THE METAPHASE CHECKPOINT IN CELLS UNDERGOING MITOSIS WITHOUT
CHROMOSOME DUPLICATION

By

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Chinese hamster ovary cells (CHO) were arrested with hydroxyurea at the beginning of DNA synthesis. Subsequent treatment with caffeine induced cells to bypass S-phase and undergo mitosis with unreplicated genomes (MUG). Treated cells built a normal spindle and distributed unattached kinetochores to daughter cells. To determine if MUG cells obey the metaphase checkpoint, we used immunofluorescence to detect and localize known metaphase checkpoint and motor proteins. In addition, the drug taxol was used to stabilize microtubules in MUG cells. The localization of CENP- E, the presence of anaphase A, taxol arrest, and taxol release acted in a similar manner as in controls. The localization of kinesin differed from the controls and that of MAD2 was inconclusive. These results imply that MUG kinetochores behave similarly to controls and probably have an operational metaphase checkpoint.

Key words: metaphase checkpoint, CENP- E, MAD2, kinesin
DEDICATION

This manuscript is dedicated to my parents, Ray and Judy Johnson. When I was a child you pushed me to work hard and taught me right from wrong. When I was a teenager, you pulled your hair out trying to get me to study. When I was an undergraduate, you helped with physics problems over the phone at midnight. When I was a graduate student, you did as you have always done- supported, advised, encouraged, and loved. Thank you.
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CHAPTER I
INTRODUCTION

The Cell Cycle

The cell cycle is a series of events that takes place in order for division to occur. The cycle is composed of two events, interphase, which consists of G1, S, G2, and mitosis (Wise and Brinkley, 1997). Mitosis begins after G2 and consists of prophase, prometaphase, metaphase, anaphase, and telophase.

In prophase, the sister chromosomes condense and become visible and the nuclear envelope disappears. Afterwards, in prometaphase, the chromosomes attach to the spindle at the centromere and begin to congress to the metaphase plate. In metaphase, the chromosomes are aligned at the metaphase plate and the kinetochores face towards opposite poles of the spindle. Anaphase occurs in two stages. In the first, anaphase A, the sister chromatids separate and move toward the poles. Anaphase B consists of the elongation of the spindle. Finally, telophase occurs when the chromatids reach the poles and the microtubules disappear. After telophase, cytokinesis takes place and the two identical daughter cells are formed.

The cell is regulated through the cell cycle by proteins known as cyclins, which appear and bind to various cyclin dependant kinases (CDKs). The CDKs are perpetually active, but cyclins are synthesized and degraded. Mitosis begins with an accumulation of
cyclin B, which is synthesized in G2 and degraded after the metaphase-anaphase transition (Holloway et al., 1993). The cyclins, through interactions with the appropriate CDKs, are responsible for the cell cycling from one phase to the next.

The Kinetochore Structure

The kinetochore is a region on the chromosome which attaches to the microtubules. From this region, mechanical forces such as plus-end microtubule depolymerization, treadmilling of the microtubules, and motor proteins such as CENP-E, guide the chromosome through the stages of mitosis. The kinetochore is a trilaminar plate-like structure consisting of an outer region which binds to microtubules, an inner domain which attaches to the chromosome, and a middle domain which is electron lucent and of an unknown material (Brinkley et al., 1992; Rieder and Salmon 1998; Maiato et al., 2004; Lui et al., 2006). Fibrous projections, called corona, extend from the outer plate. The kinetochore itself is composed of a large number of various proteins, some constitutive, which remain on the centromere through the entire cell cycle, and some passenger, which localize and depart after the metaphase-anaphase transition (Earnshaw and Cooke, 1991). Alphoid DNA (α-satellite) is found on the kinetochore and thought to be the foundation on which the kinetochore is built.

Taxol and Microtubule Dynamics

Microtubules are made from the dimerization of α and β tubulin. The microtubules elongate outward from the minus end to the plus end of the spindle and some eventually connect with the kinetochore. Multiple microtubules connect with each
kinetochore forming a kinetochore bundle. Microtubules act in two manners, both
dynamic. The first is the continuous growing and shorting (or rescue and catastrophe) of
microtubules (Mitchison, 1984). This is known as dynamic instability. There is a GTP
region on the end of the microtubule. As long as this GTP cap remains, the microtubule
grows.

The second dynamic mannerism is the growing of the plus end and shortening of
the minus end (treadmilling) of the spindle microtubules. Both are critical for the
movement of kinetochores. Taxol suppresses both the rescue and catastrophe mechanism
by stabilizing the microtubules.

Tension at the kinetochore is important for the metaphase- anaphase transition
(Waters et al., 1998; Kelling et al., 2003). Before anaphase, sister kinetochores have
poleward movements which stretch the chromatin and produce tension at the kinetochore.
In the presence of high tension, microtubules are often in a polymerization state. When
the sister kinetochores separate at anaphase, the tension on the kinetochores is reduced.
This reduction in tension switches the microtubules into a depolymerization state which
causes the kinetochores to be pulled towards spindle pole (Maiato et al., 2004).

Taxol is an anti- cancer agent used in a variety of medical treatments. Taxol
stabilizes and prevents depolymerization on the plus end of the spindle microtubules
without inhibiting the disassembly at minus-ends. At high concentrations, taxol enhances
microtubule polymerization. In addition to microtubule polymerization and the
prevention of depolymerization, taxol was also found to reduce tension at the kinetochore
(Kelling et al., 2003; Waters et al., 1998). The combination of the reduction in
microtubule dynamics along with reduction of tension at the microtubule-kinetochore junction arrests the cell in metaphase.

