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Mitotic Response to DNA Damage in Early Drosophila Embroyos: a Dissertation

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MITOTIC RESPONSE TO DNA DAMAGE IN EARLY *DROSOPHILA* EMBRYOS

A Dissertation Presented

By

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ABSTRACT

DNA damage induces mitotic exit delays through a process that requires the spindle assembly checkpoint (SAC), which blocks the metaphase to anaphase transition in the presence of unaligned chromosomes. Using time-lapse confocal microscopy in syncytial *Drosophila* embryos, we show that DNA damage leads to arrest during prometaphase and anaphase. In addition, functional GFP fusions to the SAC components MAD2 and Mps1, and the SAC target Cdc20 relocalize to kinetochore through anaphase arrest, and a null *mad2*mutation blocks damage induced prometaphase and anaphase arrest. We also show that the DNA damage signaling kinase Chk2 is required for damage induced metaphase and anaphase arrest, and that a functional GFP-Chk2 fusion localizes to kinetochores and centrosomes through mitosis. In addition, in the absence of Chk2, we find that DNA damage sufficient to fragment centromere DNA does not delay mitotic exit. We conclude that DNA damage signaling through Chk2 triggers Mad2-dependent delays in mitotic progression, both before or after the metaphase-anaphase transition.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER II

LIST OF FIGURES

CHAPTER I

CHAPTER II

APPENDIX 1

APPENDIX II

APPENDIX III

CHAPTER I

INTRODUCTION

Cell division must be tightly regulated to protect genomic integrity. In response to DNA damage, conventional cell cycle checkpoints prevent cell cycle progression into S phase or mitosis, allowing time for repair or elimination by apoptosis. Defects of these checkpoints can result in developmental lethality or cancer progression. In organisms from yeast to mammals, mitotic delays or death are frequently observed in G2-checkpoint-deficient cells following DNA damage. This process, known as mitotic catastrophe, is poorly understood. My thesis focuses on the mechanism of DNA damage-induced mitotic delays that are often associated with mitotic catastrophe. I will begin by reviewing cell cycle regulation in normal conditions and following DNA damage, and then describe my thesis research in understanding mitotic exit delays in response to DNA damage, using the syncytial *Drosophila* embryo as a model.

Mitosis

After DNA replication and a pause in G2, cells enter into mitosis to generate two identical daughter cells. Mitosis begins with nuclear envelope break down and chromosomes condensation. Bi-polar mitotic spindles then form, and chromosomes align at the metaphase plate. Spindle elongation and chromosomes separation mark the metaphase-anaphase transition (M-A transition). Finally, chromosomes decondense and the nuclear envelope forms during telophase in a process known as mitotic exit. Mitotic exit is followed by cytokinesis, which cleaves the cytoplasm to generate two cells. The Cdk1-cyclin B complex controls mitotic entry and exit (Pines, 1995). A number of mitotic regulators are essential for the dynamics of mitotic spindle assembly, chromosome movement, and progression through mitosis. The opposing activities of the spindle assembly checkpoint (SAC) and anaphase promoting complex/cyclosome (APC/C) at metaphase-anaphase transition (M-A transition) play a critical role in accurate chromosome segregation (Musacchio and Salmon, 2007).

Regulation of Cdk1/Cyclin B at entry into and exit from mitosis.

Cdk1 is the major player in mitotic progression, since Cdk1 activation is required for mitotic entry, while Cdk1 inactivation is necessary for mitotic exit (Pines, 1995). The regulation of Cyclin B level is a central event controlling Cdk1 activity. Cyclin B is synthesized in S phase and its levels increase during G2, and Cyclin B binding is required for Cdk1 activity (Pines and Hunter, 1989). After Cyclin

B binding to Cdk1, the activity of Cdk1/Cylin B complex is regulated by a number of Cdk1 regulators including Wee1 kinase and Cdc25 phosphatase (Lew and Kornbluth, 1996). Wee1 phosphorylates Cdk1 to inhibit the Cdk1-CyclinB complex and delay mitosis (Russell and Nurse, 1987). In contrast to Wee1 function, Cdc25 phosphatase activates Cdk1/Cyclin B complex by dephosphorylating Cdk1, which triggers entry into mitosis (Lundgren et al., 1991). When chromosomes are aligned at the metaphase plate, Cyclin B destruction starts to inactivate Cdk1 for mitotic exit (King et al., 1995).

In addition to temporal regulation, Cyclin B is also appears to be spatially regulated during mitosis. Initially, Cyclin B localizes in the cytoplasm during S and G2 phases, concentrates on spindles at prometaphase, and disappears after chromosomes alignment before anaphase initiation (Clute and Pines, 1999). Destruction of Cyclin B on the spindle and in the cytoplasm have been proposed to be differentially regulated (Huang and Raff, 1999).

Regulation of the metaphase to anaphase transition

Mitosis evenly distributes duplicated chromosomes to two daughter cells, and chromosome separation is tightly regulated by opposing activities of the spindle assembly checkpoint (SAC) and anaphase promoting complex (APC/C) (Musacchio and Salmon, 2007).

APC/C (anaphase promoting complex/cyclosome)

The APC/C is an E3 ubiquitin ligase that promotes securin and Cyclin B degradation by the 26S proteosome on mitotic exit (King et al., 1995; Shirayama et al., 1999; Yu et al., 1996). APC/C is activated by interaction with Cdc20 (Lim et al., 1998), which leads to Cyclin B and securin ubiquitination and destruction (Morgan, 1999). Securin inhibits activation of separase, a protease that cleaves the cohesin complex, which holds sister chromatids together. Cohesin cleavage is therefore required for anaphase initiation (Uhlmann et al., 1999). Cyclin B degradation is required to inactivate Cdk1, leading to exit from mitosis (King et al., 1996). Non-degradable Cyclin B expression leads to arrest at mid-anaphase, indicating that Cyclin B degradation is not required for chromosome segregation (Holloway et al., 1993; Su et al., 1998; Wolf et al., 2006)

Spindle Assembly Checkpoint (SAC)

The SAC monitors attachment and tension between kinetochores and microtubules. To ensure accurate chromosome separation, the active SAC negatively regulates Cdc20 to prevent APC/C activation and the metaphase-anaphase transition (M-A transition). The SAC is inactivated when all chromosomes have become bi-oriented on the metaphase plate, allowing APC/C activation, securin and Cyclin B destruction, and mitotic exit (Musacchio and Salmon, 2007) (Figure1).

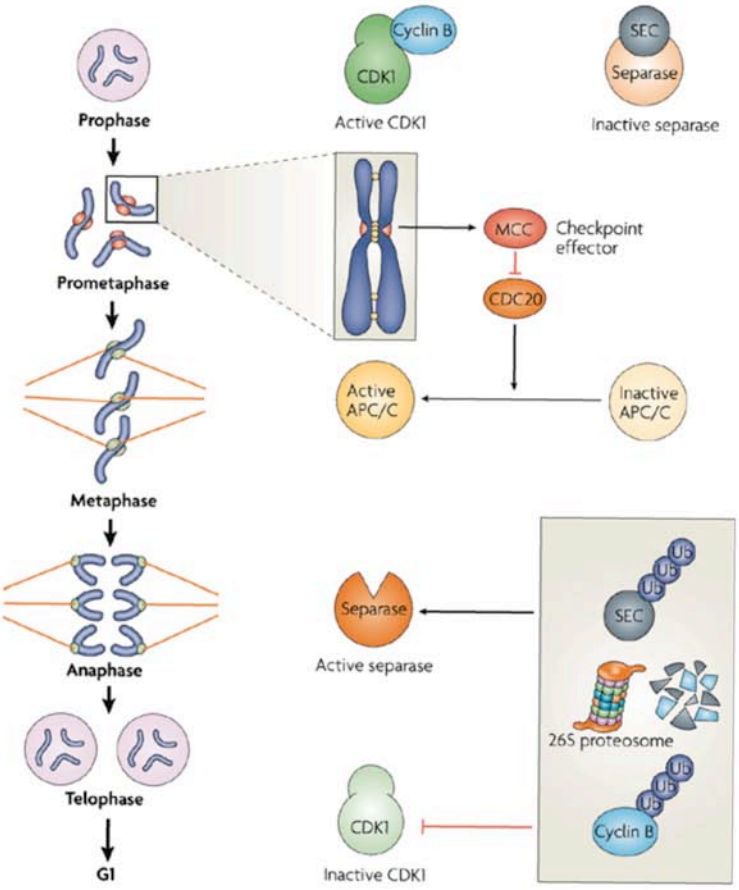

Figure 1. Regulation of the metaphase to anaphase transition. The SAC inhibits APC/C activity until all chromosomes attach to microtubules from opposite spindle poles. SAC inactivation leads to ubiquitination of Cyclin B and securin by active APC/C. 26S proteosome destroys ubiquitinated-Cylin B and -securin resulting in Cdk1 inactivation and chromosome separation, respectively, resulting in mitotic exit.
Reproduced from Musacchio and Salmon, Nature reviews, 2007

The Kinetochore.

During prometaphase, all SAC components concentrate at kinetochores, and kinetochore localization disappears following chromosome alignment, before anaphase initiation (Elledge and Harper, 1994; Howell et al., 2004). The kinetochores play a critical role for SAC activity (Shah et al., 2004). Kinetochores consist of centromeric, inner kinetochore, and outer kinetochore regions. Cohesin holds sister-chromatids together in centromeric DNA regions (Cleveland et al., 2003). It is unclear if centromeric DNA sequences are important for SAC activity. In yeast, specific centromeric sequences are important for kinetochore function (Meluh and Koshland, 1997; Westermann et al., 2003). In other organism, however, kinetochore assembly appears to be regulated by epigenetic factors such as CENP-A chromatin, rather than a specific DNA sequence (Smith, 2002; Van Hooser et al., 2001). CENP-A, a Histone H3-like protein is found in centromere nucleosomes and appears to specify kinetochores and recruit other kinetochore proteins (Van Hooser et al., 2001). The inner kinetochore also includes microtubule binding proteins, including CENP-E (Mao et al., 2003). CENP-E appears to sense unattached kinetochores and activate BubR1 for chromosome bi-orientation (McEwen et al., 2001). The Outer kinetochore contains many microtubule binding proteins, microtubule motor proteins, and SAC components (Weaver et al., 2003). The kinetochore is a protein complex that regulates cohesion of sister chromatids, microtubule dynamics, chromosome movements, and SAC activity during mitosis.

SAC activity at kinetochores

Most proteins in the SAC component complex (SCC) are conserved and share sequence and functional similarity, including Bub1, Bub3, Mad2, BubR1, MPS1, Zw10, and Rod (Roughdeal) (Musacchio and Salmon, 2007). As mentioned above, SCC components concentrate at kinetochores before the M-A transition. Mad2 among them directly binds Cdc20 to inhibit APC/C activity (Chan et al., 2000; Hoyt, 2001). Zw10, Mps1, Bub1, and BubR1 are required for Mad2 recruitment to kinetochores (Buffin et al., 2005; Chen, 2002; Sharp-Baker and Chen, 2001). Depletion of any one of these proteins inhibits Mad2 localization at kinetochores, disrupting SAC function and leading to premature chromosome segregation, lagging chromosomes at anaphase, and aneuploidy (Buffin et al., 2005; Chen, 2002; Sharp-Baker and Chen, 2001; Zhao and Chen, 2006). By contrast, Mad2 depletion does not affect BubR1 and Zw10 localization, although it abolishes SAC activity, suggesting that Mad2 plays a critical role in transmitting SAC activity (Buffin et al., 2005).

Mitotic timing is regulated by mad2 and BubR1

In addition to SAC function at unattached kinetochores, recent biochemical analyses suggest that active SAC components are present in the cytosol, and this cytosolic SAC may control mitotic progression in a kinetochore-independent manner (Sudakin et al., 2001). Recently, cytosolic SAC function has been analyzed in mammals (Meraldi et al., 2004). These studies show that complete disruption of kinetochores inhibits SAC-dependent mitotic delays, but does not affect on mitotic

timing. By contrast, they observed that Mad2 and BubR1 depletion triggers both decreased mitotic timing and failure of SAC activation. Moreover, Mad2 depletion combined with disruption of kinetochores prevented spindle checkpoint activation and decreased mitotic timing, suggesting that cytoplasmic Mad2 and BubR1 inhibit APC/C activation in the absence of functional kinetochores.

Spindle damage response.

Spindle disruption or stabilization activates the SAC to prevent securin and Cyclin B degradation, resulting in metaphase arrest before chromosomes segregation. In addition to kinetochore attachment, proper tension is critical to SAC inactivation. Kinetochore tension is generated by chromosome bi-orientation. Taxol, a microtubule-stabilizing drug that generates attached kinetochores without tension, activates the SAC. Taxol treatment leads to kinetochore localization of BubR1, but not Mad2, suggesting SAC protein localization and activation are differentially regulated by attachment and tension (Shannon et al., 2002). However, depletion of either Mad2 or BubR1 leads to SAC inactivation and mitotic progression in the presence of Taxol. Both Mad2 and BubR1 are therefore required for SAC activation in response to tension or attachment defects (Waters et al., 1998).

DNA damage response

DNA damage checkpoint

To maintain genomic integrity, DNA damage checkpoints monitor DNA lesions and prevent cell cycle progression. The cell cycle arrests at G1, S, or G2 phase, depending on the phase in which the DNA damage is sensed (Lobrich and Jeggo, 2007). ATM/ATR-Chk2/Chk1 signaling pathways prevent Cdk activation through inhibition of Cdc25 phosphatase (Figure 2). A number of proteins are required for the DNA damage checkpoint, including ATM, ATR, Chk1, Chk2, the MRN complex, p53, 53BP1, BRCA1 and Cdc25. Here, I will describe the regulation and function of the DNA damage sensors ATM/ATR, and the signal transducers Chk1/Chk2, which phosphorylate and activate effectors during the DNA damage checkpoint response.

Checkpoint components

Ataxia Telangiectasia Mutated (ATM)

Mutations in the human ATM gene lead to Ataxia Telangiectasia (AT), which is characterized by genome instability, neurodegeneration, and predisposition to cancer (Abraham, 2001). $ATM^{-/-}$ mutant mice are viable, indicating that ATM is not essential for normal cell cycle progression or development (Shiloh, 1997). However, ATM is essential for DNA damage signaling.

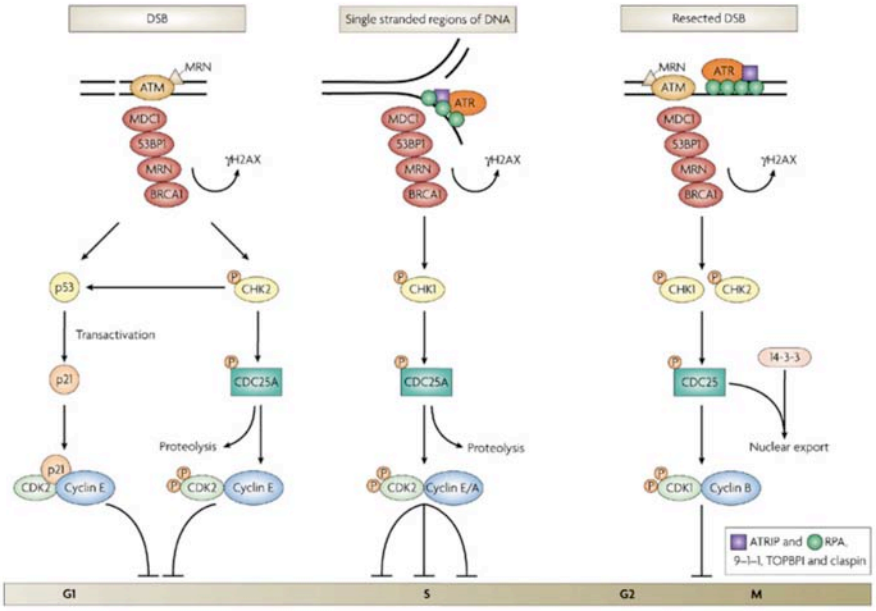

Figure 2. Overview of the DNA damage checkpoint for cell cycle arrest.
DNA damage triggers ATM/ATR activation to phosphorylate the signal transducers, Chk1 and Chk2. Active Chk1/Chk2 inhibits Cdc25 phosphatase to block cell cycle progression at G1, S or G2/M. *Reproduced from Lobrich and Jeggo, Nature reviews, 2007*

ATM activity is regulated by two distinct events – autophosphorylation and localization to DNA break sites. In normal cells, ATM forms an inactive homodimer. This homodimer dissociates upon DNA damage, which triggers autophosphorylation (Bakkenist and Kastan, 2003). The other event is recruitment of ATM at DSBs sites by the MRN complex (Carson et al., 2003; Uziel et al., 2003). The highly conserved MRN complex consists of the proteins Mre11, Rad50 and Nbs1 (Jackson, 2002; Tauchi et al., 2002). The MRN complex is involved in the initial processing of DSBs by interacting with phosphorylated H2AX (Kobayashi et al., 2002). Activated ATM then phosphorylates serine and threonine sites in the SQ/TQ motif of many substrates, including BRCA1, NBS1, Chk2, and p53, leading to G1, S and G2 arrest or apoptosis (Gatei et al., 2000; Kang et al., 2005; Matsuoka et al., 1998; Matsuoka et al., 2000).

ATR

ATR, like ATM, appears to function as a signal transducer in DNA damage signaling. ATR deficiency in mice results in early embryonic death, indicating ATR is essential for viability (Brown and Baltimore, 2000). Unlike ATM, ATR kinase appears to constitutively phosphorylate its substrates, suggesting that ATR may be regulated through changes in subcellular localization in response to DNA damage (Ball and Cortez, 2005). As with ATM, ATR phosphorylates serine or threonine residues in SQ/TQ sequences (Abraham, 2001).

Chk2

Chk2 is a kinase that is conserved from yeast to mammals. Rad53, the Chk2 homolog in budding yeast, was found as a kinase involved in the DNA damage response (Elledge and Harper, 1994). Chk2 contains SQ/TQ, Forkhead-associated (FHA), and C-terminal kinase domains. The SQ/TQ domain in the N-terminus of Chk2 is the site for phosphorylation by ATM/ATR kinases. In human cells, DNA damage leads to Chk2 activation through ATM phosphorylation of threonine residue at 68. (Melchionna et al., 2000). The FHA domain is required for homo-oligomerization of p68T-Chk2, which appears to trigger autophosphorylation and full activation (Ahn et al., 2002). The kinase domain occupies the C-terminal region.

Like ATM, Chk2 is dispensable for viability in mice, indicating that it is not essential for normal development (Takai et al., 2002). However, Chk2 is involved in cell cycle arrest and p53-dependent apoptosis in response to DNA damage (Takai et al., 2002). Activated Chk2 also phosphorylates Cdc25A to arrest the cell cycle at G1(Bartek et al., 2001). Chk2 function in the G2-M checkpoint, however, depends on cell types and the nature of the DNA damage. Cells derived from Chk2 knockout mice arrest in G2-M following DNA damage, suggesting that Chk2 is not necessary for the G2-M checkpoint (Takai et al., 2002). Elledge and coworkers showed that active Chk2 phosphorylates the serine-216 residue of Cdc25C, and phosphorylation of Cdc25 on S216 is known to block the G2-M transition (Matsuoka et al., 1998). Recently, it was also reported that ATM and ATR induce Chk2-mediated G2-M arrest in response to irradiation (Li et al., 2008; Park and Avraham, 2006).

Chk1

Chk1 was first identified in yeast as essential for cell cycle arrest before mitosis in response to DNA damage in a Rad3 (ATR)-dependent manner (Walworth et al., 1993; Walworth and Bernards, 1996). Chk1 deficient mice die during early embryogenesis, with abnormal nuclei. Similar lethality and nuclear abnormalities are found in ATR deficient mice (Takai et al., 2000), suggesting that ATR and Chk1 function in the same pathway. Cells from Chk1 deficient mice do not arrest the cell cycle following DNA damage or incomplete DNA replication, indicating Chk1 is required for DNA damage and replication checkpoint control (Takai et al., 2000). Active Chk1 phosphorylates a number of substrates to induce cell cycle arrest (Capasso et al., 2002).

Functional redundancy in checkpoint components

It was generally thought that ATM and ATR function independently and regulate two distinct pathways via the Chk2 and Chk1 effectors, respectively (Niida and Nakanishi, 2006). However, a recent report shows that ATM activates ATR by recruiting ATR at DNA damaged sites, suggesting ATR is activated in an ATM-dependent manner (Jazayeri et al., 2006). Moreover, ATM and ATR overlap in their phosphorylation of Chk2 and Chk1 as well as other substrates such as BRCA1 and the MRN complex, in contrast to earlier proposals (Gatei et al., 2000; Goodarzi et al., 2003; Sapkota et al., 2002; Shiloh, 1997). Therefore Chk1 and Chk2 appear to be

functionally redundant and regulated by both ATM and ATR in response to DNA damage. It appears that dominant function of a kinase effector (Chk1 or Chk2) may depend on cell types or organism.

Mitotic response to DNA damage

Mitotic delays and death in response to DNA damage have been observed in organisms from yeast to mammals. The mechanism is best understood in budding yeast, which delays in a metaphase state in response to DNA damage. Recently, however, several studies have explored the mechanism of damage induced mitotic delay in fission yeast, *Drosophila*, and mammalian cells.

Mitotic response to DNA damage in yeast

In budding yeast, DNA damage leads to a block in cell cycle progression at the metaphase-anaphase transition (M-A transition), through stabilization of Pds1, the securin homolog (Sanchez et al., 1999; Wang et al., 2001). The ATR homolog, Mec1, plays a critical role in DNA damage response in budding yeast (Sanchez et al., 1999; Wang et al., 2001). In the presence of DNA damage, Mec1 phosphorylates Chk1 and Chk2 to inhibit chromosome separation. Chk1 phosphorylates Pds1 to prevent APC/C recognition of Pds1, and thus stabilize Pds1 (Agarwal et al., 2003). Rad53/Chk2 prevents Pds1-Cdc20 interactions to prevent Pds1 ubiquitination (Agarwal et al., 2003). Chk1 and Chk2 thus block mitotic progression through parallel DNA damage response pathways, and both pathways are required for complete mitotic arrest. In

addition, a recent study showed that PKA (cyclin-AMP dependent protein kinase) pathway also participates DNA damage response, in parallel with Chk1 and Rad53 (Searle et al., 2004). PKA controls Cdc20 phosphorylation to prevent mitotic progression in response to DNA damage. Taken together, three distinct signaling pathways control mitotic arrest following DNA damage to prevent genomic instability in budding yeast.

