* mutant ovaries, suggesting that the effects of loss of *myt1* activity are not limited to differentiated follicle cells.

4.9 Loss of *myt1* affects accumulation of Rux and Fzr in epithelial follicle cells

As my previous results suggest that dMyt1 is important for cell exit in stalk cells and polar cells but that this is not a G2 arrest, a question is raised concerning what molecular mechanisms might be used by dMyt1 to help cells enter and/or remain in quiescent G0 or G1 phase? At least two different proteins: Rux (the gene product of *roughex*) and Fzr (the gene product of *fizzy-related*), are known to be involved in promoting entry into G1 phase in *Drosophila*. Rux functions by inhibiting Cdk1/CycA activity during mitotic exit (Foley and Sprenger, 2001), whereas Fzr helps to target the degradation of Cdk1/CycB during G1 phase (Sigrist and Lehner, 1997). If either or both of these two proteins are involved in establishing and/or maintaining the quiescent G1 phase in stalk cells and polar cells, loss of dMyt1 activity might disrupt this process to allow the stalk cells and polar cells to divide again. Thus, the unique phenotype of *myt1* mutants in stalk cells

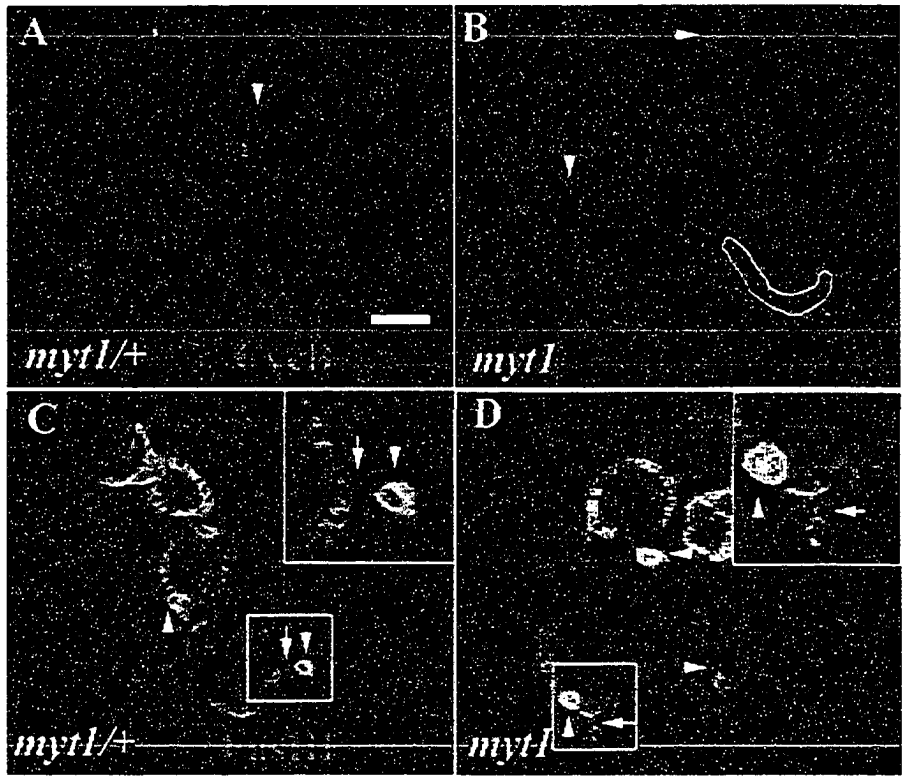

Fig. 4-15 Stalk cells and polar cells ectopically express S phase and G2 phase markers in *myt1* mutants. Control (A, C) and *myt1* mutant (B, D) ovarioles were double labeled for Cyclin B (red in A, B) and DNA (blue in A, B) or FasIII (a marker for polar cells, red in C, D) and DUP (green in C, D). (A, B) Cyclin B staining (red) was only found in polar cells of *myt1* mutant stage 6 or older egg chambers (B), but not in the control (A). The outlined region highlights epithelial follicle cells other than polar cells that also show ectopic Cyclin B staining in some *myt1* mutant egg chambers after stage 6. (C, D) DUP is normally down-regulated in differentiated stalk cells (arrow) and polar cells (arrowhead) in controls (C, inset); whereas it is still expressed in some *myt1* mutant stalk cells (D, inset, arrow) and polar cells (D, inset, arrowhead). Bar for all panels: 20 μm.

and polar cells offers an excellent opportunity to investigate another potentially novel role for Wee1-like kinases.

As a pilot experiment, I first tested whether Rux and Fzr accumulation in stalk cells and polar cells was disrupted by *myt1* mutation. In control ovaries, I hardly detected any signal above background levels for Rux and Fzr in the follicle cells, including the stalk cells and polar cells (Fig. 4-16A', Fig. 4-17A'). Using identical settings for image capture and image analysis, I observed relatively stronger nuclear staining for Rux and Fzr in *myt1* mutant ovaries compared with controls, in cycling follicle cells, stalk cells, and less frequently polar cells (Fig. 4-16B', Fig. 4-17B'). It also appeared that the Rux or Fzr–positive cells were Cyclin A-positive. These results suggest that developmental up-regulation of Rux and/or Fzr is not required to maintain stalk cells and polar cells in a quiescent G0 or G1 phase. Nonetheless, if the immuno-staining results for Rux and Fzr are representative of the levels of the respective proteins, my results suggest that Rux and Fzr are up-regulated or stabilized to compensate for mis-regulated Cdk1 activity at G1 (or G0) phase, as a consequence of loss of dMyt1 in cycling follicle cells, as well as in differentiated stalk cells and polar cells. In other words, the up-regulation or stabilization of Rux and Fzr could be an indication of ectopic Cdk1 activity in *myt1* mutant stalk cells and polar cells. Further experiments will be required to test this idea.

4.10 *myt1* may affect cycling follicle cells and post-mitotic follicle cells through different mechanisms

If uninhibited Cdk1 activity is responsible for the ectopic cell division phenotype in stalk cells and polar cells, the expression of a non-inhibitable *Cdk1AF* transgene should be capable of mimicking these phenotypes in the ovaries. When I investigated this possibility however, ovaries from heat-shock treated *Cdk1AF* flies only contained ectopic PH3-positive germline cells and epithelial follicle cells, but not polar cells and stalk cells (data not shown). This result suggested that mis-regulation of Cdk1-associated kinase activity in *myt1* mutants might not be responsible for the observed ectopic mitosis in all post-mitotic cells. Alternatively, expression of *Cdk1AF* alone is not sufficient to induce

