TESTING THE RELATIONSHIP BETWEEN CHECKPOINT ADAPTATION AND MICRONUCLEI IN HUMAN FIBROBLASTIC GLIOMA AND NORMAL LUNG CELLS

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Bachelor of Science with Distinction, University of Lethbridge, 2012

A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfilment of the
Requirements of the Degree

MASTER OF SCIENCE

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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Date of defense: August 7, 2014

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Thesis Abstract

This thesis examines the relationship between checkpoint adaptation and micronuclei in human cancer cells with damaged DNA. When cancer cells are exposed to genotoxic agents they can form micronuclei, although the precise process has yet to be described. I examined glioma cells (M059K) and found that 48% of the cells contain micronuclei after treatment with cisplatin. Treated cells also underwent checkpoint adaptation: they signalled damaged DNA, had active Chk1, arrested in G2-phase, and then entered mitosis. The relationship between checkpoint adaptation and micronuclei was confirmed by use of chemical inhibitors against Chk1 or Cdk1, which have key roles in checkpoint adaptation. When Chk1 was inhibited in treated cells, additional micronuclei formed, whereas if Cdk1 was inhibited, no additional micronuclei formed. I treated normal cells (WI-38) with cisplatin and found that they did not undergo checkpoint adaptation or form micronuclei. These data demonstrate that micronuclei are an outcome of checkpoint adaptation.

Acknowledgment

I would like to thank my supervisor Dr. Roy Golsteyn for his patience, support, and expertise on this research project. Through his guidance, I have gained invaluable research skills and acquired the technical training to work inside the laboratory. I would like to give special thanks to the members of my thesis examination committee (Dr. Marc Roussel and Dr. Elizabeth Schultz) for their advice throughout this project and thesis defense chair (Dr. Theresa Burg) and for her participation in the examination process.

I wish to also thank several individuals who also helped make this research project and thesis writing possible: Dr. Igor Kovalchuk and Dr. Li Dongping for the use of and assistance with the flow cytometer; Dr. Brent Selinger and Dr. James Thomas for the use of their plate readers; Bruce McMullin, Linda Weaver, and John Eng for their technical assistance and use of equipment; Lucy Swift, Theresa Kisko, and Daniel Hoot for their time editing my thesis; and Dr. Sophie Kérneis-Golsteyn and Tanzila Rahman for their encouragements and useful discussions.

I would also like to thank Alberta Innovates Technology Futures, the Alberta Scholarship Programs, the University of Lethbridge, and the School of Graduate Studies for financial assistance either directly or through scholarships.

Lastly, I thank both my family and friends for their continued support and encouragement throughout my M. Sc. Program.

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List of Abbreviations:

AP - Alkaline phosphatase

ATCC - American Tissue Culture Collection

ATM - Ataxia-telangiectasia mutated

ATP - Adenosine triphosphate

ATR - ATM and Rad3-related

ATRIP - ATR interacting protein

APH - Aphidicolin

BBB - Blood brain barrier

BFB - Bridge-fusion-bridge

BGC823 - Human gastric adenocarcinoma cell line

BrdU - 5-bromo-2'-deoxyuridine

Brca1 - Breast cancer 1

BSA – Bovine serum albumin

CAK - Cdk activating kinase

CCL 220 - Human colon carcinoma cell line

Cdc25C - Cell division cycle 25C

Cdk1 - Cyclin dependent kinase 1

Cdk2 - Cyclin dependent kinase 2

Cdk4 - Cyclin dependent kinase 4

Cdt1 - Chromatin licensing and DNA replication factor 1

Chk1 - Checkpoint kinase 1

Chk2 - Checkpoint kinase 2

Colo 320DM - Human colon adenocarcinoma cell line

CPT - Camptothecin

CTP - Cytidine triphosphate

DAPI - 4',6-diamidino-2-phenylindole

Dido MEF - Mouse embryonic fibroblast cell line

DK-MG - Human glioblastoma cell line

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DSB - Double-stranded break

DTT - Dithiothreitol

DU 145 - Human prostate carcinoma cell line

EGTA - Ethyleneglycol-bis (beta-aminoethylether)-*N*,*N'*-tetraacetic acid

ESCC - Esophageal squamous cell carcinoma

FISH - Fluorescent *in situ* hybridization

GBM - Glioblastoma multiforme

GMA32 - Chinese hamster fibroblast cell line

G_1-phase - Gap 1 phase

G_2-phase - Gap 2 phase

HeLa - Human cervical adenocarcinoma cell line

HCT-116 - Human colon carcinoma cell line

HMEC - Human mammary epithelial cells

HT1080 - Human colon carcinoma cell line

HT-29 - Human colon carcinoma cell line

H1299 - Human lung carcinoma cell line

K562 - Human lymphoblast cell line

M-phase - Mitosis phase

Mcm2 - Minichromosome maintenance 2

Mer11 - Meiotic recombination 11

MDA-MB-231 - Human breast adenocarcinoma cell line

MOLT4 - Human lymphoblastic leukemia cell line

MRN - Mer11-Rad50-Nbs1

mRNA - Messenger RNA

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

M059K - Human glioblastoma cell line

Nbs1 - Nibrin 1

NSCLC - Non-small cell lung carcinoma

PBS - Phosphate-buffered saline

PIKK - Phosphoinositide 3-kinase related kinase

NPB - Nucleoplasmic bridge

PP1Cα - Protein phosphatase 1Cα

Rb - Retinoblastoma

RPA - Replication protein A

SAC - Spindle assembly checkpoint

SCC - Squamous-cell carcinoma cell line

SKOV-3 - Human ovarian carcinoma cell line

S-phase - Synthesis phase

SSB - Single-stranded break

TBS-T - Tris buffered saline with Tween20

TMZ - Temozolomide

TCGA - The Cancer Genome Altas

Topo I - Topoisomerase I

UTP - Uridine triphosphate

UCN-01 - Small molecule and kinase inhibitor

U2OS - Human osteosarcoma cell line

U87MG-E6 - Human glioblastoma cell line

WHO - World Health Organization

WIL2-NS - Human lymphoblast cell line

WI-38 - Human lung fibroblast cell line

WS1 - Human normal skin cell line

Chapter 1 - Literature review

This thesis is about the relationship between damaged DNA, checkpoint adaptation, and micronuclei formation in brain cancer (glioma) cells. Here I provide a review on these processes and how they contribute to genomic change in glioma cells.

I hypothesize that micronuclei form from cells that undergo checkpoint adaptation, a process in which cells enter mitosis with damaged DNA. Secondly, I hypothesize that micronuclei continue to damage their DNA as a result of aberrant DNA replication.

1.1. The cell cycle

The cell cycle is composed of four distinct phases: gap-1 (G_1), synthesis (S), gap-2 (G_2), collectively known as interphase, and M-phase (mitosis and cytokinesis) [1]. In G_1-phase, cells nearly double in size and synthesize proteins and mRNAs required for S-phase. Importantly, human cells in G_1-phase contain 2n DNA, which corresponds to 23 chromosome pairs. When the cell enters S-phase the DNA is duplicated by a process of a semi-conservative replication. When DNA replication is completed the cell enters G_2-phase with 4n DNA. Although the DNA content has doubled, the replicated chromosomes remain connected by the centromere and are defined as sister chromatids until they are separated in mitosis. In G_2-phase cells continue to grow and synthesize additional proteins and mRNAs that are required for M-phase.

The final phase of the cell cycle is M-phase, which corresponds to both mitosis (separation of chromosomes) and cytokinesis (division of cytoplasm). Mitosis is subdivided into five stages: prophase, prometaphase, metaphase, anaphase, and telophase.

Prophase and prometaphase represent the earliest stages of mitosis. They are characterized by the condensation of sister chomatids and disassembly of the nuclear envelope. Condensation occurs after chromatin modifications, which include the phosphorylation of histone H3 on serine 10 [2]. After condensation, the sister chromatids become attached to the mitotic spindle, a structure composed of an array of microtubules. In metaphase, the sister chromatids are aligned at the equatorial plate with the aid of a spindle apparatus that originates at each pole. In anaphase, the spindle apparatus separates the sister chromatids by pulling the chromosomes towards the poles. The chromosomes decondense during telophase, while the nuclear envelope reforms to produce two distinct daughter nuclei. As telophase is occurring, cytokinesis divides the cytoplasm, which contains the nuclei and other organelles equally into two daughter cells.

Understanding the changes that occur in cells during mitosis allows us to distinguish mitotic cells from those in interphase. For example, condensed chromosomes and the phosphorylation of histone H3 on serine 10 can be used to confirm mitosis [2]. In addition, the shape of a cell can be used to distinguish mitosis. Cells in interphase are flat, but during mitosis they become rounded [3]. The round shape is caused by changes in the polymerization of actin filaments, which are a structural network of proteins in the cytoplasm [4].

1.2. Regulation of the cell cycle

The cell cycle is controlled by a family of threonine/serine kinases known as cyclin dependent kinases (Cdks) [5]. As their name suggests, they are dependent on the presence of cyclin regulatory subunits. There are four classes of Cdks involved in the cell

cycle: G_1/S-Cdks, S-Cdks, G_2-Cdks, and M-Cdks. Collectively, G_1/S-Cdks, S-Cdks, and G_2-Cdks manage the transitions between phases in interphase, whereas M-Cdks regulate mitosis.

Cdk1 is the M-Cdk that is required for mitosis [5]. It is highly regulated and must undergo three activation steps before mitosis can begin. First, Cdk1 must bind to its regulatory subunit cyclin B1. The amount of cyclin B1, unlike Cdk1, oscillates throughout the cell cycle. Cyclin B1 is absent during most of interphase; its levels increase in late G_2-phase until a peak is reached in mitosis. However, cyclin B1 is rapidly degraded by the proteasome complex during anaphase.

The second activation step of the Cdk1/cyclin B1 complex is a conformational change to allow adenosine triphosphate (ATP) to enter the catalytic site [6]. This occurs after Cdk1 has been phosphorylated on threonine 161 by the Cdk-activating kinase (CAK). This activation step is held in check by the presence of inhibitory phosphates on amino acids (threonine 14 and tyrosine 15), which maintain Cdk1 in an inactive state [6]. These inhibitory phosphates are added by the protein kinases Wee1 and Myt1 in G_2-phase [5, 6]. In the final activation step, the protein phosphatase cell division cycle 25C (Cdc25c) removes the phosphates on threonine 14 and tyrosine 15 from the Cdk1/cyclin B1 complex.

Dysregulation of the Cdk1 activation steps can lead to uncontrolled cellular proliferation. For example, induced overexpression of cyclin B1 in esophageal squamous carcinoma cells (ESCC) [7] and overexpression of Cdc25 in osteosarcoma cells (U2OS) [6] has been reported to accelerate the G_2/M-phase transition. In these cells, Cdk1 remains active regardless of the presence of Wee1. In addition to the overexpression of

these proteins, ectopic expression of cyclin B1 during G_1-phase or S-phase has been reported to trigger cell death by apoptosis [8]. Therefore controlling the Cdk1 activation steps is crucial to preventing premature mitosis. Furthermore, these steps provide a control mechanism to prevent cells with damaged DNA from entering mitosis, a point that will be discussed later in my thesis.

Cdk1 phospho-activity can also be used as a method for detecting mitosis. Cdk1/cyclin B1 phosphorylates at least 75 different proteins [5]. For example, Cdk1/cyclin B1 phosphorylates protein phosphatase 1 (PP1Cα) on threonine 320 [9]. With this information, I developed an assay to measure Cdk1 protein kinase activity in human cancer cells. I found that the phosphorylation levels of PP1Cα in mitotic colon carcinoma cells (HT-29) are approximately ten times higher than in interphase cells [10]. Therefore the presence of phospho-Thr^{320}-PP1Cα can also be used to confirm mitosis.

1.3. Cdk1 inhibitors

Because Cdk1 activity is required for mitosis, it has become an attractive therapeutic target for anticancer drugs. Since 1993, over 140 Cdk inhibitors have been developed [11, 12]. One of these inhibitors is the small molecule CR8 [12, 13]. CR8 is an analogue of roscovitine, which is currently undergoing phase II clinical trials for non-small cell lung carcinoma (NSCLC) [12]. CR8 and roscovitine are 2,6,9-trisubstituted purine and are ATP competitors that bind to the active site of Cdk1. This binding blocks the phospho-activity of Cdk1. HT-29 cells treated with 10 μM CR8 do not enter mitosis as confirmed by the absence of phospho-Thr^{320}-PP1Cα and the absence of cell rounding [3]. Using the Cdk1 assay I developed, I was able to confirm that treatment with 10 μM

CR8 reduces the levels of phospho-Thr^{320}-PP1Cα by approximately 60% [10]. By contrast, an inactive methylated form of CR8 does not prevent cell rounding or the phosphorylation of PP1Cα [3]. These studies confirm that treatment with CR8 can be used as a reliable method to block mitosis in human cells.

1.4. Cell-cycle checkpoints

In addition to the regulation by Cdks, the cell cycle is also controlled by a set of biochemical pathways called cell-cycle checkpoints. They prevent entry into the next phase of the cell-cycle when the preceding phase has not been completed. Importantly, cell-cycle checkpoints also prevent cell cycle progression if DNA is either damaged or incorrectly organized [14]. Cells have one M-phase checkpoint, the spindle assembly checkpoint (SAC) [15], and two interphase checkpoints, which are both referred to as DNA damage checkpoints [16].

The SAC is active during mitosis and leads to a cell-cycle arrest in metaphase. Its role is to ensure that chromosomes are accurately distributed to daughter cells. To accomplish this, chromosomes must attach to microtubules such that each sister kinetochore is attached to opposite poles of the bipolar spindle apparatus (amphitelic attachment). Then the chromosome must align along the equatorial plate, which requires equal tension between kinetochore microtubules. If these conditions are not met, cells arrest in metaphase. However, if the spindle structure and chromosome alignment can be re-established, the checkpoint is ended and cells will progress into anaphase.

In contrast to the SAC, the DNA damage checkpoints monitor the transition between G_1/S-phase and G_2/M-phase [16]. DNA damage checkpoints protect cells from

transmitting genomic changes to daughter cells by ensuring that damaged DNA is repaired before entry into the next phase of the cell cycle. Importantly, most cancer cells contain a defective G_1/S DNA damage checkpoint due to mutations in the p53 pathway [17, 18], misregulation of the G_1/S-Cdks or their respective cyclins [18], or mutations to the retinoblastoma (Rb) gene [18, 19]. As a result, cancer cells with damaged DNA are more likely to accumulate in G_2-phase than in the G_1-phase of the cell cycle.

1.5. G_2/M DNA damage checkpoint

In this thesis, I will be examining cells that arrest in G_2-phase at the DNA damage checkpoint prior to entering mitosis with damaged DNA. To understand how this occurs, a comprehensive understanding of the G_2/M DNA damage checkpoint is required.

The G_2/M DNA damage checkpoint is comprised of sensors, transducers, and effector molecules [20]. Together, these molecules initiate a phosphorylation cascade that causes a cell-cycle arrest in G_2-phase. The two enzymes that are responsible for the phosphorylation cascade are ataxia-telangiectasia mutated (ATM) and ATM Rad3-related (ATR) [14]. ATM and ATR are signal transducer molecules and belong to the phosphoinositide 3-kinase-related kinase (PIKK) super family. Although both ATM and ATR checkpoint pathways can be activated at the same time, each responds to a different type of damaged DNA; ATR responses to single-strand breaks (SSB), while ATM responds to double-strand breaks (DSB).

In the presence of SSB, DNA becomes coated by replication protein A (RPA) [14]. RPA binds to and stabilizes single-stranded DNA (ssDNA). When the RPA-ssDNA complex is formed it becomes a binding site for ATR interacting protein (ATRIP).

ATRIP is a sensor molecule that participates with the RPA-ssDNA complex to recruit inactive ATR homodimers to sites of damaged DNA. Once ATR binds with ATRIP, ATR undergoes autophosphorylation, which causes it to separate into two active monomer units. In contrast to SSB, DSB are detected by Mer11-Rad50-Nbs1 (MRN) complex [16], which recruits ATM to sites of damaged DNA. Like ATR, ATM exists as an inactive homodimer until it is activated by the MRN complex. Once ATM becomes active, repair complexes convert DSB to SSB, which subsequently activates ATR [14]. Together, ATM and ATR have been reported to phosphorylate approximately 700 different protein substrates on 900 different sites [21]. These substrates have important roles in signalling DNA damage repair and checkpoint activation.