Mitotic response to DNA damage in mammals

In mammals, the combination of G2/M checkpoint deficiency and DNA damage leads to mitotic exit delays, cell death during mitosis, or division failure (Castedo et al., 2004a; Roninson et al., 2001). The diversity of mitotic damage responses could reflect the genetic variability of cultured mammalian cells, or distinct responses to different levels of DNA damage.

In mammals, DNA damage-induced metaphase delays appear to be regulated by the SAC, as found in fission yeast (Mikhailov et al., 2002; Nitta M, 2004). Mikhailov et al. suggested that damage disrupts the kinetochore, indirectly triggering the SAC and Mad2-dependent mitotic delay, and that DNA damage signaling is not required for these delays (Mikhailov et al., 2002).

In contrast, Huang et al. reported that Chk1 and BRCA1-DNA damage checkpoint components are required for mitotic exit delay in response to DNA damage. They proposed that Chk1 and BRCA1 inhibit Cdh, an APC/C activator (Huang et al., 2005).

Another study has suggested that Chk2 is a negative regulator of mitotic catastrophe, which was induced by fusion of S-phase and M-phase HeLa cells (Castedo et al., 2004b). The physiological significance of division failure following fusion is unclear, and the mechanism of DNA damage associated mitotic delays in mammals remains poorly understood.

Cell cycle control during embryonic development in *Drosophila melanogaster*

During early *Drosophila* embryogenesis, the first 13 nuclear divisions are synchronous and occur in a common cytoplasm, or syncytium (Figure 3). These syncytial divisions are extremely rapid and consist of DNA synthesis (S phase) and mitotic phases (M phase) without gap phases (G1 and G2) (Foe and Alberts, 1983). The initial nuclear divisions occur in the interior of the embryos, and then the majority of nuclei migrate to the embryo cortex during cycle 9 and 10. Nuclei divide during the syncytial blastoderm stage (cycle 11-13) at the cortex. At this stage, the length of interphase gradually increases from 5 to 15 minutes. It has been proposed that maternally derived factors, such as the DNA replication machinery, are titrated by the increasing number of nuclei in the embryo to increase interphase length (Edgar et al., 1986; Sibon et al., 1997). Following mitosis 13, cellularization incorporates the cortical nuclei into cells. This process requires zygotic transcription, and marks the

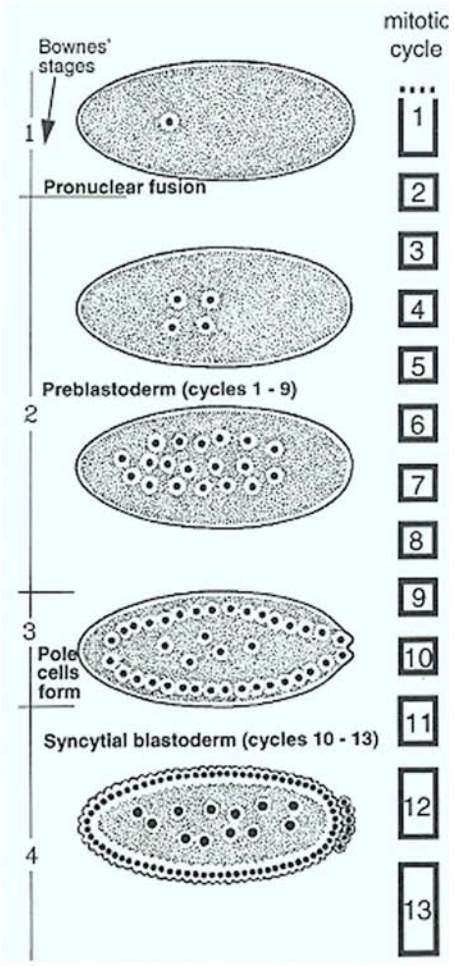

Figure 3. Syncytial *Drosophila* embryogenesis.
The first 13 divisions are synchronous and occur in a common cytoplasm. Initial nuclear divisions occur in the interior of the embryos. During cell cycle 9 and 10, nuclei move to the cortex. In syncytial blastoderm stages (cycle 11-13), nuclear divisions occur at the cortex. *Reproduced from Bate and Martinez Arias, Cold Spring Harbor Laboraory Press, 1993*

midblastula transition. After cellularization, the cell cycle becomes asynchronous as a variable G2 phase is introduced (Edgar and O'Farrell, 1990; Foe, 1989; Vassin et al., 1985).

Regulation of mitotic exit during *Drosophila* embryogenesis

The Spindle Assembly Checkpoint.

The SAC controls APC/C activity during mitotic progression in syncytial embryos, as if does in other organisms. Homologues of most components of the spindle checkpoint complex (SCC) have been identified in *Drosophila*, including BubR1, Zw10, Rod, Mad2, and Cdc20. Dynamic localization of Zw10, Rod, BubR1, and Mad2 have been examined with GFP fusions in neuroblast and/or syncytial embryos (Basto et al., 2004; Buffin et al., 2005). All these proteins concentrate at kinetochores until the M-A transition, and disappear after chromosomes have aligned at metaphase plate, consistent with *in vivo* analyses in mammals (Howell et al., 2004). Like other organisms, spindle damage (by microtubule depolymerizing drugs or stabilizing drugs) triggers SAC-dependent metaphase arrest in *Drosophila* embryos, indicating that the SAC monitors attachment and tension between microtubules and kinetochores to ensure proper chromosome segregation (Orr et al., 2007). The role of the SAC in *Drosophila* development and cell cycle progression has been tested through analysis of mutations in genes encoding the SCC components *zw10*, *rod* and *bubR1* (Basto et al., 2000; Perez-Mongiovi et al., 2005; Williams et al., 1992).

Previous studies showed that the SCC is essential for the viability in most organisms except in yeast (Dobles et al., 2000; Kitagawa and Rose, 1999). The mutations in *zw10*, *rod*, and *bubR1* also cause chromosomes segregation failure, aneuploidy, and lethality in *Drosophila* (Basto et al., 2000; Perez-Mongiovi et al., 2005; Williams et al., 1992). These findings suggest that the SAC functions in normal mitosis, as well as in response to spindle damage. However, Mad2 is not essential for viability, but is required to arrest the cell cycle in prometaphase in response microtubule depolymerization (Buffin et al., 2007). This observation suggests that Mad2 has a single function in controlling the APC/C activation by interacting with Cdc20, while other SCC components, including Zw10, Rod, BubR1, have function outside the SAC that are essential for viability. For example, Zw10 is required for Mad2 and dynein recruitment to kinetochores, and *zw10* mutations disrupt spindle organization (Starr et al., 1998).

Spatial and temporal regulation of Cyclins in syncytial embryos.

During the syncytial divisions, which have a simple S-M phase division cycle, Cyclin B levels remain high, although Cyclin B degradation is required for mitotic exit. Biochemical studies indicate that Cyclin B is not completely destroyed at the end of each syncytial mitosis (Edgar et al., 1994). However, Cyclin B degradation appears to be spatially regulated, and centrosomes and spindle associated Cyclin B may be more efficiently destroyed than cytoplasmic Cyclin B during the syncytial divisions (Huang and Raff, 1999). As soon as the nuclear envelope breaks

down (NEB), Cyclin B accumulates on centrosomes and spindles. Spindle-associated Cyclin B starts to disappear from centrosomes at late-metaphase, and is completely gone from spindles at anaphase (Huang and Raff, 1999).

In addition to Cyclin B, Cyclin A and Cyclin B3 also contribute to mitotic progression during early embryogenesis. It has been shown that high levels of Cyclin A, B and B3 block the mitosis at metaphase, early anaphase, and late anaphase/telophase, respectively, when experimentally manipulated by injecting or overexpressing stable Cyclins (Parry and O'Farrell, 2001; Sigrist et al., 1995; Su et al., 1998). This suggests that sequential destruction of Cyclins may control mitotic progression.

DNA damage response in the Drosophila embryos

During *Drosophila* embryogenesis, the checkpoint machinery induces cell cycle arrest in response to unreplicated and/or damaged DNA at different phases, depending on developmental stage. Checkpoint genes that are conserved from yeast to humans have also been identified in *Drosophila*. Mutations in *mei-41* (ATR), *grp* (Chk1), *atm*, *mnk* (Chk2), and *p53* have been characterized and found to disrupt DNA damage checkpoint control, repair, or apoptosis as described below.

Checkpoint components in Drosophila

mei-41 and grp

The *mei-41* and *grp* genes encode ATR and Chk1, respectively, and mutations in *mei-41* and *grp* lead to similar phenotypes. The mutations block the increases the interphase length that normally accompany the later syncytial divisions (cycle 11-13), resulting in mitotic entry with incomplete DNA replication. All embryos derived from *mei-41* or *grp* mutant females fail to cellularize (Sibon et al., 2000; Sibon et al., 1999; Sibon et al., 1997; Takada et al., 2007). Recently, Takada presented evidence indicating that accumulation of DNA damage, presumably caused by checkpoint failure, leads to the observed cellularization failure (Takada et al., 2007). In neuroblast, mutants for *mei-41* or *grp* are defective in checkpoint control (Brodsky et al., 2000; Jaklevic and Su, 2004)

ATM

Recently, the role of ATM has been addressed in *Drosophila*. A null mutation in the *atm* gene causes larval lethality (Silva et al., 2004), and *Drosophila* ATM appears to be upstream of Chk2-p53 apoptosis signaling (Song et al., 2004). ATM also appears to function in telomere maintenance, since *atm* mutations lead to telomeric fusions and loss of HP1 localization to telomere ends (Oikemus et al., 2004).

Mnk

DmMnk, the *Drosophila* Chk2 homologue, was originally identified as a nuclear protein kinase enriched in ovaries (Oishi et al, 1998). *mnk* null mutants are viable and fertile, indicating that Chk2 is not essential for normal development, consistent with observations in mouse (Takai et al., 2002). However, *mnk* mutants disrupt the p53 dependent damage response in somatic cells (Brodsky et al., 2004; Peters et al., 2002). p53 is required for DNA damage-induced apoptosis in *Drosophila*. (Ollmann et al., 2000). Chk2 phosphorylates and activates p53 to trigger apoptosis in response to DNA damage (Brodsky et al., 2004; Peters et al., 2002). Chk2 appears to be the primary regulator of p53-dependent apoptosis in *Drosophila*. However, Chk2 function in cell cycle control is controversial. Xu et al. suggested *mnk* mutants show an increased mitotic index following DNA damage, consistent with a function in G2-M phase cell cycle control (Xu et al., 2001). By contrast, Chk2 knock-down in S2 cells did not compromise damage induced cell cycle arrest (de Vries et al., 2005).

Cell cycle checkpoint control of the syncytial cycles

The syncytial divisions consist of S-M cycles, and inhibition of DNA replication delays progression into M phase, but does not completely block mitotic entry (Sibon et al., 1997). Entering mitosis with incomplete DNA leads to severe mitotic defects (Sibon et al., 2000; Sibon et al., 1999) By contrast, DNA breaks do not activate checkpoint dependent delays in progression into mitosis (Takada et al.,

2003). As described below, DNA damage triggers severe defects in spindle assembly and function.

Mitotic catastrophe

In the syncytial embryos, DNA damage agents and replication checkpoint mutations (*grp* and *mei-41*), lead to a dramatic loss of centrosome function and defects in spindle assembly during mitosis (Takada et al., 2003). These defects are linked to loss of γ-tubulin, γ-TuRC components, CP190 and Dgrip84 from centrosomes. The spindles are barrel-shaped and anastral, and anaphase chromosome segregation fails. These mitotic defects are dramatically suppressed in embryos derived from female homozygous for a null mutation in *mnk*, indicating Chk2 is required for this mitotic response to DNA damage. Moreover, *mnk* mutations suppress "nuclear dropout," a process that shunts damaged nuclei to the interior of the embryo following division failure. Chk2 activation may therefore disrupt the link between centrosomes, which appear to anchor nuclei at the cortex, and damaged nuclei. (Figure 4)

During post cellularization cycles 14-16, irradiation triggers delays in mitotic entry followed by mitotic arrest (Garner et al., 2001). In *dup* (double parked) mutants, which disrupt DNA replication, the cell cycle arrests in mitosis 16, following no delay in mitotic entry, and this arrest requires Bub1. In larval neuroblasts, DNA damage also delays progression into mitosis and leads to delays in the M-A transition (Royou et al., 2005). These delays appear to required both BubR1 and Chk1.

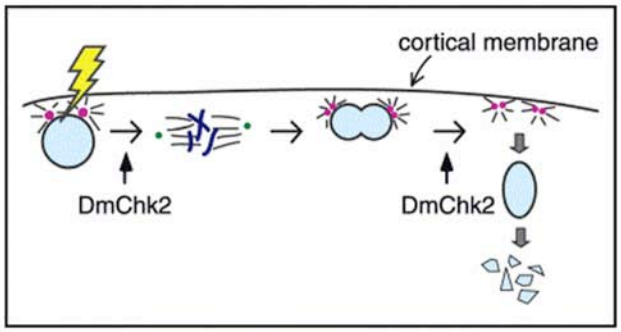

Figure 4. Mitotic response to DNA damage in early *Drosophila* embryos. DNA damage triggers centrosome inactivation in a Chk2-dependent manner during mitosis. Following division failure, damaged nuclei drop into the embryo interior at interphase in a Chk2-dependent manner. *Reproduced from Takada S and Theurkauf W, Cell, 2003*

Accordingly, Chk1 and BubR1 have been proposed to function in parallel to delay in mitosis, such that non-kinetochore DNA breaks induce Chk1-mediated mitotic delays, while kinetochore breaks triggers BubR1-induced mitotic delay (Royou et al., 2005).

These findings suggest that the SAC has a conserved function in delaying mitosis following DNA damage. However, the molecular events leading to SAC activation in response to DNA damage are poorly understood. To gain insight into the cellular and molecular mechanisms controlling the mitotic response to DNA damage, I therefore examined this process in syncytial embryos, which support a powerful combination of genetic approaches and direct *in vivo* imaging of mitotic progression. My studies indicate that DNA damage can trigger arrest at both prometaphase and anaphase, through a process that requires both the DNA damage signaling kinase Chk2 and the SAC component Mad2. These studies also show that anaphase arrest is linked to Cyclin B stabilization and rapid relocalization of SAC components to kinetochores, and the damage to centromere DNA is not sufficient to activate the SAC.

CHAPTER II

DNA DAMAGE TRIGGERS CHK2- AND MAD2-DEPENDENT ANAPHASE ARREST IN EARLY *DROSOPHILA* EMBRYOS

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Author Contributions Kwak S. conducted the experiments, analyzed the data and wrote the paper. Kelkar A. injected DNA damaging agents and replication inhibitors into embryos (Figure 1) and performed live analysis with *grp* and *mei-41* embryos (Figure 5). Takada S. generated Chk2 expressing transgenic flies. Koppetsch B. analyzed Chk2 localization (Figure 14 II). Theurkauf W. directed the project, interpreted the data, and edited the paper.

INTRODUCTION

DNA damage checkpoints monitor DNA integrity and delay cell cycle progression until lesions are repaired. Failure of the G2-M checkpoint and progression into mitosis with DNA lesions leads to mitotic exit delays, which are often associated with cell death during mitosis or division failure through a poorly defined process known as mitotic catastrophe (Castedo et al., 2004a; Chan et al., 1999; Roninson et al., 2001). DNA damage-induced mitotic delays are observed in a wide range of systems, but details of the molecular mechanism are only known in budding yeast, where activation of protein kinase A, Chk1 and Chk2 appear to inhibit destruction of Pds1 in distinct pathways (Agarwal et al., 2003; Searle et al., 2004). In higher eukaryotic systems, DNA damage-induced mitotic exit delays require components of the spindle assembly checkpoint (SAC), which is active and delays metaphase-anaphase transition (M-A transition) until all kinetochores are aligned and properly oriented at the metaphase plate (Collura et al., 2005; Mikhailov et al., 2002; Nitta M, 2004; Royou et al., 2005). However, it is unclear if DNA damage directly activates the SAC, or indirectly triggers this checkpoint by fragmenting centromere DNA and disrupting kinetochore structure.

Synthesis of the Cdk1 activator Cyclin B is essential for entry into mitosis, and Cyclin B destruction and Cdk1 inactivation leads to exit from mitosis (King et al., 1995). During mitotic exit, Cyclin B is destroyed through ubiquitin-dependent proteolysis, which is controlled by the anaphase promoting complex/cyclosome

(APC/C) that is activated by Cdc20 (Yu, 2002). To ensure the fidelity of chromosome segregation during mitosis, the SAC sequesters Cdc20 and prevents activation of APC/C until all chromosomes are aligned at the metaphase plate. Chromosome alignment leads to SAC inactivation, and Cdc20 release then activates the APC and triggers M-A transition (Shirayama et al., 1999).

The spindle assembly checkpoint requires a number of proteins, termed the spindle checkpoint complex (SCC), which includes Mps1, BubR1, Zw10, and Mad2. Mad2 directly interacts with and inhibits Cdc20, preventing APC/C activation (Chan et al., 2000; Hoyt, 2001). All of the SCC components, along with Cdc20, localize to unaligned kinetochores, and all of these proteins dissociate from kinetochores following bipolar chromosome attachment and alignment. Kinetochore localization of SCC proteins and Cdc20 is therefore linked to activation, and is used to assay for SAC activity in the cell (Musacchio and Salmon, 2007).

A number of studies have shown that the SCC proteins BubR1 and Mad2 are required for damage-induced mitotic delays (Collura et al., 2005; Fang et al., 2006; Mikhailov et al., 2002; Nitta M, 2004). Rieder and colleagues, working on mammalian cells, have proposed that only extensive damage triggers mitotic exit delays, and that this extent of damage physically disrupts kinetochores, leading to SAC activation through a process that is independent of damage signaling (Mikhailov et al., 2002). Other studies suggest that DNA damage activates a signaling pathway that delays mitotic exit (Collura et al., 2005; Garner et al., 2001; Royou et al., 2005). Chk1 has been implicated in DNA damage-induced mitotic delays in fission yeast, Drosophila

neuroblasts, and mammalian cells (Collura et al., 2005; Garner et al., 2001; Huang et al., 2005; Royou et al., 2005). By contrast, Castedo et al. proposed that Chk2 is negative regulator of mitotic catastrophe in fusion of HeLa cells, following fusion of mitotic and interphase cells (Castedo et al., 2004b). In budding yeast, both Chk1 and Chk2 appear to be required for damage induced metaphase delays (Agarwal et al., 2003). Agarwal suggested that Chk1 and Chk2 function in distinct pathways that control DNA damage-induced mitotic arrest. Chk1 appears to block mitotic exit by phosphorylating Pds1 (securin), which is proposed to block ubiquitination. Chk2, by contrast, blocks Pds1 destruction by preventing interactions with Cdc20. In higher eukaryotes, however, the role of Chk1 and Chk2 in damage signaling during mitosis is still the subject of debate.

To define the molecular, genetic, and cellular mechanisms controlling mitotic progression following DNA damage, we have focused on syncytial *Drosophila* embryos which have a simplified S-M cell cycle, support high temporal resolution time-lapse imaging, and allow genetic characterization of this damage response. Surprisingly, our time-lapse and immunocytochemical studies indicate that DNA damage triggers cell cycle arrest both before and after M-A transition. Significantly, we show that both Chk2 and Mad2 are required for mitotic arrest at prometaphase and anaphase, and that anaphase arrest is associated with stabilization of Cyclin B and relocalization of SCC components to kinetochores. We also show that DNA damage sufficient to fragment centromeric DNA triggers mitotic exit delays in wild type embryos, but not in embryos that lack Chk2, despite the presence of a functional

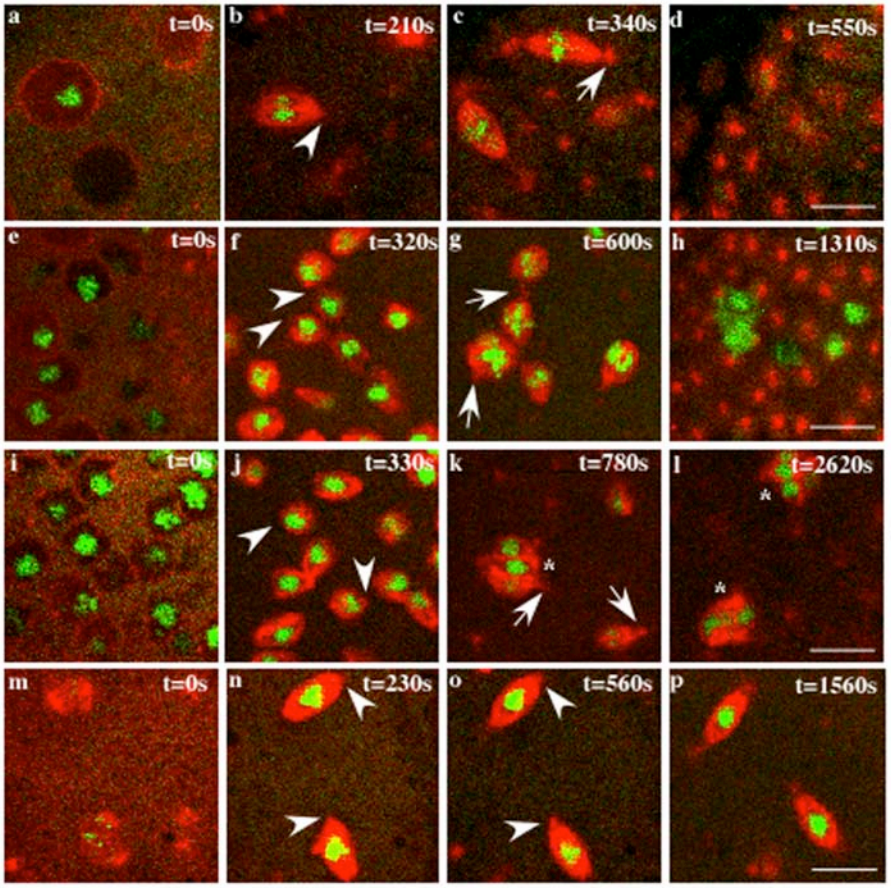
SAC. Furthermore, we show that a functional GFP-Chk2 fusion localizes on kinetochores through mitosis, suggesting that this kinase could act directly on SCC proteins. Taken together, these studies indicate that damage-induced delays in mitotic exit are not a simple consequence of kinetochore fragmentation, but result from DNA damage signaling through Chk2. Furthermore, these studies show that this signaling pathway can delay syncytial blastoderm cell cycle progression in both prometaphase and anaphase.