The Metaphase Checkpoint

The metaphase checkpoint operates by inhibiting the anaphase promoting complex (APC). The APC is an E3 ubiquitin ligase which, when activated, targets the anaphase inhibitor, securin, for ubiquitination by the 26S proteasome. Securin binds and inactivates the protein separase. When released from securin, separase is activated, and cleaves a subunit of the cohesin complex. Cohesin is a protein which binds the sister chromatids together. When separase cleaves a cohesin subunit, the chromatids can be pulled apart and anaphase begins. The activated APC complex also leads to the degradation of cyclin B (Holloway et al., 1993). The APC is activated by the protein Cdc20 and Cdh1, but Cdc20 is the accessory protein necessary for the ubiquitination of the both securin and cyclin B.

One checkpoint protein necessary for APC inhibition is CENP-E, a passenger protein that binds to the outer kinetochore plate and the microtubules, accumulates in G2, and is degraded in telophase. After the metaphase-anaphase transition, CENP-E is found at the midplate (Cooke et al., 1997; Yen et al., 1991). CENP-E is a member of the kinesin family, and a plus-end directed motor protein (Chan et al., 2005).

BubR1 is a kinase active only during mitosis and bound to the outer kinetochore. It phosphorylates and binds to Cdc20, inhibiting the APC complex. CENP-E association with BubR1 is stimulated when microtubules are not attached to the kinetochore (Weaver et al., 2003). After BubR1 stimulates CENP-E binding to the kinetochore, the CENP-E
and BubR1 complex activates the BubR1 kinase which phosphorylates a variety of proteins including Cdc20, which is responsible for inhibiting the anaphase promoting complex. BubR1 also acts as a kinase on CENP-E. After CENP-E facilitates the binding of microtubules to the kinetochore, the BubR1 kinase activity is removed, Cdc20 is released, and anaphase begins (Mao et al., 2003). Thus, CENP-E is indirectly responsible for the metaphase checkpoint by silencing BubR1.

CENP-E is responsible for the capture of microtubules by the kinetochore and is essential for mitosis (Mao et al., 2003). When CENP-E is disrupted, the microtubule-kinetochore binding at aligned kinetochores is reduced, and those unaligned are severely reduced (McEwan et al., 2001). The kinetochore is normally dynamic; it captures and releases microtubules. Without CENP-E the kinetochore is thought to continue to release microtubules, but is unable to capture them efficiently, resulting in a net microtubule loss at the kinetochore.

Since CENP-E is critical for microtubule stability, and correctly aligned amphetamine microtubule-kinetochore attachments are important to tension maintenance, CENP-E is also critical for tension across the centromeres (McEwan et al., 2001).

Further, CENP-E is important for correct chromosome alignment (Yao et al., 2000; Cooke et al., 1997). When CENP-E is depleted in a cell, most chromatin arrives at the metaphase plate without CENP-E. However, while most kinetochores arrive at the correct location without CENP-E, multiple kinetochores are found near the spindle poles and are unable to move towards the plate, resulting in aneuploidy (McEwan 2001). In one line of mice, mitosis consistently resulted in aneuploidy because the organism was heterozygous for the CENP-E gene. The mice, perhaps due to aneuploidy, often showed
tumorgenesis. Homozygosity for the lack of CENP-E is lethal (Weaver et al 2007). Without CENP-E, chromosomes frequently fail to become bi-oriented, align properly, and pass through the metaphase checkpoint (Cooke et al., 1997).

Mad2 is a passenger protein found on the outer plate of those kinetochores that are not attached to spindles during prometaphase. In interphase and prophase, Mad2 is localized to the nuclear envelope but not to kinetochores. After the proper amphetelic kinetochore-microtubules attachments, Mad2 leaves the kinetochore (Waters et al., 1998). Mad1 is a checkpoint protein bound to the outer plate of the kinetochore. Mad-2 binds to Mad-1 and Mad-2 inhibits the APC complex by binding to Cdc20 (Fang et al., 1998).

Mad2 seems to act based on microtubule-kinetochore attachment rather than tension. When taxol is used to stabilize the microtubules, tension is lost, but Mad2 does not re-localize (Waters et al., 1998). Likewise, when micromanipulation was used to remove tension, Mad2 did not localize to the kinetochore. The protein exists in two different confirmations, an open form or O-Mad2, and a closed form or C-Mad2. The closed form has a high affinity for Cdc20 and thus is more effective in inhibiting the APC complex. A possible mechanism for the conversion is termed the exchange model. Mad-1 is thought to facilitate the conformational change. The binding sites of Mad-1 and Cdc20 are similar, so Cdc20 could be a competitor with Mad-1 for C-Mad-2. As long as there are unattached kinetochores and Cdc20 binding sites, C-Mad2 is thought to be generated to inhibit that APC activation. One acknowledged problem with this model is that the affinities for Mad1-Mad2 and Mad2-Cdc20 might be too high for O-Mad2 to be converted to C-Mad2 by Mad1 (Luo et al., 2002).
A second model is the Template model which states that Mad1 and C-Mad2 form a complex. O-Mad2 binds to the complex and is converted to the closed form. In the C-Mad2 form, it binds to Cdc20 and inhibits the APC and the initiation of anaphase (Fang et al., 1998).

A sub-theory related to the Template model suggests that the C-Mad2- Cdc20 complex can convert cytosolic O-Mad2 into the more Cdc20 compatible C-Mad2 (Chan et al., 2005). The signal would be greatly amplified with the combination of the Template theory and the complex conversion for Mad2.