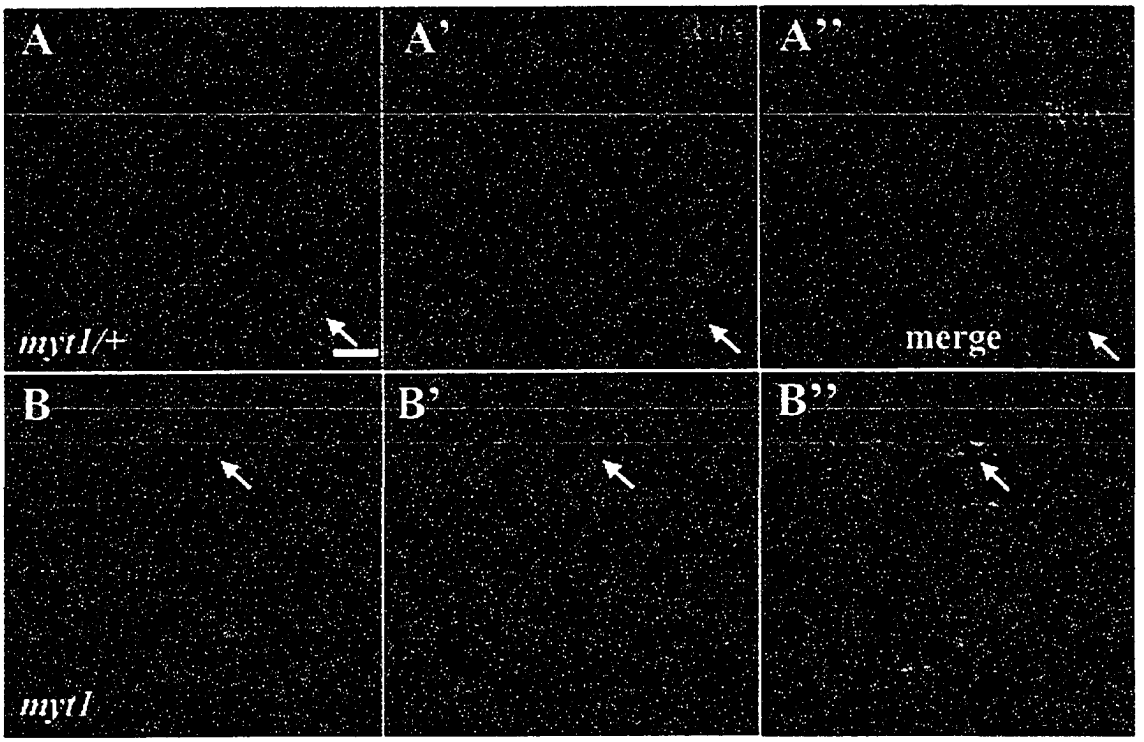

Fig. 4-16 Up-regulation of Rux and Cyclin A in epithelial follicle cells of *myt1* mutants. Cyclin A staining (green) and Rux staining (red) in control egg chambers (A, A') and *myt1* mutant egg chambers (B, B'), respectively. A'' and B'' are merged images of Cyclin A (green), Rux (red) and DNA (blue) for control and *myt1* mutants. Arrows point to stalk cells. Mutant epithelial follicle cells (on the surface of the egg chambers) and stalk cells (arrow) show much stronger Cyclin A and Rux staining in the *myt1* mutants, compared with controls (A, A'). Bar for all panels: 8 μm.

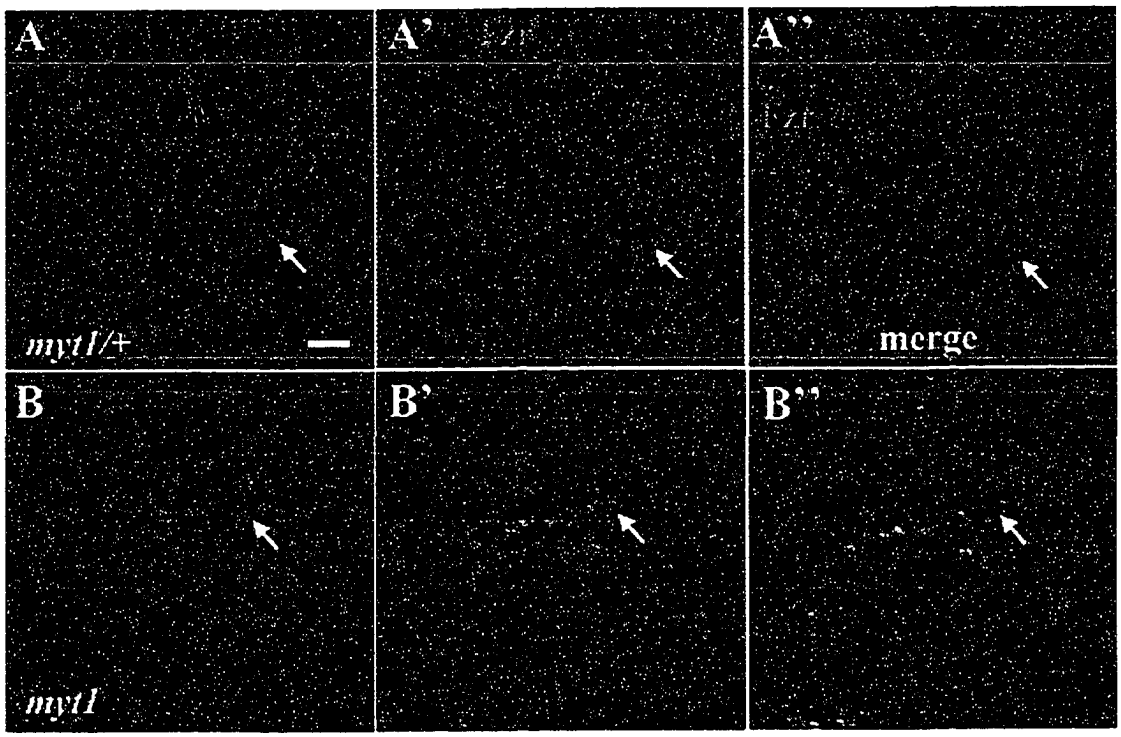

Fig. 4-17 Up-regulation of Fzr and Cyclin A in epithelial follicle cells of *myt1* mutants. Cyclin A staining (green) and Fzr staining (red) in control egg chambers (A, A') and *myt1* mutant egg chambers (B, B'), respectively. A'' and B'' are the merged images. Arrows point to stalk cells. Mutant epithelial follicle cells (on the surface of the egg chambers) and stalk cells (arrow) show much stronger Cyclin A and Fzr staining in the *myt1* mutants, relative to controls (A, A'). Bar for all panels: 8 μm.

ectopic cell division in stalk cells and polar cells, to account for these results.

If failure to inhibit Cdk1 activity is responsible for the over-proliferation defect in one type of cells but not the other, loss of *cycB* expression in *myt1* mutant ovaries should affect the over-proliferation phenotypes in cycling epithelial follicle cells and post-mitotic stalk cells and polar cells differentially, since loss of *cycB* should only affect Cyclin B–associated Cdk1 kinase activity. As predicted, many PH3-positive differentiated follicle cells (including polar cells) were still found at the anterior end of egg chambers from *cycB; myt1* double mutants, similar to results shown previously from *myt1* mutants alone (compare Fig. 4-18G and H). This result indicates that the ectopic mitosis seen in *myt1* mutant cycling follicle cells, stalk cells and polar cells can take place independent of Cdk1-Cyclin B activity.

As previously described, *cycB* mutant females have rudimentary ovaries (Jacobs et al., 1998), consisting of germaria with no obvious egg chambers or, occasionally, with 1-2 egg chambers that only have 3-4 germline cells each (Fig. 4-18B, F). Some PH3-positive cells were still present in the *cycB* mutant ovaries, however (Fig. 4-18B, F). Loss of *cycB* limits oogenesis development beyond the pupal stage in females, since germline cells and follicle cells prematurely stop dividing in the absence of Cyclin B. The PH3 staining pattern in epithelial follicle cells of *cycB; myt1* double mutant ovaries was essentially the same as seen in *myt1* mutants, however (Fig. 4-18D and C). Moreover, the ovaries from *cycB; myt1* double mutants actually developed further than *cycB* mutants alone, often containing 4 egg chambers with 7 nurse cell nuclei (ie. developing up to stage 5 or 6, see Fig. 4-18D). Why is over-proliferation in cycling follicle cells not affected by loss of *cycB* activity in the *myt1* mutants? One possible explanation is that Cyclin A or Cyclin B3 is up-regulated or stabilized in response to loss of *myt1* (as shown in Fig 4-16B and 17B), consequently the extra amount of Cyclin A or Cyclin B3 can sustain the development of ovaries further in the double mutants. Despite the possible up-regulation of Cyclin A or Cyclin B3 in *cycB; myt1* double mutants, the proliferation ability of germline cells and follicle cells remained dependent on a sufficient supply of Cyclin B, as revealed by the lack of 16-cell cysts and older (than stage 7) egg chambers in these mutants.

These results provide interesting clues as to how dMyt1 may be implicated in regulating cycling follicle cells and post-mitotic cells via different mechanisms. For example, lack of *myt1* may cause up-regulation or stabilization of Cyclin A or Cyclin B3 and thus paradoxically promote mitosis in the absence of Cyclin B, in addition to mis-regulation of inhibitory phosphorylation of Cdk1. In post-mitotic cells, however, *myt1* may be required to prevent ectopic mitosis during cell cycle exit independently of its role in negatively regulating Cdk1. Perhaps Myt1 is required for negatively regulating Cdk2, whose activity is important for promoting S phase (Stern et al., 1993). The exact mechanisms governing cell cycle exit remain poorly understood, thus further studies of disrupted cell cycle exit in *myt1* mutants should shed more insights into this unsolved puzzle.