In the presence of either SSB or DSB, ATM and ATR phosphorylate the histone variant H2AX on serine 139 [22], which then becomes γH2AX. Paull *et al.* reported that histone γH2AX foci typically appear one to three minutes after a DNA break is introduced [23]. Histone γH2AX is involved in recruiting repair proteins, such as breast cancer 1 (Brca1), to damaged sites [23] and stimulating chromatin relaxation [24]. If damaged DNA is successfully repaired, histone γH2AX is subsequently dephosphorylated by protein phosphatase 1 (PP1) [25] and protein phosphatase 2 (PP2) [26]. The detection of histone γH2AX is a reliable marker for damaged DNA in cells because it is present immediately after the introduction of DNA damage and is dephosphorylated when damaged DNA is repaired.

In addition to signalling for DNA damage repair, ATM and ATR also trigger checkpoint activation (Figure 1). In normal cells, ATM and ATR have two main checkpoint pathways that can arrest cells in interphase: the p53-p21 dependent pathway

and the checkpoint kinase (Chk) dependent pathway. The p53-p21 dependent pathway initiates the G_1/S DNA damage checkpoint. This requires activation of the tumour suppressor protein p53 by ATM and ATR. In addition to initiating the DNA damage checkpoint, p53 triggers cell death through apoptosis, and repair of DSB [17, 27-29]. In the absence of damaged DNA, p53 activity is inhibited by the binding of minichromosome maintenance 2 (Mcm2) [30]. Mcm2 is an E3 ubiqiutin ligase that mediates the degradation of p53 by the ubiquitin-proteasome pathway. ATM and ATR phosphorylate p53 on threonine 15 and serine 20, which inhibits the binding of Mcm2. This allows p53 to be imported into the nucleus where it acts as a transcription factor for several genes, including one that encodes the Cdk inhibitor p21. Once synthesized, p21 binds to G_1/S-Cdks and S-Cdks (Cdk4/6/cyclin D [31] and Cdk2/cyclinA/E [32]). This binding initiates a cell-cycle arrest in either G_1-phase or S-phase.

In at least 80% of cancer cells, the p53-p21 pathway is non-functional [17]. In these cells, damaged DNA in G_1-phase and S-phase might not initiate a cell-cycle arrest. Instead, in the presence of damaged DNA, the checkpoint kinase dependent pathway is required to arrest cells in G_2-phase. In this pathway, ATM and ATR phosphorylate effector molecules checkpoint kinase 1 (Chk1) on serine 317 and serine 345 [33] and checkpoint kinase 2 (Chk2) on threonine 26, threonine 68, and serine 50, respectively [34]. These phosphates are required for Chk1 and Chk2 activation and are only present if DNA is damaged. Chk1 and Chk2 inhibit entry into mitosis by two mechanisms: down-regulating the activity of Cdc25C and increasing the activity of Wee1. As shown in Figure 1, Chk1 and Chk2 participate in cell-cycle regulation by interacting with Cdc25 and Wee1. Chk1 and Chk2 phosphorylate Cdc25C on serine 216 [35], which causes

Cdc25C to bind with a protein complex named 14-3-3. This causes Cdc25C to be sequestered in the cytoplasm where it will eventually be degraded by the proteasome complex. Simultaneously, Chk1 phosphorylates Wee1 on serine 642 so that it remains active [36].

Although both Chk1 and Chk2 can be activated in response to damaged DNA, active Chk1 (but not Chk2) is crucial to maintaining the G_2-phase arrest. Chen *et al.* reported that down-regulation of Chk1 by RNA interference was sufficient to abrogate the G_2/M DNA damage checkpoint in human lung cancer cells (H1299) treated with the genotoxic agent doxorubicin [37]. In a subsequent study, Carrassa *et al.* reported that down-regulation of Chk1 also eliminated the G_2/M DNA damage checkpoint in up to 50% of human colon carcinoma cells (HCT-116) exposed to ionizing radiation or *cis*-diamminedichloroplatinum (cisplatin) [38]. In the same study it was reported that knockdown only of Chk2 by RNA interference did not have an effect on the G_2/M DNA damage checkpoint in these cells. In recent complementary experiments, Bo *et al.* reported that overexpression of Chk1 (but not Chk2) in human gastric cancer cells (BGC823) treated with diallyl disulfide increased the number of cells in G_2-phase [39]. They also showed that knockdown of Chk1 (but not Chk2) with siRNAs specific to Chk1 partially prevented the down-regulation of Cdc25C and Cdk1/cyclin B1 activity. Together these reports suggest that Chk1 is more important than Chk2 in maintaining the G_2-phase in arrest in the presence of damaged DNA. Therefore, for this thesis the phosphorylation of Chk1 on serine 317 and serine 345 will be used as a marker to detect the activation of the DNA damage checkpoint.

1.6. Chk1 inhibitors

Recently, there has been an interest in developing compounds that inhibit Chk1 or other proteins required to maintain the DNA damage checkpoint. Chk1 inhibitors prevent cell-cycle arrest due to damaged DNA and promote mitotic cell death. Experimentally, the DNA damage checkpoints can be inhibited with high concentrations of caffeine. Caffeine inhibits ATM and ATR, however, the concentration of caffeine required to abrogate the DNA damage checkpoints in humans is not clinically attainable [40]. As such, small molecules with a higher specificity for a protein kinase, such as Chk1, have been developed.

An example of a commonly used Chk1 inhibitor is the indolocarbazole UCN-01 (7-hydroxystraurosporine). It is an ATP competitor that is approximately 100,000 times more potent than caffeine [40]. Busby *et al.* reported that UCN-01 treatment prevented Chk1 from phosphorylating Cdc25C on serine 216 in erythroblast leukemia cells (K562) after treatment with 8 Gy (Gray) of ionizing radiation [41]. Cells did not arrest in G_2-phase and instead entered mitosis. However, clinical studies have reported that UCN-01 has a high affinity for human plasma protein (α_1-acid glycoprotein), which extends its half-life and limits its bioavailability [40, 42]. Cell culture studies have confirmed that cells incubated with human serum instead of bovine serum, which is more commonly used in laboratories, required 100 times more UCN-01 to abrogate the DNA damage checkpoint [40]. Therefore other Chk1 inhibitors have been developed to avoid these undesirable features.

A promising alternative to UCN-01 is the indolocarbazole derived molecule, Gö6976. Gö6976 is another ATP competitor molecule that binds to the active site of

Chk1 and Chk2, but not α_1-acid glycoprotein. In addition, Gö6976 has been reported to have a higher specificity for Chk1 compared to UCN-01 [43]. Kohn *et al.* reported that 30 nM to 100 nM Gö6976 abrogated the S-phase and G_2-phase arrest induced by SN-38 (the active component of the topoisomerase I inhibitor irinotecan) in human breast carcinoma cells (MDA-MB-231) [40]. As reported with UCN-01, Gö6976 prevents Chk1 from inhibiting Cdc25C [40, 43], which then allows cells to enter mitosis. Due to its high specificity for Chk1, and its low affinity for α_1-acid glycoprotein [40], Gö6976 treatment can be used as a reliable method to study the effects of abrogating the DNA damage checkpoints in human cells.

1.7. *DNA damaging agents*

If damaged DNA is so severe that it cannot be repaired during the cell-cycle arrest, cell death will occur [16]. This is the basis of genotoxic anticancer treatments. To date, ionizing radiation is the most frequently used anticancer treatment [44]. Approximately 50% of all cancer patients are treated with ionizing radiation; however, many genotoxic chemical agents, which also damage the DNA, have been developed to treat cancer patients [45]. In the experiments described in this thesis, I have selected three genotoxic agents as examples of anticancer drugs: camptothecin (CPT), S23906-1, and cisplatin (Table 1). Notably, each genotoxic agent has a different mechanism of action.

CPT is a quinoline alkaloid that was first isolated from *Camptotheca acuminata* bark extracts in 1966 while screening for natural anticancer compounds [46]. Since its discovery, two CPT analogues (topotecan and irinotecan) have been approved as chemotherapic agents to treat ovarian, cervical, colorectal, brain, and small cell lung

cancers [46, 47]. CPT and its analogues inhibit topoisomerase I (Topo I). Topo I is an enzyme that relieves torsional strain in DNA by removing supercoils during DNA replication and transcription. Topo I introduces single-stranded nicks along the phosphodiester backbone of the DNA, passing the uncleaved strand through this nick, and then religating the relaxed DNA [46]. When CPT is present, it forms hydrogen bonds between aspartate 533 and arginine 364 on Topo I, and the amine on cytosine, after the DNA nick has been introduced [48]. These hydrogen bonds stabilize the binding of Topo I to the 3' end of the DNA and prevent religation. Eventually, when the replication fork collides with the CPT-Topo I-DNA complex, the SSB induced by Topo I is converted into a DSB [46, 48].

Like topotecan and irinotecan, S23906-1 is an analogue of a natural product isolated from a plant extract. It was synthesized from its parent molecule acronycine, which was extracted from *Sarcomelicope simplicifolia* [49]. S23906-1 has been tested in clinical studies and has been shown to exhibit anticancer activity towards multiple myeloma [50] as well as several solid tumours. S23906-1 is an atypical alkylating agent that covalently binds to the N^2 of guanine in the minor groove of DNA [51, 52]. It also has a unique ability to promote DNA strand separation. Interestingly, the effects of S23906-1 are only detected when DNA replication begins. Léonce *et al.* reported that S23906-1 induced DSB during S-phase, and that treatment with the DNA polymerase α inhibitor aphidicolin prevents this damage [51]. In 2010, Cahuzac *et al.* reported that treatment with S23906-1 induced cell death by mitotic catastrophe [52].

In contrast to CPT and S23906-1, cisplatin is an inorganic molecule, based on platinum. It was first synthesized in 1845 [53], but was not discovered to have anticancer

properties until the 1960s [54]. Rosenberg *et al.* first reported that cisplatin treatment inhibited cell division in *Escherichia coli* [55] and then later reported that cisplatin injections also caused tumour regression in mice harboring transplanted sarcomas [56]. Cisplatin was subsequently approved as an anticancer drug for humans in 1978 [53]. Currently, cisplatin is used to treat testicular, lung, ovarian, and head and neck cancers [53]. It is also used in combination with other drugs such as temozolomide (TMZ) to treat brain cancers [57, 58]. Cisplatin induces intra-strand, inter-strand, and DNA-protein crosslinks [53, 59]. The chlorine atoms of cisplatin are replaced with the N^7 of either guanine or adenine. Cisplatin binds more tightly to nitrogen, because nitrogen balances the platinum charge more effectively than chlorine. Eastman reported that 90% of DNA crosslinks induced by cisplatin occur between two adjacent guanines (1,2-GpG) [60]. When the crosslinks are encountered by either the DNA replication or transcription machinery, they are subsequently converted into either SSB or DSB following collision [53].

1.8. Micronuclei

A common outcome of treating cells with genotoxic agents including cisplatin and CPT is formation of micronuclei. Micronuclei can form in cells with damaged DNA that enter mitosis. As such, it has been suggested that micronuclei formation may require checkpoint adaptation, a process that will be discussed later in this thesis. Here I provide a detailed review of micronuclei and their relation to mitosis, damaged DNA, and genomic change in cells.

1.8.1. Micronuclei and their relationship to mitosis

Micronuclei form from whole chromosomes [61, 62] or chromosome fragments [63] that do not attach properly to the spindle apparatus, and fail to be incorporated into daughter nuclei after completion of telophase. Eventually, the excluded chromosome or chromosome fragments are encased by smaller nuclei that function independently of the main nucleus.

There are four related mechanisms that are known to disrupt the attachment of chromosomes or chromosome fragments to the spindle apparatus: (1) unrepaired (or incorrectly repaired) DNA breaks [64, 65], (2) dysfunctional mitotic spindle complexes or microtubules [66-68], (3) defects in kinetochore proteins or assembly [69-71], and (4) a defective mitotic checkpoint [71]. Although genotoxic treatment may cause micronuclei to form as a result of all four of these mechanisms, unrepaired DNA breaks are expected to be the biggest contributor.

1.8.2. Micronuclei arise from unrepaired DNA breaks

Damaged DNA can interfere with the attachment of a chromosome to the spindle apparatus, if the centromere is missing or damaged (Figure 2). This is because centromeric DNA provides the binding site for the kinetochore proteins, which are required to connect the chromosomes to the spindle microtubules. Acentric (centromere lacking) chromosome fragments, which are generated through ionizing radiation and/or genotoxic chemical agents, are known to cause the formation of micronuclei. Cornforth and Goodwin reported that up to 84% of micronuclei that form in skin fibroblasts obtained from live donors and then treated with 6 Gy of ionizing radiation, contained chromosome fragments that lacked centromeric DNA [72]. In another study, Jirsova *et al.*

reported that skin fibroblasts treated with 2-80 μM cisplatin gave rise to micronuclei containing acentric chromosome fragments [64]. Utani *et al.* reported that high concentrations of CPT (14 μM) also induced chromosome fragmentation and micronuclei formation in human colon carcinoma cells (COLO 320DM) [73].

In cases where a centromere is damaged, missegregation of whole chromosomes may also occur. Guerrero *et al.* reported that localized damage to the centromeric DNA of mouse embryonic fibroblasts (Dido MEF) led to the generation of merotelic chromosome attachments (kinetochores that are attached to both spindles) and resulted in 2.6 times more micronucleated cells than those without damaged centromeres [74]. It has been suggested that damage to centromeric DNA in human cells disrupts the binding of the histone H3 variants CENPA and CENPB, which are required for kinetochore assembly [71].

In some cases, DNA damage can lead to the generation of dicentric (two centromeres) chromosomes (Figure 2) [71, 75, 76]. Typically, this occurs when the telomere regions of two chromosomes, which are either damaged or eroded, fuse. When anaphase begins, the centromeres of the dicentric chromosome are pulled to opposite poles. This process stretches the chromosome and forms what is known as a nucleoplasmic bridge (NPB) between the daughter nuclei. Eventually, the NPB breaks in cytokinesis and the broken DNA fragments are enclosed in micronuclei. Pampalona *et al.* reported that chromosome fusions arise in primary epithelial cells (HMEC) that have damaged telomere ends [77]. In this study, the researchers observed that NPB formed between daughter nuclei after the spindle apparatus failed to separate a dicentric chromosome. Approximately 40% of micronuclei examined later harboured dicentric

chromosome fragments. In another study, Thomas *et al.* reported that lymphoblasts (WIL2- NS) treated with 2 Gy of radiation were 25 times more likely to form NPB containing dicentric chromosomes than non-treated cells [76].

1.8.3. Micronuclei induce aneuploidy

Micronuclei can arise from cells with damaged DNA that enter mitosis, but they also can induce further genomic changes once present. The most common type of genomic change reported in micronucleated cells that undergo mitosis is whole chromosome aneuploidy. This can occur if the chromosomes in micronuclei are not replicated, actively degraded, or exported out of the cell. Okamoto *et al.* reported that human colon carcinoma (CCL 220) and cervical carcinoma (HeLa) cells harbouring micronuclei that lack nuclear lamina do not replicate DNA and give rise to daughter cells with a different chromosome number [78]. After analysis by the terminal dUTP nick-end labelling (TUNEL) assay, Terradas *et al.* reported that chromosomes are degraded by an apoptotic-like pathway [63]. In some cases micronuclei undergo nuclear-envelope collapse due to either absent or discontinuous lamina [79]. Hatch *et al.* reported that 60% of micronuclei lacking lamin B1 in U2OS cells ruptured and degraded DNA prior to mitosis [79]. Similar observations were made using cervical (HeLa), breast (MDA-MB-231), and prostate (DU 145) cancer cells in this study.