Mad2 seems to be necessary for a working metaphase checkpoint. Waters (1998) depleted PtK1 cells of Mad2 and caused the cells to enter premature anaphase. The results were even more convincing in the taxol experiments. Waters used taxol to reduce the tension at the kinetochores and then depleted the cells of Mad2. Even though the cells had reduced tension they entered anaphase prematurely. In addition, knock-out mice for the Mad2 protein do not obey the metaphase checkpoint and displayed chromosome missegregation (Dobles et al., 2000). These data suggest that Mad2 is important for the metaphase checkpoint.

Kinesins are motor proteins that are critical for a variety of cellular processes. They use ATP hydrolysis to move various molecules along the microtubule. Many kinesins exist and they are necessary to the processes of mitosis. Mitotic kinesin is critical to the formation and function of the bi-polar spindle, chromosome segregation, chromosome transport, the metaphase checkpoint, and cytokinesis (Wittmann et al., 2001). In humans and mice there are more than 45 different kinesins that are differentiated by where the motor domain is located. Kinesins with the motor domain
near the N-terminus move towards the plus-end of microtubules are by far the most common, those with the motor domain near the C-terminus migrate towards the minus-end, and those with a motor domain located in the center are responsible for the destabilization of microtubule ends (Sarli and Giannis, 2006, Miki et al., 2001).

Kinesins (Sarli and Giannis, 2006, Miki et al., 2001) are essential in bipolar spindle formation. Cells depleted of Eg5 or Kif2A form monopolar spindles. Cells depleted of KifC1 form multipolar spindles that divide into daughters (Zhu et al., 2005).

Kinesins are necessary for chromosome congression to and alignment on the metaphase plate. Depletion of the kinesin proteins, KifC1, MCAK, Kif14, Kif18, and Kid disturb normal chromosome movement in mitosis. Depletion of MCAK or Kid leads to misaligned chromosomes. Cells depleted of Kid showed a delay in the metaphase-anaphase transition (Zhu et al., 2005).

When Kif14 is depleted, chromosomes congress to the metaphase plate, but then move towards the spindle and then back to the metaphase plate, causing an anaphase delay. In Kif18 depleted cells, chromatin did not congress, but otherwise showed similar movement. Both depletions lead to cells which pass the metaphase to anaphase transition, but are delayed, and then fail to achieve cytokinesis. Depletions of Kif14 and Kif18 lead to a reduction in tension at the kinetochore. These depletions show that these proteins are not essential for the kinetochore-microtubule attachments, but they are necessary for the alignment and congression of chromosomes to the metaphase plate (Zhu et al., 2005).

Kinesins are essential for anaphase and cytokinesis in mitotic cells. The Kif4A and Kif4B proteins are necessary for both anaphase and cytokinesis. Depletion of the kinesins Kif4A and Kif4B result in cells with over-elongated spindles during anaphase B.
and with chromosomes abnormally close to the spindle poles. These cells fail to complete cytokinesis (Zhu et al., 2005). The kinesins MKLP1 and MKLP2 are critical to cytokinesis. Depletions of either protein results in cells that failed to accomplish cytokinesis and instead form bi-nucleated cells (Zhu et al., 2005).

Mitosis with Unreplicated Genomes

Normal progression through the cell cycle is necessary for regulation of the cellular mechanisms critical for a successful cellular division. However, certain treatments have been shown to allow cells to bypass the normal progression of cellular events.

The xanthine, caffeine, is a drug that can change the normal series of cellular events. Unlike the normal cell cycle, in which cells go through interphase and then mitosis, replicating their DNA and dividing into daughter cells, the addition of the drug, caffeine, disrupts the standard cycle, forcing the cell into mitosis when the cell should be halted in S phase (Schlegel and Pardee 1986).

When a cell is irradiated in the G2 phase, the cell cycle is normally arrested due to damaged chromosomes and the activation of the DNA damage checkpoint. With the addition of caffeine, however, the cell proceeds through the cycle despite this damage (Jung and Streffer 1992).

Hydroxyurea inhibits the enzyme ribonucleotide reductase by destroying free radicals that are necessary for the enzyme to convert ribonucleotides to deoxyribonucleotides, which are necessary for the synthesis of DNA. This arrests the cell in early S phase due to the lack of nucleotides. In addition, hydroxyurea uncouples
centrosome duplication from the cell cycle (Balczon et al 1995). Even though the DNA remains unreplicated after hydroxyurea treatment, when caffeine was added the cells proceeded into mitosis (Schlegel and Pardee 1986).

These cells were named MUG cells or cells that undergo mitosis with unreplicated genomes (Zinkowski et al., 1989). MUG cells have extensive DNA damage, in which the DNA detaches from the kinetochore region and spreads genetic material throughout the cell. This produces centromere-kinetochore fragments (CKFs), or fragments of the kinetochore with a small amount of DNA (Wise and Brinkley, 1997; Brinkley et al., 1988). In Chinese hamster ovary (CHO) cells the kinetochores were shown to be highly fragmented (Zinkowski et al 1991). However, in HeLa cells the kinetochores are relatively intact with the DNA about 130-160 kbs in size and attached to the kinetochore region (Balczon et Al., 2002).

The CKFs bind to the microtubules and progress through mitosis normally, with the exception of anaphase. Anaphase B, or the considerable elongation of the spindle, is a phenomenon that occurs in control CHO cells. The spindle can elongate up to 20 μm from the normal metaphase spindle length of 9-10 μm in CHO cells. In MUG cells the average spindle length was 9 μm, and no anaphase elongation occurred (Wise and Brinkley, 1997).

Colcemid is a drug that destabilizes microtubules, halting the cell in prometaphase. When colcemid is removed, the cells proceed through mitosis. As in control cells, when the drug is added to MUG treated cells, they arrest and recover once the colcemid is removed (Brinkley et al., 1998). The spindles in MUG cells are smaller

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than normal at the beginning of recovery, but they quickly catch up to the average metaphase CHO spindle length (Wise and Brinkley, 1997).