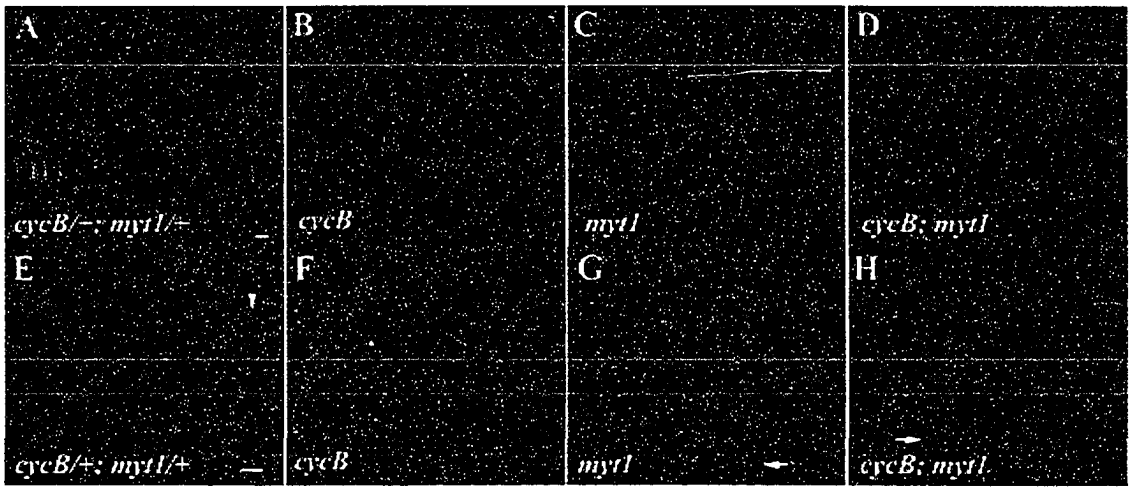

Fig. 4-18 Loss of *cycB* has no discernible effect on the ectopic PH3 staining pattern in *myt1* mutant egg chambers. Ovarioles and egg chambers from *cycB/+; myt1/+* (A, E), *cycB* mutants (B,F), *myt1* mutants (C,G) and *cycB; myt1* double mutants (D,H) were stained for PH3 (red) and DNA (blue). (A, B, C, D) Ovarioles of the indicated genotypes. Very few PH3-positive cells could be seen in *cycB/+; myt1/+* (A) or *cycB* mutants (B). Ovarioles from *cycB* mutants contain mostly germarium (B, F), occasionally with 1 or 2 egg chambers (B). Ovaries from *myt1* mutants (C) and *cycB; myt1* double mutants (D) had many PH3-positive cells. (E, G, H) Egg chambers of the indicated genotypes. Compared to the control (E), PH3-positive cells were present in the anterior of egg chamber from both *myt1* mutants (G) and *cycB; myt1* double mutants (H). Bar for A-D; 20 μm; bar for E-H: 20 μm.

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Chapter 5 General Discussion and Conclusions

Drosophila myt1 mutants exhibit multiple defects in bristles, testes and ovaries as adults; however, none of these phenotypes were seen in mutants for *wee1*, a gene that encodes a nuclear Wee1 that also regulates Cdk1 by inhibitory phosphorylation. The phenotypic differences between *myt1* and *wee1* probably stem from the distinct sub-cellular localization and Cdk1 phosphorylation site specificity of Wee1 and Myt1. The functional redundancy between these two Cdk1 inhibitory kinases can be attributed to the fact that both gene products phosphorylate Y15 of the protein kinase of Cdk1, which shuttles between the cytoplasm and nucleus in association with Cyclin B. Here, the possible functions of dMyt1, in relation to those of dWee1, will be discussed, particularly with respect to their differences in localization and biochemical properties. Models regarding how dMyt1 functions to regulate the G2/M transition and cell cycle exit will also be proposed.

5.1 dMyt1 and dWee1 have both unique and redundant functions in *Drosophila*

Maternal *wee1* is absolutely required for progressively lengthening interphase during the last few cell cycles of syncytial embryos (Stumpff et al., 2004). Without a maternal supply of dWee1, mutant embryos are thought to encounter problems as a result of incomplete DNA replication before the onset of mitosis, leading to a terminal late syncytial arrest phenotype (Price et al., 2000). The idea that dWee1 functions to delay mitosis during S phase in response to ongoing DNA replication implicates dWee1 as a critical component of the S/M checkpoint. It is unlikely that this role would be essential at later developmental stages, since post-syncytial cell cycles are much slower and do not impose such stringent temporal requirements for the timing of S phase completion and the onset of mitosis, to coordinate cell proliferation with developmental events. Consistent with this idea, no developmental zygotic phenotypes were found in *wee1* mutants, although they remain S/M checkpoint defective with respect to sensitivity to the DNA replication inhibitor hydroxyurea (Price et al., 2000). *wee1* transheterozygotes or hemizygotes are also viable and fertile as adults (although eggs laid by females do not hatch due to the maternal requirement); indicating that regulatory constraints on Cdk1 activation before entry into mitosis/meiosis remain sufficient in *wee1* mutants. These

observations hint that the other Wee1-like kinase in *Drosophila*, dMyt1, may substitute for or be redundant with dWee1 during zygotic development.

Indeed, my studies of zygotic *myt1* mutants revealed developmental defects in several tissue types, including embryos, larvae and adults. *myt1* mutants display bristle defects, male sterility, partial female sterility and a complex set of defects during embryogenesis. A defect seen in several tissues affected by loss of *myt1* was an apparent increase in cell proliferation, characterized by more dividing cells (relative to controls). These defects were a consequence of mis-regulated Cdk1 activity, that can affect cell fate changes and the rate of cell proliferation in cells that would normally be undergoing developmentally programmed mitotic cycles or allow cells that would normally exit the cell cycle while undergoing terminal differentiation, to undergo ectopic cell divisions. Despite these multiple defects throughout development, *myt1* mutants are viable, implying that the constraints on Cdk1 activation before entry into mitosis are sufficient for maintaining viability. Presumably, dWee1 activity is sufficient for regulating Cdk1 activity in *myt1* mutants. These findings suggest that *myt1* or *wee1* can compensate for each other when one is absent. Evidence presented in this study supports this concept in three aspects: 1) *wee1; myt1* progeny were never recovered in my genetic crosses; 2) *wee1* was haplo-insufficient in *myt1* mutants for viability and bristle development but not *vice versa* (, which implies that *myt1* is more important as a negative regulator of Cdk1 in zygotes); 3) the severity of *wee1* alleles determined the decrease in viability of *myt1* mutants in an allele-specific manner.

Since Myt1 and Wee1 reside in very different compartments within the cell and phosphorylate Cdk1 at different site(s), it is interesting to speculate how these two kinases could be acting to compensate for each other, to what extent this occurs, and under what conditions. The different cellular effects of *myt1* and *wee1* mutations on primary spermatocytes shed some insights into this issue in several respects. No defect was ever seen in the primary spermatocytes of *wee1* mutants alone, suggesting that dWee1 is entirely dispensable for the development of these cells when dMyt1 is available. Without dMyt1 however, the primary spermatocytes show premature centrosome separation and splitting, as well as fusome fragmentation in the cytoplasm, although these cells do not proceed prematurely into meiosis with respect to nuclear criteria. Induction

of *Cdk1AF* in primary spermatocytes triggers a similar centrosomal defect, strongly arguing that these defects are caused by failure to regulate Cdk1 activity, rather than another, unknown target of dMyt1. Taken together these results suggest that dMyt1 is required in the cytoplasm to inhibit Cdk1 (and thereby affect Cdk1 phosphorylation-dependent processes such as centrosome separation), a function which cannot be compensated for by nuclear dWee1 in the primary spermatocytes.

Nonetheless, my data also suggest that dWee1 is able to protect nuclei of primary spermatocytes from cytoplasmically activated Cdk1/Cyclin complexes in *myt1* mutants, presumably by inhibiting Cdk1 locally in the nucleus. If the *wee1* gene copy number is reduced to one, however, the protection of nuclei provided by dWee1 becomes insufficient, as indicated by my observations of apparent premature chromosome condensation in the nuclei. Therefore, nuclear dWee1 is the last line of defense for these cells to delay entry into meiosis when dMyt1 is not functional, although dWee1 is dispensable in the presence of dMyt1. In the absence of dMyt1, dWee1 also becomes a determining factor for viability in a dosage-dependent and allele-dependent manner. For instance, after removing one copy of *wee1* approximately 10% ($wee1^{ES1}$ allele) or 30% ($wee1^{DS1}$ allele) of *myt1* flies can survive to adulthood, with enhanced bristle phenotypes in the microchaetae as well as enhanced meiotic defects in primary spermatocytes.

Primary spermatocytes are probably affected the most in *myt1* mutants because they have an extremely long pre-meiotic G2 phase, a stage that specifically requires Myt1 for complete inhibition of Cdk1 activation in the cytoplasm. Similarly, precursor cells for macrochaetae and microchaetae also have a relatively long G2 phase compared to other somatic cells, and the G2 phase in the former is longer than the latter (Huang et al., 1991), consistent with these cells being more sensitive to loss of Myt1 function. Although normal levels of Wee1 activity can fully compensate in cells without such developmentally regulated protracted G2 phases in *myt1* mutants, the lethality of the double mutants demonstrates that there is an essential requirement for Cdk1 regulation in all cells that must be provided by either Wee1 or Myt1. Although it remains unclear exactly how cell division is affected without both dWee1 and dMyt1, it is tempting to predict that the phenotype might recapitulate what was seen in *wee1/+; myt1* primary

spermatocytes, where premature chromosome condensation during entry into meiosis presumably results in cell lethality.