RESULTS

DNA damage triggers prometaphase and anaphase delays

To directly analyze progression through mitosis following DNA damage, we injected syncytial blastoderm stage *Drosophila* embryos with a mixture of the DNA dye Oligreen, rhodamine-tubulin (R-tubulin), and a DNA damaging agent. Immediately following injection, time-lapse laser-scanning confocal microscopy was used to monitor nuclear envelope breakdown (NEB), spindle assembly and chromosome alignment, sister chromosome separation and spindle elongation at the metaphase-anaphase transition (M-A transition), and mitotic exit as indicated by nuclear envelope formation (NEF) (see experimental procedures). The X-ray mimetic bleomycin and the topoisomerase inhibitors etoposide and camptothecin (CPT) were used to induce DNA double-strand breaks (DSB), and all three agents produce a similar spectrum of defects (Figure 1). CPT stably traps a DSB reaction intermediate and produced a consistent mitotic response, and was therefore chosen for the majority of our studies.

Syncytial blastoderm stage embryos do not delay progression into mitosis following DNA damage, but show a robust mitotic response to damage that includes "centrosome inactivation", assembly of anastral spindles, and chromosome segregation failures (Sibon et al., 2000; Takada et al., 2003). Time-lapse analysis demonstrated that these defects link to a delay in mitotic exit, and to arrest at both prometaphase and during anaphase, after sister chromosome separation and spindle



blm, 100µg/ml Aphidicolin

blm, 20s UV (at beginning of preceding S-phase)

blm, 10µM Etoposide

blm, 1µg/ml Bleomycin

Figure 1. DNA double-strand breaks lead to anaphase arrest. Replication inhibitor (aphidicolin; a-d) as well as DNA damaging agents (UV, etoposide and bleomycin; e-h, i-l and m-p, respectively) cause centrosome inactivation after NEB that persists through metaphase (open arrowheads; b,f,j and n) but centrosome activity resumes at the beginning of anaphase (open arrows; c, g, k and o). **(a-d)** The replication inhibitor aphidicolin does not induce anaphase arrest. The *blm* embryo injected with aphidicolin (100μg/ml), Oli-green and rhodamine-labeled tubulin was observed live with confocal microscope. Aphidicolin injection causes slight increase in the overall length of mitosis. **(e-h)** UV irradiation of *blm* embryos can induce anaphase arrest that may be followed by mitotic exit. The embryo was injected with a mixture of DAPI, Oli-green and rhodamine-labeled tubulin was analyzed *in vivo*. The embryo irradiated with UV for 20 sec exits from mitosis after prolonged anaphase (~12 min; about 3 times longer than the control *blm* embryos). **(i-l & m-p)** Injection of *blm* embryos with 10uM etoposide (i-l) or 1ug/ml bleomycin (m-p) induces arrest in anaphase. Induction of double strand breaks in the *blm* embryos with either etoposide or bleomycin injection leads to a delay in anaphase initiation followed by an arrest in anaphase.

elongation (Figure 2E). In 8 of 13 embryos injected with 100 μM CPT, nuclei delayed in a prometaphase configuration before exiting mitosis as indicated by NEF. Diffusion of CPT from the injection site produces a gradient of the DNA damaging agent in the syncytial embryo, allowing a rough correlation between the extent of DNA damage and the mitotic response. In 5 of 13 embryos injected with 100 μM CPT, we observed mitotic arrest near the injection site and mitotic delays followed by mitotic exit in more distant regions. By contrast, mitotic arrest was observed in only 1 of 9 embryos injected with 50 μM CPT, and mitotic delay shown in the remaining embryos (Table1). The Blooms helicase (Blm) has been implicated in double-strand break repair (Adams et al., 2003; Adams MD, 2003; Kusano et al., 1999; Kusano K, 1999), and mitotic arrest was observed in 100% of *blm* mutant embryos injected with either 50 μM or 100μM CPT (Table 1). These findings indicate that lower levels of damage trigger mitotic delays, while higher levels of damage induce mitotic arrest. When damage was followed by mitotic exit, cell cycle delays were primarily in prometaphase (Table 1). The total length of mitosis (NEB to NEF) averaged 4.1± 0.9 min in control embryos, compared to 7.7±2.2 min. in 100 μM CPT injected embryos. The M-A transition is marked by sister chromosome separation, an abrupt increase in astral microtubule length, and spindle elongation. In the absence of DNA damaging agents, the M-A transition was initiated 3.1± 0.5 min after NEB (Fig 2A-C, Table 1). Following 100 μM CPT injection, NEB was followed by a drop in centrosome-organized microtubule nucleation and assembly of spindles with reduced asters. When mitotic exit was observed, these reduced asters and short spindles

Table 1. DNA damage induces Chk2- and Mad2-mediated mitotic delays in syncytial *Drosophila* embryos

	control		50 □M camptothecin			100 □M camptothecin			250□M colchicine
	total embryo No.	NEB-NEF (M-A) min	total embryo No.	NEB-NEF (M-A) min	Total No. Arrested embryos (M /A/M-A)	total embryo No.	NEB-NEF (M-A) min	Total No. Arrested embryos (M /A/M-A)	mitotic arrest
w1118	10	4.12±0.9 (3.1±0.5)	9	7.8±1.6 (6.2±2.5)	1 (1/-/-)	13	7.7±2.2 (6.4±2.1)	5 (1/4/-)	yes
mnk	5	3.8±1.1 (3.2±0.7)	6	4.3±0.5 (2.9±0.29)	0	10	4.5±0.8 (3±0.6)	0	yes
blm	6	5.9±1 (6)	7	-	7 (2/3/2)	8	-	8 (3/2/3)	yes
mnk;blm	7	5.7± 0.9 (4±0.8)	N/D			10	7.03±1.5 (6.1±1.6)	0	N/D
mad2	4	4.4±0.8 (3.14±0.7)	N/D			10	6.4±1.1 (4.9±1.3)*	0	No
zw10	8	4 ~ 10 (8)	3	≥6	0	5	≥8	0	No

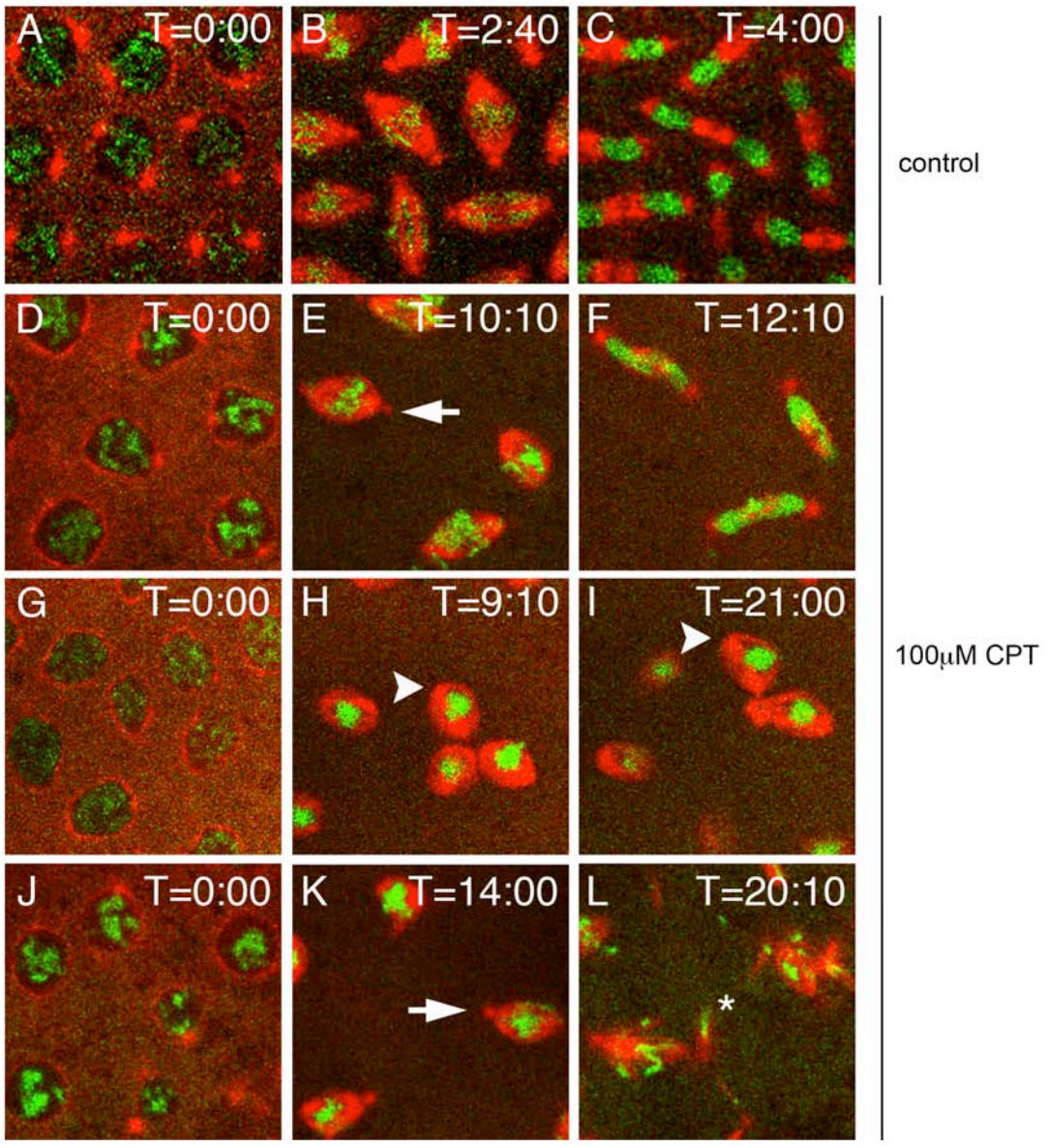
NEB-NEF : the duration of mitosis

M-A : metaphase-anaphase transition timing

Total No. Arrested embryos (M /A/M-A) ; Total number of embryos arrested following drug injection (metaphase arrest /anaphase arrest/unsynchronized)

N/D : not determined

* : 6/10 embryos determined for time of M-A transition. 4 embryos appear to completely fail chromosomes segregation.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B T=2:40
C T=4:00
control
D T=0:00
E T=10:10
F T=12:10
G T=0:00
H T=9:10
I T=21:00
100μM CPT
J T=0:00
K T=14:00
L T=20:10
*

Figure 2. DNA damage-induced mitotic exit delays in *Drosophila* syncytial blastoderm stage embryos. (A-L) Selected frames from the live analysis of control w^{1118} *Drosophila* embryos injected with Oli-green and rhodamine-conjugated tubulin (R-tubulin). The embryos were analyzed immediately after injection by laser scanning-confocal microscopy. (**A-C**) A control w^{1118} injected embryo. (A) Nuclear envelope breakdown (NEB) is marked as time zero (T=0:00). (B) Anaphase initiates in about 2.5 minutes (min) following NEB. (C) Nuclear envelope reformation (NEF) occurs about 1.5 min after anaphase initiation. **(D-L)** Control embryos (w^{1118}) injected with 100μM camptothecin (CPT) along with Oligreen and R-tubulin. (D-F) DNA damage induced by CPT injection causes a delay in anaphase initiation (10 min vs 2.5 min), but not in duration of anaphase (2 min vs. 1.5 min) as compared to the control embryo (compare E-F with B-C). Note that centrosomes regain microtubule nucleation activity at anaphase initiation (E, arrows). (G-I) DNA damage causes an embryo to arrest at metaphase until recording is terminated. The embryo also displays persistent centrosome inactivation (arrowhead). Chromosomes are neither completely aligned nor separated in the absence of NEF. (J-L) DNA damage induces anaphase arrest. After prolonged metaphase, anaphase initiates with active centrosomes and chromosome separation (K). After chromosome separation, apparent anaphase arrest occurs (L). Chromosomes or chromosome fragments are generally found attached to the spindle pole and oscillate occasionally between the main spindle body and the attached pole (L, asterisk).

persisted for an average of 6.4 ± 2.1 min, when astral microtubule assembly abruptly increased, the spindles elongated, some chromosome movement toward the poles was observed. The time from this apparent M-A transition to NEF was 1.7 ± 0.4 min, compared to 1.5 ± 0.6 min in control embryos. Under these conditions, DNA damage thus delays the M-A transition, but does not significantly alter progression through anaphase (Figure 2 D-F. and Table 1).

As noted above, 100μM CPT injection induces mitotic arrest in 5 of 13 of WT embryos and in 100% of *blm* mutants (8 of 8)(Table 1). Mitotic arrest is defined as the absence of NEF for a minimum of 20 minutes after NEB, when recordings were generally terminated. Two distinct classes of mitotic arrest were observed. In the first class, centrosome based microtubule nucleation dropped at NEB, relatively short barrel-shaped spindles assembled, chromosomes did not fully align, and this configuration was maintained for the duration of the recording (Figure 2G-I). In the majority of embryos, however, anastral spindles initially assembled, but a delay with this configuration was abruptly terminated by an increase in centrosome organized microtubule nucleation, spindle elongation, and pole-ward movement of at least a subset of chromosomes (Fig 2K) (Figure 2J-L). The separated chromosomes then remained condensed and oscillated between the poles of the elongated spindles, and NEF was not observed. During this apparent anaphase arrest, chromosomes or chromosome fragments and associated microtubule bundles occasionally splayed from the spindle, but generally remained attached to one pole and often rejoined the main spindle mass (Fig. 2L, asterisks).

To confirm that damage could lead to arrest in anaphase, we assayed localization of a functional GFP-MEI-S332 fusion protein (Moore et al., 1998). MEI-S332/Shugoshin helps maintain sister chromatid cohesion and accumulates on centromeres at prometaphase until the metaphase-anaphase (M-A) transition, when it is rapidly lost from centromeres. This abrupt loss from centromeres is clearly observed in control embryos expressing GFP-MEI-S332 and injected with rhodamine-tubulin to visualize microtubules (Figure 3A-C) (Moore et al., 1998). Following CPT injection, however, nuclei in a subset of embryos showed progressive accumulation of GFP-MEI-S332 on centromeres associated with stable, anastral spindles (Figure 3D-F). These embryos thus appear to be arrested in prometaphase or metaphase. In the final group of embryos, GFP-Mei-S332 also accumulated on centromeres. However, after a delay with anastral spindles, GFP-MEI-S332 localization to centromeres abruptly dropped, and this drop precisely correlated with an increase in astral microtubule assembly and spindle elongation (Figure 3H). Strikingly, GFP-MEI-S332 then rapidly relocalized to distinct foci that oscillated on the elongated spindles, and this configuration was stable until the recording was terminated (Figure 3I). These observations indicate that DNA damage can lead to arrest both before and after the M-A transition.

Interestingly, the DNA replication inhibitor aphidicholin and UV light did not induce mitotic arrest, but did consistently trigger mitotic delays (Figure 1). These observations suggest that DSBs and stalled replication forks or UV activate distinct mitotic damage responses.

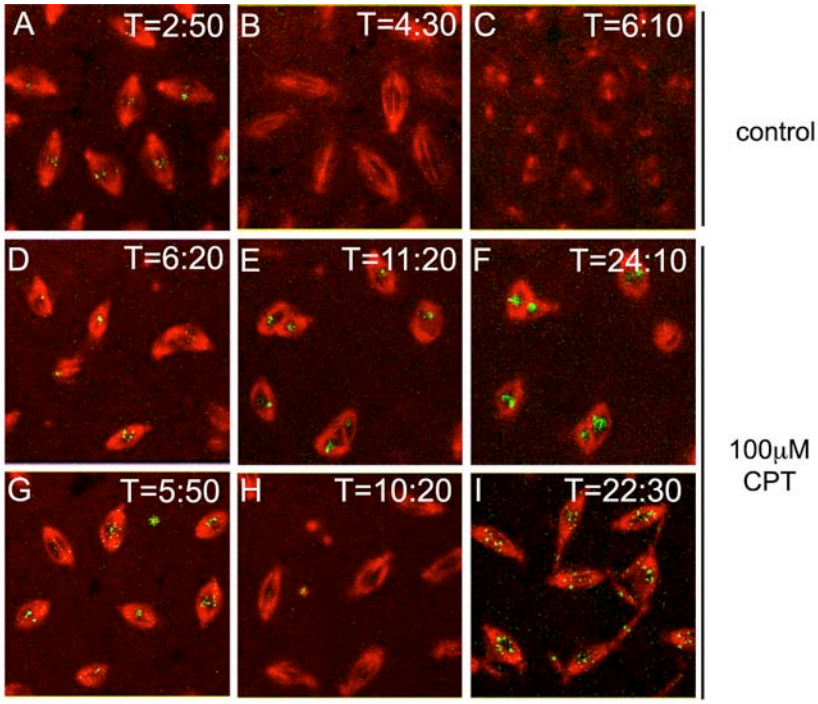
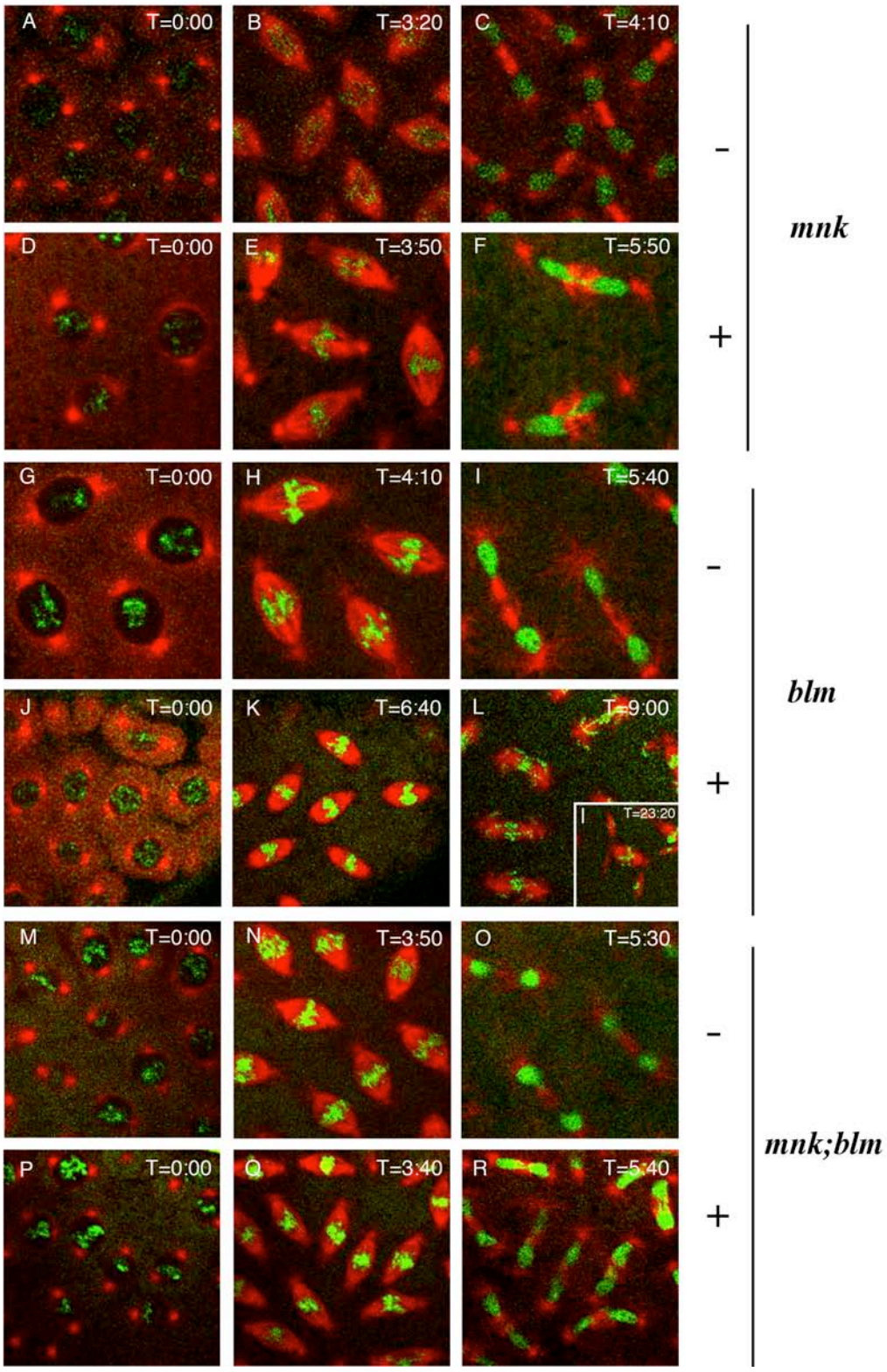


Figure 3. DNA damage triggers delays in metaphase and anaphase.
Localization of functional Mei-S322-GFP fusion proteins was examined by in vivo analysis after injecting rhodamine-tubulin with or without CPT. **(A-C)** Localization of Mei-S322 in a control embryo. Mei-S332 localizes on the centromere during metaphase (A) and disappears from mid-anaphase (B) (Moore et al., 1998). Nuclei exit from mitosis 6 min after NEB (C). **(D-I)** Localization of Mei-S322-GFP in embryos injected with 100μM CPT. (D-F) Nuclei fail to exit from mitosis in response to DNA-damage. Mei-S322-GFP persists at centromeres during the entire recording and anastral barrel-shaped spindles are observed, indicating metaphase arrest. (G-I) Mei-S322-GFP localizes to the centromere at metaphase (G), disappears at anaphase (H), and then relocalizes and remains on chromosomes and fragmented DNA (I), indicating anaphase arrest.