When the cells divide into daughter cells, the DNA is fragmented and randomly dispersed. The numbers of kinetochores in each daughter MUG cell ranged from 14 to 64. When the daughter cells were observed, cells that displayed CKFs near the number of 44 segregated evenly into daughter cells more often than their cellular counterparts with fewer CKFs. However, CKFs that displayed lower fragmentation, and thus lower kinetochore number, were less likely to segregate their kinetochores evenly into daughter cells (Brinkley et al, 1998).

Experiments on CHO, PtK, and muntjak cells produced MUG cells (Zinkowski et al., 1989; Brinkley et al., 1988), but human cells failed to MUG cells. The levels of cyclin E and A were evaluated in HeLa cells compared to CHO cells. Cyclin E levels seemed to be equivalent, while levels of cyclin A were found to be lower in HeLa cells. When cyclin A was over-expressed, the HeLa cells became MUG competent (Balczon 2001).

Research Objectives

Observational

A. Observe the presence of anaphase in MUG cells

B. Observe presence or absence of CENP-E, kinesin, and MAD2 proteins in MUG cells through immunofluorescence technique

C. Observe the localization of CENP-E, kinesin, and MAD2 proteins in MUG cells through immunofluorescence technique

D. Compare the localization in MUG cells to that of controls
Experimental

A. Treat MUG cells with taxol to determine if arrest occurs

B. Release MUG cells from taxol to observe rate of recovery
Cell Culture Technique

The Chinese hamster ovary (CHO) cell line K1 was grown in McCoy’s 5A medium with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. Cultures were kept in a 37 °C incubator with an atmosphere of 5% CO₂. The cell cycle time of CHO cells from the beginning of interphase to the end of telophase is 12 hours. G1 takes 1 hour, S phase takes 7 hours, G2 takes 2 hours, and mitosis takes 1 hour (Wise and Brinkley, 1997).

MUG Technique

Cells were treated with trypsin-EDTA and plated on coverslips in 60 x 15 mm Petri dishes with culture medium. The Petri dishes were incubated for twenty-four hours and the medium replaced with medium containing 2 mM hydroxyurea (HU) for 20 hours. After 20 hours, the medium was removed and replaced with medium containing 2 mM HU and 5 mM caffeine. The cells were incubated for another 7 hours.
Taxol Block Technique

Cells were treated with trypsin-EDTA and plated on coverslips in 60 x 15 mm Petri dishes in culture medium. The Petri dishes were incubated for twenty-four hours and the medium replaced with medium containing 2 mM hydroxyurea (HU) for 20 hours. After 20 hours, the medium was removed and replaced with medium containing 2 mM HU, 5 mM caffeine, and 5 µg/ml taxol. The cells were then incubated for discrete time periods. After 7, 9, 11, 13, and 15 hours, the cells were removed and prepared for lysis and fixation. After immunofluorescence, the cells were viewed with confocal microscopy and percentages of cells with spindles and midbodies in the total cell population were obtained.

Taxol Release Technique

Cells were treated with trypsin-EDTA and plated on coverslips in 60 x 15 mm Petri dishes with medium. The Petri dishes were incubated for twenty-four hours and the medium replaced with medium containing 2 mM hydroxyurea (HU) for 20 hours. After 20 hours, the medium was removed and replaced with medium containing 2 mM HU, 5 mM caffeine, and 5 µg/ml taxol. The cells were incubated for 9 hours after which the taxol-medium was discarded and fresh culture medium was added. Coverslips were removed at 5, 10, 20, 30, and 40 minutes and prepared for lysis and fixation. After immunofluorescence, the cells were viewed with confocal microscopy and percentages of cells with spindles and midbodies in the total cell population were obtained.
Immunofluorescence Technique

**Cold Methanol Fixation**

For coverslips that were to be stained with anti-MAD2 (Mitotic Arrest Defective 2) and with CREST (calcinosis, Raynaud’s phenomenon, oesophageal dismotility, sclerodactyly, telangiectasia) antiserum, Petri dishes were removed from the incubator, and the coverslips containing the CHO cells were immersed in PBS (0.0137 M NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, and 1 mM KH$_2$PO$_4$, pH of 7.2) for 30 seconds, twice. Coverslips were then immersed in -20 ºC methanol for 10 minutes. The cover slips were submerged in PBS again for five minutes, twice. At this stage, the coverslips were either stored at 4 ºC in PBS, or stained.

**Formaldehyde Fixation**

Petri dishes were removed from the incubator, and the coverslips to be stained for CENP-E (centromere protein E) and kinesin were fixed in formaldehyde. The medium was removed from the Petri dishes and PEM + PEG (80 mM PIPES, 1 mM EGTA, 1 mM MgCl and 6H$_2$O, + 4% polyethylene glycol, pH of 6.9) was added for 30 seconds. After 30 seconds, the PEM + PEG solution was removed and 0.5% Triton-X 100 in PEM was added for 2 minutes. Then, the coverslips were rinsed in 3% formaldehyde in PEM for 20 minutes. After 20 minutes, the coverslips were rinsed twice in PBS for 30 seconds each. The coverslips were either stored in PBS overnight at 4 ºC, or stained.
Tubulin, CREST, CENP-E, MAD2, and Kinesin Staining