1.8.4. Micronuclei induce break-fusion-bridge cycle

In addition to causing chromosome aneuploidy, micronuclei containing chromosomes that lack telomeres can initiate a break-fusion-bridge (BFB) cycle [71, 80].

BFB cycles occur when a chromosome lacking telomeres is replicated into two sister chromatids, which also lack telomeres [71]. The two sister chromatids then fuse, creating a dicentric chromosome. During anaphase the dicentric chromosome is broken, but at a site different from that of the original fusion. The daughter cells receive unequal chromatid fragments, which again lack telomeres. As a result of the absent telomeres, the BFB cycle continues. Notably, during the BFB cycle some genes can be amplified whereas others are lost depending on the location of the chromosome break. This mechanism has been observed in Chinese hamster fibroblasts (GMA32) whereby a BFB cycle lead to the amplification of several genes on chromosome 1 as detected by fluorescence *in situ* hybridization (FISH) [80].

1.8.5. Protein recruitment is impaired in micronuclei

Although micronuclei appear to be morphologically similar to the main nucleus, several studies have reported that micronuclei are impaired in the recruitment of proteins involved in transcription, DNA replication, and DNA repair. Reduced protein recruitment in micronuclei denotes that DNA sequences will be less efficiently transcribed, replicated, and repaired, which will alter biological processes in the cell.

As a result of impaired protein recruitment, most micronuclei are transcriptionally inactive. Hoffelder *et al.* reported that 96% of micronuclei in oral squamous-cell carcinoma cells (SCC) did not incorporate fluorescently labelled UTP (uridine triphosphate), which is used to measure transcriptional activity, despite high levels of UTP in the main nucleus [81]. In this study, only 13% of micronuclei contained the RNA Polymerase II holoenzyme, which is required for the elongation phase of transcription.

Thus even though the DNA sequences may be present, it is as if the genes are lost since they cannot be expressed in most micronucleated cells.

In addition to impaired transcriptional activity, DNA replication is also reported to be less efficient in micronuclei [61, 63]. Crasta *et al.* reported that at least 20% of micronuclei in RPE-1 cells do not begin DNA replication until late G_2-phase [61]. This delay in DNA replication was associated with a significant reduction in the helicase components, Mcm2 and Mcm3, as well as chromosome licensing and DNA replication factor 1 (Cdt1). If DNA inside a micronucleus is not replicated before mitosis, it is impossible to pass on genomic information to both daughter cells. Therefore impaired DNA replication in micronucleated cells will enhance chromosome aneuploidy [61, 63].

Damaged DNA inside a micronucleus is also not efficiently repaired. Interestingly, in the presence of damaged DNA, micronuclei are able to recruit ATR, ATM, and histone γH2AX [61, 63], but other downstream constituents involved in the DNA damage response such as 53PB1 [61, 63, 82, 83], Rad50 [61, 82], Brca1 [61] and Mer11 [63] are not efficiently recruited. Importantly, both transcription and DNA replication have been reported to induce DNA breaks [61]. Therefore micronuclei can continuously damage their own DNA in the absence of a genotoxic agent. Consequently, unrepaired DNA breaks increase the number of chromosomal rearrangements, gene deletions, and nucleotide polymorphisms.

1.8.6. Micronuclei have fewer nuclear pore complexes

The reduced recruitment of proteins involved in transcription, DNA replication, and repair is thought to be due to defective nucleo-cytoplasmic transport. Micronuclei are

reported to have a reduced density of nuclear pores compared to the main nucleus [61, 63, 81]; however the reason for the reduction in nuclear pores density is not known. Nuclear pores are large protein complexes that span the nuclear envelope and allow the passage of large proteins with nuclear import signals into the nucleus. Since there are fewer nuclear pores in a micronucleus the recruitment of proteins is thought to be much slower compared to that in the main nucleus. Hoffelder *et al.* measured nuclear uptake using exogenous glucocorticoid-GFP and reported that only 10% of micronuclei were able to import this protein at a detectable level [81]. In another study, Crasta *et al.* reported that nuclear import in a micronucleus was on average 25% less efficient compared to that of the main nucleus [61].

1.8.7. Micronuclei induce chromothripsis

Micronuclei containing whole chromosomes (or chromosome fragments) have also been reported to induce intrachromosomal rearrangements and deletions [61]. Micronuclei have been suggested to induce chromothripsis [61, 84]. Chromothripsis occurs when a chromosome is shattered into tens or hundreds of pieces [85]. The chromosome pieces are eventually religated, but not always in the original order. In some cases a chromosome may lose DNA sequences by nuclease degradation, before religation can occur. Cells that undergo chromothripsis can be distinguished by three features: (1) complex rearrangements that are usually localized to a single chromosome or chromosome arm; (2) variation in gene copy number states; and (3) the loss of heterozygosity [85]. Chromothripsis occurs in 2-3% of all cancer cells, but has been reported to be as high as 25% in osteosarcoma cells. Stephen *et al.* sequenced 30 cancer

genomes and reported that the genomes in one leukemia patient and five osteosarcoma patients displayed the features of chromothripsis [85]. In this study, chromothripsis was also confirmed in 96 out 746 cancer cell lines including 3 glioma cell lines. Although different models have been proposed which address how chromothripsis occurs [84], asynchronous DNA replication that occurs in a micronucleus is the only confirmed mechanism [61].

Chromothripsis is thought to occur in micronuclei due to delays in DNA replication. Recall that some micronuclei do not begin DNA replication until late G_2-phase [61, 63]. Crasta *et al.* reported that DNA replication in micronuclei was not completed prior to mitosis in at least 30% of RPE-1 cells. This is a problem, because if DNA replication is not completed before mitosis, the chromosomes cannot be separated properly. Crasta *et al.* reported that replicating DNA that was in a micronucleus prematurely condensed and shattered when cells entered mitosis [61]. When the chromosomes were examined after religation, they contained non-reciprocal translocations and truncations. In addition, chromosome derivatives were detected in several cells, which suggest that major genomic rearrangements had occurred.

1.9. Outcomes of the G_2/M DNA damage checkpoint

Although much work has gone into characterizing micronuclei, the pathway that allows cells to enter mitosis with damaged DNA has not been thoroughly studied. Micronuclei form from cells that enter mitosis with damaged DNA; as such they are thought to be an outcome of checkpoint adaptation [19]. Here I will review checkpoint adaptation and the other outcomes of the G_2/M DNA damage checkpoint.

After damaged DNA has been detected and the G_2/M DNA damage checkpoint has been engaged, one of three outcomes may occur in cells: (1) checkpoint recovery, (2) cell death, (3) or checkpoint adaptation.

Checkpoint recovery occurs when the damaged DNA is repaired. It is characterized by the dephosphorylation of histone γH2AX, deactivation of checkpoint proteins (ATM, ATR, Chk1, and Chk2), and re-entry into the cell cycle [16, 86]. Alternatively, if damaged DNA cannot be repaired cell death will occur through apoptosis (programmed cell death), necrosis, senescence, or mitotic catastrophe [19]. By contrast, checkpoint adaptation occurs if cells that are unable to repair damaged DNA after a prolonged arrest deactivate the DNA damage checkpoint and enter into mitosis with damaged DNA [87].

Importantly, checkpoint recovery and cell death prevent damaged DNA from being transferred to cell progeny [16]. However, during checkpoint adaptation cells have an opportunity to transfer damaged DNA to progeny. Therefore, damaged DNA can give rise to genomic change.

1.9.1. Checkpoint adaptation

Checkpoint adaptation is defined by three steps: (1) a cell cycle arrest after detection of damaged DNA; (2) overcoming the arrest; and (3) re-entering the cell cycle with damaged DNA [87]. An overview of the steps required for checkpoint adaptation in human cancer cells is provided in figure 3.

Checkpoint adaptation was first reported in studies of *Saccharomyces cerevisiae* (budding yeast) [87, 88] and was thought to be a process that could allow single-celled

organisms to continue dividing instead of permanently arresting if their DNA was damaged but not to a lethal level [87]. Sandell and Zakain reported that if a telomere was eliminated on a non-essential chromosome, yeast cells would undergo a cell-cycle arrest mediated by the checkpoint protein Rad9 [88], which is an exonuclease involved in sensing and repairing damaged DNA. However, after a prolonged arrest, approximately 30% of these cells underwent cell division without repairing the broken chromosome. Interestingly, these cells were able to undergo up to ten cell divisions, but each time the chromosome size decreased, suggesting that genetic material was lost.

In a subsequent study, Toczyski *et al.* [87] reported that if a DSB was introduced into a mutant yeast strain defective for a DNA repair protein such as Rad52, cells would arrest in G_2-phase for approximately 10 hours (eight times their normal cell cycle), but then resume cell division if the DNA damage could not be repaired.

Initially it was thought that checkpoint adaptation would not occur in multicellular organisms. In 2004, Yoo *et al.* reported that checkpoint adaptation could occur in *Xenopus* oocyte extracts [89]. They observed that if DNA replication was blocked using the DNA polymerase α inhibitor aphidicolin, cells would temporarily arrest in interphase and then enter mitosis despite having only partially replicated chromosomes. In this study, entry into mitosis was characterized by the inactivation of the checkpoint mediator protein claspin and the dephosphorylation of Xchk1, which is a homologue of human Chk1. There are several limitations with the *Xenopus* oocyte extract system, but it provided the first evidence that cells from a multicellular vertebrate are capable of engaging checkpoint adaptation.

Yoo *et al.* thus raised the question of whether this pathway could also occur in human cells [89]. One important distinction between *Xenopus* oocytes and somatic human cells is that *Xenopus* oocytes do not contain defined gap/growth phases, but instead rapidly alternate between S-phase and mitosis [90]. As such, it has been suggested that cell division in *Xenopus* oocytes progresses "unchecked" if either DNA replication is blocked [91] or if there is damage to the DNA [92]. Furthermore, *Xenopus* oocyte extracts are a cell free system since the cell membranes are not intact.

1.9.2. Checkpoint adaptation in human cells

In 2006, Syljuåsen *et al.* reported the first observation of checkpoint adaptation in human cells [93]. In this study, U2OS cells were exposed to 6 Gy of ionizing radiation. After 20 h most cells accumulated in G_2-phase. These cells were reported to contain histone γH2AX and Chk1 that was phosphorylated on serines 317 and 345. After 30-40 h, cells began to enter mitosis as determined by a rounded morphology, condensed chromosomes, high levels of cyclin B1, and histone H3 phosphorylated on serine 10. Importantly, histone γH2AX remained present in these cells, but Chk1 was dephosphorylated (inactive). This led Syljuåsen *et al.* to the hypothesis that Chk1 dephosphorylation was required for cells to enter mitosis [93]. When Chk1 was inhibited with the small molecule UNC-01, the percentage of cells with damaged DNA that entered mitosis after 18 h increased from 2% to 12%.

The study conducted by Syljuåsen and colleagues had left open several critical questions about the relationship between damaged DNA and checkpoint adaptation. For example, a lethal dose of ionizing radiation (6 Gy) was used to induce checkpoint

adaptation. Since humans cannot tolerate exposure to this amount of radiation it was not clear if this process could actually occur in the cells of a cancer patient. However, through a follow up study Řezáčová *et al.* confirmed that checkpoint adaptation occurred in cells exposed to a medically relevant dose of ionizing radiation (1 Gy) [94]. In this study, lymphocytic leukemia cells (MOLT4) were exposed to fractionated doses of ionizing radation (1 Gy every 24 h for a total of 96 h). Approximately 26% of cells arrested in G_2-phase after 48 h with damaged DNA. Of the cells that entered mitosis after the G_2-phase arrest, 76% contained damaged DNA, as measured by histone γH2AX detection. Importantly, this paper provided evidence that non-lethal doses of ionizing radiation, such as those administered to cancer patients during treatment regimens, can trigger checkpoint adaptation.

In 2012 Kubara *et al.* reported that both HT-29 cells and M059K cells undergo checkpoint adaptation in response to treatment with pharmacological concentrations of CPT [3]. They reported that 90% of HT-29 cells entered mitosis with damaged DNA after treatment with 25 nM CPT. Of the cells that entered mitosis, 2% survived. Consistent with previous reports, inactivation of Chk1 by dephosphorylation was necessary for cells to enter mitosis with damaged DNA [93, 94]. Mitotic cells were identified using similar methods as those used by Syljuåsen *et al.* (cell rounding, phospho-Ser^{10}-Histone H3, and high levels of cyclin B1) [93] and by measuring Cdk1 activity. Kubara and colleagues reported that Cdk1 did not contain the inhibitory phosphate on tyrosine 15 and was able to phosphorylate the substrate PP1Cα on threonine 320 in mitotic cells. They also showed that if Cdk1 activity was blocked using the small

molecule CR8, cells were unable to enter mitosis and thus undergo checkpoint adaptation.

Checkpoint adaptation is very difficult to study in normal (non-cancerous) human cells because they have a fixed number of cell divisions, whereas cancer cell lines undergo an unlimited number of cell divisions. One attempt to study checkpoint adaptation in normal cells has been reported. Řezáčová *et al.* reported that normal skin fibroblasts exposed to 1.5 Gy of ionizing radiation entered mitosis [94]; however, most mitotic cells only had one or two histone γH2AX foci. Löbrich and Jeggo reported that the G_2/M DNA damage checkpoint requires a threshold of 10-20 DSB before becoming activated [95]. In addition to only reporting a few histone γH2AX foci in mitotic cells, Řezáčová *et al.* did not confirm either a cell-cycle arrest or activation of Chk1 in these cells, which are two responses that must also occur during checkpoint adaptation. Therefore, it is not clear if checkpoint adaptation occurred in these cells.

1.9.3. Significance of checkpoint adaptation

Cells that undergo checkpoint adaptation and survive generate daughter cells with a modified genome. Recall that chromosome size decreased in *Saccharomyces cerevisiae* with each subsequent division after checkpoint adaptation [88] and that *Xenopus* oocytes extracts entered mitosis despite that DNA replication was incomplete [89]. In both cases, the transfer of genomic information was not complete.

Kubara *et al.* reported that at least 2% of HT-29 cells that undergo checkpoint adaptation survived [3]. Analysis of cells treated with CPT showed that mitotic chromosomes had been shattered into several pieces [96]. Afterwards, the survivor cells

had an average of 35 chromosomes compared to non-treated cells, which had 65 chromosomes. Furthermore, at least 45% of these cells were missing telomeres and 28% had telomere sequences that were not positioned at the chromosome ends. Although chromosome aneuploidy often occurs in cells that are micronucleated, micronuclei were not examined in this study. These data suggest that cells can acquire genomic changes after checkpoint adaptation.

During the course of anticancer treatments, both normal and cancer cells are exposed to DNA damaging agents. Therefore, if normal cells undergo checkpoint adaptation there is a potential for them to acquire genomic changes that could promote further mutagenesis in cells not directly associated with a primary tumour.

1.10. Cancer

Profound genomic diversity is one of the features of cancers cells; however the mechanism responsible for this genomic diversity is not well understood. I will investigate if cells that survive checkpoint adaptation acquire genomic change. As described above, micronuclei are commonly found in cancer cells and are one type of genomic change; however, their relationship to checkpoint adaption has not been investigated. I will study this relationship in brain cancer cells, since they exhibit profound genomic diversity and are among the most difficult tumours to treat.

1.10.1 Glioblastoma multiforme

The Canadian Cancer Society has predicted that in 2014 approximately 2900 Canadians will be diagnosed with a primary brain tumour and at least 1950 will die from

their disease [97]. The World Health Organization (WHO) has listed more than 120 different types of brain cancers [98]. Typically, brain cancers are named after the type of cell from which they develop and are categorized by tumour grades (I to IV). Grades describe the aggressiveness and growth rate of a tumour. For example, grade I tumours typically describe benign or slow growing tissue, which if completely removed by surgical resection, do not return. By contrast, grade II to IV tumours are defined by malignant fast growing tissue which even after being surgically removed, often return.

The most common type of brain tumours are gliomas [99]. Gliomas develop from glial cells (non-neuronal support cells), which include astrocytes, oligodendrocytes, ependymocytes, and radial glia cells [100, 101]. Approximately 51% of gliomas are classified as being highly malignant (grade IV tumours) [101] and are referred to as glioblastomas multiforme (GBM). Their high rate of proliferation and aggressive invasiveness make GBM one of the deadliest cancers to humans worldwide [101, 102]. In fact, a patient with untreated GBM has a median survival time of approximately 16 weeks after diagnosis [103].