Chk2 is required for DNA damage induced mitotic arrest

Chk2 functions in multiple DNA damage signaling pathways and is required for DNA damage induced centrosome inactivation in *Drosophila* syncytial blastoderm embryos (Takada et al., 2003). To determine if Chk2 is required for DNA damage associated mitotic delays and arrest, we assayed cell cycle progression in embryos derived from females homozygous for a null mutations in *mnk,* which encodes Chk2 (Figure 4A-F, Table1). In contrast to *w-1118* and *Oregon-R* control embryos, 100% of *mnk* mutant embryos injected with 100 μM CPT progressed through mitosis without significant delay. The total time in mitosis (NEB-NEF) was 3.8 ± 1.1 min. in *mnk* control embryos, and 4.5 ± 0.8 min following injection with 100 μM CPT. As noted above, 100% of *blm* mutant embryos arrest in mitosis following injection of either 100 μM or 50 μM CPT (Figure 4 D-I, Table 1). The *blm* mutant background thus provides a particularly stringent test for Chk2 function in mitotic exit. We therefore generated *mnk;blm* double mutant embryos and assayed cell cycle progression following 100 μM CPT injection. In striking contrast to *blm* single mutants, 100% of *mnk;blm* double mutant embryos injected with CPT progressed through mitosis (Figure 4 P-R Table 1). The total time in mitosis (NEB to NEF) was 5.7 ± 0.9 min in *mnk;blm* mutants injected with carrier control solutions, and 7.0 ± 1.5 min following CPT injection (Figure 4 M-R, Table 1). This modest delay was not statistically significant. In the absence of damage, *blm* mutants progress through mitosis more slowly than wild-type controls (5.9 ± 1vs 4.1 ± 0.9 in WT control). We initially speculated that this was due to repair defects and Chk2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B T=3:20
C T=4:10
D T=0:00
E T=3:50
F T=5:50
G T=0:00
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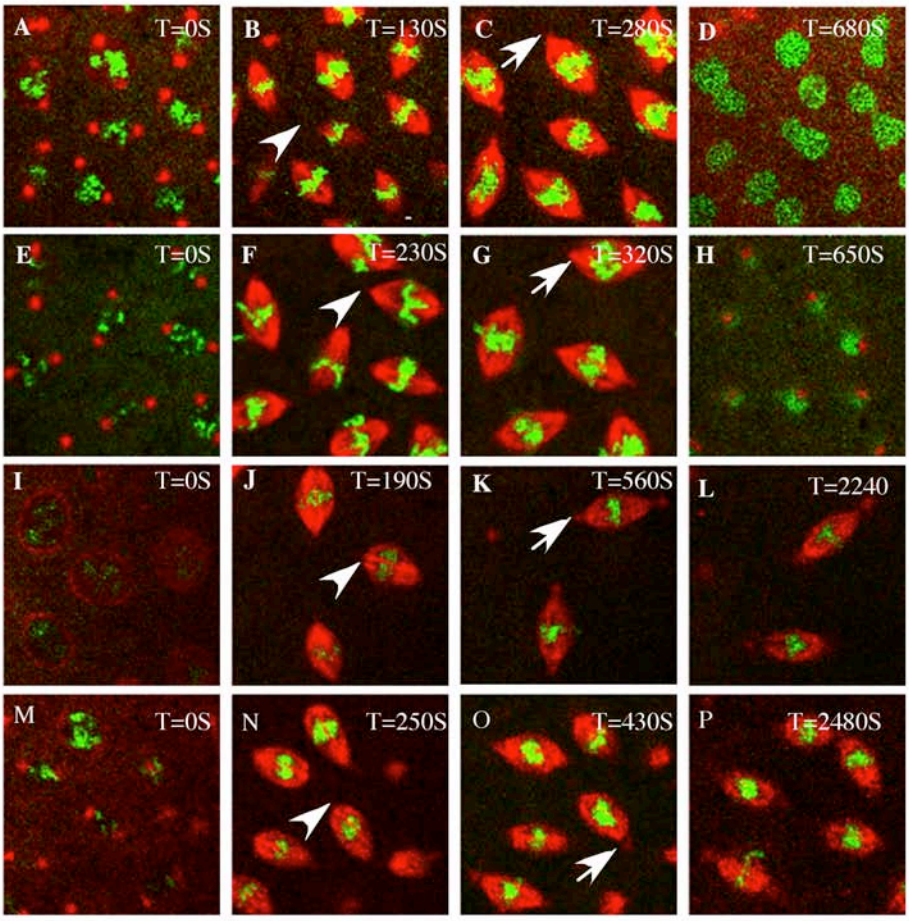
I T=5:40
J T=0:00
K T=6:40
L T=9:00
I T=23:20
M T=0:00
N T=3:50
O T=5:30
P T=0:00
Q T=3:40
R T=5:40
-
+
mnk
blm
mnk;blm

Figure 4. Chk2 is involved in DNA damage-induced mitotic arrest. *mnk*, *blm* and *mnk;blm* mutant embryos were injected with Oli-green and rhodamine-conjugated tubulin with or without 100μM CPT. **(A-F)** *mnk* embryos (A-C) Control *mnk* embryos have astral mitotic spindles and normal duration of mitosis as w^{1118} control embryos shown in Figure 1. (D-F) By contrast to CPT-injected wild type embryos (Figure 1), DNA damage induced by CPT injection does not induce centrosome inactivation and does not increase mitotic length in *mnk* embryos. **(G-L)** *blm* DNA repair mutants are hypersensitive to camptothecin. A control *blm* embryo undergoes normal cell cycle (G-I) whereas a camptothecin-injected blm displays centrosome inactivation and anaphase arrest (J-L). NEF fails until the recording is terminated (L inset). (DNA damage induced-metaphase arrest also occurs but phenotype is not shown. See Table 1 and supplementary movies). **(M-R)** *mnk;blm* double mutants. Non-treated (M-O) and CPT-injected (N-R) *mnk;blm* mutants are phenotypically indistinguishable, indicating the *mnk* mutation suppresses hypersensitivity to camptothecin caused by the *blm* mutation.

activation, but we subsequently found that the *mnk* mutation does not suppress this phenotype (5.7 ± 0.9). The modest increase in mitotic length in *blm* is therefore Chk2-independent. By contrast, damage-induced prometaphase and anaphase arrest in *blm* mutant shows absolute dependence on Chk2.

The ATR and Chk1 kinases have conserved functions in DNA damage signaling and Chk1 has been implicated in DNA damage associated metaphase to anaphase delays in *Drosophila* somatic cells (Huang et al., 2005; Royou et al., 2005). We therefore assayed mitotic progression following DNA damage in embryos derived from females mutant for *mei-41* and *grp,* which encode ATR and Chk1 respectively. Both *grp* and *mei-41* mutant embryos consistently delayed or arrested in mitosis following CPT injection. In addition, CPT induced mitotic arrest in 100% of *mei-41;blm* and *grp;blm* double mutant embryos (Figure 5). Chk2 functions upstream of p53 in damage-induced apoptosis (Brodsky et al., 2004), and we therefore assayed mitotic progression following damage in p53 null mutant embryos. These embryos also showed a normal range of mitotic exit delay and arrest (Kelkar and Theurkauf, personal communication). DNA damage induced mitotic arrest thus required Chk2, but is independent of ATR, Chk1, and p53.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C
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D
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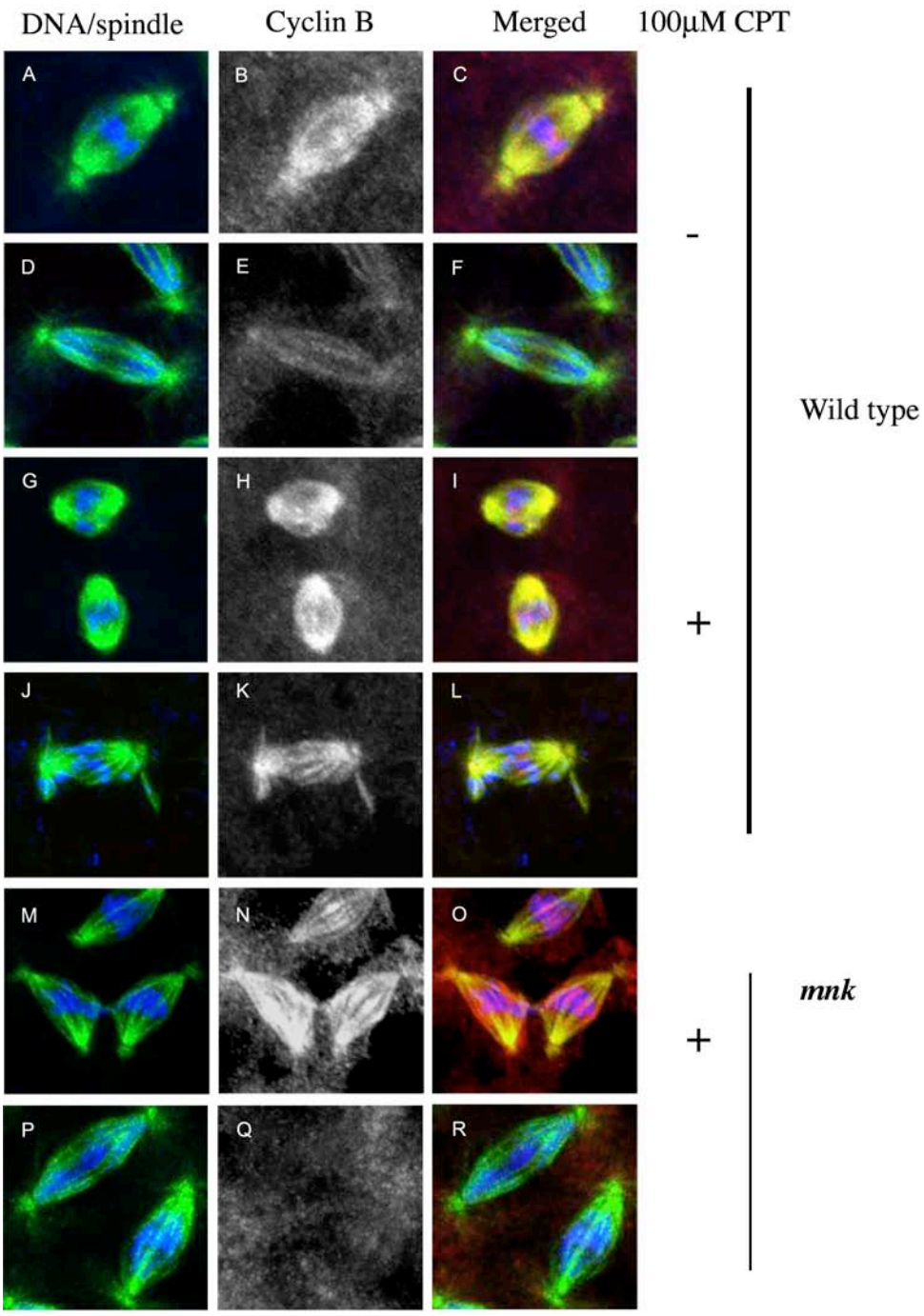
G
T=320S
H
T=650S
grp
I
T=0S
J
T=190S
K
T=560S
L
T=2240
mei-41;blm
M
T=0S
N
T=250S
O
T=430S
P
T=2480S
grp;blm

Figure 5. ATR and Chk1 are not required for anaphase arrest. Embryos from *mei-41* (ATR), *grp* (Chk1), *mei-41;blm* and *grp;blm* mutants were injected with 50μM or 25 μM camptothecin, respectively, along with Oligreen and rhodamine-tubulin and followed immediately by in vivo confocal imaging. **(A-L)** *mei-41 (*A-D) and *grp* (G-H). The total length of mitosis 12 in the camptothecin-injected mutant embryos is ~11 min. This is approximately double the mitotic length of the control mutant embryos injected with oligreen and rhodamine- tubulin (Mitosis 12 takes about 5-6 min in *mei41* and 6-7 min in *grp* mutant embryos, *see* Sibon et al, 1999). Both *mei41* and *grp* mutant embryos exhibit centrosome inactivation at metaphase (b and f, open arrowheads). The centrosome foci come back at initiation of anaphase (c and g, open arrows). The time spent in anaphase increases in response to camptothecin injection in the both mutants (6.6 min in mei41 and 5.5 min in grp vs. ~3 min in controls for which data is not shown). **(I-O)** *mei-41;blm* (I-L) and *grp;blm* (M-O). Mutations in either *mei-41* or *grp* do not suppress *blm* hypersensitivity in response to DNA damage.

DNA damage stabilizes Cyclin B

Destruction of Cyclin B is required for mitotic exit and normally begins late in metaphase, such that only low levels of Cyclin B are detectable during anaphase. A non-degradable cyclin-B mutation leads to anaphase arrest in Drosophila embryos, cycling Xenopus extracts, and mammalian cells (Holloway et al., 1993; Su et al., 1998; Wolf et al., 2006), leading us to speculate that DNA damage may lead to Cyclin B stabilization. We therefore assayed Cyclin B by immunofluorescence labeling in control and CPT treated embryos. Consistent with earlier findings (Huang and Raff, 1999), we found that Cyclin B levels begin to drop late in metaphase, when the chromosomes are aligned at the metaphase plate but sister chromatids have not separated, and that Cyclin B levels are near background levels during anaphase (Fig 6 A-F). In embryos treated with the DNA damaging drugs, by contrast, we observe robust Cyclin B accumulation on centrosomes and the spindles, even after chromosomes had started to move to the poles and the spindle had elongated (Fig 6 J-L). As noted above, mnk mutant embryos do not delay mitosis following CPT injection. Consistent with this observation, we never observed high levels of Cyclin B during anaphase in mnk or mnk;blm mutant embryos treated with CPT (Figure 6 M-R, Figure 7). Thus DNA damage stabilizes Cyclin B through the M-A transition by means of a process that requires Chk2.


DNA/spindle
Cyclin B
Merged
100µM CPT
A
B
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D
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M
N
O
P
Q
R
-
+
+
Wild type
mnk

Figure 6. DNA damage triggers Chk2-dependent cyclin B stabilization. w^{1118} and *mnk* embryos were methanol fixed following 20μM CPT treatment or without treatment (control), and labeled for spindle (green), DNA (blue), and Cyclin B (Red). (**A-F)** w^{1118} control embryos. (A-C) Cyclin B localizes at spindle, centrosomes and metaphase plate at metaphase. (D-F) Cyclin B disappears from centrosomes and the spindle during anaphase (**G-L)** CPT-treated w^{1118} embryos. (G-I) Cyclin B localizes to anastral spindles and the metaphase plate, but not at centrosomes during metaphase. (J-L) High levels of Cyclin B persist on the spindle and centrosomes during anaphase, indicating DNA damage blocks Cyclin B degradation. A subset of chromosomes are dispersed and spindle structure is abnormal. This spindle and chromosome morphology is indicative of anaphase arrest. **(M-R)** CPT-treated *mnk* embryos in metaphase (M-O) and anaphase (P-R). Cyclin B dynamics is similar to w^{1118} control embryos, indicating the Chk2 mutation abolishes DNA damage-induced Cyclin B stabilization.

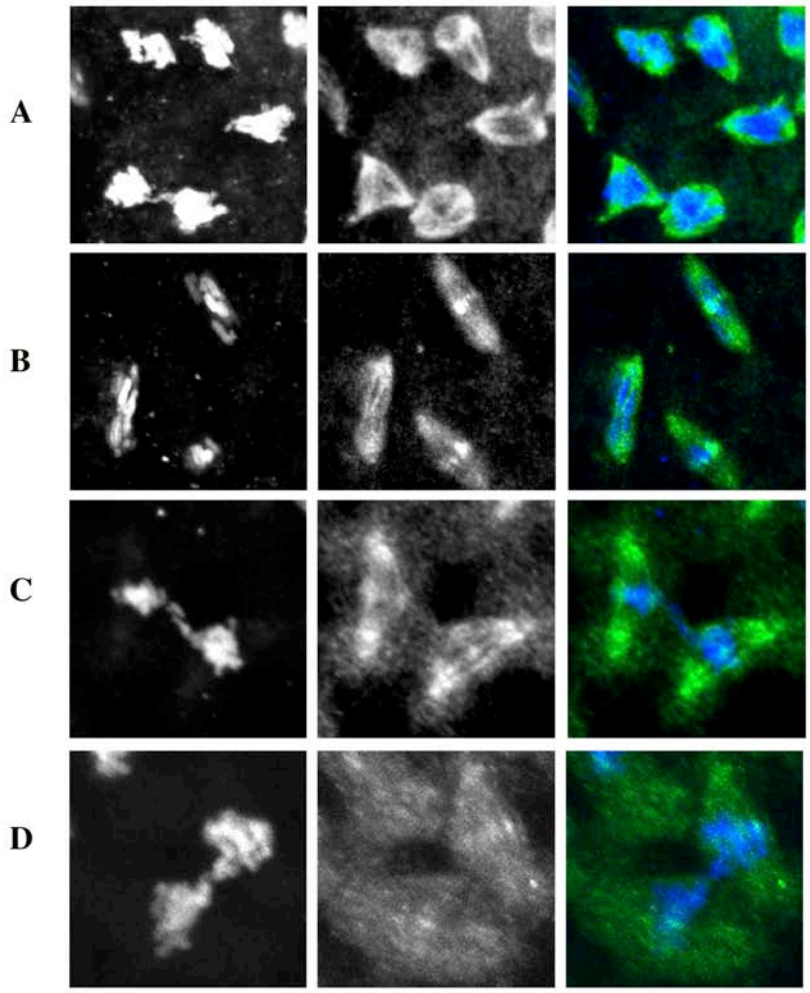


Figure 7. DNA damage triggers Chk2-dependent cyclin B stabilization.
blm and *mnk;blm* embryos were methanol fixed following 20μM CPT treatment and labeled for DNA (blue) and cyclin B (green). (A, B) CPT-treated *blm* embryos. (A) Cyclin B localizes at anastral spindle and metaphase plate, but not at centrosome during metaphase.(B) High levels of Cyclin B persist on the spindle and centrosome after sisterchromatid seperation, indicating DNA damage blocks cyclin B degradation.
(C, D) CPT treated *mnk;blm* embryos. Cyclin B localizes on the spindles and centrosomes at metaphase (C) and disappears at anaphase (D), indicating Chk2 mutation abolishes DNA damage-induced cyclin B stabilization in *blm* mutants.

Mad2 required for damage induced prometaphase and anaphase arrest.

The SAC is a surveillance system that inhibits the anaphase promoting complex/cyclosome (APC/C) until all chromosomes are aligned at the metaphase plate. Chromosome alignment leads to activation of Cdc20/Fizzy, which promotes APC/C dependent destruction of proteins, including Securin and Cyclin B, thus driving mitotic exit (Chan et al., 2000; Hoyt, 2001). To determine if the SAC is required for mitotic delays in early embryos, we assayed mitotic progression and Cyclin B localization in embryos derived from females homozygous for a null mutation in *mad2* (Buffin et al., 2007). We confirmed that Mad2 is required for SAC function by injecting control and *mad2* mutant embryos with the microtubule assembly inhibitor colchicine and monitoring cell cycle progression. 100% of wild type embryos injected with colchicine arrested in mitosis for over 20 minutes. Similarly, 100% of *mnk* mutant embryos arrested in mitosis following colchicine injection, indicating that Chk2 is not required for the SAC (Table 1, Figure 8). By contrast, 100% of colchicine injected *mad2* mutant embryos exited mitosis (Figure 9 III). However, these embryos did show a modest but statistically significant delay in mitosis. Microtubule disruption can therefore trigger modest Mad2-independent mitotic delays. *mad2* mutants that progressed into mitosis following DNA damage showed centrosome inactivation and anastral spindles assembled, indicating that Chk2 signaling is intact (Figure 9C). Nonetheless, 100% of mutant embryos initiated anaphase and exited mitosis, indicating Mad2 is required for metaphase and anaphase arrest (Figure 9I, Table 1).