Parafilm was placed on a Petri dish and four drops of 100 μL of 1% bovine serum albumin (BSA) in PBS were then placed on the parafilm. One coverslip was placed face down on each drop and incubated for 45 minutes at 37 ºC. After incubation, the parafilm was cleaned and a 50 μL drop of 1% BSA in PBS with the correct dilution of primary antibody was added (Table 1). All primary antibodies were obtained commercially except anti-CENP-E, donated and tested by Dr. Tim Yen, and CREST antiserum, donated and tested by Dr. Bill Brinkley. The coverslips were placed faced down on the drops and incubated for 45 minutes at 37 ºC in a humidified chamber. After the 45 minutes, the coverslips were rinsed in PBS twice for 5 minutes each. The parafilm was cleaned with distilled water and a 50 μL drop of 1% BSA in PBS with the correct amount of secondary antibody was added (Table 1). The coverslips were placed faced down on the drops and incubated for 45 minutes at 37 ºC in a humidified chamber. After the 45 minutes, the coverslips were rinsed twice in PBS for 5 minutes each. DAPI (diluted 1:10,000) was added for 15 minutes and the coplin jars were stored in the dark for 15 minutes. Then, the coverslips were rinsed three times in PBS for 5 minutes each and mounted on slides using Vectashield® mounting medium.

Confocal Microscopy

After staining, the coverslips were observed using a Carl Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Microimaging, Inc.) on an Inverted Zeiss Axiovert 200 M light microscope. A Plan-Apochromat 63x/1.4 oil objective was used. The cells were scanned using a multi-track option to reduce bleed-through and
were captured with 512 x 512 pixel scans averaged 8 times. This produced a series of
through the cell and allowed for a three-dimensional image to be formed using Zeiss
LSM Image Browser version 3,5,0,233.
<table>
<thead>
<tr>
<th>antigen</th>
<th>Tubulin</th>
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CREST Stained Cells

In both control and MUG cells, CREST, an anti-kinetochore antiserum taken from patients with scleroderma, was used to stain cells to observe their progress through mitosis. Also, α-tubulin was stained in order to observe the microtubules to determine the spindle length. In control cells, the kinetochores are about 1 μm in size (Figure 1) and the cells divide to form daughters.

In MUG treated cells, however, differences were obvious. One of these is that the kinetochores in treated cells were much smaller and more numerous than those in the controls (Figure 2 and 3). Also, as shown in Figure 2, many, but not all, of the treated cells had multiple spindle poles. In anaphase, MUG cells failed to elongate into anaphase B (Figure 3). The DNA in these cells was fragmented and scattered away from the kinetochore region (Figure 4). While these differences did occur, the treated cells progressed from metaphase to anaphase to telophase and then divided into daughters (Figures 2, 3, and 4).
CENP- E Stained Cells

Control and MUG cells were both stained with HX1 antiserum that localized to CENP- E, a protein found on the kinetochore. They were also stained with anti- α-tubulin. In control cells, during prophase and prometaphase, CENP- E is bound to the kinetochore. Figure 5 shows the cell in very late prometaphase; the DNA was almost aligned at the metaphase plate and some CENP- E was still found on the kinetochore region. During anaphase, however, a transition occurred. In Figure 6, the DNA showed the cell to be in anaphase. The CENP- E delocalized from the kinetochore and was found instead at the metaphase plate in the region with the microtubules.

In MUG treated cells, the localization of CENP- E seemed likely to be similar to that found in controls. In Figure 7 and 8, both α- tubulin and CENP- E were stained in MUG cells. CENP- E was found at the center of the spindle towards the metaphase plate. Unlike the controls, however, CENP- E seemed to be more concentrated in the MUG cells.

To ensure that CENP- E was actually localizing to the kinetochore region in MUG cells as it did in control cells, DAPI, CREST antiserum, and HX1 antiserum were all applied. The DNA was found to be fragmented. Figure 9 displays a cell possibly in late prometaphase and the CENP- E was concentrated at the metaphase plate. Figure 10 shows a MUG cell, possibly in prometaphase or anaphase, in which CENP- E colocalized with the kinetochores. Figure 11 shows a cell possibly in prometaphase.
Kinesin Stained Cells

Control and MUG cells were stained with anti- general kinesin, DAPI, and anti- α tubulin in order to visualize kinesin localization. In control cells, no matter the stage of mitosis, kinesin was found to be scattered throughout the cell (Figure 12).

As shown previously with other staining, in MUG cells stained for kinesin, the DNA was found fragmented (Figure 13 and 14). Unlike control cells, in MUG cells kinesin localized in a different manner. Rather than the kinesin being evenly distributed, as in control cells, it was found to be concentrated in ‘spots’ (Figure 13 and 14). These spots did not appear to be colocalized with either the spindle or the DNA.

MAD2 Stained Cells

Both Control and MUG treated cells were stained with anti-Mad2, anti- α tubulin, and DAPI. In control cells MAD2 localized before the metaphase to anaphase transition. Figure 15 shows a cell in late prometaphase with MAD2 localization at the chromatin furthest away from the metaphase plate.

Unlike control cells, in MUG cells, MAD2 was undetectable. There was a faint red background visible in cells undergoing mitosis, but no sharp localization was evident (Figure 16). The DNA was found fragmented as with other MUG cells.

Taxol Block

As a control, MUG cells on coverslips were left in caffeine and HU for 7, 9, 11, and 13 hours. They were then removed and stained with CREST antiserum, DAPI, and anti- α tubulin. 900 to 1100 MUG control cells were observed at each time point. The
spindles and midbodies were observed at the various time points. After being in caffeine and HU for 7 hours, 14.9% of cells showed spindles while only 2.4% had midbodies. As the coverslips with cells on them were left in caffeine for longer periods of time, the numbers of spindles were reduced and the midbodies increased. After 9 hours, 3.2% of the population showed spindles, while 4.4% had midbodies.