1.10.2. GBM treatments

Treating GBMs is difficult. This is partly because the brain has a limited repair capacity and is highly sensitive to damage from genotoxic agents [100]. Furthermore, many anticancer drugs are unable to cross the blood-brain barrier (BBB) due to their size or polarity. Nearly 98% of small molecules used in treatments do not cross the BBB [104]. Two anticancer drugs that are used to treat GBM include CPT [105] and cisplatin [57, 58].

In addition to chemotherapy, GBM are also treated with a combination of surgery and radiation [100]. The goal of surgical resections is to remove as much of the tumour mass as possible, while the remaining tumour cells are targeted with chemotherapy and radiation. However, despite available treatments, median patient survival is only 12-14 months [100, 101] with only 3-5% surviving more than 3 years [106].

1.10.3. Significance for investigating micronuclei and checkpoint adaptation in GBM

To improve survival rates of GBM patients, much research has gone into characterizing tumours cells obtained from GBM patients with the hope of identifying common underlying causes. The Cancer Genome Altas (TCGA) reported that the DNA sequences of 206 tumours were substantially different from each other [107]. These differences were greater than the variations that exist in tissues from healthy individuals. The differences between genomes included: nucleotide substitutions, gene amplifications, deletions, and chromosomal rearrangements. In a follow up study another 128 tumours were analyzed, it was reported that their genomes also exhibited chromosome aneuploidy [108]. The genomic diversity and instability within these tumours cells is the likely the reason they respond poorly to anticancer treatments. In many cases glioblastomas initially respond to genotoxic treatments and either stop growing or decrease in size; however, cells resistant to chemotherapy and/or radiation eventually allow the tumour to continue growing. One hypothesis for how this occurs is that tumour cells acquire changes to their genome following exposure to chemotherapy and/or radiation [109]. It is therefore important to understand how cells acquire genomic changes so that the mechanisms responsible can be targeted.

1.10.4. Checkpoint adaptation and glioblastoma cells

Several studies have confirmed the activation of the DNA damage checkpoint in glioblastoma cells following genotoxic treatment [110], however few studies have examined the events that occur after the DNA damage checkpoint is activated. I propose that glioblastoma cells undergo checkpoint adaptation and acquire genomic changes such as micronuclei.

Micronuclei are commonly observed in glioblastoma cells following genotoxic treatment [111, 112]. Akudugu and Böhm reported that five glioblastoma cell lines (G120, G60, G28, G44, and G62) formed micronuclei after exposed to ionizing radiation [111]. At a therapeutic dose of 2 Gy approximately 25-50% of these cells acquired micronuclei [111]. In another study of 8 human glioblastoma cell lines, Slowinski *et al.* observed micronuclei after exposure to 2 Gy of ionizing radiation [112]. In this study 78% of DK-MG cells that underwent mitosis produced at least one daughter cell with a micronucleus. Importantly, these studies did not confirm if the DNA damage checkpoint was active or if cells entered mitosis with damaged DNA prior to cell death, therefore it is not known if checkpoint adaptation occurred.

Checkpoint adaptation has been reported to occur in a glioblastoma cell line (M059K) following treatment with CPT [3]. Evidence of checkpoint adaptation was also reported in another glioblastoma cell line (U87MG-E6) [113]. In this study, mitotic death occurred in cells treated with the DNA methylating agent temozolomide (TMZ) after a prolonged arrest in G_2-phase, but there was no confirmation of damaged DNA in mitotic

cells. Importantly, micronuclei were not reported in either of the studies using M059K cells or U87MG-E6 cells.

1.10.5. M059K cells

M059K cells have been previously established as a cellular model for checkpoint adaptation in our laboratory [3]. They exhibit several chromosomal rearrangements [114], contain a variable chromosome number (65 to 79), and at least 22% of cells are polyploid [115]. Cells treated with 25 nM CPT recruited histone γH2AX and contained Chk1 phosphorylated on serine 345 [3]. Approximately 48 h post treatment, cells accumulate in G_2-phase of the cell cycle as determined by flow cytometry. After accumulating in G_2-phase, cells begin to enter mitosis as detected by cell rounding, and by the presence of the phospho-Ser^{10}-histone H3. While in mitosis, cells retained histone γH2AX and dephosphorylated Chk1.

M059K cells have also been previously shown to acquire micronuclei following exposure to silver nanoparticles (Ag-np) [116]. Ag-np induced DSB and chromosome aberrations including acentric and dicentric chromosomes. Lim *et al.* reported that cells treated with 100 μg/mL of Ag-np form 50% more micronuclei than non-treated cells [116]. Therefore, even novel genotoxic agents can induce micronuclei. Because M059K cells form micronuclei in the presence of damaged DNA and undergo checkpoint adaptation they are a useful model to study the relationship between these processes.

Chapter 2 - Introduction

The prognosis for glioblastoma patients is dismal, with an average survival rate of 12 to 14 months [100, 101] and a 3 year survival of only 3% to 5% even after treatment with radiation and chemotherapy [106]. It has been suggested that existing treatments may be ineffective due to the profound genomic diversity present in glioblastoma cells [107]. After sequencing the tumour genomes from 206 glioblastoma patients, The Cancer Genome Atlas (TCGA) reported that every genome had different combinations of nucleotide substitutions, gene amplifications, deletions, and chromosomal rearrangements [107]. In follow-up study of another 128 glioblastomas, chromosome aneuploidy was also reported in the tumour genomes [108]. To improve patient outcome, it is necessary to understand how these differences arise in glioblastoma genomes.

One way cells can acquire genomic change is through checkpoint adaptation (mitosis with damaged DNA) [16, 19]. In 2012, it was reported that human glioblastoma cells (M059K) underwent checkpoint adaptation following treatment with 25 nM camptothecin (CPT) [3]. CPT is a topoisomerase I inhibitor, which is used to treat glioblastoma patients [105].

Checkpoint adaptation is characterized by a cell-cycle arrest after detection of damaged DNA, overcoming this arrest and entry into mitosis with damaged DNA [87]. Cells signal the presence of damaged DNA by activating histone γH2AX [22, 23]. This leads to the activation of the DNA damage checkpoint, which is characterized by the phosphorylation of checkpoint kinase 1 (Chk1) on serine 345 [14]. Phospho-Ser^{345}-Chk1 prevents the activation of Cdk1 by up-regulating the activity of Wee1 [36] and down-regulating the activity of Cdc25C [35]. This causes cells to arrest in G_2-phase with 4n

DNA [33]. Eventually, cells enter mitosis despite having high levels of histone γH2AX [3, 93]. Mitosis can be detected by the presence of cyclin B1 and activation of cyclin-dependent kinase 1 (Cdk1) [5, 6].

Although most cells that undergo checkpoint adaptation die, at least 2% survive [3]. Survivor cells have been reported to contain fewer chromosomes, which are smaller in size and have telomeres that are either missing or at positions other than the chromosome ends [96].

Several studies have demonstrated that genotoxic treatments can induce micronuclei, another type of genomic change. Micronuclei form from lagging anaphase whole chromosome [61, 62] or chromosome fragments [63] that did not attach properly to the spindle apparatus. This can occur if the centromere is either damaged [64, 71, 72] or missing [71, 74] as is the case for acentric chromosome fragments. In addition, damaged chromosomes may fuse and create a dicentric chromosome [71], which becomes attached to both spindle poles. Dicentric chromosomes form a nucleoplasmic bridge (NPB) between daughter nuclei after anaphase. The NPB is subsequently broken in cytokinesis and the resulting DNA fragments are then incorporated into micronuclei.

Once present, micronuclei induce additional damage to DNA. In 2012, Crasta *et al.* reported that chromosomes inside micronuclei acquire DNA breaks and, in some cases, undergo chromothripsis (chromosome shattering) due to aberrant DNA replication [61]. This process can be detected by the asynchronous incorporation of 5-bromo-2'-deoxyuridine (BrdU) in place of thymine during DNA replication and by the presence of histone γH2AX foci in the micronuclei [61, 63]. Chromosomes that undergo this process are shown to acquire genomic changes including translocation and truncations [61].

Despite the formation of micronuclei from cells that enter mitosis with damaged DNA, the relationship of micronuclei to checkpoint adaptation has not been studied. This is mainly because checkpoint adaptation was only recently observed in human cancer cells. In this thesis I will determine if the formation of micronuclei requires checkpoint adaptation [63, 71]. To study this relationship I will examine M059K cells since they have been previously shown to undergo checkpoint adaptation. Furthermore, these cells have also been reported to form micronuclei after genotoxic treatment with silver nanoparticles (Ag-np) [116].

In addition to determining the relationship between micronuclei and checkpoint adaptation in M059K cells, I will also determine if either of these processes occurs in the normal lung fibroblast cell line (WI-38). Checkpoint adaptation has not been reported in normal cells. Normal cells are difficult to culture and have a limited number of cell divisions [117].

WI-38 cells contain two functional DNA damage checkpoints that regulate the transition between G_1/S and G_2/M-phases. At least 80% of cancer cells (including M059K cells) have a defect in the p53 pathway [17], which impairs the activation of the G_1/S DNA damage checkpoint. This causes M059K cells to accumulate in G_2-phase with damaged DNA, a response that is required for checkpoint adaptation [3]. Since WI-38 cells have a functional G_1/S DNA damage checkpoint, it is not known if they can undergo checkpoint adaptation. Understanding if checkpoint adaptation can occur in normal cells is important since genomic instability in normal cells may contribute to the development of secondary tumours; a process that commonly occurs in glioblastoma patients.

Research Objectives

The main objective of this thesis is to determine if checkpoint adaptation is required for the formation of micronuclei in human cancer cells. Checkpoint adaptation and the formation of micronuclei have been reported in human cancer cells with damaged DNA; however the relationship between these processes has not been studied. To study this relationship, it will be necessary to complete the following:

1. Develop an experimental model to study micronuclei using a glioma cell line (M059K) and compare them to a normal cell line (WI-38). This is important because many cancer cells already have micronuclei and therefore it is necessary to characterize how many are micronucleated before genotoxic treatment.
2. It is not known to which genotoxic molecule the M059K and WI-38 cells will be sensitive to, therefore it is necessary to characterize different genototxic molecules (CPT, S23906-1, and cisplatin). Then I will test if the selected genotoxic agent induces micronuclei in M059K cells.
3. If micronuclei form in M059K cells, I will test if cells also exhibit the steps of checkpoint adaptation: signal damaged DNA, activate Chk1, accumulate in G_2 phase, and enter into mitosis with damaged DNA.
4. I will examine if WI-38 cells undergo the steps of checkpoint adaptation and/or form micronuclei. This is important because checkpoint adaptation has not been reported in normal cells. Answering this question is important in explaining how normal cells become cancerous.

Chapter 3 - Materials and methods

3.1. Cell Culture

Human M059K and WI-38 cells were obtained from the American Type Culture Collection (CRL-2365; CCL-75). M059K cells were maintained in Dulbecco's Modified Eagle medium (DMEM) F-12 (Gibco; 11320-082), supplemented with 10% heat inactivated fetal calf serum (Gibco; 12484028), 2 mM non-essential amino acids (Gibco; 11140050), and 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. WI-38 cells were maintained in DMEM/high glucose (Sigma; D6546) supplemented with 10% heat inactivated fetal calf serum (Gibco; 12484028), 2 mM non-essential amino acids (Gibco; 11140050), and 1.6 mM GlutaMAX (Gibco; 35050-061). Cells were grown at 37°C in 5% CO_2 and media was changed every three to four days. M059K and WI-38 cells were plated at a density of 5.0 x 10^5 cells/75 cm^2 flask and cultured for 48 h prior to treatment. The compound cisplatin (Sigma; 479306-1G) was dissolved in dimethyl sulfoxide (DMSO) (Sigma; D2438) to a concentration of 100 mM. CR8 (Tocris Biosciences; 2706/10), Gö6976 (Tocris Biosciences; 2253), CPT (Sigma; 7689-03-4) and S23906-1 (Servier) were dissolved to a concentration of 10 mM. Nocodazole (Sigma-Aldrich; M1404-10MG), and aphidicolin (Santa Cruz; sc-201535) were dissolved in DMSO at concentrations of 200 μg/mL and 10 mg/mL respectively. All compounds listed were stored at -20°C until use.

3.2. Cytotoxicity Assay

Cytotoxicity of CPT, S23906-1, and cisplatin on M059K and WI-38 cells was measured by the micro-culture tetrazolium assay (MTT; (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich; M2128-1G)) [3, 118]. Cells were plated at 4.0 x 10^5 cells/96 well assay plate and cultured for 48 h prior to treatment. All measurements were performed in triplicate at 48 h and 96 h unless otherwise indicated. After the desired treatment time, 20 μL of MTT solution (5 mg/mL of MTT in PBS) was added to each well without removing media for 3.5 h. Afterwards, the media was aspirated and 100 μL of MTT solvent (4 mM HCl, 0.1% IPEGAL (octylphenoxypolyethoxyethanol), in isopropanol) added. Plates were placed on shake tray for 20 min and then the absorbance of each well was measure at 590 nm using the BioTek™ microplate spectrophotometer powered by Eon™ software. Results were expressed as IC_{50} concentrations; the genotoxic molecule concentration that reduced absorbance of the MTT by 50%, compared to 0.1% DMSO treated cells. The normalized percent absorbance was calculated using the equation:

$$Normalized\ \%\ absorbance = (absorbance/DMSO\ absorbance) \times 100\%$$

The log concentration of the genotoxic molecule was then plotted against the normalized percent absorbance and analyzed by GraphPad Prism 5 software using non-linear regression (log (inhibitor) versus normalized response) to estimate the IC_{50} concentration. Standard curves were then plotted using the equation:

$$Y = maximum + (maximum - minimum) / (1 + 10^{X - LogIC50})$$

Where the maximum is the percentage of viable cells after treatment with 0.1% DMSO and the minimum is the percentage of viable cells after treatment with the highest concentration of the genotoxic molecule.

3.3. Flow Cytometry

After desired treatment time, total cell cultures were trypsinized, washed in phosphatase-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4) and fixed in 90% ethanol (-20°C) for at least 24 h. Fixed cells were rinsed once with PBS and twice with wash buffer (PBS, 1% BSA, 0.1% sodium azide) [3]. For analysis, samples were incubated for 20 min in wash buffer containing 0.02 mg/mL propidium iodide (Life Technologies; P1304MP) and 0.2 mg/mL RNAse A (Sigma; R6513-250MG), and analyzed with a FACS Canto II™ flow cytometer (BD Biosciences) using BD FACSDiva™ software. Gating was set using non-treated and nocodazole samples. Experiments were repeated at least twice.

3.4. Extract preparation

Cells were trypsinized, passed through a 26 gauge needle 5 times, and resuspended in extraction buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 50 mM NaF, 10 mM EGTA (ethyleneglycol-bis(beta-aminoethylether)-N,N′-tetraacetic acid), 50 mM β-glycerophosphate, 1 mM ATP, 1 mM (DTT) dithiothreitol, 1% Triton X-100, 10 µg/mL RNAse A (Sigma; R6513-250MG), 0.4 U/mL DNAse I (Invitrogen, I354Ba), with protease inhibitors (Roche; 11836170001)) at a concentration of 20,000 cells/µL on ice for 30 min. The suspension was centrifuged at

10,000×g for 10 min at 4 °C [3]. Extracts were boiled for 5 min in the presence of 2× SDS sample buffer (20% Glycerol, 10% DTT, 6% SDS, 500 mM Tris, pH 6.84).