In general, the fact that Myt1 and Wee1 share the same target, Cdk1, may ensure robustness of cell cycle regulation to *Drosophila*, since either Wee1 or Myt1 is sufficient for inhibiting Cdk1 activity, at least in vital tissues. On the other hand, the differences between Myt1 and Wee1 allow flexibility in Cdk1 regulation in different developmental contexts: dWee1 primarily regulating the S/M transition during early embryogenesis (Price et al., 2000), whereas dMyt1 being the major G2/M regulator throughout the remainder of *Drosophila* development . Since Myt1 is also found in many other metazoans, it will be of great interest to see if what has been learned in *Drosophila* can also apply to these organisms.

5.2 Loss of dMyt1 activity has different effects on male and female meiosis

Meiosis in males and females shares common features, albeit these two processes also have some overt differences, such as a failure to undergo meiotic recombination in male *Drosophila.* The study of *Drosophila myt1* mutants has disclosed another type of difference between male and female meiosis. The involvement of Myt1 in regulating meiosis was initially identified in *Xenopus* oocyte extracts, where a MAP kinase-dependent signaling pathway inactivates Myt1 in stage VI oocytes to release them from pre-meiotic "G2-like" prophase I arrest, implying that Myt1 is responsible for prophase I arrest during *Xenopus* female meiosis (Palmer et al., 1998). A similar meiotic role for Myt1 was also reported in the starfish, *A. pectinifera* (Okumura et al., 2002). Reciprocally, a gain-of-function mutation of a Myt1 ortholog in *C. elegans* is also male sterile, apparently because Cdk1 inhibitory activity from the mutated form of Myt1 cannot be overcome, blocking meiotic progression (Lamitina and L'Hernault, 2002). In contrast, Wee1 is required to be absent during the MI to MII transition in *Xenopus*, since low levels of residual Cdk1 activity are needed to inhibit the initiation of DNA synthesis at that time (Iwabuchi et al., 2000; Nakajo et al., 2000). Therefore, it was postulated that Myt1 is the sole Cdk1 protein kinase that functions during meiosis I, although it was not clear whether this applied to both males and females in all metazoans (Nakajo et al., 2000).

My phenotypic analysis of *Drosophila myt1* mutants indicates that meiosis is affected differently by loss of dMyt1 activity in males and females. For example, primary spermatocytes in *myt1* mutants show premature centrosome separation and splitting and fusome fragmentation defects but no apparent effects on chromosome condensation and nuclear envelope breakdown. By regulating centrosome behavior during male pre-meiotic G2 phase, *myt1* is essential for faithful segregation of chromosome, ensuring the formation of a properly formed spindle apparatus. On the other hand, female meiosis appeared normal in *myt1* mutants (in the absence of a FM7 balancer chromosome) from prophase I to metaphase I (data not shown). Female meiosis was not always successful in the absence of dMyt1 however, as indicated by elevated rates of X chromosome non-disjunction (~11%) in *myt1* mutants. Animal oocytes are acentrosomal, and so it was no surprise that centrosome defects were not seen in *myt1* female oocytes. Genetic data indicated that non-disjunction in *myt1* females occurs primarily during meiosis I and primarily involves achiasmate chromosomes. Considering that the *myt1* mutation shows a strong effect at the G2/M transition on regulating Cdk1 activity, which is important for spindle assembly in somatic cells (Nigg et al., 1996), it is not surprising that most non-disjunction events in *myt1* mutant females occur at meiosis I. Two kinesin-like motor proteins, Ncd (*non-claret disjunctional* gene product) and Sub (*subito* gene product), are involved in meiotic spindle pole formation (Giunta et al., 2002; Matthies et al., 1996), and mutations in the corresponding genes cause non-disjunction events in meiosis I. It is possible, then, that *myt1* mutations cause non-disjunction defects in meiosis I by affecting the normal function of these proteins. How would loss of Myt1 disrupt the function of Ncd and Sub during female meiosis? One possible scenario is that Myt1 may do so through Axs (the gene product of *aberrant X segregation*), a novel ER-residing transmembrane protein ensheathing the meiotic spindle (Kramer and Hawley, 2003). This idea is attractive because of the following facts: 1) EGFP-tagged Myt1 was also seen to be localized to the meiotic sheath structure (J. Kramer, pers. comm.); 2) *Axs* mutations cause high frequency of NDJ in achiasmate chromosomes during female meiosis (Kramer and Hawley, 2003) and 3) the meiotic spindle is abnormal in *Axs* mutants (Kramer and Hawley, 2003). Another challenge for future investigation then,

will be to determine if Myt1 interacts with Axs and how Axs interacts with motor proteins such as Ncd and Sub.

Meiosis in *myt1* males and females also shows distinct responses towards lowering *wee1* copy number. In addition to *myt1*-specific centrosome and fusome defects, primary spermatocytes from *wee1/+; myt1* males apparently initiate chromosome condensation prematurely, leading to an aberrant meiosis with fragmented and highly condensed nuclei. Oocytes from females of the same genotype, however, look normal with no sign of premature chromosome condensation (data not shown). Curiously, in a small fraction of *wee1/+; myt1* oogonia, the mitotic divisions were reduced from four cycles to three prior to meiosis, generating egg chambers with only 8 germline cells in a cyst rather than 16 germline cells. The cause of this defect was not determined.

It was unexpected that *myt1* mutations would affect meiosis so differently in *Drosophila* males and females. The meiotic function of dMyt1 in *Drosophila* males seems to be well-conserved with that reported in other species, consistent with the fact that males have a very long (~90 hr) pre-meiotic G2 phase (Fuller, 1993). In contrast, females normally arrest in prophase I, a difference that may account for the failure to detect any obvious cytological defects in the *myt1* mutant females (Spradling, 1993). A similar discrepancy of meiotic behavior in different sexes caused by the same mutation had also been reported for *cycB* and *twine* mutants in *Drosophila* (Courtot et al., 1992; Jacobs et al., 1998; White-Cooper et al., 1993). Namely, primary spermatocytes in *twine* mutants do not enter meiosis; whereas oocytes in *twine* mutants initiate meiosis I but fail to arrest at metaphase I and progress further in an aberrant meiosis (Courtot et al., 1992; White-Cooper et al., 1993). *cycB* mutants have rudimentary ovaries, with 3-4 germline cells in each cyst residing in a germarium or, very rarely, a stage 2-3 egg chamber (Jacobs et al., 1998), whereas *cycB* mutant primary spermatocytes arrest at metaphase I of meiosis (Trunova et al., 1998).

These different meiotic phenotypes in males and females described in *myt1*, *cycB* and *twine* mutants explicitly indicate that there are fundamental differences in how the cell cycle machinery operates within the two sexes during meiosis. Since dMyt1 negatively regulates G2 Cyclin-associated Cdk1 activity, the effect of lacking dMyt1 may be masked under circumstances where G2 Cyclins are limited. This is probably the

reason why *myt1* mutation exhibits differential effects on male meiosis and female meiosis. Primary spermatocytes contain abundant *cyclin B* transcripts that are produced by testis-specific machinery that also synthesizes many other transcripts required for meiosis and sperm differentiation (Dalby and Glover, 1992; Dalby and Glover, 1993; White-Cooper et al., 1998). Immuno-fluorescent staining indicated that Cyclin B proteins start accumulating in young primary spermatocytes, peaking at late prophase I, just prior to metaphase I (White-Cooper et al., 1998). In females, there are two transcripts of *cyclin B* containing different 3'-UTRs that were detected with different expression patterns: the small transcript (2.3kb) can be seen in the germarium, stage 2-7 oocytes and overlying follicle cells; whereas the larger one (2.7kb) is limited in stage 9-10 nurse cells (Dalby and Glover, 1992; Dalby and Glover, 1993). Despite the abundance of these *cyclin B* transcripts, Cyclin B proteins were not detectable except in the germarium and proliferating follicle cells, suggesting that translation of the smaller transcripts in stage 2-7 oocytes and the larger transcripts in stage 9-10 nurse cells is suppressed (Dalby and Glover, 1992; Dalby and Glover, 1993).