The taxol treated MUG cells had taxol, caffeine, and HU added for 7, 9, 11, and 13 hours before the coverslips were removed and stained as the controls were. 700 to 5,000 cells taxol blocked cells were observed at each time point. After 7 hours, there were 12.3% spindles and 0.9% midbodies found in the cell population. After 9 hours, there were 15.8% of the total population with spindles and 0.48% with midbodies. After 11 and 13 hours, there were 14% and 7% of the population with spindles while only 1.1% and 0.47% had midbodies (Figure 17). Error bars denote calculated standard experimental error.

**Taxol Release**

Cells were grown on coverslips and after being immersed in HU for 20 hours, taxol, caffeine and HU were added for 9 hours. After 9 hours, fresh medium was added; cells were taken out at discrete time points, and stained with CREST antiserum, DAPI, and anti-α tubulin. 750 to 1250 cells were observed at each time point. After being released from the taxol medium for 5 minutes, 10.3% of cell population had spindles and 1.1% had midbodies. After 10 minutes, 4.67% of the population had spindles while 1.23% had midbodies. At 20 and 30 minutes after taxol release, 3.88% and 0.8% of cells had spindles while 3.3% and 5.7% displayed midbodies. After 40 minutes of release, 2%
of the population showed spindles and 5.83% had midboies (Figure 18). Error bars
denote calculated standard experimental error.
Figure 1. Confocal micrograph of an anaphase control cell stained with CREST. The fixed CHO cell was stained with anti-α tubulin (green) (top right panel) and CREST, an anti-kinetochore antiserum (red) (top left panel). The composite image (bottom left panel) shows the images merged.
Figure 2. Confocal micrograph of a metaphase MUG cell stained with CREST. The fixed cell was stained with anti-α tubulin (green) (top left panel) and CREST, an anti-kinetochore antiserum (red) (top right panel). The composite image (bottom left panel) shows the images merged.
Figure 3. Confocal micrograph of an anaphase MUG cell stained with CREST. The fixed cell was stained with anti-α tubulin (green) (top right panel) and CREST, an anti-kinetochore antiserum (red) (top left panel). The composite image (bottom left panel) shows the images merged.
Figure 4. Confocal micrograph of a telophase MUG cell stained with CREST. The cell was stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel), and CREST, an anti-kinetochore antiserum (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 5. Confocal micrograph of a prometaphase control cell stained with HX1. The fixed cell was stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel) and anti-CENP-E (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 6. Confocal micrograph of an anaphase control cell stained with HX1. The fixed cell was stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel) and anti-CENP- E (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 7. Confocal micrograph of a prometaphase MUG cell stained with HX1. The fixed cell was stained with anti-α tubulin (green) (top left panel) and anti-CENP-E (red) (top right panel). The composite image (bottom left panel) shows the images merged.
Figure 8. Confocal micrograph of a prometaphase MUG cell stained with HX1. The fixed cell was stained with anti-α tubulin (green) (top left panel) and anti-CENP-E (red) (top left panel). The composite image (bottom left panel) shows the images merged.
Figure 9. Confocal micrograph of MUG cell stained with HX1 and CREST. The fixed cell was stained with DAPI (blue) (top left panel), anti-CENP-E (green) (top right panel) and CREST, an anti-kinetochore antiserum (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 10. Confocal micrograph of MUG cell stained with HX1 and CREST probably in prometaphase or anaphase. The fixed cell was stained with DAPI (blue) (top left panel), anti-CENP-E (green) (top right panel) and CREST, an anti-kinetochore antiserum (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 11. Confocal micrograph of MUG cell stained with HX1 and CREST probably in prometaphase. The fixed cell was stained with DAPI (blue) (top left panel), anti- CENP- E (green) (top right panel) and CREST, an anti- kinetochore antiserum (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 12. Confocal micrograph of a control cell stained for kinesin. The fixed cell was stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel) and anti-kinesin (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 13.  Confocal micrograph of a MUG cell stained for kinesin. The fixed cell was stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel) and anti-kinesin (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 14. Confocal micrograph of MUG cells stained for kinesin. The fixed cells were stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel) and anti-kinesin (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 15. Confocal micrograph of a control cell stained for MAD2. The fixed cell was stained with DAPI (blue) (top left panel), anti- α tubulin (green) (top right panel) and anti- MAD-2 (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 16. Confocal micrograph of a MUG cell stained for MAD2. The fixed cell was stained with DAPI (blue) (top left panel), anti-α tubulin (green) (top right panel) and anti-MAD-2 (red) (bottom left panel). The composite image (bottom right panel) shows the images merged.
Figure 17. Graph of percentages of total cells in a population that show spindles and midbodies during a taxol block. For purposes of this experiment, mitosis was defined as any cell with a spindle.
Figure 18. Graph of percentages of total cells in a population that show spindles and midbodies during taxol release. The medium containing taxol was removed from the population and replaced with fresh medium. For purposes of this experiment, mitosis was defined as any cell with a spindle.
Zinkowski (1989) and Wise and Brinkley (1997) found that even though MUG cells have fractured chromatin and CKFs, they undergo an apparently normal mitosis to produce daughter cells. Wise (Wise, unpublished data) then discovered that the kinetochores were distributed evenly into the two daughter cells. Because of the ability of MUG cells to divide and distribute the CKFs evenly, my hypothesis is that MUG cells obey the metaphase checkpoint. In doing so, they retain the necessary checkpoint proteins, localize these proteins in a similar manner as do CHO control cells, arrest in taxol, and divide normally once released from the taxol block.

To test this hypothesis, we used indirect immunofluorescence to stain for kinetochore proteins and the checkpoint proteins, CENP-E, kinesin, and Mad2. Then, we blocked with taxol to determine whether or not the cell arrested, after which, we released the cells from the taxol block to determine whether or not they could recover and finish mitosis.