3.5. Electrophoresis and western blotting

Reaction mixtures were separated in triplicate on an 8% SDS gel with a 4% stacking gel at 200 volts for 35 min. Proteins were transferred to nitrocellulose with a wet electroblotter system (BioRad) for 17.5 h at 30 volts and 90 mA. Subsequently, the membrane was blocked with either 5% low fat milk in Tris buffered saline TBS-0.1% Tween-20 (TBST) or 2% BSA in TBS-0.1% Tween-20 (TBS-T), and incubated overnight with the indicated primary antibody as follows: anti-Chk1 (Santa Cruz; SC-8408; 1:200), anti-phospho ser345 Chk1 (Cell Signalling; 2348S; 1:2000), or anti-actin (Santa Cruz; sc-58673; 1:200). After washing, the membrane was incubated with alkaline phosphatase-coupled anti-mouse (Promega; S3721; 2500) or anti-rabbit antibodies (Millipore; AP132A; 1:2500). Western blots were performed at least two times [3, 10].

3.6. Immunofluorescence microscopy

Cells were plated on glass coverslips for 48 h prior to treatment, then fixed in 3% formaldehyde for 20 min at room temperature. Cells were quenched with 50 mM NH_4Cl in PBS, permeabilized for 5 min in 0.2% Triton X-100, and blocked with 3% BSA for 30 min. Cells were incubated with primary antibodies for 2 h at room temperature as follows: anti-histone γH2AX (Millipore; 05-636; 1:400), anti-cyclin B1 (Santa Cruz; SC-752; 1:100), or anti-lamin A/C (Santa Cruz; SC-6215; 1:150). After washing, cells were incubated with secondary antibodies at room temperature for 2 h as indicated: Alexa488

anti-mouse (Life Technologies; A11059; 1:400) for anti-histone γH2AX, Alexa488 anti-rabbit for cyclin B1 (Life Technologies; A11008; 1:400), and Alexa488 anti-goat (Life Technologies; A11059; 1:150) for anti-lamin A/C. Nuclei were stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) in PBS for 15 min prior to mounting [3]. Cells were observed on an Olympus microscope operated by Infinity Capture Imaging software. Images were collected by the Infinity3 camera within the linear dynamic range. The linear dynamic range represents the range in which the relationship between a signal intensity and amount of material is likely to be linear. Images were prepared with ImageJ (IJ 1.46r) and Adobe Photoshop (CS3 10.0) software. Cells positive for histone γH2AX, cyclin B1, or Lamin A/C were counted using ImageJ (IJ 1.46r) software. At least 200 cells were counted per experiments and experiments were repeats at least twice.

3.7. Scoring micronuclei

Micronucleated cells, individual micronuclei, and nucleoplasmic bridges (NPBs) were counted manually from DAPI stained cells. DAPI positive micronuclei were confirmed by additional staining methods: lamin A/C, histone γH2AX, or BrdU. At least 200 cells were counted for each experiment and experiments were repeated at least twice. Criteria for scoring micronuclei included the following: (1) separate (non-overlapping) extra-nuclear structures that were positive for DAPI; (2) intensity of DAPI staining in micronucleus did not exceed the main nucleus; (3) encased by nuclear envelope composed of the protein lamin A/C; and (4) a rounded shape [71, 78, 119]. DAPI staining intensity and shape of micronuclei were confirmed by visual observations.

3.8. 5-bromo-2'-deoxyuridine (BrdU) assay

Cells were pre-labelled with 10 μM BrdU for 1 h at 37°C and then fixed with ethanol and permeabilised according to the manufacturers' conditions (5-bromo-2-deoxyuridine Labelling and Detection Kit 1; Roche; 11296736001). Cells were then stained with anti-BrdU for 1 h at 37°C (1:10) and then incubated with the anti-mouse-Ig-fluorescein secondary antibody for 1 h at 37°C (1:10). Slides were then processed for immunofluorescence [61].

3.9. Statistics

Graphing and statistics were completed using Microsoft Excel 2010 software. Data were collected and plotted as means ± standard error of the means. Significance was determined using ANOVA and student *t*-test for two paired sample means unless otherwise indicated.

Chapter 4 - Results

To examine the relationship between damaged DNA, checkpoint adaptation, and micronuclei, I organized experiments into four parts: (1) develop a new experimental model using glial cancer cells; (2) measurement of micronuclei formation under different treatment conditions; (3) testing the relationship between damaged DNA and micronuclei; and (4) examine micronuclei in a non-cancer cell line.

4.1. Characterization of micronuclei in glial cancer cells

It was important to characterize our experimental model prior to performing experiments because most cancer cells already contain micronuclei [71, 73, 120]. Therefore, I first determined the percentage of M059K cells with either micronuclei or nucleoplasmic bridges (NPB). Micronuclei were defined as separate (non-overlapping) extra-nuclear structures with a rounded shape that contained DNA encased by a nuclear envelope composed of the protein lamin A/C [61, 63, 71, 119, 121-123]. I stained cells with DAPI to detect DNA and observed them by immunofluorescence microscopy (Figure 4). I then counted rounded extra-nuclear structures that were DAPI positive. In an exponentially growing culture of M059K cells, 25% ± 1% contained at least one DAPI stained structure in addition to the main nucleus. Cells also had NPBs but at a much lower frequency (0.5% ± 0.1%). To confirm that the DAPI positive structures were contained by a nuclear envelope, I stained cells with an anti-lamin A/C antibody. I examined 200 micronucleated cells and confirmed that 94% ± 8% of extra-nuclear structures identified by DAPI also contained lamin A/C. This experiment was repeated twice. Since almost all DAPI structures had lamin A/C, it was not required for subsequent

experiments for identifying micronuclei in these cells. Therefore, in our experimental model, approximately 24% of the M059K cells have micronuclei, which is consistent with a cancer phenotype [116, 124-127]. If checkpoint adaptation is linked to micronuclei production, then I could expect that there will be changes in either the number of cells that have micronuclei and/or NPBs, or the number of micronuclei and/or NPBs in cells that undergo checkpoint adaptation.

Micronuclei are rarely detected in normal, non-cancerous human cells [126, 128]. I confirmed this by examining the normal human lung cell line WI-38, which are fibroblastic, as are M059K cells. I was obliged to use lung cells since normal neural cells are extremely difficult to cultivate. Cells were stained with either DAPI or anti-lamin A/C antibodies and observed by immunofluorescence microscopy (Figure 5). In contrast to M059K cells in which 25% ± 1% had DAPI stained structures, only 2% ± 2% of WI-38 cells contained DAPI stained structures. No NPBs were detected in these cells. Cells were also analyzed for the presence of lamin A/C, but only 47% ± 5% of extra-nuclear DAPI stained structures were lamin A/C positive. Since over half of extra-nuclear DAPI stained structures in WI-38 cells were not enclosed by a nuclear envelope, they were not considered to be true micronuclei [61, 67, 78, 129]. These results confirmed that normal cells have few micronuclei [126], compared to cancer cells.

4.2. Characterization of genotoxic agents

To test the relationship between a genotoxic treatment and changes in the status of micronuclei, I first needed to select a genotoxic agent for use in our experiments. I tested three agents, camptothecin (CPT), S23906-1, and cisplatin (Table 1), with a different

mechanism of action. Our criteria for selection were an agent that has an IC_{50} concentration to cultured cells that was similar to the concentration used to treat human cancer patients [19, 130], and a difference in cell viability between early and late times after treatment [3]. Different concentrations of either CPT, S23906-1, or cisplatin were added to cells and cell viability was measured at 48 h and 96 h by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 6; Table 2). Data were normalised to those obtained by treatment with 0.1% DMSO, which was the solvent. I observed that cell viability declined in both M059K cells and WI-38 cells as the concentration of each genotoxic agent was increased; the lowest viability was observed at 96 h and the highest viability was observed at 48 h. I found that M059K cells treated with CPT had IC_{50} values of 49.4 nM at 48 h and 7.4 nM at 96 h. WI-38 cells were less sensitive to CPT and had IC_{50} values of 376.4 nM at 48 h and 94.6 nM at 96 h. Whereas the IC_{50} value of CPT after 96 h in M059K cells was within the pharmacologically relevant concentration range (2.8-17.3 nM) [131], the IC_{50} concentration in WI-38 cells was five times higher than the maximum concentrations received by human patients. CPT was therefore excluded from further experiments. M059K cells treated with S23906-1 had IC_{50} values of 7.6 μM at 48 h and 2.2 μM at 96 h; these concentrations were within the cytotoxic range of previously published IC_{50} values (1-10 μM) [51, 52, 132, 133]. WI-38 cells were less sensitive to S23906-1. The calculated IC_{50} values were 48.1 μM at 48 h and 14.8 μM at 96 h, which were outside our experimental treatment range (0.01-10 μM) and were higher than concentrations previously published. On the basis of this, S23906-1 was also excluded from further testing. M059K cells treated with cisplatin had IC_{50} values of 58.8 μM at 48 h and 15.4 μM at 96 h. By contrast, WI-38 cells treated with

cisplatin had an IC_{50} value 12 times higher than M059K cells at 48 h (681 μM), but had a similar IC_{50} value of 18.5 μM at 96 h. Based on these data, 30 μM cisplatin was selected for subsequent experiments because it was a cytotoxic concentration in both M059K cells and WI-38 cells and was within the pharmacologically relevant treatment range (12-40 μM) for human cancer patients [19, 134-136]. I noted that 40% ± 5% of M059K cells and 40% ± 11% of WI-38 cells treated with 30 μM cisplatin for 96 h were still alive. To further demonstrate the cytotoxicity of 30 μM cisplatin, I treated cells with cisplatin for an additional 24 h (120 h total). I observed that cell viability declined to 19% ± 5% in M059K cells and 27% ± 2% in WI-38 cells treated with cisplatin for 120 h indicating that longer treatments with cisplatin results in more cell death. Importantly, at this concentration 80-90% of cells were alive at 48 h, but most (73-81%) had died by 120 h. This type of cellular response is characteristic of checkpoint adaptation [3], a point that will be described later in my thesis. Based upon the outcome of these tests, I then selected cisplatin as the genotoxic agent to investigate micronuclei.

4.3. Cisplatin induces micronucleation in M059K cells

Having confirmed that cisplatin induced the desired cytotoxic response in M059K cells, I then tested if treatment with cisplatin would increase either the number of cells that had micronuclei and/or NPBs, or the number of micronuclei and/or NPBs per cell. Cells were treated with 30 μM cisplatin for up to 120 h and then stained with DAPI and observed by immunofluorescence microscopy (Figure 7A). I observed that the percentage of cells with micronuclei was 24% ± 2% at time 0, but doubled to 48% ± 7% by 96 h ((t = 2.13; d.f. = 4; $p < 0.05$) Figure 7B). I also observed an increase in the

number of micronuclei per cell (Figure 7C). At time 0, 72% ± 2% of the micronucleated cells had one micronucleus whereas only 2% ± 0.4% of cells had six or more micronuclei. However, after 120 h the percentage of cells with one micronucleus dropped to 47% ± 1% and those containing six or more micronuclei had significantly increased to 33% ± 1% (t = 2.13; d.f. = 4; $p < 0.05$). The number of cells containing two to five micronuclei did not change in a significant manner. I also observed additional NPBs. Between 0 and 72 h, the proportion of cells with NPBs was less than 1%, but after 120 h they had increased to 3% ± 1% (t = 2.13; d.f. = 4; $p < 0.05$). These data confirm that treatment with cisplatin induces the formation of micronuclei in M059K cells, an observation that had been previously reported in experiments using other human cancer cells [64, 68, 137]. Having observed that an increase in treatment time led to an increase in micronucleation, I then tested the effects of increases in cisplatin concentrations. Cells were either non-treated or treated with 10-300 μM cisplatin for 48 h, and then stained for DNA and observed by immunofluorescence. I observed that the percentage of cells with micronuclei increased from 23% ± 1% in non-treated cells to 60% ± 8% in cells treated with 300 μM cisplatin (t = 6.31; d.f. = 1; $p < 0.05$ (Figure 8)). These data demonstrate a dose-dependent response between cisplatin and micronuclei formation.

4.4. Micronuclei persist for at least eight days after treatment with cisplatin

Knowing that cisplatin increased the percent of cells with micronuclei, I examined whether the micronuclei that formed persisted after cisplatin treatment. I treated M059K cells with 30 μM cisplatin for 120 h, and then cultured them for an additional 8-10 days with fresh (non-treated) media. Cells were then stained with DAPI and observed by

immunofluorescence microscopy (Figure 9). I observed that the percentage of micronuclei in surviving cells (cells analyzed 8 to 10 days after cisplatin removal), was significantly higher than in non-treated cells, 47% ± 6% compared to 23% ± 5% (t = 2.02; d.f. = 5; $p < 0.05$). Interestingly, the percentage of surviving micronucleated cells remained at a similar level to that of cells analyzed directly after treatment with 30 μM cisplatin for 120 h (Figure 7). Together, these data suggest that once a micronucleus is formed, it can be stably maintained in cells for at least 8-10 days after cisplatin treatment is stopped.

4.5. Micronuclei arise in M059K cells that have undergone checkpoint adaptation

Since M059K cells treated with cisplatin formed additional micronuclei, I then asked if this increase required checkpoint adaptation. Checkpoint adaptation is composed of three steps: (1) an arrest in G_2-phase following detection of damaged DNA; (2) bypass of the G_2-phase arrest; and (3) entry into mitosis in the presence of damaged DNA [87]. I prepared a series of experiments to investigate if these steps had occurred.

Cells that undergo checkpoint adaptation signal the presence of damaged DNA through the phosphorylation of the histone variant H2AX on serine 139, then becoming γH2AX. M059K cells were either non-treated or treated with different concentrations of cisplatin (10 μM to 300 μM) and then stained with DAPI and anti-histone γH2AX antibodies for detection of DNA and damaged DNA, respectively (Figure 10A). I observed that both the number of cells positive for histone γH2AX and intensity of histone γH2AX staining increased with an increase in the concentration of cisplatin. No histone γH2AX signals were detected in non-treated cells. The percentage of cells that

were positive for histone γH2AX after treatment with 10 μM cisplatin was 61% ± 3%, but this percentage significantly increased to 94% ± 2% with higher concentrations of cisplatin (30 μM to 300 μM) ((t = 6.31; d.f. = 1; p < 0.05) Figure 10B). These data are consistent with previously published reports [52, 138], which show that cisplatin damages DNA, a response necessary for the first step of checkpoint adaptation [3].

Damaged DNA leads to a second checkpoint adaptation response: phosphorylation of checkpoint kinase 1 (Chk1) on serine 345. This phosphorylation activates Chk1 and causes cells to initiate a cell-cycle arrest. To examine this, I prepared protein extracts from M059K cells that were either non-treated or treated with 30 μM cisplatin for 24 h and then tested them for phospho-Ser^{345}-Chk1 by western blotting (Figure 11A). Phospho-Ser^{345}-Chk1 was present in cells treated with cisplatin, but absent in non-treated cells. Comparison of non-phosphorylated Chk1 levels in treated and non-treated cells confirmed that the total amount of Chk1 protein were similar. Comparison of actin levels between extracts confirmed equal loading of proteins. These data indicated that cells treated with 30 μM cisplatin activate Chk1, which is also necessary for the first step of checkpoint adaptation.

I then examined if a cell-cycle arrest had occurred in cisplatin-treated cells by the technique of flow cytometry, which measures DNA content. M059K cells were either non-treated, treated with 200 ng/mL of nocodazole (G_2/M-phase control), or treated with 30 μM cisplatin, and then analyzed at 24 h by flow cytometry (Figure 11B and C). As expected, non-treated cells were predominantly in the G_1-phase (67% ± 1%) with the remaining cells in either the S-phase (14.6 ± 1%) or G_2/M-phase (18% ± 2%) [3]. By contrast, 49% ± 1% of cells treated with nocodazole were in the G_2/M-phase [3].

Treatment with cisplatin resulted in 47% ± 1% of cells in G_1-phase, 24% ± 0.3% cells in S-phase, and 29% ± 1% cells in G_2/M-phase, which confirmed that a cell-cycle arrest had occurred in some cells[139].