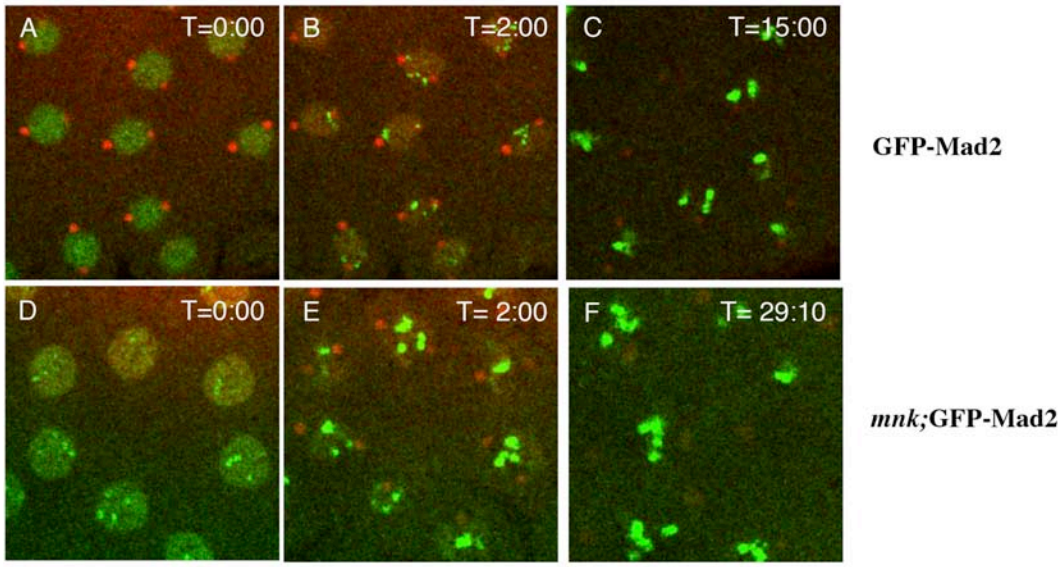
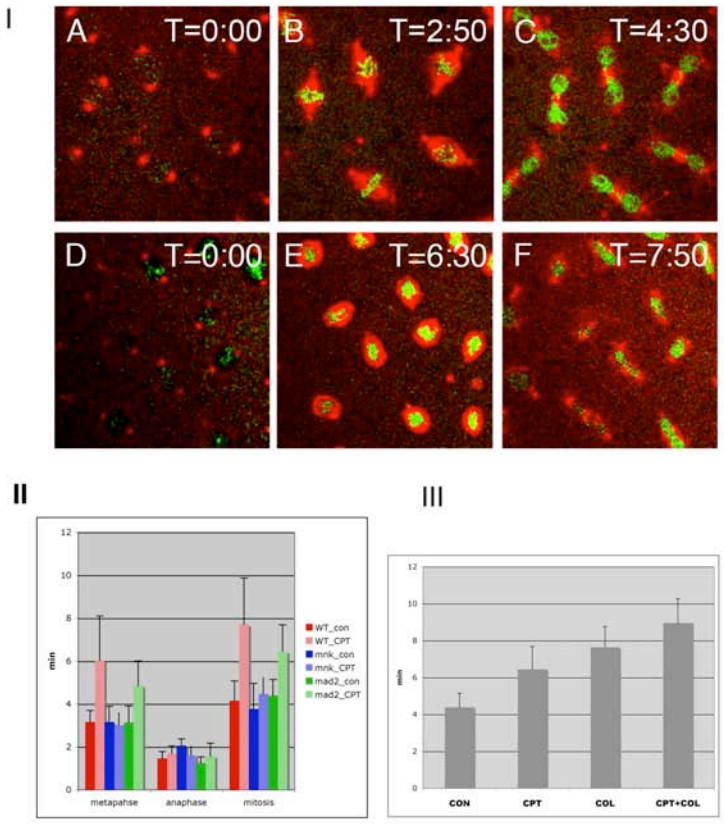


Figure 8. Chk2 is not required for spindle checkpoint activation.
(A-C) In w^{1118} embryos, GFP-Mad2 accumulates at kinetochores and cell cycle arrests during mitosis following colchicine injection. (D-F) In *mnk* embryos, colchicine injection triggers mitotic arrest with GFP-Mad2 accumulation at kinetochores.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II
min
12
10
8
6
4
2
0
metapahse
anaphase
mitosis
WT_con
WT_CPT
mnik_con
mnik_CPT
mad2_con
mad2_CPT
III
min
12
10
8
6
4
2
0
CON
CPT
COL
CPT+COL

Figure 9. Mad2 is required for mitotic arrest in response to DNA damage. (I) Mad2 is required for mitotic arrest in response to DNA damage (A-C) In control *mad2* embryos, total duration of mitosis is ~ 4 to 5 min and centrosomes actively nucleate microtubule asters throughout mitosis. (D-F) CPT injected *mad2* embryos show centrosome inactivation (E), delay before the M-A transition, and then exit from mitosis, suggesting Mad2 is not required for mitotic delay. However, Mad2 is essential for mitotic arrest (See Table 1). **(II)** DNA damage triggers Chk2-mediated but Mad2-independent mitotic delay. Duration of mitosis (NEB-NEF), metaphase length (NEB-M/A) and anaphase length (M/A-NEF) are compared in control- and damaged-w^{1118}, *mnk* and *mad2* embryos. In damaged w^{1118} and *mad2,* prometaphase length increases but anaphase length is unaffected. Mitotic length in *mnk* embryos does not increase upon DNA damage. **(III)** DNA damage may trigger a mitotic delay in a spindle checkpoint (SAC)-independent manner. To determine whether SAC is required for mitotic delay, total duration of mitosis following double injection of CPT and colchicines in *mad2* embryos is compared to mitosis length after either single drug injection in *mad2* mutant embryos. Double drug injection slightly increases mitotic length compared with either single injection, although the difference was mild (t test P = 0.17).

To determine if additional SAC components are required for damage induced mitotic delays, we assayed *zw10* null mutant embryos for progression through mitosis following DNA damage. Null mutations in *zw10* are lethal, so we used germ-line clones to generate mutant embryos (Williams et al., 1992). These embryos show high rates of spontaneous mitotic defects, which complicated interpretation of these experiments. Nonetheless, we did not observe mitotic arrest in any of the control or CPT-injected *zw10* mutant embryos analyzed, suggesting that an intact SAC is essential for DNA damage-induced mitotic arrest (Figure 10).

However, CPT did trigger modest delays in mitotic exit in *mad2* mutant embryos (6.4 ± 1.1 min vs. 4.4 ± 0.8 min. in controls) (Figure 9 II, Table 1). Similar delays were observed following microtubule depolymerization, suggesting that the mitotic exit delays following CPT treatment may be secondary to spindle disruption. Alternatively, damage signaling and microtubule disruption could delay mitotic exit by independent mechanisms. Consistent with this hypothesis, *mad2* mutant embryos injected with colchicine and CPT delayed in mitosis longer than *mad2* mutant treated with either agent alone (Figure 9 III). However, the additional delay was modest (t-test P= 0.17) and somewhat difficult to interpret. By contrast, both *mnk* and *mad2* mutations consistently suppressed damage induced mitotic arrest, demonstrating that both Mad2 and Chk2 are required for this DNA damage response.

To genetically assay for interactions between Mad2 and Chk2, we attempted to construct *mnk;mad2* double mutants. Less than 1% of the expected double mutant adults were recovered, and these adults were sterile. The lethality appears to occur

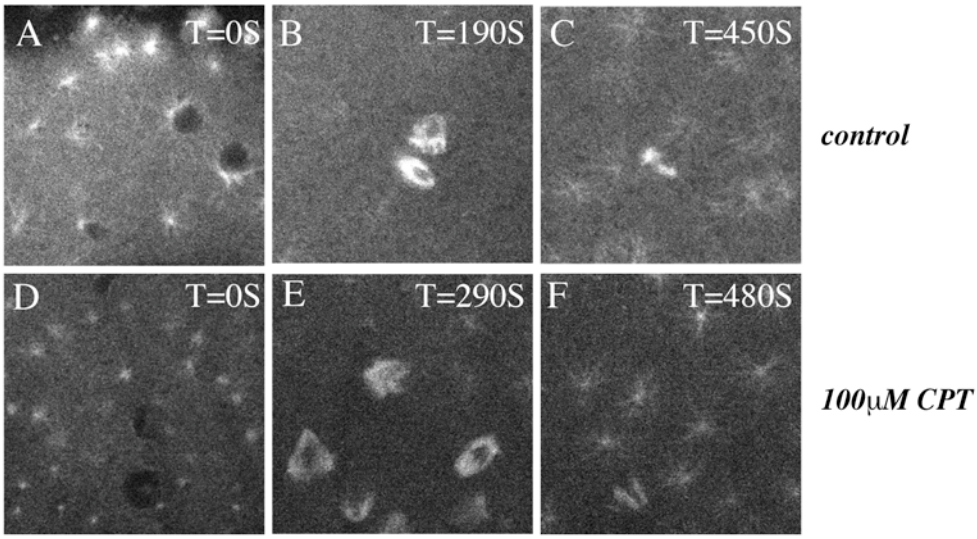


Figure 10. Zw10 is required for DNA damage-induced mitotic arrest.
(A-C) In *zw10* mutant embryos, centrosome inactivation spontaneously occurs, but nuclear envelop forms after delays in mitosis. (D-F) Damaged *zw10* embryos show similar mitotic defects to the control embryos. Control and damaged *zw10* embryos do not arrest during mitosis.

during embryogenesis, larval and pupal development. This negative genetic interaction is striking, as animals homozygous for null alleles of either *mnk* or *mad2* are viable and fertile. This zygotic lethal phenotype is currently under investigation.

Spindle checkpoint proteins and Cdc20/Fizzy relocalize to kinetochores during anaphase arrest.

Active SAC proteins localize to unattached kinetochores, and dissociate from kinetochores following chromosome alignment at the metaphase plate (Musacchio and Salmon, 2007). The SAC inhibits Cdc20/Fizzy, which also localizes to unattached kinetochores and dissociates from kinetochores after alignment (Raff et al., 2002). Localization of Cdc20/Fizzy thus provides an indicator of SAC activity. To gain further insight into the role of the SAC during damage induced mitotic delays, we therefore directly monitored localization of a functional GFP-Fizzy fusion protein by time-lapse confocal microscopy. Embryos were injected with rhodamine-tubulin and CPT, allowing direct visualization of spindle assembly and mitotic progression. In control embryos, GFP-Fizzy localized to kinetochores through the metaphase to anaphase transition, and localization rapidly declined during anaphase (Figure 11A-D) (Raff et al., 2002). During CPT induced prometaphase arrest, by contrast, GFP-Fizzy remained localized to kinetochores for a minimum of 20 minutes (Figure 11E-H). As noted above, GFP-MEI-S332 is also retained at centromeres during prometaphase arrest (Figure 2). In CPT-treated embryos that ultimately arrested in

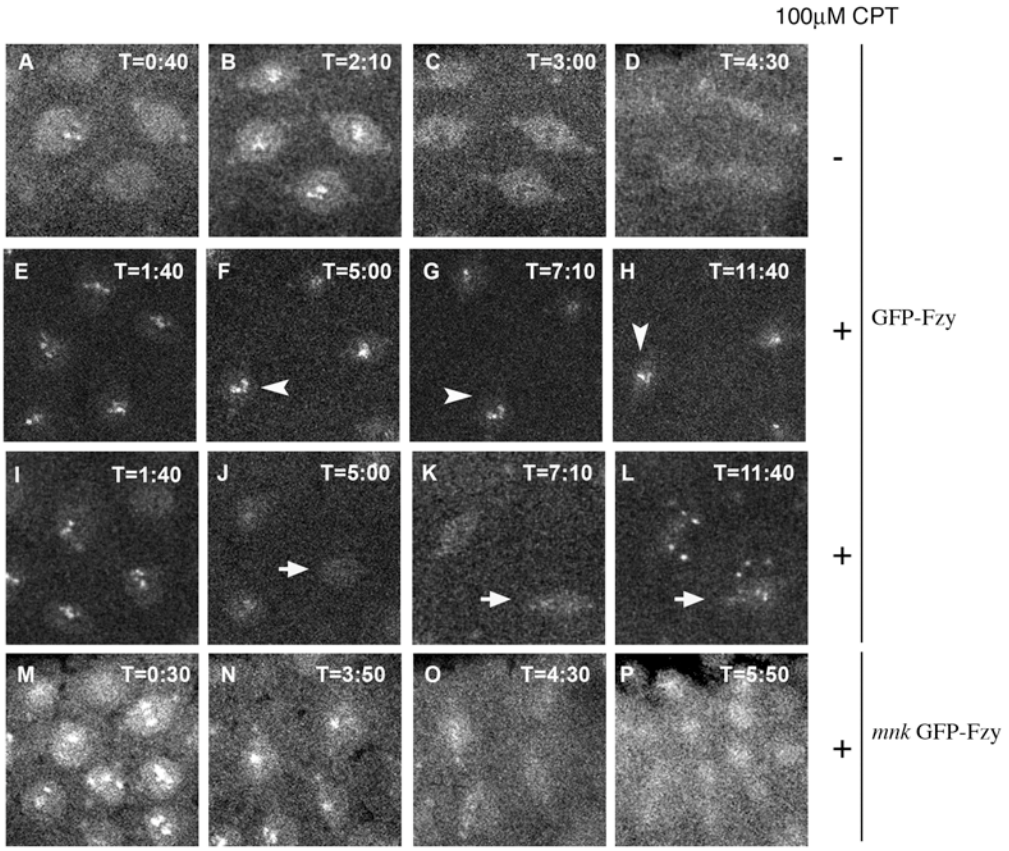


Figure 11. Cdc20/Fizzy relocalizes to kinetochores after M-A transition in a Chk2-dependent manner during DNA damage induced anaphase arrest. (A-L) Localization of GFP-Fizzy fusion protein in control embryos with or without CPT injection. (A-D) Fizzy concentrates at kinetochores during metaphase (B) and disappears from kinetochores during anaphase (C) without DNA damage. (E-H) Fizzy continuously localizes at kinetochores during DNA damage-induced metaphase arrest. (I-L) During DNA damage-induced anaphase arrest, Fizzy is found at kinetochores at prometaphase (I), disappears at M-A transition (J) and relocalizes and stays at kinetochores (L). (M-P) Fizzy is not relocaliz at kinetochores in CPT-injected *mnk* embryos. Fizzy localizes to kinetochores during metaphase (N) and disappears at anaphase (O).

anaphase, GFP-Fizzy localized to kinetochore through metaphase, and kinetochore localization

dropped dramatically as the spindles elongated at the onset of anaphase. However, the fusion protein rapidly localized to distinct foci as the cell cycle arrested in anaphase. These foci oscillated on the elongated spindle during anaphase arrest (Figure 11. I-L). By contrast, GFP-Fizzy did not relocalize to kinetochores in *mnk* mutants treated with CPT (Figure 11. M-P). An essentially identical relocalization pattern was observed with GFP-Mad2 and GFP-Mps1, although the signal for these fusions was weak and kinetochores were somewhat difficult to track (Figure 12). These findings confirm that DNA damage can lead to anaphase arrest, and suggest that this arrest is the result of SAC reactivation.

Centromere integrity and mitotic arrest

Damage to centromere DNA is proposed to disrupt kinetochore structure and function, and could therefore indirectly activate the SAC (Mikhailov et al., 2002). To directly assay the integrity of centromere DNA in embryos treated with CPT, we used fluorescent in situ hybridization (FISH) to localize dodeca satellite sequences, which are largely restricted to centromeric heterochromatin on chromosome 3 (Abad et al., 1992; Abad JP, 1992). During metaphase in controls of w^{1118} and *mnk* embryos, we observed two prominent pairs of closely spaced foci associated with the replicated maternal and paternal third chromosomes, and one or two more minor foci. During anaphase, two major foci were present on each half spindle (4 dots per nuclei)(Figure

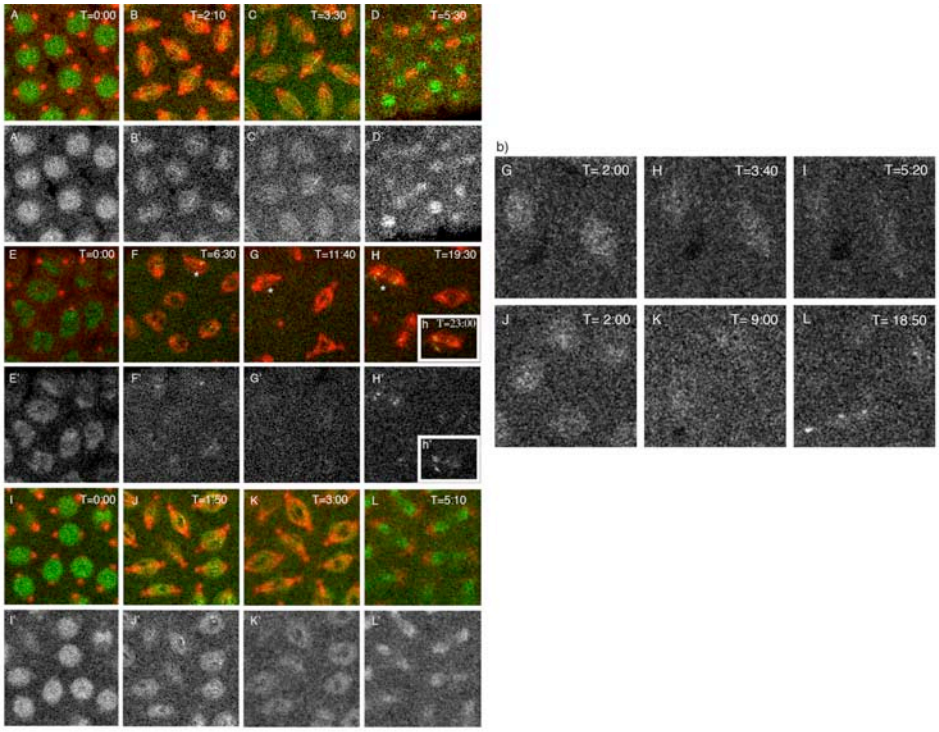
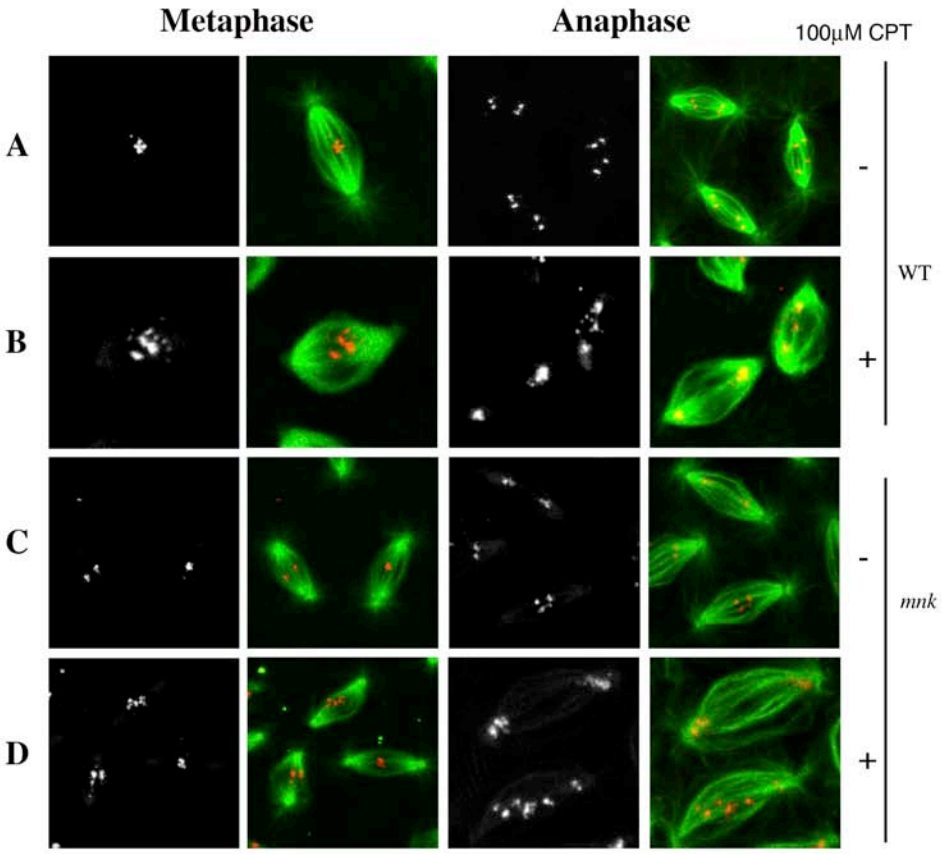


Figure 12. Mad2 and Mps1 relocalize at kinetochore after M-A transition during anaphase arrest. (a) Mad2 localization **(A-H)** (A-D) Mad2 disappears from kinetochores during anaphase followed by kineotochore localization during metphase in controls. (E-H) During DNA damage-induced anaphase arrest, Mad2 disappears at M-A transition and relocalizes and stays at kinetochores **(I-L)** Mad2 does not relocalized at kinetochores in CPT-injected *mnk* embryos. (b) (A-C) Mps1 concentrates at kinetochores during mitosis and dissociates from kinetochores at anaphase in control embryos. (D-F) Mps1 relocalizes in DNA damage-induced anaphase arrested embryos.

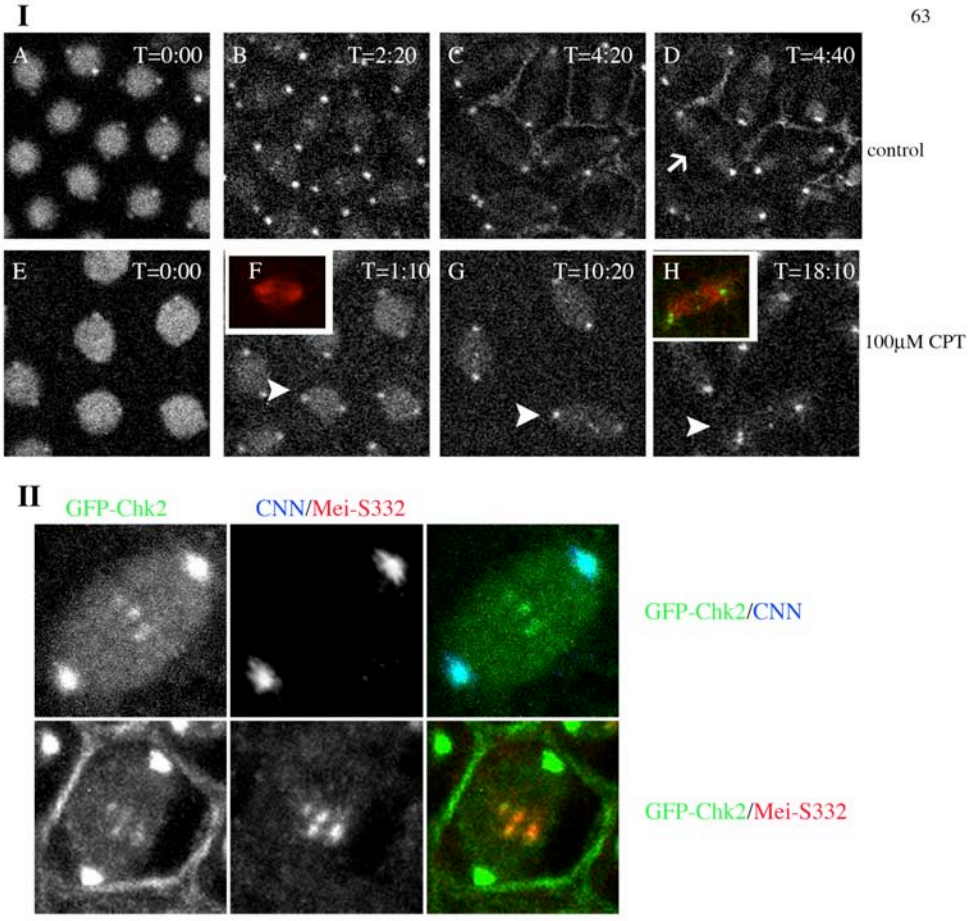


Figrue 13. Centromere fragmentation is not sufficient for mitotic delays. To determine if centromere fragmentation upon DNA damage triggers SAC-mediated mitotic delays, w^{1118} and *mnk* embryos were fixed without drug treatment (control) or after CPT treatment, followed by flourescent-in situ hybridization (FISH) with dodeca satellite probe (red) and labeling spindles (green). (A, C) 2 pairs foci during metaphase and 4 dots during anaphase are detected in w^{1118} (A) and *mnk* (C) embryos, representing replicated 3rd chromosomes (4N). (B, D) After CPT treatment, many smaller dots are detected during metaphase and anaphase in w^{1118} (B) and *mnk* (D) embryos, indicating DNA damage fragmentizes centromere/kinetochore in w^{1118} and *mnk* embryos. Mutation in mnk supresses DNA damage-induced mitotic delays (Figure 3), indicating kinetochore fragmentation is not sufficient to trigger mitotic delays.