**CREST Staining**

Previous research determined that MUG cells undergo metaphase and then divide into daughter cells (Wise and Brinkley, 1997). We used CREST antiserum to stain the...
kinetochores in previously identified stages. The images of MUG cells that we obtained from cells in prophase, prometaphase, metaphase, telophase, and interphase were equivalent to those results previously obtained. However, no stage resembling anaphase has been detected previously, though it was thought to exist because of the fidelity of the separation of the kinetochores.

The MUG anaphase-like image that we captured on the confocal microscope appears to have smaller and more fragmented kinetochores than controls, as is seen in other stages of MUG mitosis. In a normal anaphase cell, the kinetochores are about 1 µm in length whereas in the MUG anaphase cell, the kinetochore fragments are more numerous and thus much smaller. As predicted by Wise and Brinkley (1997), it seems that anaphase B, or the elongation of a cell spindle during anaphase, did not occur. Wise and Brinkley (1997) found that the average MUG spindle was 9 µm in length and the anaphase spindle we detected was under 10 µm. The presence of anaphase in MUG cells means that a metaphase to anaphase transition must occur.

**CENP- E Staining**

In control cells, CENP- E localized to the kinetochore until the metaphase-anaphase transition. After the transition, the protein relocalized to the metaphase plate and appeared in a line-like configuration. Staining for tubulin and CENP- E together in MUG cells revealed that, probably during late prometaphase, CENP- E is concentrated at the metaphase plate, as in controls. In MUG cells, we have found that CENP- E colocalized with kinetochores stained by CREST antiserum. Figure 10 appeared to be a MUG cell in prometaphase or in anaphase. If the cell was in prometaphase, then the
CENP-E localization is similar to that of controls. However, if the cell was in anaphase, then CENP-E did not relocalize in MUG cells after the metaphase-anaphase transition. Without tubulin or intact chromatin as a guide to determine the mitotic stage of the cell, it was difficult to determine.

There was one major difference between CENP-E in MUG cells and the controls. In MUG cells, CENP-E was more concentrated in late prometaphase than in controls. One possibility that could account for this difference is the number of kinetochores. In control cells, CENP-E binds to each sister kinetochore. In MUG cells, while there are no sister kinetochores, due to the lack of a successful S phase, there is a large number of CKFs and each of those probably have CENP-E in order to segregate properly. Zinkowski (1991) showed that, in CHO cells, the kinetochores were highly fragmented. While the numbers of CKFs were difficult to accurately count due to the small size, Zinkowski suggested that in many cases there were at least as many as double the haploid number of kinetochores.

We were unable to find any CENP-E and tubulin stained cells in anaphase. However, it is possible that Figure 10 shows a cell in anaphase with a different localization pattern from those seen in controls. The reduced numbers of anaphase cells were probably due to the rarity of anaphase MUG cells in the population.

CENP-E probably behaves in the same manner as in controls. It colocalizes to the kinetochore in prometaphase and is found at the metaphase plate in late prometaphase and metaphase. While there is a greater concentration of CENP-E at the metaphase plate than in controls, this could be due to numbers of CKFs. One difference may be in the
localization of CENP-E at anaphase in MUG cells. It is possible that the protein does not leave the kinetochore during the metaphase-anaphase transition in MUG cells.

**Kinesin Staining**

General kinesin, an important motor protein necessary to many cellular functions and was found distributed throughout the control cell. In MUG cells, kinesin did not behave as it did in controls. In MUG cells, kinesin was spotty in appearance and concentrated in areas found in both the chromatin and the spindle. Probably, at least some of this kinesin was chromo-kinesin, which was unbound due to the fragmentation of the chromatin. It is unlikely that all of the kinesin observed was chromo-kinesin because spots of kinesin were found in the area of the spindle in each of the cells observed. Also, in control cells, kinesin was scattered throughout the cell, not concentrated in the chromatin, as it would be if the antibody was only localizing to chromo-kinesin. Some of the kinesin was probably mitotic kinesin which is necessary for the formation and function of the bi-polar spindle, chromosome segregation, chromosome transport, the metaphase checkpoint, and cytokinesis (Wittmann et al., 2001).

It is difficult to tell which of the kinesins were abnormal due to the number of different proteins in this group. However, there are some possibilities. Perhaps a kinesin necessary for chromosome congression to the metaphase plate was different between MUG cells and controls. Depletions of Kid, a kinesin protein, lead to a delay in the metaphase-anaphase transition. However, a loss of Kid also leads to misaligned chromosomes and since the kinetochores segregated evenly in MUG cells, Kid was unlikely to be affected. A loss of Kif14 and Kif18 led to a delay in the metaphase-
anaphase transition, but reduced tension at the kinetochore causing the cell to fail to undergo cytokinesis (Zhu et al., 2005). MUG cells can undergo cytokinesis and previous studies (Wise, unpublished data) showed the presence of the 3F3 epitope, which responds to tension. While these three candidates are unlikely options to the kinesin localization differences found in MUG cells, they have a commonality. They are responsible for a delay of the metaphase- anaphase transition by allowing the chromosomes to move towards the spindle pole and back again before anaphase begins. In a population of MUG cells, prometaphase and metaphase stages were more common than cells in prophase, and even more common than those in anaphase. If the transition were delayed in this manner, more cells would be in the prometaphase or metaphase stages. Perhaps another similar kinesin is the culprit.

Though it is not known why kinesin localization was different in MUG cells, it is evident that kinesin did not behave similarly as in controls.