Having shown that M059K cells treated with cisplatin had damaged DNA, active Chk1, and had undergone a cell-cycle arrest, I then examined the population for mitotic cells. This was important because in the final step of checkpoint adaptation, cells enter mitosis with damaged DNA [87]. I first tested if cells contained the cyclin B1, which is an essential protein that forms a complex with the mitotic enzyme cyclin dependent kinase 1 (Cdk1) [6]. Cells were either non-treated or treated with 30 μM cisplatin for 48 h and then stained for cyclin B1 and observed by immunofluorescence microscopy (Figure 12). In non-treated cell populations, only 13% ± 3% of cells were cyclin B1 positive. By contrast, treatment with 30 μM cisplatin for 48 h increased the percentage of cyclin B1 positive cells to 75% ± 3% (t = 6.31; d.f. = 1; $p < 0.05$). The accumulation of cyclin B1 positive cells following cisplatin treatment suggests that these cells were "primed" for entry into mitosis despite the fact that 94% ± 2% had damaged DNA at 48 h (Figure 10).

The analysis of cyclin B1 and histone γH2AX revealed that most cells were competent to enter mitosis with damaged DNA. Under the current model of checkpoint adaptation, the inhibition of either Chk1 or Cdk1 with chemical inhibitors can be used control the number of cells that enter into mitosis.

Chk1 controls the DNA damage checkpoints that are responsible for the cell-cycle arrest. Inhibiting Chk1 activity causes cells to enter mitosis with damaged DNA [40]. I tested if blocking the activity of Chk1 with the small molecule Gö6976 [140] would result in an increase in micronucleated M059K cells. Cells were either non-treated,

treated with 15 nM Gö6976 for 24 h, 30 μM cisplatin for 48 h, or with 30 μM cisplatin for 24 h and then co-treated with 15 nM Gö6976 for an additional 24 h. Cells were stained with DAPI and then observed by immunofluorescence microscopy (Figure 13). I observed that 28% ± 1% cisplatin-treated cells had micronuclei. By contrast, 38% ± 3% of cells co-treated with Gö6976 and cisplatin had micronuclei, which represented a significant increase ($t = 2.92$; d.f. = 2; $p < 0.05$). There was no significant change in the percentage of cells with micronuclei after treatment with Gö6976 alone compared to non-treated cells ($t = 2.92$; d.f. = 2; $p = 0.052$). These data suggest that inhibiting Chk1 enables more cells with damaged DNA to enter mitosis and subsequently form new micronuclei.

Having shown that enabling cells to enter mitosis with damaged DNA increases the number of micronuclei, I then tested if preventing mitosis in cells with damaged DNA would stop new micronuclei from forming. Cdk1 controls entry into mitosis. Inhibiting Cdk1 with CR8 prevents cells with damaged DNA from entering mitosis [3]. Cells were treated with either 30 μM cisplatin or co-treated with 30 μM cisplatin and 500 nM CR8 for up to 120 h. Cells were then stained with DAPI and observed by immunofluorescence microscopy (Figure 14). As shown in Figure 7, treatment with cisplatin increased the percentage of micronucleated cells over time. By contrast, no significant change in micronucleation was observed in the cell population that was co-treated with cisplatin and CR8. This confirmed our prediction that cells with damaged DNA cannot form new micronuclei if Cdk1 activity is blocked.

4.6. Micronuclei undergo aberrant DNA replication

In 2012, Crasta *et al.* examined micronuclei in RPE-1 (epithelial) and U2OS (osteosarcoma) cells and showed that DNA in micronuclei is damaged by aberrant DNA replication [61]. They showed that DNA replication in micronuclei was not synchronized with the DNA replication in the main nucleus. They also showed that DNA replication induced histone γH2AX foci in micronuclei. Since I had an established experimental model that had a high percentage of cells with micronuclei, I was well poised to extend the observations by Crasta *et al.* [61]. To examine if DNA in the micronuclei of M059K cells undergoes asynchronous DNA replication and if replication damages the DNA I designed a series of experiments in three steps: (1) characterization of DNA replication in micronuclei; (2) examination of micronuclei for damaged DNA; and (3) testing the relationship between replication and damaged DNA in micronuclei.

It was not known whether the DNA of micronuclei in M059K cells would undergo DNA replication, therefore I first needed to answer this. I examined DNA synthesis by a method that measures the incorporation of the nucleoside analogue 5-bromo-2'-deoxyuridine (BrdU) in place of thymidine during DNA replication. Cells were incubated with 30 μM BrdU for 30 min and then stained with DAPI and anti-BrdU antibodies and observed by immunofluorescence microscopy (Figure 15). I detected BrdU incorporation in 35% ± 4% of non-treated cells, consistent with cell-cycle phase values obtained by flow cytometry (Figure 11B and C). I observed that BrdU signals in the micronucleus and main nucleus were not correlated in 20% ± 7% of non-treated cells (Table 3). These cells were either positive for BrdU in the micronucleus and not in the main nucleus or vice versa.

To address if DNA in micronuclei was damaged, I then examined micronuclei for histone γH2AX. Consistent with previously published reports [61], I detected that 47% ± 9% of micronuclei in non-treated cells were positive for histone γH2AX staining whereas the main nuclei were negative for histone γH2AX staining (Figure 16, Table 4). These data support that the DNA in micronuclei can be damaged in the absence of genotoxic treatment with cisplatin, and that this damage might be caused by DNA replication.

To test whether DNA replication was inducing damaged DNA in micronuclei, I set up experiments to block DNA replication using aphidicolin (APH), a DNA polymerase α inhibitor [141, 142]. First, I needed to determine the optimal APH concentration that could inhibit DNA replication in these cells. Cells were non-treated, treated with 200 ng/mL of nocodazole to set a 4n DNA amount, or treated with 0.1 μg/mL, 1 μg/mL, and 10 μg/mL of APH for 24 h and then analyzed by flow cytometry for DNA content (Figure 17). Higher APH concentrations resulted in fewer cells in S-phase and G_2/M-phase compared to non-treated cells (Figure 17B). Treatment with 10 μg/mL APH resulted in the highest amount of cells in G_1-phase (82% ± 3%), which suggested that DNA replication was blocked. Therefore, 10 μg/mL APH was used in subsequent experiments.

To confirm that APH treatment could inhibit DNA replication, I treated M059K cells with 10 μg/mL APH for 24 h, added BrdU to the media, and then examined the incorporation of BrdU by immunofluorescence microscopy (Figure 18). In the absence of APH, 30% ± 1% of cells incorporated BrdU in their nuclei whereas only 5% ± 1% of APH-treated cells had BrdU in their nuclei ($t = 6.31$; d.f. = 1; $p < 0.05$) confirming that DNA replication was inhibited. Treatment with APH also reduced asynchronous BrdU

incorporation in micronuclei from 17% ± 4% in non-treated cells to 4% ± 1% (t = 6.31; d.f. = 1; $p < 0.05$). These data confirmed that that BrdU incorporation is dependent upon DNA replication and that blocking replication prevents BrdU incorporation in both the main nuclei and micronuclei of M059K cells.

By characterizing several of the parameters of micronuclei formation and by validating methods to study micronuclei using inhibitors, I could now test the relationship between DNA replication and damaged DNA in micronuclei. I tested if inhibiting DNA replication by APH treatment would change histone γH2AX levels within micronuclei. Cells were either non-treated or treated with 10 μg/mL APH and then stained with histone γH2AX antibodies, and observed by immunofluorescence microscopy (Figure 19). The percentage of micronuclei that were positive for histone γH2AX was 54% ± 5% in non-treated cells and was reduced to 32% ± 10% (t = 6.31; d.f. = 1; $p < 0.05$) in APH-treated cells. These data indicated that DNA replication has a role in damaging DNA in micronuclei. Therefore, micronuclei can acquire damage in the absence of a genotoxic treatment.

I then explored the effects of a genotoxic treatment upon histone γH2AX signals in micronuclei in M059K cells. Cells were either non-treated, treated with 30 μM cisplatin for 48 h, or with 30 μM cisplatin for 24 h and then co-treated APH for an additional 24 h. Cells were then stained with DAPI and anti-histone γH2AX antibodies and observed by immunofluorescence microscopy (Figure 19B and C). I observed that 83% ± 6% of micronuclei contained histone γH2AX signals in the presence of cisplatin. By contrast, only 67% ± 8% of micronuclei had histone γH2AX signals in cells co-treated with cisplatin and APH (t = 2.92; d.f. = 2; $p < 0.05$). Under these conditions, the

percentage of cells with micronuclei after treatment with APH and cisplatin was similar to that of cells in the non-treated populations (Figure 19C). These data confirmed that micronuclei acquire additional damage in the presence of cisplatin and that treatment with APH can both limit damage to DNA by cisplatin and prevent cells from forming new micronuclei.

4.7. WI-38 cells treated with cisplatin do not form additional micronuclei

I established a model system using M059K cells in which I could study micronuclei in cells that undergo checkpoint adaptation in response to cisplatin treatment. During our investigation I observed that micronuclei acquired damaged DNA as a result of aberrant DNA replication. With this information, I was now ready to examine if WI-38 cells, which are not cancerous, undergo a similar response to cisplatin as observed in M059K cells.

I observed that new micronuclei arise in M059K cells treated with cisplatin, but these are cancer cells and known to have impairments to the DNA damage checkpoint and DNA repair pathways [143-145]. Since WI-38 cells are normal cells and have functional DNA damage checkpoints and repair pathways, I predicted that additional micronuclei would not form following genotoxic treatment [126]. To test this prediction, I treated WI-38 cells with 30 μM cisplatin for up to 120 h. Cells were stained with DAPI and then observed by immunofluorescence microscopy (Figure 20). During the first 48 h, no morphological changes were observed in cells treated with 30 μM cisplatin. However after 72 h, I observed cells undergoing apoptotic cell death as they displayed discrete, brightly-stained chromatin structure [68, 119, 122, 128]. Between 96 and 120 h the

number of apoptotic cells increased, as well as material from dead cells. The highest percentage of micronucleated cells observed was 5% ± 2% at 96 h but this was not statistically significant ($p > 0.05$). In addition, I did not detect cells with NPBs. These data suggested that cisplatin does not induce micronuclei formation in WI-38 cells but rather causes apoptotic cell death.

4.8. WI-38 cells do not undergo checkpoint adaptation at a detectable frequency

Since micronuclei were not observed in WI-38 cells treated with cisplatin, I predicted that these cells do not undergo checkpoint adaptation. To address this, I examined cells for the steps of checkpoint adaptation. First, I examined WI-38 cells treated with cisplatin for levels of histone γH2AX. Cells were either non-treated or treated with different concentrations of cisplatin (10-300 μM) for 48 h, stained with DAPI and anti-histone γH2AX antibodies, and then observed by immunofluorescence microscopy (Figure 21). The results were similar to those of M059K cells; the percentage of WI-38 cells positive for histone γH2AX after treatment with 30 μM cisplatin was 79% ± 3%, but it significantly increased to 95% with treatments of 100 μM or greater ($t = 6.31$; d.f. = 1; $p < 0.05$). At concentrations lower than or equal to 30 μM, most cells had histone γH2AX foci but at concentrations greater than 30 μM cisplatin, pan-nuclear staining was observed. The presence of histone γH2AX pan-nuclear staining indicates that these cells have acquired more damage than those with histone γH2AX foci. Non-treated WI-38 cells did not stain for histone γH2AX. Nearly all WI-38 cells died after treatment with greater than 100 μM cisplatin; therefore it was not possible to study micronuclei formation in survival cells, as I did with M059K cells. These data revealed

that although the DNA of WI-38 cells can be damaged by cisplatin treatment, only a very small percentage of cells form micronuclei.

To better understand the differences in the production of micronuclei that I observed between WI-38 and M059K cells, I investigated the response by WI-38 to cisplatin and corresponding damaged DNA. I tested if cisplatin induced the activation of the DNA damage checkpoint in WI-38 cells. I prepared protein extracts from cells that were either non-treated or treated with cisplatin for 24 h and examined them for phospho-Ser^{345}-Chk1, via western blotting (Figure 22A). Similarly to M059K cells, phospho-Ser^{345}-Chk1 was present in cisplatin treated cells but absent in non-treated cells. Comparison of total Chk1 levels revealed the difference in the phosphorylation was also accompanied with an increase in Chk1 levels. Actin levels showed equal loading of protein extracts. These data support that WI-38 cells initiate the DNA damage checkpoint by activating Chk1 after treatment with 30 μM cisplatin.

I tested if WI-38 cells underwent a cell cycle arrest after treatment with cisplatin. Cells were non-treated, treated with 200 ng/mL of nocodazole (G_2/M-phase control), or treated with 30 μM cisplatin, and then analyzed at 24 h by flow cytometry (Figure 22B and C). Non-treated cells were predominantly in the G_1/G_0-phase with the remaining cells in either the S-phase or G_2/M-phase. Cells treated with nocodazole accumulated in the G_2/M-phase. In contrast to M059K cells (Figure 11), there were no differences in the percentages of WI-38 cells in S-phase or G_2/M-phase after treatment with cisplatin compared to non-treated cells.

I then examined if cisplatin treated WI-38 cells express cyclin B1, which is required for mitosis. Cells were either non-treated or treated with 30 μM cisplatin for 48

h and then stained with anti-cyclin B1 antibodies and observed by immunofluorescence microscopy (Figure 23). I observed that 13% ± 1% of non-treated cells and 22% ± 1% of cells treated with 30 μM cisplatin for 48 h were positive for cyclin B1 (t = 6.31; d.f. = 1; $p < 0.05$). These data confirm that the majority of WI-38 cells were not "primed" to enter mitosis although most had damaged DNA, which was consistent with the lack of micronuclei observed during cisplatin treatments.

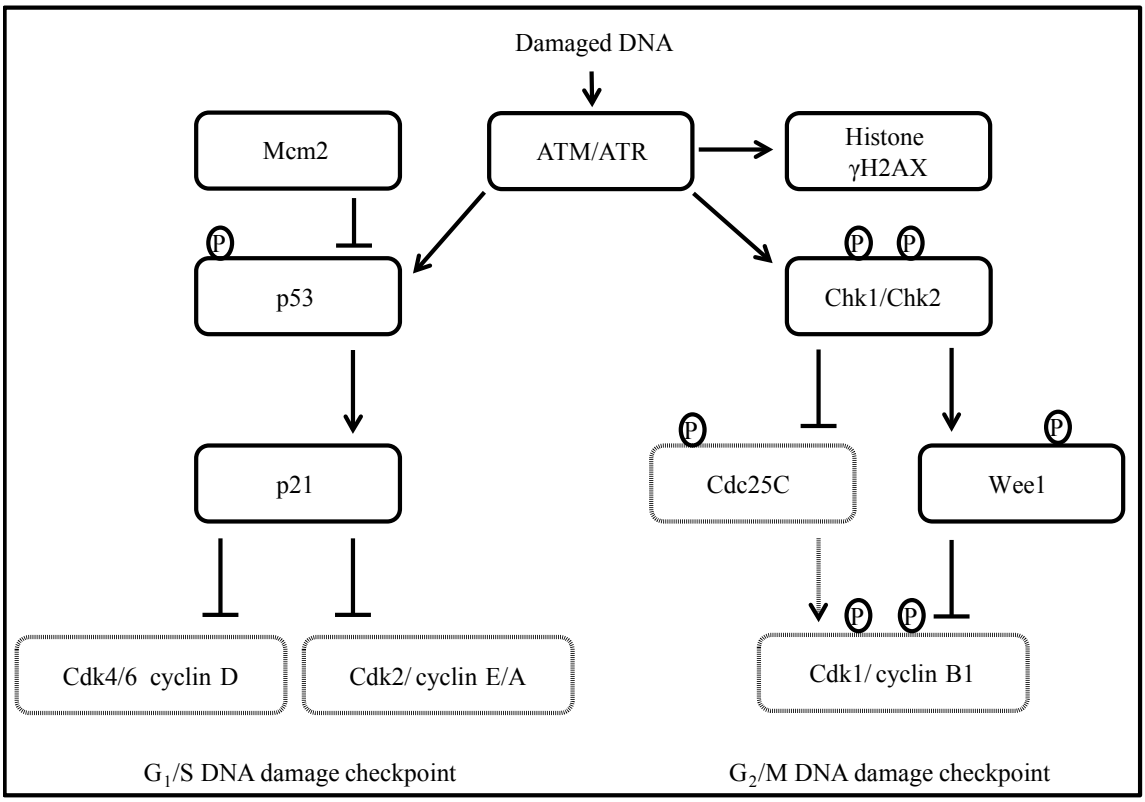
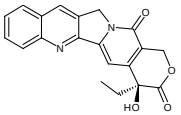
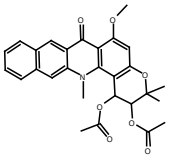
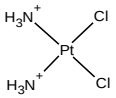


Figure 1. An overview of the DNA damage checkpoints. In the presence of damaged DNA the ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) kinases are activated and initiate a phosphorylation cascade. This causes cells to arrest in either G_1/S (left) or G_2/M phase (right) of the cell cycle through inhibition of cyclin dependent kinases (Cdks). Bold lines indicate active proteins whereas dotted lines show inactive proteins. Phosphorylation events are marked by the letter P.