13 A, C). Following CPT treatment, by contrast, we observed multiple smaller foci during both metaphase and anaphase (figure 13B). Essentially identical fragmentation of the dodeca satellite signal was observed in CPT treated *mnk* mutants (Figure 13D), which do not delay in mitosis. Significantly, *mnk* mutants arrest in mitosis indefinitely in response to microtubule depolymerization, indicating that the SAC is functional. We therefore conclude that fragmentation of centromere DNA is not sufficient to activate the SAC, and that damage-induced mitotic delays in syncytial embryos results from DNA damage signaling through Chk2.

A functional GFP-Chk2 fusion localizes to centromeres and centrosomes

We previously found that Chk2 functions in a pathway that disrupts centrosome function and spindle assembly in response to DNA damage (Takada et al., 2003). Here we show that Chk2 functions with SAC components to control mitotic exit. Chk2 could directly modify the division machinery and SAC components, or activate intermediate factors that disrupt mitosis. To begin to address the mechanism of Chk2 damage signaling to the division machinery, we generated at GFP-Chk2 fusion and assayed localization and function during mitosis. For these studies, we crossed a GFP-Chk2 transgene into *mnk* mutants. The resulting embryos were injected with CPT and rhodamine-tubulin and assayed for centrosome inactivation and mitotic delays by time-lapse confocal microscopy. In contrast to parental *mnk* mutants, these embryo showed centrosome inactivation and delayed in mitosis following DNA damage (Figure 14E-H, centrosome inactivation Figure 14F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C T=4:20
D T=4:40
control
E T=0:00
F T=1:10
G T=10:20
H T=18:10
100µM CPT
II
GFP-Chk2
CNN/Mei-S332
GFP-Chk2/CNN
GFP-Chk2/Mei-S332

Figure 14. Chk2 localizes to centromeres and centrosomes. (I) live analysis was performed after Rhodamin-tubulin injection with or without CPT in the *mnk* embryos expressing GFP-Chk2. (A-C) In the control embryo, Chk2 is found in the nucleus and on centrosomes during interphase to NEB (A). After NEB, Chk2 localizes at kinetochore regions at metaphase (B), disappears from kinetochores at the M-A transition (C), and then is found in a dot that appears to be midbody (D, arrow). Throughout mitosis, Chk2 is detected on centrosome (E-H) In the CPT-injected *mnk* embryo expressing GFP-Chk2, DNA damage triggers centrosome inactivation (F inset) and anaphase arrest, indicating GFP-Chk2 is functional. Chk2 dynamic localization is similar to the control except continuous localization at kinetochore regions (G and H). Chk2 is found on centrosome when centrosome inactivation occurs (F). **(II)** To determine Chk2 localization on centrosomes and centromeres, the *mnk* embryos expressing GFP-Chk2 were fixed and labeled with CNN (blue) or MEI-S332 (Red). Chk2 colocalizes with CNN on the centrosome and with Mei-S332 at centromeres during mitosis.

inset), indicating functional rescue by the GFP fused transgene. The functional GFP fusion was largely restricted to the nucleus during interphase, although signal was also observed at foci that appeared to be centrosomes. Following nuclear envelope breakdown, centrosome localization increased, and smaller foci that moved to the metaphase plate and appeared to represent kinetochores were observed (Figure 14B). Colocalization with MEI-S322 and CNN confirmed that GFP-Chk2 localizes to centromeres and centrosomes (Figure 14 II). During anaphase, kinetochore localization was lost (Figure 14. C), and the fusion protein transiently accumulated in a dot that appeared to be at the center of the midbody (arrow, Figure 8D). The chromosomal passenger proteins Aurora B, CENP-C and Survivin show a remarkably similar localization pattern. These findings suggest that Chk2 controls mitotic progression by regulating the SAC or passenger protein complex.

We also analyzed GFP-Chk2 localization after CPT injection (Figure 14E-H). Consistent with previous immunocytochemical studies, GFP-Chk2 persisted on centrosomes through metaphase. During progression into anaphase and during anaphase arrest GFP-Chk2 continuously localized to kinetochores. These findings suggest that Chk2 could directly modify centrosome proteins and components of the SCC to trigger centrosome inactivation and mitotic exit arrest.

DISCUSSION

Studies in a number of systems indicate that DNA damage leads to delays in mitosis that required the spindle assembly checkpoint. The mechanism of these mitotic delays and SAC activation, however, remains controversial. Several recent studies has implicated signaling through Chk1 or Chk2 in this process, while Rieder and colleagues have proposed that SAC activation is secondary to DNA break formation in centromere DNA (Mikhailov et al., 2002). Here we analyze the mitotic response to DNA damage in syncytial *Drosophila* embryos, which support time-lapse analysis of mitotic progression and genetic dissection of DNA damage signaling. These studies show that both Chk2 and the SAC component Mad2 are required for damage induced mitotic exit delays, and that these delays can be imposed both before and after the M-A transition. Furthermore, we present evidence that fragmentation of centromere DNA is not sufficient to activate the SAC. These observations lead us to propose that mitotic progression in syncytial embryos is controlled by a Chk2-dependent DNA damage signaling pathway that delays mitotic exit by activating the SAC.

DNA damage triggers mitotic delays in syncytial embryos

Our *in vivo* studies revealed three different phenotypes associated with damaging agents, which appear to represent dosage dependent responses to DNA breaks. In regions of the embryo distant form the injection site, which presumably accumulate lower levels of DNA damage, we observed delays in prometaphase followed by mitotic exit with anaphase chromosome segregation defects. Near the injection site, by contrast, we often observed prolonged prometaphase arrest, stable anastral spindles, and no indication of anaphase onset. Finally, at intermediate regions of the embryo, astral spindles formed, but a delay in this configuration was abruptly terminated as the spindles elongated, astral microtubule assembly increased, chromosomes moved toward the pole. However, mitotic exit was not completed and chromosomes oscillated on the elongated spindles in a prolonged anaphase arrest.

Anaphase arrest was confirmed by time-lapse analysis of GFP fusions to Mei-S332, Fizzy, Mad2 and Mps1. All of these proteins normally dissociate from centromeres or kinetochores at the M-A transition and do not relocalize to centromeres or kinetochores until the following mitosis. Following damage, however, all of these proteins transiently dissociate from kinetochores and rapidly reassociate with the separated chromosomes, which oscillate on elongated "anaphase" spindles.

Mitotic arrest after chromosome separation does not appear to be specific to the syncytial embryo. In yeast, cell cycle arrest at mid-anaphase is observed when dicentric chromosomes are present, and non-degradable Cyclin B triggers anaphase

arrest in *Drosophila*, *Xenopus*, and mammalian cells (Holloway et al., 1993; Su et al., 1998; Wolf et al., 2006; Yang et al., 1997). Consistent with these observations, Cyclin B levels remain high during DNA damage-induced anaphase arrest in syncytial embryos. In mammalian cells, DNA damage is reported to trigger pseudo-metaphase arrest with high levels of Cyclin B, pole-ward movements of at least a subset of chromosomes (Nitta M, 2004). As observed here, this arrest appears to require the SAC. These observations suggest that anaphase arrest and Cyclin B stabilization is a conserved response to DNA damage. Arrest at this late mitotic phase prevents propagation of damage-induced mutations, and may therefore provide an important "last chance" genome maintenance mechanism.

Chk2 is required for DNA damage induced mitotic arrest in syncytial embryos

To address the role of DNA damage signaling proteins in the mitotic response to DNA damage, we assayed embryos derived from females that were homozygous for null alleles of *mei-41* (ATR), *grp* (Chk1), *p53*, and *mnk* (Chk2). The *mei-41, grp,* and *p53* mutants showed a normal spectrum of mitotic delays and arrest, while *mnk* mutants that lack Chk2 were completely resistant to DNA damage induced mitotic arrest, Chk1, by contrast, functions in the syncytial blastoderm stage DNA replication checkpoint pathway(Sibon et al., 1999; Sibon et al., 1997), but does not appear to play a role in the mitotic response to damage during this developmental window. By contrast, Chk1 has been implicated in DNA damage induced mitotic exit control during the later somatic divisions in *Drosophila* embryos (Royou et al., 2005), and

Chk1 also appears to control mitotic exit in mammalian cells irradiated during inhibitor induced mitotic arrest (Huang et al., 2005). By contrast, we have found that transgenic expression of human Chk2 rescues DNA damage-triggered mitotic delays in *mnk* mutants (Appendix I). In addition, human colorectal cancer cells (HCT116) delay in mitosis following damage, while *chk2* mutant HCT116 cells do not (Hanne Varmark and Theurkauf, personal communication). Chk1 and Chk2 participate in mitotic arrest in response to DNA damage in yeast (Agarwal et al., 2003), and Chk1 and Chk2 often function redundantly in DNA damage signaling (Niida and Nakanishi, 2006). Taken together, these findings suggest that both of these kinases control mitotic exit, and that the dominant kinase mediating varies between cells and is organisms specific (Rhind and Russell, 2000).

How is Chk2 activated during mitosis? ATM activates Chk2 in other contexts (Burma et al., 2001), and could it control Chk2 activity during mitosis. Strong mutations in the *Drosophila atm* gene are lethal, making direct tests of this possibility in syncytial embryos difficult. Phosphorylation of histone H2Av (the H2AX homologue in *Drosophila*), by ATM appears to amplify the damage signal and is required for some DNA damage responses (Burma et al., 2001; Kang et al., 2005). We find that embryos from flies carrying H2AX phosphorylation site mutants arrest in mitosis following DNA damage arrest (Appendix III). This observation suggests that Chk2 may be activated by an ATM- independent mechanism. However, a number of ATM dependent damage responses do not required H2Av

phosphorylation. The mechanism of Chk2 activation during mitosis thus remains to be determined.

The SAC is required for prometaphase and anaphase arrest.

The SAC is required for mitotic arrest in response to DNA damage in systems ranging from yeast to mammalian cultured cells. Consistent with these observations, we find that mutations in *zw10* and *mad2* suppress damage-induced mitotic exit delays in syncytial embryos. Surprisingly, these mutations completely eliminate damage induced arrest in both prometaphase and anaphase. These functional observations suggest that damage leads to SAC activation during anaphase. SCC proteins normally localize to kinetochores through prometaphase and disappear as microtubule attachment is established and chromosomes are oriented at the metaphase plate. SCC localization at kinetochores is therefore tightly linked to SAC activity. Significantly, we find that, during damage-induced anaphase arrest, GFP-Mad2 and the APC/C activator and SAC target GFP-Fizzy dissociate from kinetochores on anaphase onset, but rapidly reassociate with chromosomes in a Chk2-dependent manner (Figure 11 and figure 12). These observations suggest that DNA damage signaling through Chk2 maintains SAC activity during anaphase, or it reactivates the SAC following the M-A transition, and thus blocks Cyclin B destruction and mitotic exit.

The dynamic redistribution of GFP-Chk2 suggests that this conserved kinase may directly target proteins required for spindle assembly and mitotic progression. We show that GFP-Chk2 localizes predominantly to nuclei during interphase, consistent with previous studies. During prometaphase and metaphase, however, GFP-Chk2 associates with both centrosomes and the centromere regions of mitotic chromosomes (Figure 14). During anaphase, GFP-Chk2 maintains association with centrosomes, but dissociates from the centromeres, and transiently localizes to the center of the midbody. These findings suggest that Chk2 may directly phosphorylate centrosome proteins required for microtubule nucleation, including components of the γ Tubulin ring complex (TuRC), and SCC proteins or Cdc20, which localizes at kinetochores. The dynamic mitotic localization pattern of Chk2 is strikingly similar to that of chromosomal passenger proteins, which control multiple aspects of chromosome segregation, spindle assembly and mitotic progression. It is therefore possible that Chk2 modulates passenger protein activity to control mitotic progression.

The kinetochore has a central role in sensing chromosome alignment and activating the SAC (Cleveland et al., 2003). DNA damage has been proposed to delay mitosis by fragmenting centromere DNA, and thus disrupting kinetochore function. However, there is no data directly linking centromere DNA integrity to kinetochore function. Using FISH for a third chromosome satellite sequence, we show that CPT treatment does lead to fragmentation of centromere DNA. However, in *mnk* mutant embryos this does not lead to mitotic exit delays. Significantly, we show that *mnk*

mutant embryos arrest indefinitely following microtubule depolymerization, demonstrating that the SAC is functional. These studies show that functional kinetochores can assemble on a fragmented DNA scaffold, and that centromere DNA damage alone is not sufficient to activate the SAC.

Is SAC activity spatially regulated?

As embryos progress into DNA damaged anaphase arrest, MEI-S332 dissociates from centromeres, and centromeric satellite sequences appear to separate. In addition, Mad2, MPS1, and Mad2 temporally dissociate from kinetochores as MEI-S332 during anaphase arrest. These findings suggest that chromosomes have aligned, the SAC has been inactivated, leading to APC/C activation and separase-dependent sister chromosome disjunction. Cyclin B is also a target for the APC/C and normally begins to degrade prior to sister chromatid separation. However, following damage, we find that Cyclin B levels remain high through mitosis and into anaphase (figure 4). This correlates with relocalization of SCC proteins to kinetochores. These findings lead us to propose a two-step model for DNA damage control of mitotic exit. In this model, damage signaling through Chk2 first disrupts centrosome function and spindle assembly, blocking chromosome alignment and activating the SAC. In response to high levels of damage, spindle function is so compromised that bipolar chromosome orientation is never achieved, leading to prometaphase arrest. With lower levels of damage, however, sufficient spindle function is recovered to achieve chromosome orientation after a delay. This satisfies

the kinetochore-associated SAC, leading to local APC/C activation and chromatid disjunction. However, we propose that continued damage signaling though Chk2 maintains SAC function in other regions of the cell, blocking Cyclin B destruction in the cytoplasm and on the spindle, thus blocking mitotic exit. In this model, the SAC is not "reactivated" during anaphase. Instead, the block to Cyclin B destruction leads to SCC reassociation with kinetochores, which is observed when non-degradable Cyclin B is expressed in embryos (Parry et al., 2003). Supporting this model, Cyclin B levels normally decline before anaphase onset, but appear to remain high through metaphase following DNA damage. If the SAC were reactivated during anaphase, we would expect to see a normal decline in Cyclin B prior to anaphase, when chromosomes aligned and the kinetochore associated SAC was satisfied.

It is unclear how Chk2 activates the SAC to block mitotic exit. Key SCC components and targets, including Mad2 and Cdc20, dynamically associate with kinetochores. Chk2 also localizes to the kinetochore region, suggesting that Chk2 could modify and activate one or more SCC proteins as they transiently associate with kinetochores. However, it seems likely that kinetochore bound Chk2 could activate SCC components controlling Cyclin B destruction, while allowing SAC inactivation in the same domain, allowing chromatid separation. However, SCC proteins and Chk2 also localize to centrosomes, raising the possibility that damage dependent SAC activation takes place at the spindle pole. In supports of this model, SCC proteins were not detected on centrosomes during arrest both before and after the M-A transition, in contrast to localization at the centrosomes in normal

conditions. The dynamics of Chk2 and passenger protein localization during mitosis are strikingly similar. Moreover, Chk2 remains associated with kinetochores during DNA damage induced anaphase arrest, and INCENP retains centromere association though anaphase in the presence of non-degradable Cyclin B (Parry et al., 2003). These observations suggest that Chk2 may control mitotic exit and spindle function by modulating the activity of the passenger protein complex. The substrates for Chk2 during damage induced mitotic arrest, however, remain to be been defined.

We speculate that the biological function of this mitotic damage response is to maintain DNA integrity. Cells that arrest in mitosis indefinitely are removed from the pool of proliferating cells. The fate of cells that exit mitosis after a delay is less clear. However, in the syncytial embryos the resulting nuclei invariably dissociate from the cortex and therefore do not contribute to the blastoderm embryo (Takada et al., 2003). In somatic cells, we speculate that damage induced mitotic delays may be followed by senescence or apoptosis, which would also limit propagation of damage induced mutations.

MATERIALS AND METHODS

Mutant strains

Oreogon R was used for wild type control. w^{1118} also was used for control since most strains analyzed were w^{1118} in the background. mnk^{6006} (Abdu et al., 2002), $grp^{fs(A)4}$, $mei41^{D3}$ (Sibon et al., 2000; Sibon et al., 1997), 309^{D2}, 309^{D3} (Kusano et al., 1999), $mad2^{p}$ (Buffin et al., 2007), and $zw10^{S1}$ alleles were tested for defects in DNA damage-induced mitotic delays. Using FRT/FLP system (Chou and Perrimon, 1996), homozygous *zw10* germ-line clone was generated following the procedures previously described (Williams and Goldberg, 1994). To obtain embryos for microinjection or immunostaining, the homozygous mutant females were crossed with heterozygous male siblings. To determine the dynamics of proteins following DNA damage by live analysis, GFP-Mei-S332 (Moore et al., 1998), GFP-Fizzy (Huang and Raff, 1999), GFP-Mad2 (Buffin et al., 2005), and GFP-MPS1 (Fischer et al., 2004) were tested.

DNA damaging drugs/replication inhibitors

The DNA damaging drugs used include camptothecin (Sigma), etoposide (Sigma), bleomycin sulfate (Sigma) and aphidicolin (Sigma).

Microinjections and time-lapse confocal microscopy

For *in vivo* analysis of the effects of DNA damaging agents, embryos were injected or treated as follows. A collection of 0-2 h old embryos was hand dechorionated, arranged in a line on an adhesive-coated coverslip and dehydrated for 5 min on a bed of Drierite in a petri-dish (Theurkauf and Heck, 1999). The DNA damaging drugs were mixed with Oligreen (Molecular Probes, at 1:20 dilution) and Rhodamine-labeled tubulin (Cytoskeleton, at 5mg/ml) to observe chromatin and microtubules, respectively, and injected manually into the embryos. Immediately following the injections, live confocal imaging was initiated using a Leica TCS-SP inverted laser-scanning microscope. The frames were captured at 10 s intervals. For inflicting UV damage, DAPI was mixed with Oligreen and rhodamine-labeled tubulin and the injected embryos were briefly shined with UV light through the objective lens during the course of imaging.

Immunostaining

Immunostaining was performed as described previously (Theurkauf, 1994). For treating with a drug, embryos were bleach dechorionated, and transferred to a 1:1 mixture of octane and Robb's buffer containing the drug at a desired concentration (Theurkauf and Heck, 1999). Antibodies used for immunostaining were anti-Cyclin B (santa cruz, 1:250) and Mouse anti-Histone (1:200, Sigma). The immunostained embryos were analyzed using Leica TCS-SP inverted laser-scanning microscope.

Dodecasatellite DNA Probe Preparation

A 450bp dodecasatellite fragment was obtained by PCR amplification of plasmid DNA (generously provided by Alfredo Villasante) using T7 and T3 primers. To generate smaller fragments, amplified DNA was enzymatically digested with HaeIII, MspI or Sau3A1 (in PCR buffer conditions) overnight at 37˚C. Following overnight digestion, a 30 min digestion with RsaI was performed. The small fragments were precipitated with 0.1 volume of 3M sodium acetate, 2.5 volumes of 100% ethanol and 0.1mg/ml glycogen (1 μl -20mg/ml stock/ 200μl). The solution was placed at -80˚C for 1 hour followed by centrifugation for 30 min at 4˚C and the resulting pellet was washed with 75% ethanol and air-dried before continuing. The DNA fragments were labeled using ARES™ Alexa Fluor® 555 DNA Labeling Kit (Invitrogen) according to the manufacturer's instructions. The labeled DNA fragments were precipitated as above and resuspended in dH_2O.

Fluorescent In situ hybridization (FISH)

W^{1118} and *mnk* embryos were prepared as described previously (Theurkauf and Heck, 1999). Procedure for FISH was slightly modified from a protocol described previously for mitotic chromosome (Abdu et al., 2002). Hybridized embryos were washed with PBST and subject to immunostaining as described above using DM1A-α-tubulin (sigma, 1:500).

CHAPTER III

CONCLUSIONS

I have characterized cytological and molecular mechanisms for DNA damage-induced mitotic delays in early *Drosophila* embryos by performing *in vivo* analysis. I have shown that DNA damage triggers mitotic delays and arrest at either metaphase or anaphase, which appears to be dependent on the degree of DNA damage. Moreover, I determined that Chk2 and Mad2 mediate Cyclin B stabilization to block mitotic exit upon DNA damage.

In contrast to a previous report (Mikhailov et al., 2002), I found that centromere fragmentation is not sufficient for SAC activation. In addition, I also found that SAC components and Cdc20 dissociate from kinetochores, and rapidly relocalize and remain at kinetochores in a Chk2-dependent manner during DNA damage-induced anaphase arrest. Taken together, I have shown that DNA damage triggers Mad2-mediated mitotic arrest at both metaphase and anaphase through Chk2 in *Drosophila* embryos.

Cyclin B stabilization triggers mitotic arrest in response to DNA damage

Sequential degradation of Cyclin A, B and B3 are required for mitotic progression in *Drosophila* embryos (Sigrist et al., 1995; Su et al., 1998). Is

degradation of other Cyclins blocked in response to DNA damage, in addition to Cyclin B? We have recently shown that DNA damage caused by incomplete DNA replication triggers the accumulation of Cyclin A through active Chk2 during syncytial divisions (Takada et al., 2007). I also determined Cyclin B3 is stabilized at centrosomes and spindles following DNA damage in *Drosophila* early embryos (appendix III). Taken together, DNA damage appears to block degradation of all Cyclins including Cyclin B, Cyclin A and Cyclin B3.

Does Cyclin A and B3 stabilization block mitotic exit? Non-degradable Cyclin A, B and B3 arrest cell cycle at prometaphase, anaphase, and late anaphase/telophase, respectively (Sigrist et al., 1995; Su et al., 1998). It is possible that Cyclin A accumulation triggers prometaphase arrest and Cyclin B accumulation triggers anaphase arrest. However, recent data showed that overexpression of non-degradable Cyclin B triggers metaphase arrest (Wolf et al., 2006), suggesting Cyclin B may be a major determinant among Cyclins for mitotic arrest. In addition, Edgar et al., showed that a spindle inhibitor, colcemid blocks Cyclin B degradation, but not Cyclin A, leading to metaphase arrest (Edgar et al., 1994). They proposed that Cyclin A appears to be degraded by fully active Cdk1 following its temporal accumulation. We also observed that high levels of Cyclin B lead to mitotic arrest in response to DNA damage. Conversely, no mitotic arrest phenotypes were observed when Cyclin B levels were dropped. Taken together, it seems that stable Cyclin B is sufficient to cause mitotic arrest.