The maternally contributed *cyclin B* transcripts in *Drosophila* and *Xenopus* embryos are suppressed by a protein complex that includes Nanos, Pumilio and other unknown factors. The complex acts by shortening the length of the polyA tail of the transcripts, inhibiting their translation (Asaoka-Taguchi et al., 1999; Groisman et al., 2002). Prior to meiosis I, a similar translational repression of *cyclin B* transcripts by the Nanos-Pumilio machinery was also observed in *Xenopus* oocytes (Cao and Richter, 2002; Nakahata et al., 2003). The release of *cyclin B* translational repression is coincident with the timing of germinal vesicle break-down (GVBD), which marks the beginning of metaphase I in *Xenopus* oocytes (Nakahata et al., 2003). It is rather appealing to speculate that Cyclin B translation is also suppressed in prophase I *Drosophila* oocytes, given that *Drosophila* are equipped with the same translational silencing/activating machinery as in *Xenopus*. Assuming that this is true, the different meiotic phenotypes seen in *myt1* males and females may thus be explained by differences in the availability of Cyclin B proteins during G2 phase and prophase I of meiosis.

The availability of Cyclin B could also explain the differential responses to reduced *wee1* copy number in *wee1/+; myt1* males and females. Since Cyclin B is

already present during G2 phase and prophase I of male meiosis (White-Cooper et al., 1998), reduced levels of *wee1* were presumably unable to inhibit cytoplasmically activated Cdk1/Cyclin B activity in these *myt1* mutant primary spermatocytes, accounting for the apparent premature chromosome condensation phenotype. In *wee1/+; myt1* females, if Cyclin B proteins are not translated until metaphase I of meiosis, then premature chromosome condensation would not occur even when the level of Wee1 kinases is low. In response to a lower level of Wee1 kinases however, translational suppression of Cyclin B transcripts could be imposed in mitotically dividing oogonia to delay mitosis in *wee1/+; myt1* females. Consequently, limited production of Cyclin B in oogonia could be responsible for giving rise to 8 cell cysts in *wee1/+; myt1* females.

The hypothesis that Cyclin B translational suppression in *Drosophila* oocytes during prophase I can accounts for differences observed in male and female *myt1* mutants could be tested in several different ways. Gal4-driven germline-specific expression of Cyclin B expression in the oocytes of *myt1* mutants might generate meiotic phenotypes that would not be seen in *myt1* mutants without such ectopic expression, if limited Cyclin B levels account for the failure to observe female meiotic defects. Moreover, mutations affecting the oocyte translational repression machinery might produce a similar result as ectopic expression of Cyclin B, if this hypothesis is valid. With *Drosophila* genetics, these experiments are practical and should help us to accept or reject the hypothesis.

5.3 Involvement of dMyt1 in the DNA damage checkpoint

All eukaryotes, including unicellular organisms such as yeasts, have evolved a well-conserved DNA damage checkpoint mechanism to protect their DNA from endogenous and exogenous damage. As part of the DNA damage checkpoint, Wee1-like kinases contribute by phosphorylating Y15 of Cdk1, and hence arrest entry into mitosis until the damage is repaired. Since both Wee1 and Myt1 can catalyze this Y15 phosphorylation reaction, it remains unclear whether or not Wee1 and Myt1 are both involved in the DNA damage checkpoint.

Two important checkpoint effecter proteins: Chk1 and/or Chk2, are activated in response to DNA damage, to trigger a series of coordinated responses including DNA repair and delay of entry into mitosis or apoptosis, when the damage is too severe to be

repaired. Intensive studies in several model organisms have established that the Chk1 and Chk2-mediated DNA damage response targets both Cdc25C and Wee1 to inhibit Cdk1 activation. This involves two reciprocal effects: eliminating Cdc25C activity and Cdc25C nuclear retention, but promoting Wee1 activity in the nucleus (Kumagai et al., 1998; Lee et al., 2001; Lopez-Girona et al., 1999; Peng et al., 1997; Raleigh and O'Connell, 2000; Zeng and Piwnica-Worms, 1999). Interestingly, my results indicated that *wee1* mutant cells in 3rd instar wing imaginal discs were not defective for the DNA damage checkpoint after ionizing radiation (IR) treatment, as judged by PH3 staining for mitotic cells. These results are in agreement with the results of *wee1* knockdown in mammalian cells, which also showed no effect on the DNA damage checkpoint, assayed similarly (van Vugt et al., 2004). In contrast, many PH3-positive cells were seen after IR treatment in *myt1* mutant wing imaginal discs, however the staining pattern in mutant cells appeared to be highly fragmented, resembling the fragmentation of chromosome seen in "mitotic catastrophe" (Castedo et al., 2004). Many conditions can trigger mitotic catastrophe, one being premature entry of activated Cdk1/Cyclin B complexes into the nucleus (Heald et al., 1993). It will be interesting to confirm if loss of inhibitory phosphorylation of Cdk1 is the underlying mechanism for the apparent "mitotic catastrophe" seen in *myt1* wing discs after irradiation.

As discussed in a previous section, dWee1 and dMyt1 appear to have overlapping functions for negatively regulating Cdk1 activity at certain stages of development. For example, *myt1* mutants showed signs of premature activation of Cdk1 in primary spermatocytes but not in wing imaginal disc cells, suggesting that dWee1 is able to substitute for dMyt1 under the latter conditions. However, when challenged with IR treatment, *myt1* mutant cells could apparently no longer launch and/or maintain an effective DNA damage checkpoint, and thereby undergo mitotic catastrophe. In contrast, *wee1* mutant cells did not display such defects after DNA damage, presumably because cytoplasmic dMyt1 was sufficient to suppress the activation of Cdk1 in the cytoplasm and thus prevent the active isoform of Cdk1 from entering the nucleus. Additional genetic evidence also supports the idea that dMyt1 has a role in the G2/M checkpoint pathway. Eliminating one copy of *chk2* resulted in a 90% decrease of viability in *myt1* mutants, whereas losing both copies of *grapes* (*Drosophila* homolog of *chk1*) was

necessary to cause a similar loss of viability in *myt1* mutants. The results of these viability tests imply that *myt1* mutant cells have a compromised DNA damage checkpoint, making them sensitive to lower gene dosage of Chk2 or loss of Chk1 at a cellular level. Furthermore, the double mutant combination of *wee1* and *myt1* was lethal in flies, implying that either the Wee1-regulated DNA replication checkpoint or the Myt1-regulated DNA damage checkpoint is required for cell viability.

5.4 A model for Myt1 regulation of Cdk1 activation in *Drosophila*

Since the functions of dMyt1 discussed above are related to the regulation of Cdk1 complexes at the G2/M transition, I will present a model for Myt1 regulation of Cdk1 activation in *Drosophila* and explain how this model can be used to understand *myt1* phenotypes. The premise of my model is that Cyclin A or Cyclin B-associated Cdk1 is singly phosphorylated by Myt1 at T14 (or doubly phosphorylated at both T14 and Y15 sites, when Wee1 is missing) in the cytoplasm during G2 phase (see Fig. 5-1A). These Cdk1/Cyclin A or B complexes can then translocate into the nucleus and be phosphorylated at Y15 by nuclear Wee1 (if this site was not phosphorylated in the cytoplasm by Myt1). Therefore, Cdk1 complexes with Cyclin A or B eventually become doubly phosphorylated at both T14 and Y15 in the nucleus, and may then shuttle back into the cytoplasm in this state, as reported for mammalian cells and *Drosophila* embryos (Hagting et al., 1998; Huang and Raff, 1999; Yang et al., 1998). Accordingly, all of the Cdk1 complexes with Cyclin A or B would eventually become doubly phosphorylated through this sequential phosphorylation by Myt1 and Wee1 in different cellular compartments, ensuring complete suppression of Cdk1 activity during G2 phase.

When Myt1 is absent, Cyclin A or Cyclin B associated Cdk1 is not phosphorylated when these complexes initially form in the cytoplasm. Cdk1 can still be singly phosphorylated by Wee1 at Y15 in the nucleus, however (Fig. 5-1B). These complexes are actively exported out of nucleus, but may be partially active and thus capable of triggering certain Cdk1-regulated events prematurely (for example, centrosome separation in primary spermatocytes), but unable to reach the activation threshold necessary for triggering entry into M phase.