Table 1. Description of genotoxic agents, their mechanism of action, and the type of DNA damage they induce [19].

Genotoxic agent	Chemical structure	Mechanism of action	Type of DNA damage	Pharmacological treatment range
Camptothecin (CPT)		Topoisomerase I inhibitor. Prevents religation of DNA nicks.	DSB and SSB	2.8 nM to 17.3 nM
S23906-1		Binds to N^2 of guanine in minor DNA groove. Induces intra-stranded, and inter-stranded DNA cross-links.	DSB and SSB	1 µM to 10 µM
cis-diamminedichloro platinum (Cisplatin)		Binds to N^7 guanine and adenine. Induces intra-strand, inter-strand, and DNA-protein crosslinks.	DSB, SSB, and AP sites	12 µM to 40 µM

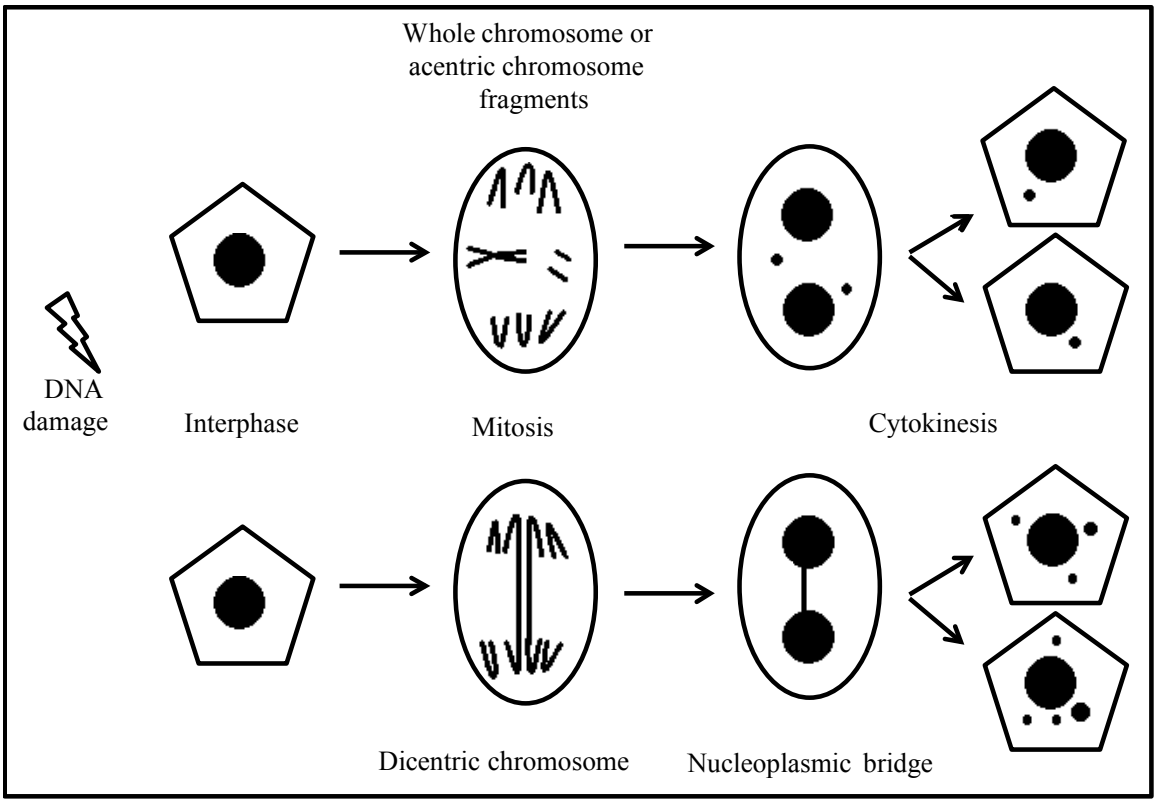


Figure 2. Overview of micronuclei formation in cells with damaged DNA. Cells with damaged DNA can enter mitosis. Lagging whole chromosomes or acentric chromosome fragments (top), which do not properly attach to the spindle apparatus, are not incorporated into daughter nuclei and instead form micronuclei. Damaged chromosomes may also fuse to form a dicentric chromosome (bottom), which is pulled to opposite poles during anaphase. This creates a nucleoplasmic bridge (NPB) between daughter nuclei, which then breaks after cytokinesis. DNA fragments from broken NPB are enclosed by micronuclei.

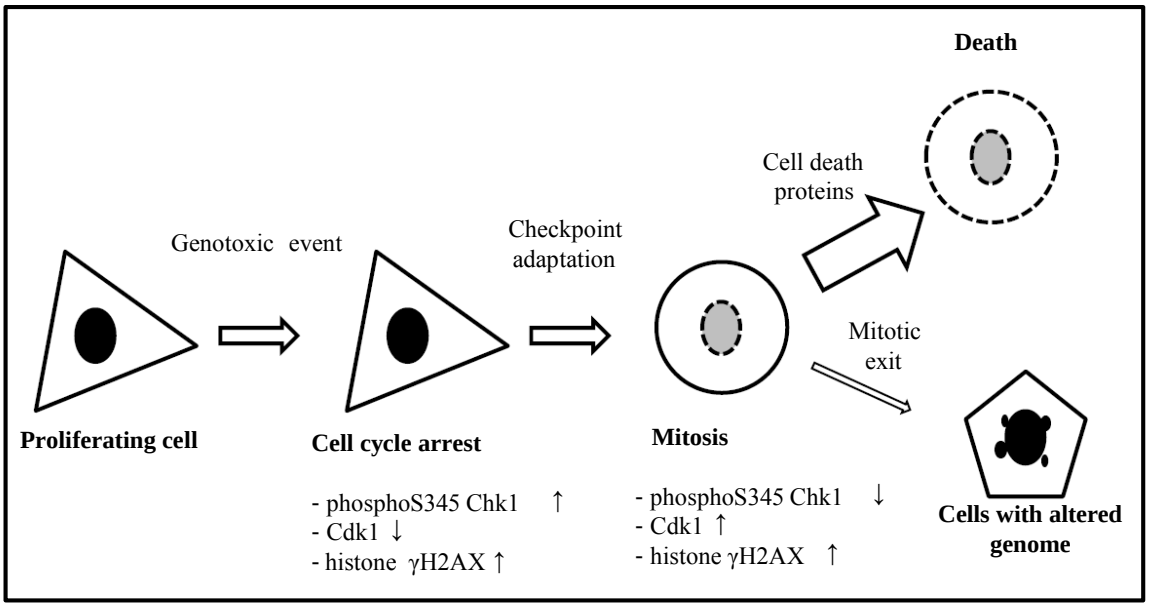


Figure 3. Overview for the steps required for checkpoint adaptation after treatment with a genotoxic agent [3]. Proliferating cells arrest in the cell cycle after detection of damaged DNA. Cells then enter mitosis with damaged DNA. Most cells die in mitosis; however a small number survive and are predicted to have a rearranged genome.

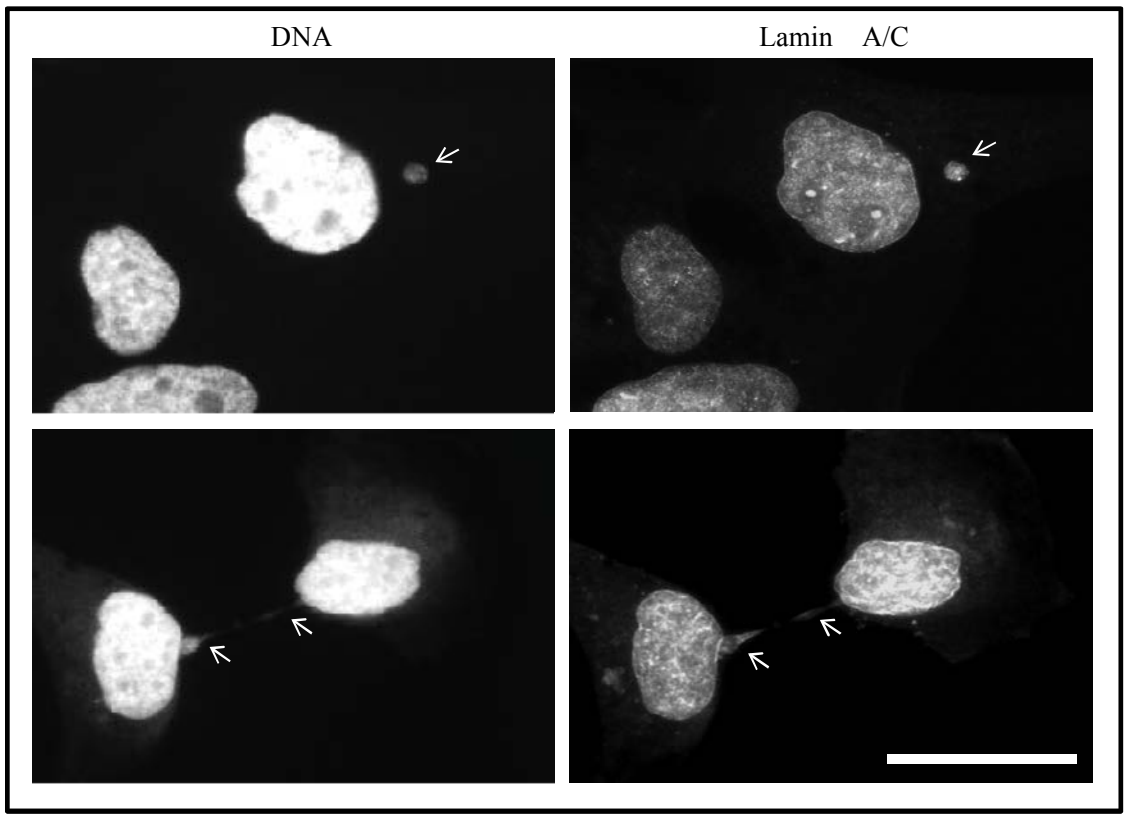


Figure 4. Human glioma fibroblasts contain micronuclei and nucleoplasmic bridges. M059K cells were stained for either DNA (left) or lamin A/C (right) and observed by immunofluorescence microscopy. Arrows indicate a micronucleus (top) or a nucleoplasmic bridge (bottom). Scale bar = 25 μm. Images are representative of two experiments.

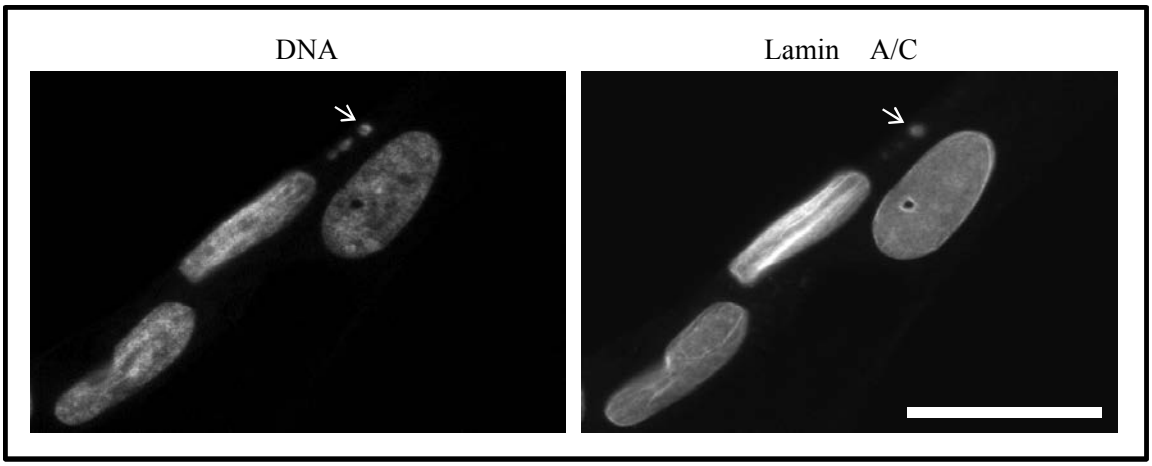


Figure 5. Normal human lung fibroblasts are unlikely to have micronuclei. WI-38 cells were stained for DNA (left) or lamin A/C (right) and observed by immunofluorescence microscropy. The nuclei of three cells are shown. Arrows indicate a micronucleus. Scale bar = 25 μm. Images are representative of two experiments.

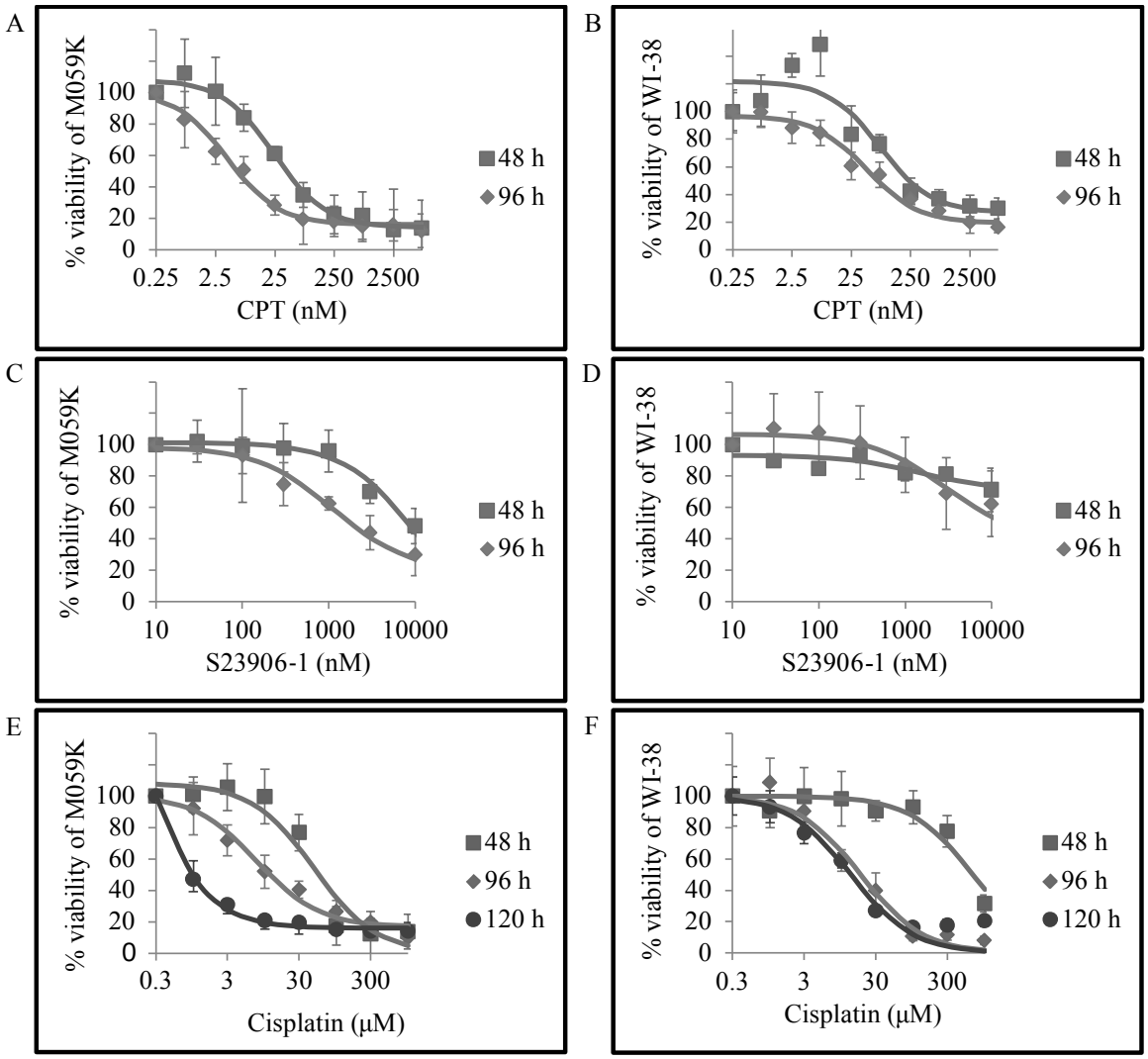


Figure 6. Cells treated with different concentrations of genotoxic agents have reduced cell viability. M059K and WI-38 cells were treated with different concentrations of either camptothecin (CPT) (A and B), S23906-1 (C and D), or cisplatin (E and F) for 48 h (squares), 96 h (diamonds), and 120 h (circles). Cell viability was measured by the MTT assay. Means of three experiments and standard error of means is shown.