Mad2-independent mitotic delay in response to DNA damage

Mutations in *mad2* abolished mitotic arrest after DNA damage in early *Drosophila* embryos, consistent with previous reports in mammals. However, I observed that the mitotic delay accompanies centrosome inactivation and defects in spindle assembly in damaged *mad2* mutant embryos. It is possible that the mitotic delay in *mad2* mutants is secondary to spindle disruption, since treatment with microtubule depolymerizing drug also triggers mitotic delay in these embryos. Alternatively, a SAC-independent machinery may be activated and trigger mitotic delay. To test these possibilities, live analyses was performed following double injection with colchicine and CPT into *mad2* mutant embryos. I found that double injection causes a longer mitotic delay than either single drug injection in *mad2* embryos, suggesting that a SAC-independent mechanism may be responsible for the mitotic delay in the presence of DNA damage. However, the difference is mild which makes it difficult to interpret.

It is possible that mad2-independent residual SAC proteins sense spindle disruption triggered by DNA damage. We observed that mutations in *zw10* cause mitotic delay with spindle disruption, suggesting that Zw10 is not required for mitotic delay. Recently, BubR1 was reported to activate the SAC in a Mad2-independent manner after chromosomes are aligned, although Mad2 plays a critical role to activate SAC by unattached kinetochore (Orr et al., 2007). We observed DNA damage-

induced mitotic delay before chromosome alignment in *mad2* mutant embryos, and it appears that BubR1 is not a player.

Centrosomin (CNN) is a core component of centrosome. Mutations in *cnn* trigger loss of centrosome function and formation of anastral mitotic spindles, leading to mitotic delay. Phenotypes of *cnn* mutants are very similar to those in DNA damaged syncytial embryos (Vaizel-Ohayon and Schejter, 1999). Interestingly, Buffin et al., reported that mutations in *mad2* completely suppress the mitotic delay caused by *cnn* mutation (Buffin et al., 2007). By contrast, they observed a mitotic delay in 18% of control *mad2* mutant neuroblast cells suggesting a SAC-independent machinery triggers a mitotic delay in *mad2* mutants neuroblasts. Taken together, I propose that DNA damage also activates a SAC-independent machinery that controls metaphase and anaphase transition, such as APC/C components, proteosome, or Cyclins.

Dose Chk2 function as a kinase to trigger mitotic response following DNA damage?

Chk2 is a functionally conserved kinase that phosphorylates its substrates to arrest the cell cycle, and it is involved in apoptosis following DNA damage as part of the conventional checkpoint pathways. Since phosphorylation events are critical for mitotic progression, we assume that Chk2 kinase activity is presumably required for mitotic delays.

To address the mechanism of Chk2 signaling to mitotic machinery and to test Chk2 kinase activity, we generated transgenic flies expressing either Drosophila Chk2 or Chk2 kinase deficiency mutant (Chk2KD), both fused to GFP Chk2KD as described previously (Peters et al., 2002). In neuroblast, Chk2 phosphorylates p53 which leads to DNA damage-induced apoptosis (Peters et al., 2002). Chk2 kinase activity is required for p53-dependent apoptosis since Chk2KD does not phosphorylate and activate p53. Surprisingly however, our preliminary data show that, in addition to GFP-Chk2 WT, GFP-Chk2KD rescues all mitotic defects in response to DNA damage in mnk mutant embryos (Takada S. and Theurkauf W, personal communication). Furthermore, human Chk2KD also rescues centrosome inactivation following DNA damage in mnk mutant background (Appendix I). Is Chk2 kinase activity dispensable for activation of the mitotic machinery in response to DNA damage? We observed Chk2 phosphorylation in response to DNA damage generated by incomplete DNA replication (Takada et al., 2007). In addition, we observed that γ-tubulin and CNN are modified in a Chk2-dependent manner in the presence of DNA damage (Appendix II). These suggest that active Chk2 modifies γ-tubulin and CNN. However, it is formally possible that the modified Chk2 (phosphorylated Chk2) without kinase activity interacts with and controls an unknown intermediate, which in turn regulates γ-tubulin and CNN modification. Unlike in ovary where Chk2 is abundant, embryos during early developmental stages may tightly regulate Chk2 protein levels for accurate signal transduction in response to DNA damage. Since overexpressing Chk2KD may generate an artificial cellular

situation, and thus being able to rescue the mnk phenotype, Chk2KD expression driven by its own Mnk promotor may be required to determine whether kinase activity is involved in this process.

Chk2-mediated parallel pathways may lead to anaphase arrest during mitosis in response to DNA damage

We have observed three different phenotypes in response to DNA damage mitotic delay, metaphase arrest and anaphase arrest accompanied by centrosome inactivation and spindle disruption. High dosage of DNA damaging drugs trigger spindle disruption and chromosome alignment failure leading to metaphase arrest. It appears that Chk2-dependent centrosome inactivation and anastral mitotic spindles lead to failure of chromosome bi-orientation that activates SAC. By contrast, in response to lower dosage of DNA damage, the spindle function is sufficient to align chromosomes following the prolonged mitotic delay. Subsequently, SAC is inactivated and chromatids are separated by active APC/C to initiate anaphase. Therefore, it appears that, in addition to SAC activation by the unattached kinetochore, a Chk2- and SAC-mediated machinery is activated to block mitotic exit in response to DNA damage.

SAC relocalization at kinetochores after anaphase initiation implies that SAC is probably reactivated to arrest at anaphase following DNA damage. Murray and coworkers showed that overexpression of Mps1 after chromosome segregation triggers anaphase arrest in a Mad2-dependent manner (Palframan et al., 2006),

suggesting that the SAC can be reactivated during anaphase. In this model, as soon as chromosomes are aligned, Cyclin B and securin are degraded. However, Chk2 reactivates the SAC, which causes cell cycle arrest during anaphase.

To test this model, we examined changes in Cyclin B levels at the M-A transition following DNA damage. Cyclin B destruction starts before chromosomes segregation in the normal condition. We carefully examined the Cyclin B levels on the spindle and centrosome and found that Cyclin B remains abundant even when chromosomes are aligned and separated. This suggests that Cyclin B level may not change at M-A transition and that SAC is probably not reactivated for anaphase arrest.

The other possibility is that both Chk2 and SAC continuously trigger inhibition of Cyclin B degradation in non-kinetochore regions such as cytoplasm and spindles. Recently, it was proposed that cytosolic SAC components (SCC) regulate mitotic timing, while kinetochore binding SCC controls the spindle checkpoint (Meraldi et al., 2004). Moreover, Buffin and coworkers suggested that Drosophila Mad2 functions for mitotic timing and spindle checkpoint (Buffin et al., 2007). These data support the model in which cytosolic SCC may be involved in blocking Cyclin B degradation. How does Chk2 maintain cytosolic SAC activity in response to DNA damage? It might be that unknown intermediate helps crosstalks between Chk2 signaling and SAC or that Chk2 directly activates cytosolic SAC at centrosomes or spindles. In this model, SCC relocalization at kinetochore is a secondary sequence of high Cdk1 activity. Even though chromosomes are segregated, high Cdk1 activity

maintains a prometaphase-like characteristic including spindle dynamics and microtubule binding motors to recruit SAC proteins at kinetochores. For example, merotelic chromosome attachment (bi-polar attachment on single kinetochore) may be generated by high Cdk1 activity, and recruit SAC to kinetochores through Aurora B. Although we could not detect kinetochore and microtubule attachment with our *in vivo* resolution, we often observed that a subset of separated chromosomes move back to metaphase plate, suggesting that merotelic attachment are generated after chromosomes segregation during anaphase arrest. Moreover, the merotelic attachment was observed in non-degradable Cyclin B expressing cells (Parry et. al., 2003).

Taken together, we hypothesize that two distinct pathways control mitotic delays in response to DNA damage. One is that Chk2-dependent centrosome inactivation and spindle disruption triggers kinetochore-dependent SAC activation. The other is that Chk2 continuously triggers activation of cytosolic SAC which prevents APC/C dependent Cyclin B degradation.

What are the Chk2 targets?

γ-TuRC complex.

The centrosome, a microtubule organizing center, consists of centrioles and pericentriolar material (PCM). A number of proteins exist in PCM including the γ-tubulin ring complex (γ-TuRC) and many mitotic regulators. γ-TuRC is the microtubule nucleation center (Doxsey et al., 2005). Chk2 is found on the centrosomes throughout mitosis and interphase in normal conditions and in the

presence of DNA damage. DNA damage triggers defects in centrosome integrity and functions in a Chk2-dependent manner in syncytial embryos (Takada et. al., 2003). γ-tubulin and Dgrip84 are lost from centrosomes following DNA damage during mitosis, but centrosomin (CNN) remains at centrosomes (Sibon et. al., 1999). However, depletion of CNN in *Drosophila* results in anastral mitotic spindles, suggesting that CNN is required for centrosome formation. Our preliminary data from 2D gel analyses suggest that Chk2 modifies γ-tubulin and CNN in response to DNA damage (Appendix II). We are currently investigating whether Chk2 induces changes in the γ-TuRC composition following DNA damage to inactivate centrosome function.

SAC components

We demonstrate that DNA damage triggers Mad2-mediated mitotic arrest through Chk2. Like SAC components (SCC) and Cdc20, Chk2 localizes at kinetochores until anaphase is initiated. However, Chk2 is not required for spindle damage-induced SAC activation. DNA damage specifically triggers Chk2 and SAC-dependent mitotic arrest. In the absence of Chk2, SAC cannot induce mitotic arrest and cannot relocalize at kinetochores after anaphase initiation, suggesting that Chk2 induces SAC activation in response to DNA damage. Chk2 may recruit SCC at kinetochores, or control the kinase activity of SAC proteins to activate SAC in response to DNA damage. In normal condition, SAC activity is regulated by the SCC localization at kinetochores as well as kinase activity.

To test for a genetic interaction between Mad2 and Chk2 (Mnk), we attempted to generate *mnk;mad2* double mutants. *mad2* and *mnk* single mutants are viable and fertile. Surprisingly, *mnk;mad2* mutants die during all developmental stages including early embryo stage, larva and pupae stage. Does Chk2 have functional redundancy with Mad2? Our preliminary data suggest that neuroblasts in double mutants do not contain more abnormal mitotic cells and aneuploids than those in wild type or single mutants, implicating that regulation of cell cycle is not affected by the double mutations. Currently, we do not understand why double mutations cause the lethality. Alternatively, we are testing interaction between SAC proteins and Chk2 via biochemical approaches.

Cdc20

Cdc20 phosphorylation is required for SAC activation (Chung and Chen, 2003). In budding yeast, Chk1 and Chk2 inhibit Cdc20 interaction with APC/C in response to DNA damage. To determine whether Chk2 controls Cdc20 to activate SAC, I performed biochemical assays and found that Chk2 does not interact with Cdc20. However, it is possible that Chk2 may transiently interact with and control Cdc20. I also examined Chk2-dependent Cdc20 modifications in response to DNA damage by performing 2D gel analysis. However, Cdc20 appears to be modified in a Chk2-independent manner following DNA damage (Appendix II).

Polo

Polo controls all mitotic events including Cdk1 activation, spindle assembly and centrosome maturation, and mitotic exit through APC/C activation (Glover, 2005). In *Drosophila*, Polo localizes at the centrosome, kinetochore and midbody, similarly to Chk2 (Moutinho-Santos et al., 1999). Interestingly, depletion of Polo caused several mitotic defects in neuroblast including loss of γ-tubulin and CP190 from centrosomes and mitotic arrest after chromosomes separation (Donaldson et al., 2001). These phenotypes are very similar to those of syncytial embryos upon DNA damage. Moreover, Polo-like kinase in mammals is inactivated by ATM following DNA damage to block progression into mitosis (van Vugt et al., 2001). These data suggest that Chk2 may negatively regulate Polo in response to DNA damage. Since null mutation in polo is lethal, we were not able to use strong *polo* mutants in syncytial embryos. Although I observed that DNA damage triggers Polo loss from centrosome and accumulation at kinetochores (Appendix III), recent preliminary data from 2D gel analysis suggests that DNA damage triggers Polo modification in a Chk2-independent manner (Appendix II).

Aurora B

Aurora B is a component of chromosomal passenger complex (CPC). Depletion of Aurora B triggers SCC accumulation leading to mitotic arrest (Kallio et al., 2002). On the contrary, Aurora B appears to be required for Mad2 and BubR1 accumulation following spindle damage (Ditchfield et al., 2003). Although the

precise mechanism is not known, Aurora B regulates SAC activity. Specifically, Aurora B has been reported to sever the bi-polar attached microtubules on a single kinetochore, generating unattached kinetochores that activate SAC (Pinsky et al., 2006; Tanaka et al., 2002). Interestingly, the dynamics of Chk2 and Aurora B localization during mitosis are similar. Non-degradable Cyclin B expression has been shown to trigger anaphase arrest in *Drosophila* cells (Parrey et al., 2003). Aurora B and INCENP consistently localize on kinetochores during anaphase arrest in non-degradable Cyclin B expressing *Drosophila* cells, similar to Chk2 localization in DNA damage induced anaphase arrest embryos.

Although our preliminary data suggest that Chk2 modifies γ-tubulin and CNN, but not Cdc20 and Polo, following DNA damage, we do not understand how Chk2 activates the SAC. To better understand the mechanism of DNA damage-induced mitotic delays, we have been performing biochemical approaches, including GST pull-down, immunoprecipitation, sucrose gradient and 2D gel analysis to identify Chk2 substrates.

Chk2 has a critical role in DNA damage-induced mitotic delay in *Drosophila* syncytial divisions. However, other reports suggest that Chk1 mediates the mitotic delay following DNA damage in fission yeast, mammals, and later stage of *Drosophila* embryos. By contrast, we found that human Chk2 triggers centrosome inactivation and mitotic delays following DNA damage in Drosophila embryos (Appendix I). Moreover, I observed that Human Chk2 rescues all mitotic defect in *mnk* mutant embryos following DNA damage (Appendix I). Both Chk1 and Chk2 are

required for mitotic delay in budding yeast, and they function redundantly in the conventional checkpoint. Therefore, I hypothesize that the functional requirement for either Chk1 or Chk2, or both may depend on cell types and organisms.

Why does DNA damage trigger anaphase arrest? Since chromosomes are very condensed and segregated after M-A transition, it seems almost impossible for DNA repair to occur during the delays. Therefore, we speculate that it may be the last defense mechanism for the damaged cells to block propagation of the mutations to their daughter nuclei**.**

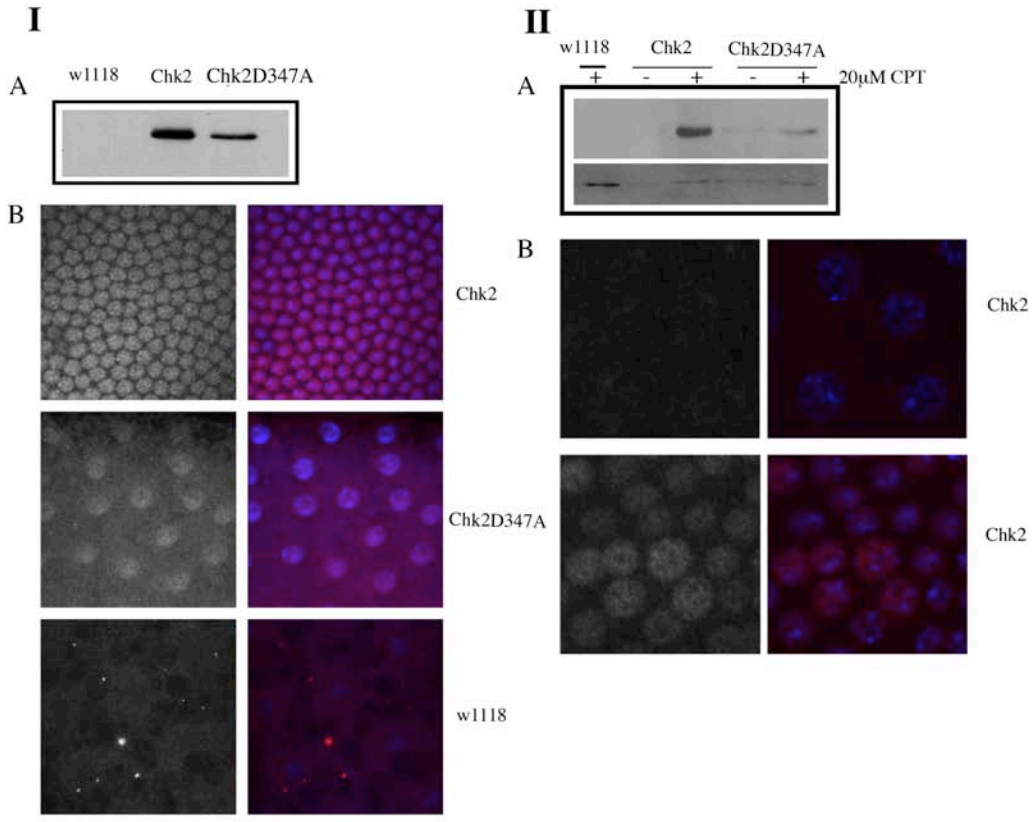
APPENDIX I

HUMAN CHK2 RESCUES DNA DAMAGE-INDUCED MITOTIC RESPONSE IN *MNK* MUTANT EMBRYOS

Mitotic response to DNA damage is frequently observed in organisms from yeast to mammals and we have demonstrated Chk2 being a central regulator for the response in early *Drosophila* embryos, consistent with reports in budding yeast. However, it has not been determined whether Chk2 is required for this process in other organisms, except a recent report where Chk2 acts as a negative regulator by studying fusion cells from interphase and mitotic cells (Castedo et al., 2004b).

Chk2 functions in the conventional checkpoint and is a structurally conserved kinase. Human Chk2 is 34% identical (45% similar) to *Drosophila* Chk2. To determine whether human Chk2 can function in *Drosophila* embryos and rescue the *mnk* phenotype, we first examined whether human Chk2 has capacity to delay during mitosis upon DNA damage in syncytial *Drosophila* embryos.

We used the UAS-Gal4 system to drive human Chk2 expression. Human Chk2 (67KDa) expression was determined by a human Chk2 antibody which does not cross react with *Drosophila* Chk2 (55KDa) (Figure 1 I.A). Human Chk2 did not affect viability and fertility of the transgenic flies. We subsequently examined its localization in *Drosophila* embryos and found it being localized at nucleus at interphase (Figure 1 I.B),

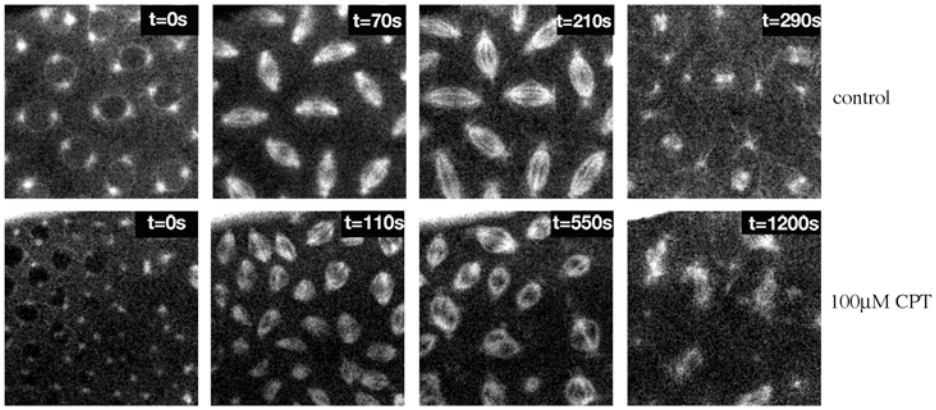


Appendix I Figure 1. Human Chk2 was expressed and phophorylated upon DNA damage in *Drosophila* embryos. (I) Westen detects Human Chk2 expression in Drosophila embryos (A), and immunostaning shows its localization in nuclei during interphase (B). **(II)** DNA damage triggers phosphorylation of human Chk2 and Chk2D347A. In merged images, DNA is shown in blue and Chk2 in Red.

consistent with the previous study in human cells (Lukas et al., 2003). During mitosis, Chk2 is uniformly distributed in cytoplasm.

Next, we determined whether human Chk2 is modified by DNA damage in embryos (Figure 1 II). Anti-phospho-Chk2-Thr 68 antibodies specifically recognized T68-phosphorylated Chk2 in Camptothecin (CPT)-treated embryos, but not in non-transformants, nor in control embryos (Figure 1 II.A), indicating that DNA damage triggers human Chk2 phosphorylation by *Drosophila* machinery. At interphase, T68-phosphorylated Chk2 was only found in the nuclei of damaged embryos (Figure 1 II.B). However, the antibody specificity was lost during mitosis, giving a false signal on centrosome in embryo that does not express human Chk2.