**MAD2 Staining**

In control cells, MAD2 binds to unattached kinetochores and is crucial in the metaphase checkpoint. After attachment, MAD2 is transported away from the kinetochore. In MUG cells, the data remain unclear. In previous studied Wise (Wise, unpublished) detected MAD2 and found that it colocalized with the kinetochore. In the present study, however, while MAD2 may have been present, we were unable to detect it in MUG cells. However because of the successful segregation of the CKFs and the detection of an anaphase stage, it seems probable that it is present.
**Taxol Block**

Taxol stabilizes microtubules and prevents depolymerization at the plus end of the microtubules. This in turn reduces tension at the kinetochore in control cells (Kelling et al 2003). In control cells, taxol leads to arrest of the cell at metaphase.

We used MUG treated cells as controls to ensure that the cells were dividing. We applied HU for 20 hours, then caffeine and HU, and removed the cells at various time points. At 7 hours, 14.9% of cells showed spindles, while 2.4% had midbodies. At 9 hours and beyond, there were more midbodies than spindles, indicating that the cells were dividing.

We applied HU as mentioned above and then added taxol, caffeine, and HU for various time points. At 7 hours, there were 12.3% spindles and 0.9% midbodies in the population. At 9, 11, and 13 hours, 15.8%, 14%, and 7% of the cell population had spindles while 0.48%, 1.1%, and 0.47% had midbodies. There was an increase in cells with spindles between 7 and 9 hours because a few late cells had not yet divided at the 7 hour time point. At the 13 hour time point, there was a reduction in the number of spindles. However, there are still a very small number of midbodies indicating that division is not rapidly occurring. More research is needed to determine if this was random or if there is a trend to be seen. However, no matter the slight drops and rises in spindle percentages, this experiment indicates that taxol, in general, arrested MUG cells because of the differences observed in spindle and midbody percentages between MUG controls and taxol- treated cells.
Taxol both reduces microtubule turnover and the tension at the kinetochore. Reduced tension alone activates the metaphase checkpoint in CHO cells and halts the cell in anaphase. This research seems to indicate that MUG cells require microtubule turnover in order to divide. This is interesting because most CKFs are attached to both spindle poles rather than one. The MUG kinetochore is attached to microtubule bundles on each side of the spindle. How the kinetochores segregate is a mystery. Due to the equal segregation of the CKFs into daughters, the cell is somehow compensating for not having sister kinetochores. Perhaps the cell can somehow non-randomly remove one side of a microtubule bundle attachment.

**Taxol Release**

Cells were placed into taxol, caffeine, and HU for 9 hours. After the 9 hours, the taxol solution was removed, fresh medium added, and samples were taken at various time points. After 5 minutes, 10.3% of cells had spindles and 0.1% of cells had midbodies. At 10, 20, 30, and 40 minutes the cells had 4.67%, 3.88%, 0.8%, and 2% of the cell population had spindles while 1.23%, 3.3%, 5.7%, and 5.83% had midbodies. Between 5 and 20 minutes most of the cells recovered from the block and divided. After 20 minutes, almost all of the cells recovered and divided.

A control cell’s spindle is about 15 µm in length at anaphase. It has been determined that during anaphase kinetochores move, on average, 1 µm per minute in most types of cells. Therefore, if MUG spindles do not undergo anaphase B, and are only on average 9 µm in length (Wise and Brinkley, 1997), then they are taking longer to divide than is to be expected.
Colcemid is a drug that destabilizes microtubules. When it was added to a MUG population, the cells were arrested and when it was removed, they recovered similarly to the controls (Brinkley et al., 1998). However, it took longer for the MUG cells to recover than it did the controls (Wise and Brinkley, 1997). This is similar to the results of the taxol release experiments in the current work.

Conclusions

My hypothesis was that MUG cells obey the metaphase checkpoint and in doing so, display the same localization and behavior of checkpoint proteins as do control cells.

In one sense, MUG cells acted as controls during mitosis and seemed to obey the metaphase checkpoint. The MUG spindles transitioned from metaphase to anaphase, although in anaphase, they did not elongate. CENP- E was found to localize in a similar manner in MUG cells as in controls. CENP- E colocalized with the kinetochore and migrated to the metaphase plate. However, CENP- E was more concentrated in MUG cells than controls, perhaps due to numerous kinetochore fragments. Anaphase was probably not found in CENP- E stained cells, perhaps due to the reduced number of anaphase cells found in MUG populations. The taxol experiments seemed to indicate that taxol did arrest MUG cells in metaphase, and so either microtubule turnover or tension at the kinetochore is required for MUG cell division. This indicates an activate metaphase checkpoint in MUG cells as in controls. When fresh medium was added to the taxol treated cells, they recovered and divided. However, they seemed to recover from arrest more slowly than expected.
In other ways, MUG cells seem to behave differently than control cells. Kinesins did not localize similarly in MUG cells as they did in controls. Instead of being scattered throughout the cell, as in controls, they were found in concentrated spots. MAD2 did not seem localized, even though other research (Wise, unpublished data) indicates that MAD2 is present and found on the kinetochore in MUG cells as in controls.

It seems possible that the lack of anaphase B, the reduced number of anaphase stages found, and the spotty appearance of kinesin may be related. There is a number of kinesins responsible for kinetochore movement and spindle function in mitosis, perhaps one of the kinesins is affected in the MUG treatment. However, from these experiments it is impossible to determine which kinesin or kinesins are affected.

More research needs to be conducted to determine the percentages of cells with anaphase in a MUG population. Time lapse video would be helpful in observing kinetochore movement to determine why anaphase stages are rare and if there is a delay in the metaphase- anaphase transition. Video would also allow observations about the way in which MUG kinetochores move. The presence and localization of MAD2 needs to be further investigated, perhaps if the experiments were conducted with different solutions of fixatives, MAD2 could be observed. There are many questions to the process of MUG kinetochore congression and the metaphase checkpoint in these cells. However, evidence suggests that MUG cells probably obey the metaphase checkpoint.


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