According to this model, entry into mitosis would then occur when positive mitotic regulators such as Aurora A, Polo and Cdc25C are produced and given enough time to accumulate and be recruited to centrosomes to act as an "organizing center" for Cdk1 activation, generating the switch-like peak in Cdk1 activity that is normally seen during G2/M transition (Fig. 5-2, green line). Without Myt1, I hypothesize that partially active pY15-Cdk1/Cyclin A or B complexes as well as their positive regulators, gradually accumulate within the cytoplasm. Before the cell reaches the threshold level for Cdk1/Cyclin activation by positive regulators situated at the centrosome, the pY15-Cdk1 activity in *myt1* mutant cells would be insufficient to promote nuclear division, owing to protection by nuclear Wee1 kinase. Conceivably, these alterations could reduce the time required to accumulate sufficient positive mitotic factors in the cytoplasm for fully activating Cdk1 activity and thus shorten the time that cells remain in G2 phase (Fig. 5-2, red line).

The extremely high mitotic index observed in *myt1* mutant germline cells is consistent with the prediction that *myt1* mutant cells might have a faster G2/M transition, although I cannot exclude the possibility that mitotic delays may also contribute to this phenotype in mutant cells. Conversely, when Myt1 is over-expressed in dividing cells, it delays mitosis significantly (Price et al., 2002). Similar results were also reported for Myt1 over-expression in *Drosophila* S2 cells (Cornwell et al., 2002).

The model I have presented also suggests that Myt1 and Wee1 regulate the timing of different cellular events during the G2/M transition, consistent with my analysis of *myt1* mutant primary spermatocytes. My model predicts that *myt1* mutant primary spermatocytes display premature centrosome separation/splitting and fusome fragmentation, because of an accumulation of partially active pY15-Cdk1 in the cytoplasm of these cells; a hypothesis that needs to be empirically tested. Since nuclear Wee1 can protect the nuclei of primary spermatocytes from pY15-Cdk1 activity, premature chromosome condensation or other nuclear events are not seen in these cells, despite changes in the cytoplasm. This protection by Wee1 is clearly revealed once the gene copy number of *wee1* is lowered in *myt1* mutants.

While maternal *wee1* is essential for syncytial cell division during embryogenesis (Price et al., 2000), *myt1* does not appear to be as important at this stage of development.

Consistent with these observations, whereas pY15-Cdk1 can be readily detected in syncytial embryos, pT14-Cdk1 is essentially absent, when assayed using phospho-specific antibodies (personal communication with E. Homola). A small amount of pY15-Cdk1 can be detected in maternal *wee1* mutant embryos, presumably due to inhibitory phosphorylation by Myt1, suggesting that the Y15 specific kinase activity of Myt1 is not sufficient to protect nuclei from inappropriate Cdk1 activity in this developmental context. It will be very interesting to further explore this proposed regulatory mechanism for differential phosphorylation of Cdk1 at T14 and Y15 in syncytial embryos.

Despite their differences in sub-cellular localization and Cdk1 target site specificity, either *myt1* or *wee1* is sufficient to inhibit Cdk1 during G2 phase at most stages of *Drosophila* development, one exception being in primary spermatocytes. The proposed biochemical basis for the observed redundancy between Myt1 and Wee1 relies on that fact that both Myt1 and Wee1 can phosphorylate Y15 of Cdk1. In response to DNA damage, Myt1 and Wee1 are also redundant for arresting cells at G2 phase by this mechanism. Once the DNA damage checkpoint is triggered, however, Myt1 is required to establish an effective suppression of Cdk1 activity in the cytoplasm. Thus, my model suggests that Myt1 is more important than Wee1 for either establishing or maintaining G2 arrest during DNA damage, consistent with *myt1* mutant cells revealing a phenotype similar to mitotic catastrophe, an abortive mitosis that can be caused by premature Cdk1 activation. Although Wee1 is not required for establishing this DNA damage checkpoint, as indicated by this study and others, Wee1 is thought to be activated in response to DNA damage and consequently has to be eliminated by a Polo-dependent proteolysis to allow reentry into mitosis after recovery from DNA damage (van Vugt et al., 2004). These dual checkpoint mechanisms provided by Myt1 and Wee1 give metazoans cells more flexibility, in terms of pausing at the G2/M transition, for meeting both intrinsic and extrinsic requirements.

Although there are alternative models that could explain the functions of Myt1 and Wee1 in *Drosophila*, the one I am presenting here is probably the simplest one. Since this working model makes explicit predictions, future experiments should allow us to decide whether or not to accept this model.

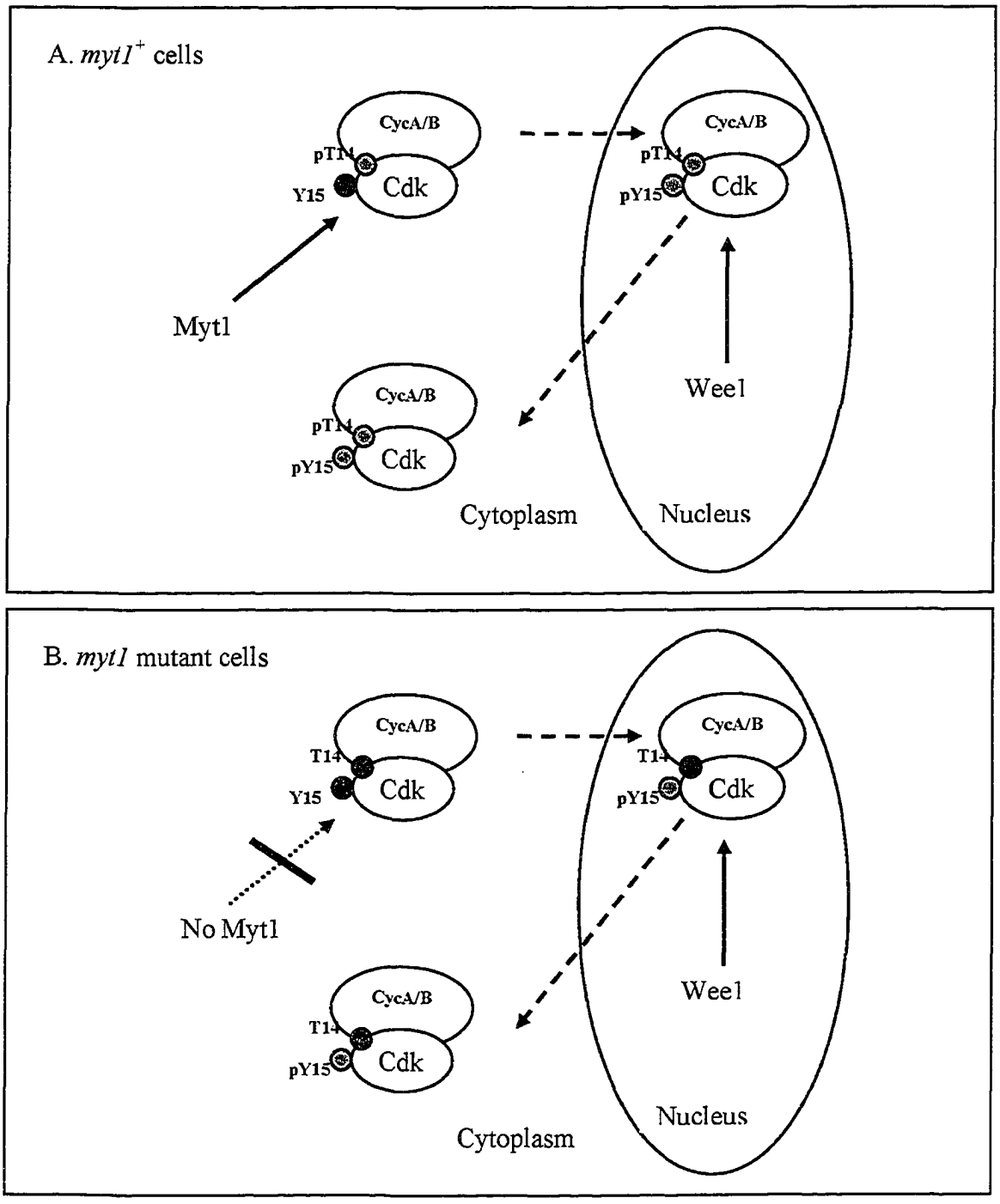

Fig. 5-1 A working model for Wee1 and Myt1 might sequentially phosphorylate Cdk1 in *myt1*$^{+}$ (A) and *myt1* mutant (B) cells. Green circles denote non-phosphorylated residues, whereas red circles denote phosphorylated residues. The hypothesis is that phosphorylation of the Y15 site alone would allow Cdk1 to be partially active in the cytoplasm, yet incapable of triggering nuclear events of mitosis because of Wee1.