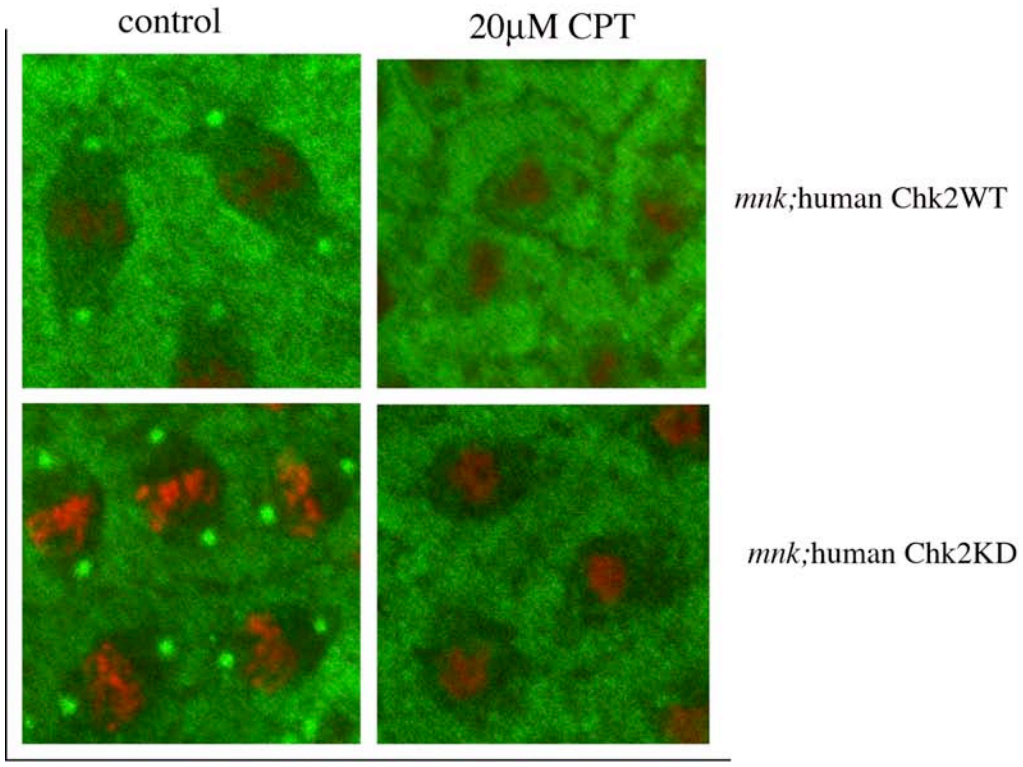
To test whether human Chk2 functions during mitosis following DNA damage, human Chk2 was expressed in *mnk* mutant embryos (Figure 2). We analyzed the duration of mitosis with time-lapse laser-scanning confocal microscopy following injection of R-tubulin (rhodamine-tubulin) and CPT into the embryos. In control embryos, human Chk2 did not affect cell cycle timing and mitotic spindle formation. However, we observed anastral barrel-shaped mitotic spindles and mitotic delay/arrest following DNA damage in *mnk*, indicating that human Chk2 can rescue DNA damage-induced mitotic defects in *mnk* mutants. To confirm that human Chk2 triggers loss of centrosome integrity in response to DNA damage, immunohistochemisty was performed (Figure 3). We found that DNA damage induces γ-tubulin loss in a human Chk2-dependent manner in *mnk* mutant embryos, indicating human Chk2 triggers centrosome inactivation in response to DNA damage



Appendix I Figure 2. Human Chk2 rescues mitotic defects following DNA damage in *mnk* embryos. Live anlysis was performed after R-tubulin injection without drug (control) or with drug in *mnk* embryos expressing human Chk2. In controls, cell cycle normally progresses with astral spindles. However, in damaged embryos, DNA damage blocks mitotic exit with severe spindle disruption, indicating that human Chk2 rescures DNA damage-induced mitotic defects in *mnk* embryos..

similar to *Drosophila* Chk2. Taken together, we suggest that human Chk2 may function to delay mitosis and to disrupt spindles in response to DNA damage. Currently, we are investigating a role for human Chk2 in mitosis in damaged human cells.

As a negative control, human Chk2 kinase deficient protein (Chk2D347A) was expressed in *mnk* embryos (Figure 1). Surprisingly however, we found that Chk2KD rescues centrosome inactivation during mitosis in response to DNA damage, a similar function to wild type human Chk2 (Figure 3). At present, it remains to be determined whether Chk2 kinase activity is required for its role in mitotic response upon DNA damage.



Appendix I Figure 3. Human Chk2 triggers centrosome inactivation following DNA damage in *mnk* mutant embryos. Control or damaged mnk embryos expressing human Chk2-WT or -KD were fixed followed by immunostaining. γ-tubulin is shown in green and DNA is in red. Both human Chk2 -WT and -KD induced loss of γ-tubulin in response to DNA damage.

Materials and methods

Construction of human Chk2

Plasmids containing human Chk2 cDNA or Chk2D347A cDNA were kindly provided by Steven Elledge. Chk2D347A was previously described (Matsuoka et al., 1998). Full length human Chk2 or Chk2D347A cDNA was amplified by PCR using oligos containing a Kpn site at the 5'end and XbaI site at the 3'end. The PCR fragments were inserted into pUASP vector (Rorth, 1998). Flies were transformed with the resulting constructs using standard techniques (Rorth, 1998). Two independent lines were generated for each construct.

Fly genetics

GAL-UAS system was used for human Chk2 expression (Rorth, 1998). The following stocks were used for the experiments; *mnk*:GAL4-VP16, *mnk* human Chk2/Cyo, *mnk*: human Chk2/TM3.

Microinjection and time-lapse confocal microscopy

For in vivo analysis of the effects of DNA damaging agents, the embryos were injected or treated as follows. A 0-2 h old embryos was hand dechorionated, arranged in a line on a adhesive-coated coverslip and dehydrated for 5 min on a bed of Drierite in a petri-dish (Theurkauf and Heck, 1999). The DNA damaging drugs were mixed with rhodamine-labelled tubulin (Cytoskeleton, at 5mg/ml) to detect microtubules and

injected manually in the embryos. Immediately following the injections, live confocal imaging was initiated using a Leica TCS-SP inverted laser-scanning microscope. The live image frames were captured at 10 s intervals.

Western blot

For western blot, 0-3hrs collected embryos were dechorionated by 50% bleach. Control embryos were directly homogenized after bleach. To induce DNA damage, dechorionated embryos were incubated in octane/Robb's medium for 10min, washed, and homogenized in 2X sample buffer. Human Chk2 is detected by mouse-anti-Chk2 antibody (DCS-273, 1:1000) (Lukas et al., 2003). Rabbit-anti-phosphop-Chk2-Thr 68 antibody (1:200) was kindly provided by Stern (Tsvetkov et al., 2003).

Immunostaining

Embryos were prepared as described previously (Takada et al., 2003). Mouse-γ-tubulin antibody (GTU88, 1:500) and TOTO3 (Molecular Probes, Inc) were used to detect γ-tubulin and DNA, respectively.

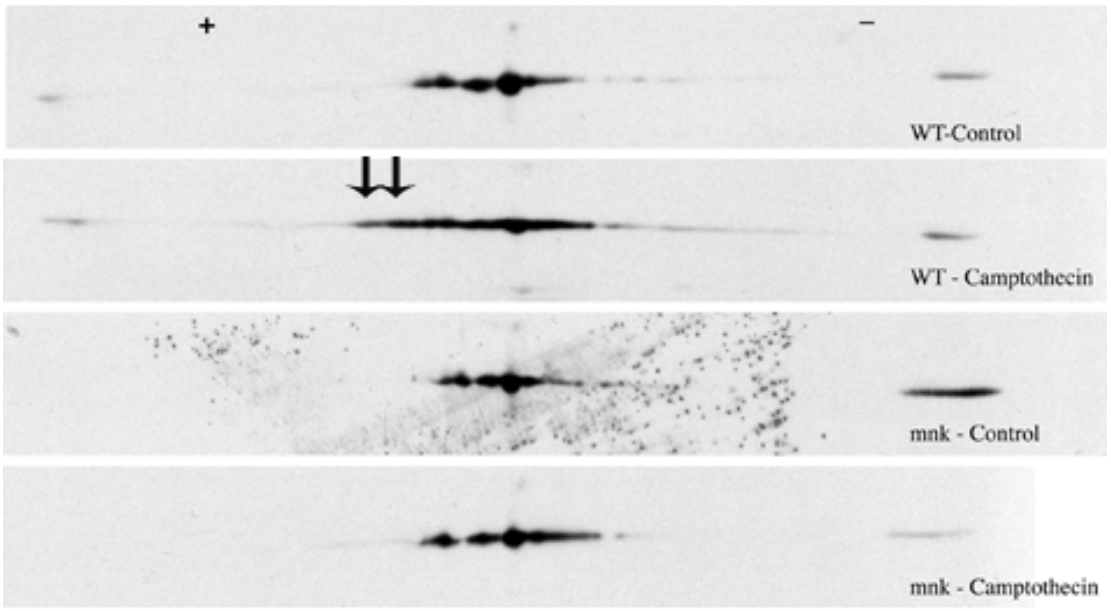
APPENDIX II

CHK2 MODIFIES γ-TUBULIN AND CNN IN RESPONSE TO DNA DAMAGE

To identify molecules that are modified by Chk2 in response to DNA damage, we performed 2D gel analysis followed by western blotting for candidates including Polo, Cdc20, Cyclin B, Zw10 (a SAC protein), and centrosomal proteins (γ-tubulin, CNN and Dgrp 84). The rational for the selection of these candidates are discussed in Chapter III. Total embryo extracts prepared from wild type and *mnk* embryos both with and without DNA damage were subject to 2D gel analysis to identify molecules regulated by Chk2 upon DNA damage. 4 sets of experiments for Cdc20 and γ-tubulin, 2 sets of experiments for Cyclin B, Zw10, and Dgrp84, and 1 set of experiment for CNN and Polo have been performed.

From the 1st set of experiment, we found Chk2-dependent Cdc20 modification in response to DNA damage. However, we were unable to detect such modification in the following 3 sets of experiments. In contrast, we found γ-tubulin being modified by Chk2 consistently upon DNA damage (Figure 1). This suggests that Chk2 may modify γ-tubulin upon DNA damage leading to centrosome inactivation.

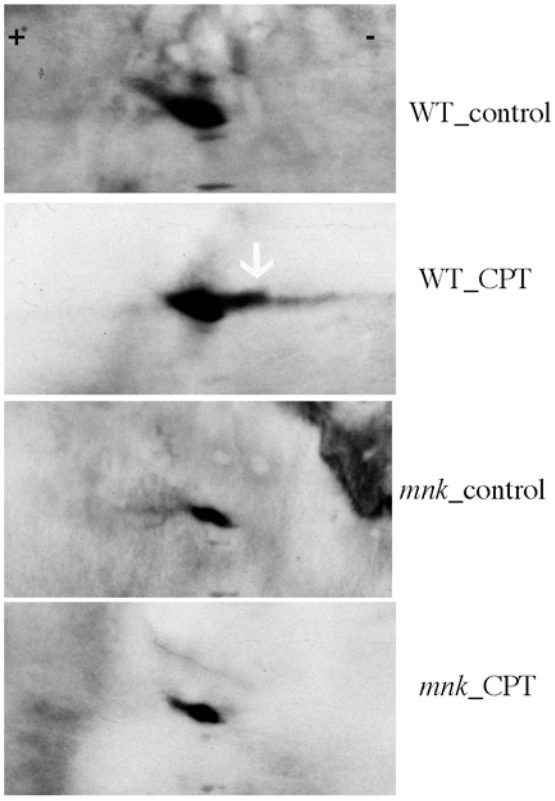
Although used for western blot following SDS-PAGE, Cyclin B antibody did not detect its antigen after 2D analysis, making our analysis impossible. Zw10, Dgrip 84 and Polo were found modified upon DNA damage. However, different isoforms were found in control *mnk* compare to control w^{1118}, making it hard to interpret.



Appendix II Figure 1. Chk2 triggers γ-tubulin modification in response to DNA damage. γ-tubulin was detected by western following 2D gel. Several isoforms of γ-tubulin exsit in WT control. DNA damage triggers γ-tubulin modification in a Chk2-dependent manner. Arrows indicate more positively charged γ-tubulins.

-

Interestingly, we found that CNN is modified by Chk2 upon DNA damage, although CNN remains on centrosome in the presence of DNA damage (Figure 2). Currently, we are investigating Chk2-dependent modification in the components of γ-TuRC complex upon DNA damage.



Appendix II Figure 2. Chk2 induces CNN modification upon DNA damage.

Materials and Methods

Preparation of embryos extracts

1-2 hour embryos were dechorionated in 50% bleach. For controls, dechorionated embryos were directly homogenized in Destreak Rehydration buffer (Amersham). For damaged extracts, embryos were first incubated in octane/Robb's medium containing 20 μM CPT for 10min, washed, and then homogenized in the buffer. Extracts were quickly frozen in liquid nitrogen and stored at -80°C and used for 2D gel analysis within five days.

2D gel analysis

2D gel analysis was performed according to the procedure described in Amersham manuals. For first dimension, 100-200μg sample was loaded on a immobilized pH gradient (IGP) strip (11 cm in length and pH3-pH10 range) followed by rehydration with rehydrating solution for 12 hours and ran 3 hours with Ettan IPGphor Isoelectric Focusing system. Subsequently, separated proteins by their own pH on the strip were equilibrated and ran on a 5-20% gradient SDS-PAGE gel for second dimension.

Western blot

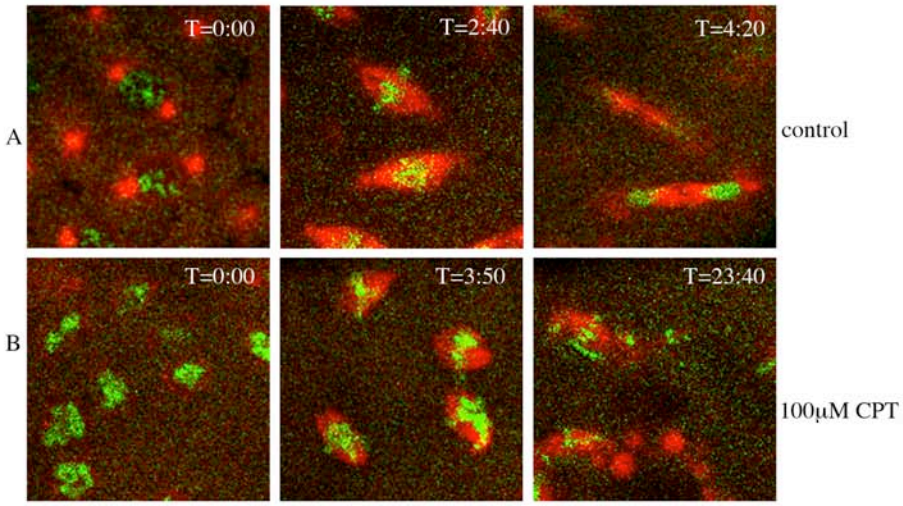
The following antibodies were used in this experiment; Mouse anti-γ-tubulin antibody (GTU88 1:500), Dgrip 84 antibody (1:2000), CNN antibody (1:500), mouse Polo antibody (1:200), Cyclin B antibody (santa cruz, 1:250) Cdc20 antibody (1:500) and Zw10 antibody (1:1000) (kindly provided by Raff J. and Glover M).

APPENDIX III

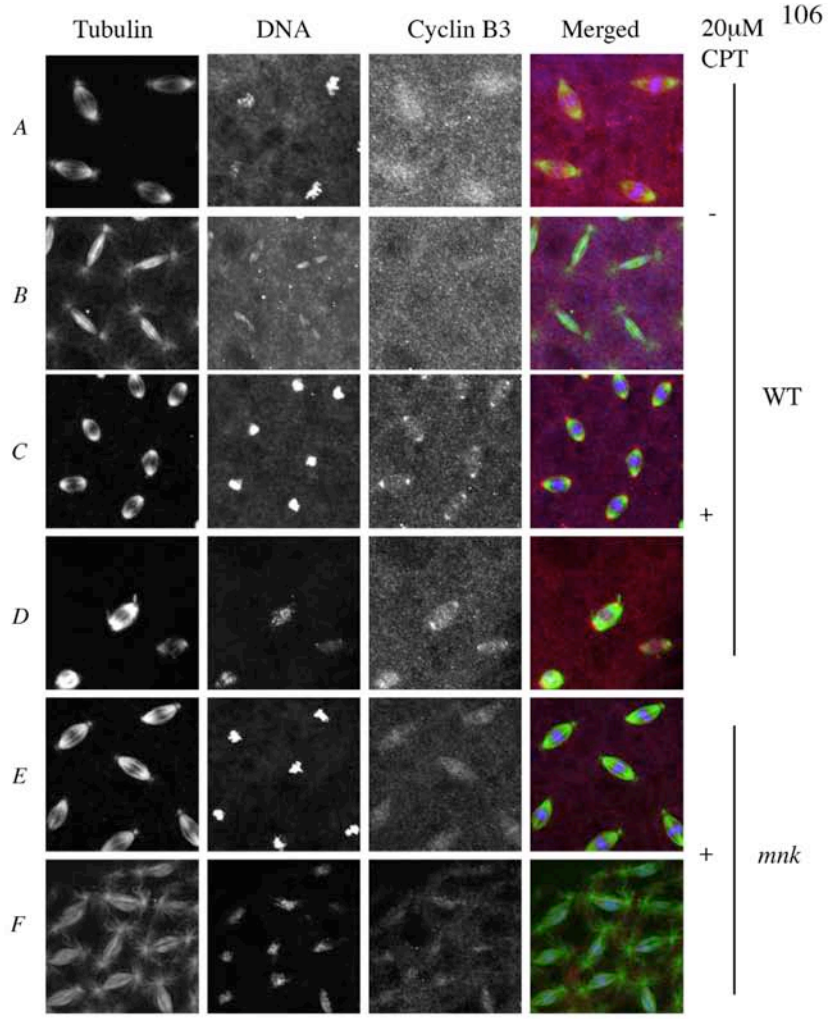
Here, I show additional supporting data that are not closely related, but mentioned previously in the main text of my thesis.

DNA damage triggers H2AX phosphorylation that amplifies DNA damage signal and is required for some DNA damage responses (Burma et al., 2001; Kang et al., 2005). H2Av, *Drosophila* homologue of H2AX is phosphorylated and required for apoptosis in response to DSBs (Leach et al., 2000). To determine whether H2Av phosphorylation is required for mitotic arrest in response to DNA damage, I injected CPT into emgryos mutant of the H2Av phosphorylation site (Figure 1). Mitotic arrest was normally observed in the mutant embryos, indicating H2Av phosphorylation is dispensable for mitotic arrest following DNA damage (Chapter II).

In *Drosophila*, Cyclins A, B and B3 controls mitotic progression (Parry and O'Farrell, 2001; Sigrist et al., 1995; Su et al., 1998). I found that Cyclin B is stabilized in a Chk2-dependent manner in response to DNA damage. We also showed that DNA damage caused by incomplete DNA replication triggers Cyclin A accumulation in a Chk2-dependent manner (Takada et al., 2007). To address whether DNA damage triggers Cyclin B3 stabilization, I performed immunoflourescence on drug treated or untreated embryos with Cyclin B3 antibody (Figure 2). Cyclin B3 accumulates on the spindles at metaphase and disappears at



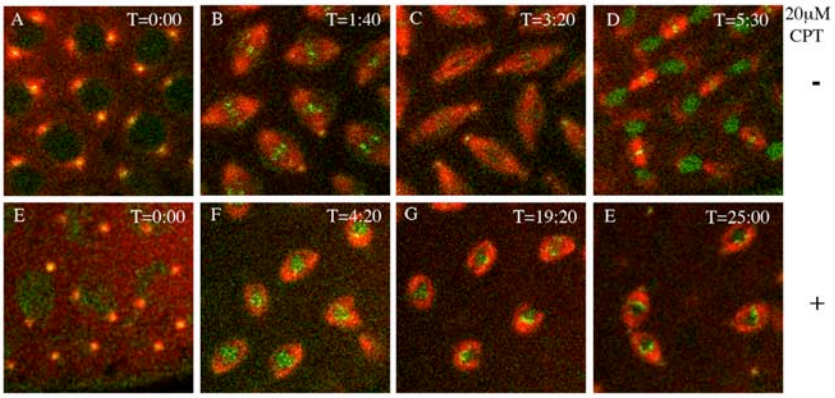
APPENDIX III Figure 1. H2Av phosphorylation is not required for mitotic arrest in response to DNA damage.
Embryos from H2Av phosphorylation site mutants were injected with Oligreen and R-tubulin without (A) and with 100uM CPT (B). Control H2Av mutant embryos have astral spindles and normal duration of mitosis (A). CPT injection triggers centrosome inactivation and mitotic arrest (B)



APPENDIX III Figure 2. DNA damage triggers Chk2-dependent Cyclin B3 localization on centrosome regions.
w^{1118} and *mnk* embryos were methanol fixed following 20uM CPT treatment (+) or without treatment (-), and labeled for tubulin (green), DNA (blue) and Cyclin B3 (red). In w^{1118} control embryos, Cyclin B3 localizes at spindles at metaphase (A) and diappears at anaphase (B). DNA damage induces Cyclin B3 localization on centrosom regions during metaphase (C) and anaphase (D). In CPT-treated *mnk* embryos, Cyclin B3 is not observed on spindle regions (E, F).

anaphase during syncytial divisions. However, with DNA damage Cyclin B3 localizes on the centrosomes during metaphase and anaphase, indicating that DNA damage triggers change cyclin B3 dynamics and accumulation (Chapter III). By contrast, the mutations in Chk2 suppress Cyclin B3 stabilization on the centrosome regions, indicating Chk2 is required for Cyclin localization on the centrosome regions in response to DNA damage.

Polo localizes at centrosomes, kinetochores and the midbody in *Drosophila* (Moutinho-Santos et al., 1999). Mitotic arrest after centromere separation was observed in *polo* mutant embryos (Donaldson et al., 2001), suggesting Polo may negatively function in mitotic arrest following DNA damage. However, to test this genetically is difficult since strong mutations in *polo* are lethal. To determine Polo localization following DNA damage, I performed live analysis in GFP-Polo expressing embryos. In control embryos, Polo localizes at centrosomes, kinetochores and the midbody (Figure 3A-D). However, DNA damage triggers loss of Polo from centrosomes when centrosome inactivation occurs. Moreover, I observed high accumulation of Polo on the kinetochores (Chapter III).



APPENDIX III Figure 3. DNA damage triggers Polo accumulation on kinetochores and its loss from centrosomes.

GFP-Polo expressing embryos were injected with R-tubulin with (+) or without (-) CPT. (A-D) Control embryos. Polo localizes on centrosome throughout mitosis. Polo localizes on kinetochores at metaphase (B) and disappears at anaphase (C). At late telophase, Polo is observed at midbody and nuclei (D). (E-H) In CPT injected embryos, Polo highly localizes on kinetochore during mitotic arrest. Polo is loss from centrosomes when centrosome inactivation occurs (F).

Materials and Methods

Fly stocks

These strains were used ; w^{1118}, *mnk*, P{His2Av$^{\Delta CTXc}$};*l*(3)His2Av^{810}/TM3 (Clarkson et al., 1999)

Live analysis

Live analysis was performed as described previously (Chapter II).

Immunostaining

The procedure of immunostaining was mentioned previously (Chapter II). Rabbit Cyclin B3 antibody (1:500) and Toto 3 were used to detect Cyclin B3 and DNA, respectively.

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