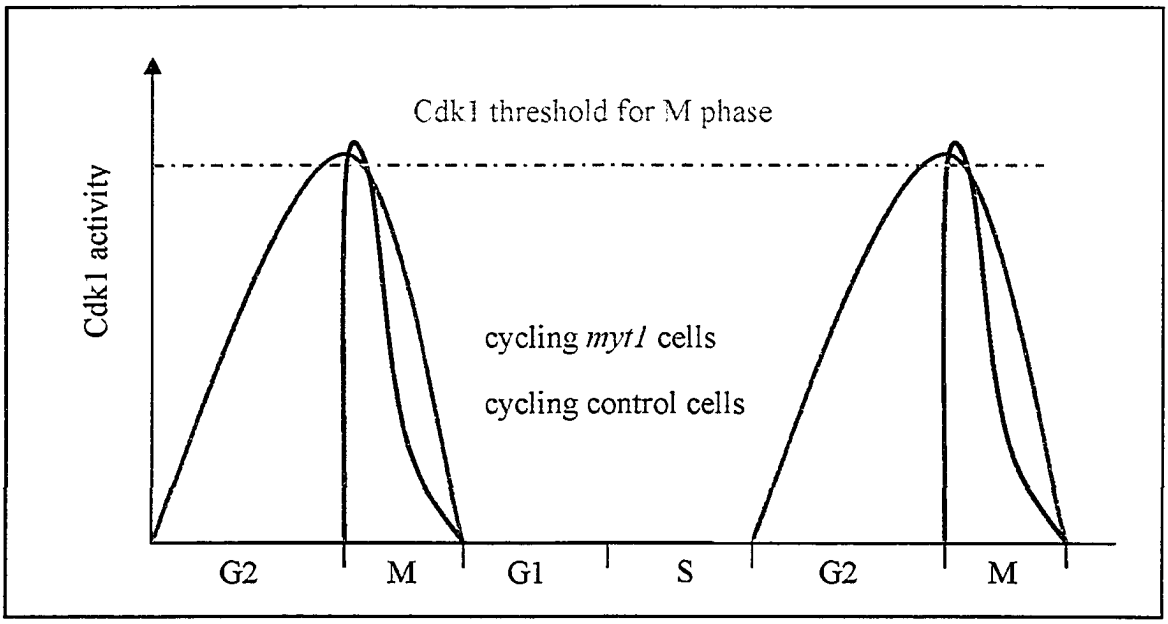

Fig. 5-2 Gradual increase of Cdk1 activity in cycling *myt1* mutant cells at the G2/M transition. See text for details.

5.5 dMyt1 has an unexpected role in cell cycle exit

In addition to the high mitotic index seen in *myt1* mutant proliferating germline cells, many terminally differentiated somatic cell types also undergo one or more extra rounds of cell division in the mutants. Initially I interpreted this phenotype as meaning that G2 phase-arrested somatic cells enter mitosis in the absence of Myt1. To my surprise, in controls the polar cells and stalk cells did not accumulate G2 Cyclins, specifically Cyclin A and Cyclin B in controls, but did in *myt1* mutants. These results suggest the possibility that these "differentiated" cells normally arrest either in G0 or G1 phase, during oogenesis. I favor the hypothesis that these cells normally arrest in G0 phase for two reasons: 1) DNA synthesis and mitosis do not occur in these cells; 2) as in G0 mammalian cells, the essential licensing factor Cdt1 (DUP in *Drosophila*) is down-regulated in stalk cells and polar cells (Xouri et al., 2004).

No matter what cell cycle phase the polar cells and stalk cells arrest in during normal development, it is worth speculating upon the mechanism that these cells use to keep dividing when dMyt1 is no longer available. One idea to explore is that dMyt1 is also involved in regulating Cdk2 (which has conserved T18 and Y19 as potential inhibitory phosphorylation sites), a Cyclin-dependent kinase that functions during S phase (Stern et al., 1993), in stalk cells and polar cells during cell cycle exit. No evidence for Cdk2 being regulated by inhibitory phosphorylation of these sites was found in a study that explicitly examined this possibility in *Drosophila* (Lane et al., 2000), however. Other alternative possibilities should also be considered, including the possibility that Cdk2 has inhibitory phosphorylation sites other than T18 and Y19.

Another possibility is that ectopic Cdk1-Cyclin A may catalyze DNA synthesis in *myt1*mutant stalk cells and polar cells, analogous to the situation observed in *fzr* mutants where ectopic Cdk1-Cyclin A and/or Cdk1-CyclinB promotes M phase in these cells (Sigrist and Lehner, 1997). This hypothesis predicts that the non-inhibitable form of Cdk1, Cdk1AF, would be able to phenocopy the cell cycle exit defect in mutant stalk cells and polar cells. My experiments showed that expression of *Cdk1AF* did not cause ectopic mitosis in the stalk cells and polar cells however, although ectopic Cdk1 activity did mimic the over proliferation phenotype of *myt1* in cycling follicle cells. Moreover, my observation that *myt1* mutation was capable of partially rescuing the developmental

defects of egg chambers caused by loss of *cycB*, suggested that *myt1* mutant cells have more mitotic Cyclins (Cyclin A and Cyclin B3 in this case, but perhaps also Cyclin B, in *myt1* mutants alone). This interpretation was supported by immunological data indicating higher levels of Cyclin A and Cyclin B in the *myt1* mutants. The fact that the induction of *Cdk1AF* alone was not equivalent to loss of *myt1* could thus perhaps also best be explained in terms of the availability of mitotic Cyclins. To test this possibility, induction of *Cdk1AF* and Cyclin A, Cyclin B or Cyclin B3 should be carried out in the future.

In conclusion, it appears that Myt1 is involved in regulating G2/M transitions during mitosis, meiosis, DNA damage induced cell cycle arrest and developmental cell cycle exit. The unifying theme that emerges behind these pleiotropic effects is that Myt1 functions to generate a temporary pause in the cell cycle that allows cells to accommodate themselves to specific developmental and environmental requirements. Thus, the work presented here extends our understanding of how Myt1 kinases operate and establishes both unique and redundant Cdk1 inhibitory roles for Wee1 and Myt1, that may also apply to other species of metazoans.

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Appendix Materials and Methods

6.1 Isolation of *myt1* mutants

As the *myt1* locus is at region 64F of the 3rd chromosome, I used a chromosomal deletion called *Df(3L)64D-F* that uncovers the *myt1* locus and ~25-30 other genes (Garcia-Bellido et al., 1994) for a genetic screen. Males from an isogenized p^p e^l stock were fed a 10 mM solution containing the mutagen ethyl methane sulfonate (EMS), then mated with females carrying a third chromosome balancer. The heterozygous F1 male progeny were then crossed to females carrying *Df(3L)64D-F* and the hemizygous non-balancer F2 progeny were scored for morphological defects, lethality and female sterility. Sibling virgins and males bearing the mutagenized p^p e^l chromosome as well as a third chromosome balancer were collected to establish/maintain these lethal and female sterile lines. A transgenic line with the genomic *myt1* sequence was then used to identify *myt1* mutants from established lethal or female sterile lines. To make the *P{myt1+}* rescue construct, a 3.6Kb SacI-EcoRI fragment, including the entire *myt1* gene and approximately 1 kb of upstream and downstream non-coding DNA, was sub-cloned from a P1 clone (DS04757) into the *pCaspeR* transposon vector (Pirrotta, 1988). Transgenic lines carrying the integrated construct were then generated by standard procedures (Spradling, 1986). After scoring ~5,000 mutagenized chromosomes, I had isolated ~140 lethal lines and ~15 female sterile lines, from which two independent lethal lines, designated as $myt1^1$ and $myt1^2$, could be rescued by the genomic *myt1* transgenic line containing rescue construct *P{myt1+}*.

When retested in a larger scale, however, $myt1^1$/*Df(3L)64D-F* or $myt1^2$/*Df(3L)64D-F* hemizygous mutants were viable with bristle defects and male sterility. Nonetheless, both defects could be rescued by the genomic *myt1* transgenic line, confirming that both lines are *myt1* mutants. Genomic sequencing indicated that $myt1^1$ and $myt1^2$ alleles possess an identical single nucleotide deletion (which is not typical for EMS mutagenesis) at position 689, implying the possibility that these two alleles might have originated from a spontaneous mutation in isogenized p^p e^l males. Unless otherwise stated, *myt1* mutants used for experiments were of $myt1^1/myt1^2$ genotype, which showed essentially similar bristle and male sterile phenotypes as $myt1^1$/*Df(3L)64D-F* hemizygous mutants.

To generate follicle cell clones in the ovaries, 2-day-old females of *hs-FLP/+*; $myt1^2$, *P[ry+;hs-neo;FRT]80B/*$myt1^+$*,GFP(nls)*, *P[ry+;hs-neo;FRT]80B* genotype were

heated shocked at 37°C for 1 hour to induce FLP-mediated mitotic recombination (Xu and Rubin, 1993). 3 or 4 days after the heat-shock, ovaries from these females were removed for immuno-staining to visualize *myt1* mutant clones.

6.2 *Drosophila* stocks and culture

Most genetic markers used in the study are as described in The Genome of *Drosophila melanogaster* (Lindsley and Zimm, 1992) and Flybase (2003). Flies for most crosses and in egg collection cages were raised on standard medium at 25°C. Embryos were collected from eggs laid by females of the desired genotype on the surface of grape juice plates at designated time intervals and aged to reach the desired developmental stages.

Apart from the fly stocks listed in materials and methods of Chapter 2 and Chapter 3, the following stocks were used in genetics crosses for this study: *cycB^{2}/CyO,ftz-lacZ*, *grp^{1}/CyO*, *chk2/CyO* (also known as *lok^{p6}*, generated by *P*-element insertion), *70C[w^{+}];P[ry^{+};hs-neo;FRT]80B*, *hs-FLP*; *GFP(nls)*, *P[ry^{+};hs-neo;FRT]80B*, *hs-FLP*, *arm-LacZ*, *P[ry^{+};hs-neo;FRT]80B*, *FM7w, y,w*; *Sp/Roi; Sb/TM6B, y,w*; *Sp/CyO*; *TM6B/TM6, y,w*; *Neu-Gal4, y,w*; *NOS-Gal4* and *y,w*.

6.3 Construction of pUASp-(myc)$_{12}$-myt1 plasmid

A fragment (~560bp in size) containing (myc)$_{12}$ was removed from pINV-spc1^{+}-myc$_{12}$ (Iacovoni et al., 1999) by *Kpn*I and *Xho*I digestion and subcloned into the pBluescript SK(+) vector to obtain an *Eco*RI site at the 3'end of (myc)$_{12}$ sequence. This construct was then used for PCR amplification of a (myc)$_{12}$ fragment with introduction of a *Kpn*I site (GGT ACC) and simultaneous removal of an endogenous *Eco*RI site (GAA TTC) at the 5' end using the primers GAA TTG GGT ACC GTG TGC T GGA ATG CG GCT (*Kpn*I-(Myc)$_{12}$ primer) and AAT TAA CCC TCA CTA AAG GG (T3 primer). PCR-based transversion of the *Eco*RI site also served to ensure the presence of a start codon (ATG) at the 5'end of the PCR product (bold in *Kpn*I-(Myc)$_{12}$ primer sequence). This (myc)$_{12}$ fragment was then subcloned into pUASp vector (Rorth, 1998, modified by Christine Walker in our lab) through *Kpn*I and *Eco*RI sites to give rise to pUASp-(myc)12 plasmid with 5'-*Kpn*I/*Eco*RI/*Bam*HI/*Xba*I-3' as (unique) multiple cloning sites.

myt1 cDNA was cloned from pUASp-Dmyt1 plasmid by PCR, using primers ATC GAA TTC **ATG GAA AAG CAT CAT CGC** and GGG GAT GCT TAA TTG TGT TCT (3'pUASp primer, which is complementary to a sequence that is 3'downstream from the multiple cloning sites of pUASp vector) to introduce an *Eco*RI site (underlined sequence) directly ahead of the beginning of the *myt1* coding sequence (bold sequence) at the 5'end. This fragment was then digested and inserted to the pUASp-$(myc)_{12}$ plasmid, which was cut with *Xba*I, blunted by Klenow enzyme, and subsequently cut with *Eco*RI. Sequencing confirmed no change in sequence of $(myc)_{12}$, *myt1* cDNA and the presence of the resulting linker (5'-*Xho*I-*Eco*RI-3' fragment from multiple cloning sites of pBluescript SK(+) vector) between them during construction.

6.4 Construction of pUASp-EGFP-myt1 plasmid

pUASp-EGFP-myt1 plasmid was constructed by PCR amplification of a fragment containing the complete EGFP coding sequence from pEGFP-C1 (Clontech), using primers CTG GGT ACC **ATG GTG AGC AAG GGC GAG** (*Kpn*I-EGFP-5'primer) and AAT GAA TTC **CTT GTA CAG CTC GTC CAT** (*Eco*RI-EGFP-3'primer), which served to introduce *Kpn*I and *Eco*RI sites (underlined sequences) at the 5' and 3'end (bold sequences), respectively. This fragment was then subcloned into pUASp-$(myc)_{12}$ vector through *Kpn*I and *Eco*RI sites, and sequenced to confirm no changes in *EGFP* and *myt1* cDNA sequences.

6.5 Irradiation treatment for DNA damage checkpoint tests

20-30 wandering 3rd instar larvae of desired genotypes in a fresh vial (diameter: 2cm), were irradiated in the center of the stage of the Gamma Cell (manufactured by Atomic Energy of Canada, Ltd.). A Co^{60} γ ray source was used to irradiate larvae for 6.5 minutes to administer a dose of 40Gy. After 30 minutes of recovery time, wing imaginal discs were removed from irradiated larvae for fixation and analysis. For shorter recovery times of 5, 10 and 20 minutes, wing discs were removed and incubated in a drop of 1X PBS solution (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) for 20 minutes prior to irradiation. Fixation of irradiated wing discs is as described in 6.5.

6.6 Fixation of embryos and wing discs

Collected embryos were rinsed twice with distilled water and then dechorionated in 50% bleach for 2.5 minutes. After being permeabilized in heptane for 30 seconds, these embryos were then transferred to a microfuge tube containing equal volume of 3.7% formaldehyde (Sigma) in 1X PBS with heptane, and mixed on a nutator at room temperature for 20 minutes. The bottom layer containing formaldehyde was then removed, replaced with 1 ml of methanol. Tube was vigorously shaken for 20 seconds, and left to stand for 1-2 minutes to allow embryos to settle. The upper layer of heptane was then removed, and the remaining embryos were washed with methanol twice. To visualize microtubules and other cytoskeletal structures, use of 37% formaldehyde for 5 minutes was done to yield better immuno-staining results. Fixed embryos were then stored in methanol at -20°C. Before immuno-staining, fixed embryos were re-hydrated with 1X PBS and then 0.1% Tween-20 in 1X PBS.

Wing discs attached to the larval cuticle were transferred into an eppendorf tube containing 3.7% formaldehyde in 1X PBS, and then fixed at room temperature for 20 minutes. Fixed discs were washed twice with 1X PBS and then permeabilized with 0.5% Tween-20 in 1X PBS for 30 minutes. After rinsing twice with 0.1% Tween-20 in 1XPBS, these discs were ready for immuno-staining. Fixation of ovaries was as described in materials and methods in Chapter 2. These protocols are modified from those described in *Drosophila* Protocls (Sullivan et al., 2000).

6.7 Immunofluorescence staining

Immuno-staining was performed as described in Chapter 2 and Chapter 3. Primary antibodies were used at the following dilutions: rabbit anti-Rux (1/200, Thomas et al., 1994), rabbit anti-Fzr (1/200, described in Sigrist and Lehner, 1997), mouse anti-cyclin B (1/2, obtained from the Developmental Studies Hybridoma Bank), mouse anti-cyclin A (1/10, obtained from the Developmental Studies Hybridoma Bank), guinea-pig anti-DUP (1/1000, described in Whittaker et al., 2000), rabbit anti-Chk2 or Dmnk (1/200, described in Oishi et al., 1998). Alexa 488 and 568-conjugated secondary antibodies (Molecular

probes) were used at 1/1000 dilution. Images were either captured with a Zeiss Epifluorescent microscope (via IP Lab imaging software) or a Leica TCS-SP2 Multiphoton Confocal Laser Scanning Microscope (TCS-MP). Unless otherwise stated, all images were acquired with the same settings for controls and mutants. Most of the images obtained were later enhanced and resized with Photoshop software.

6.8 Preparation of specimen for Scanning Electron Microscopy (SEM)

Adult flies were fixed in a microfuge tube in 800µl of fixative (1% glutaraldehyde, 1% formaldehyde and 1M sodium cacodylate, pH 7.2, with a drop of 0.2% Tween-20 to reduce the surface tension) for 2 hours. These fixed flies were rinsed with distilled water and then dehydrated through a graded ethanol series (once with 25%, 50%, 75%, and twice with absolute). After the Critical-Point-Dry procedure, flies were mounted on stubs for acquiring images directly with Philips/FEI LaB6 Environmental Scanning Electron Microscope (ESEM).

6.9 References

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