Table 2. Mean IC_{50} concentrations of different genotoxic agents used to treat either M059K or WI-38 cells for 48 and 96 h.

	M059K		WI-38	
Genotoxic agent	48 h	96 h	48 h	96 h
CPT (nM)	49.4	7.4	374.6	96.4
S23906-1 (μM)	2.2	7.6	48.1	14.8
Cisplatin (μM)	58.8	15.4	681.0	18.5

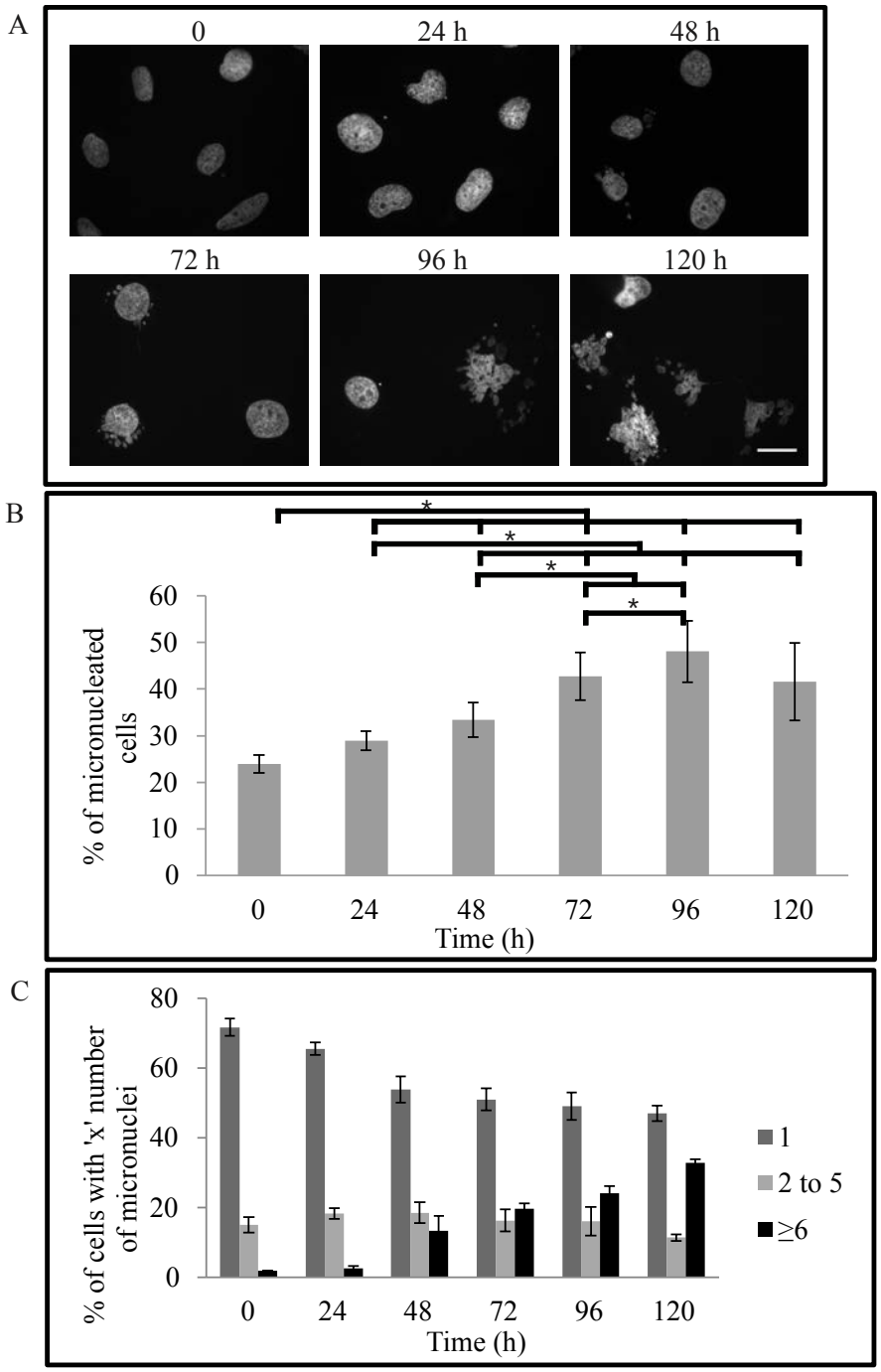


Figure 7. Treatment with cisplatin increases the percentage of micronucleated M059K cells and micronuclei per cell, over time (h). Cells were treated with 30 μM cisplatin for up to 120 h. At least 200 cells were counted and experiments were repeated five times.
A. Cells were fixed at indicated times, stained for DNA, and observed by immunofluorescence microscopy. Scale bar equals 25 μm.
B. Mean percentage of micronucleated cells and standard error of means is shown. Asterisks show significant difference. $t(4) = 2.13$, $p < 0.05$.
C. Mean percentage of micronuclei per cell and standard error of means is shown. Asterisks show significant difference. $t(4) = 2.13$, $p < 0.05$.

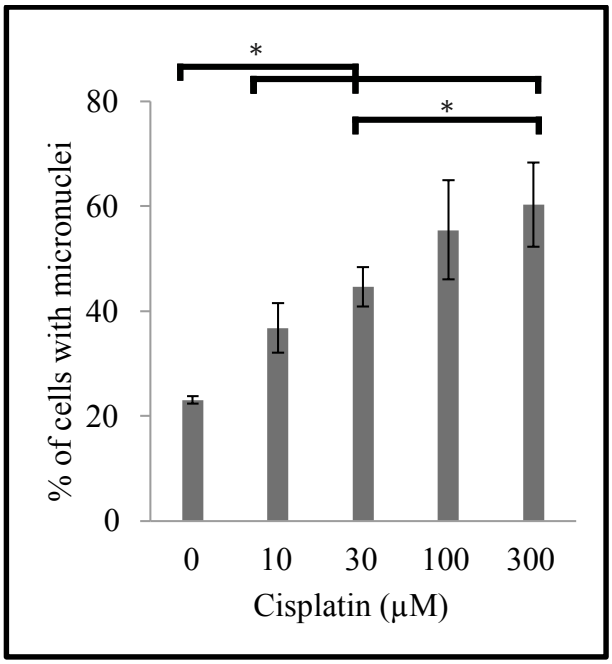


Figure 8. Increasing concentrations of cisplatin increase the percentage of micronuclei in M059K cells. Cells were treated with the indicated concentrations of cisplatin for 48 h. At least 200 cells were counted and experiments were repeated twice. The mean percentage of cells with micronuclei was calculated at each concentration. Standard error of means is shown. Asterisks show significant differences. $t(1) = 6.31$, $p < 0.05$.

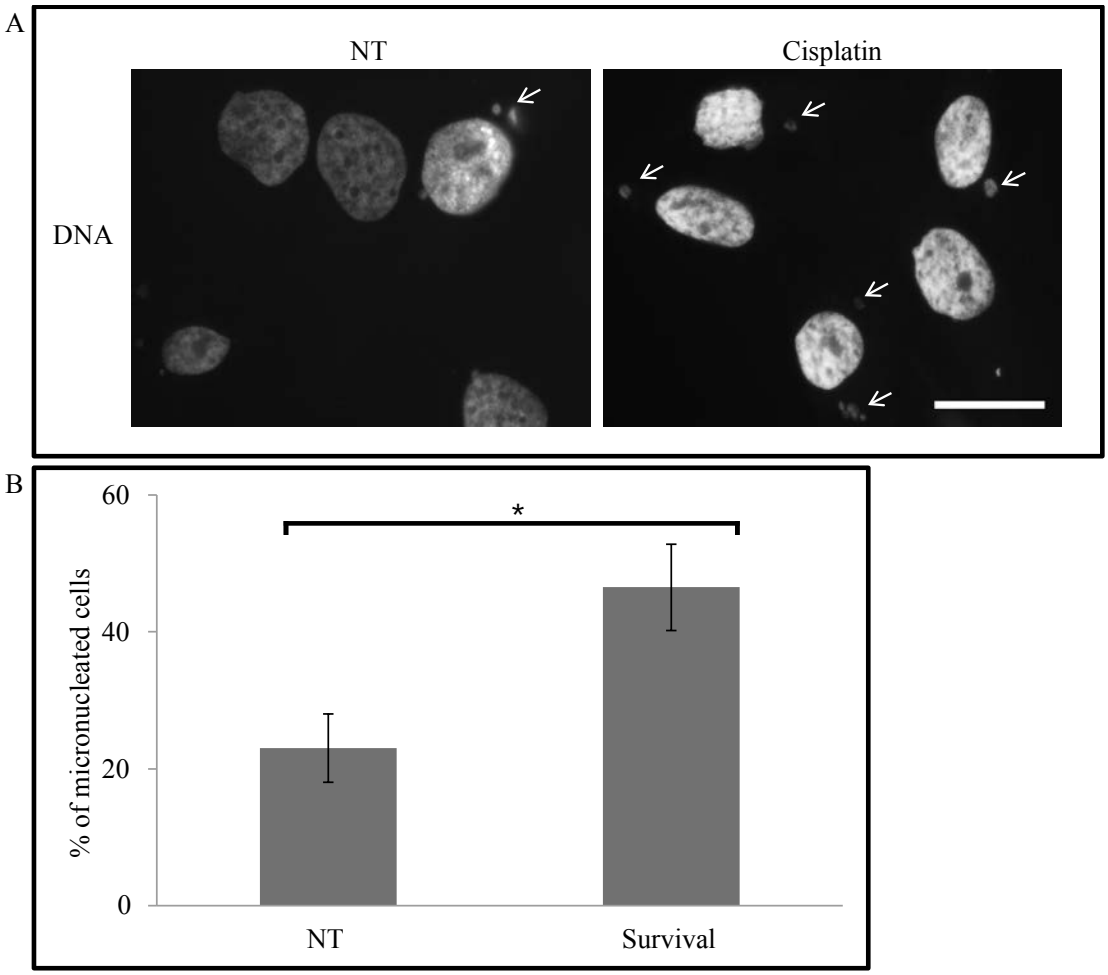


Figure 9. M059K cells retain micronuclei 8-10 days after treatment with cisplatin.
A. Cells were either non-treated (left) or treated with 30 μM cisplatin (right) for 120 h and then cultured for 8-10 days. Cells were stained to mark DNA and then observed by immunofluorescence microscopy. Arrows indicate the presence of a micronucleus. Scale bar equals 25 μm. At least 200 cells were counted and experiments were repeated at least three times.
B. The mean percentage of micronucleated cells was calculated. Standard error of means is shown. Asterisk shows significant difference. $t(5) = 2.01$, $p < 0.05$.

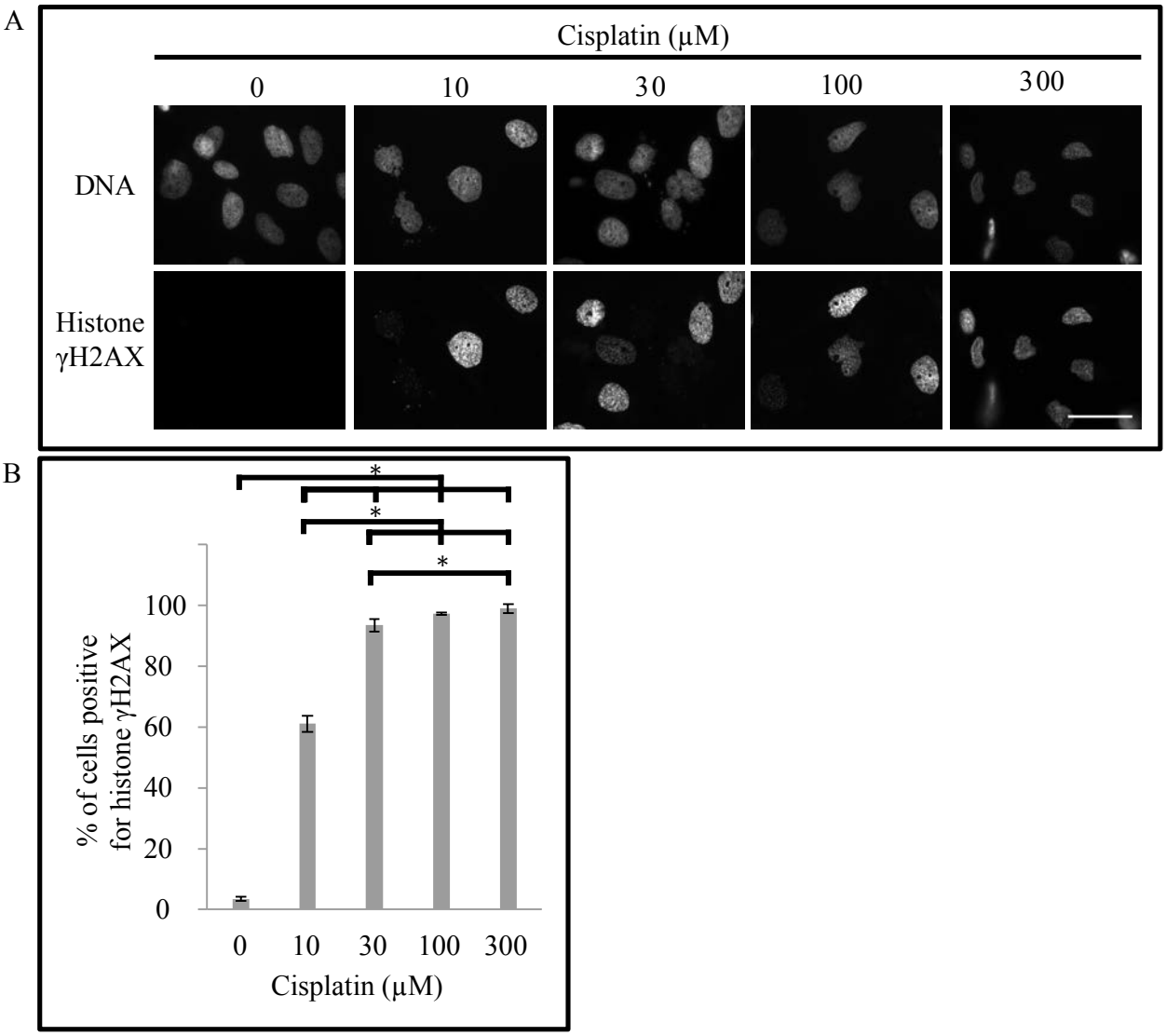


Figure 10. Cells signal damaged DNA in a dose-dependent manner following treatment with cisplatin. At least 200 cells were counted and experiments were repeated twice.
A. M059K cells were treated with increasing concentrations of cisplatin for 48 h and then stained for either DNA (top) or histone γH2AX (bottom). Cells were observed by immunofluorescence microscopy. Scale bar equals 50 μm.
B. The mean percentage of cells positive for histone γH2AX. Standard error of means is shown. Asterisks show significant differences. $t(1) = 6.31$, $p < 0.05$.

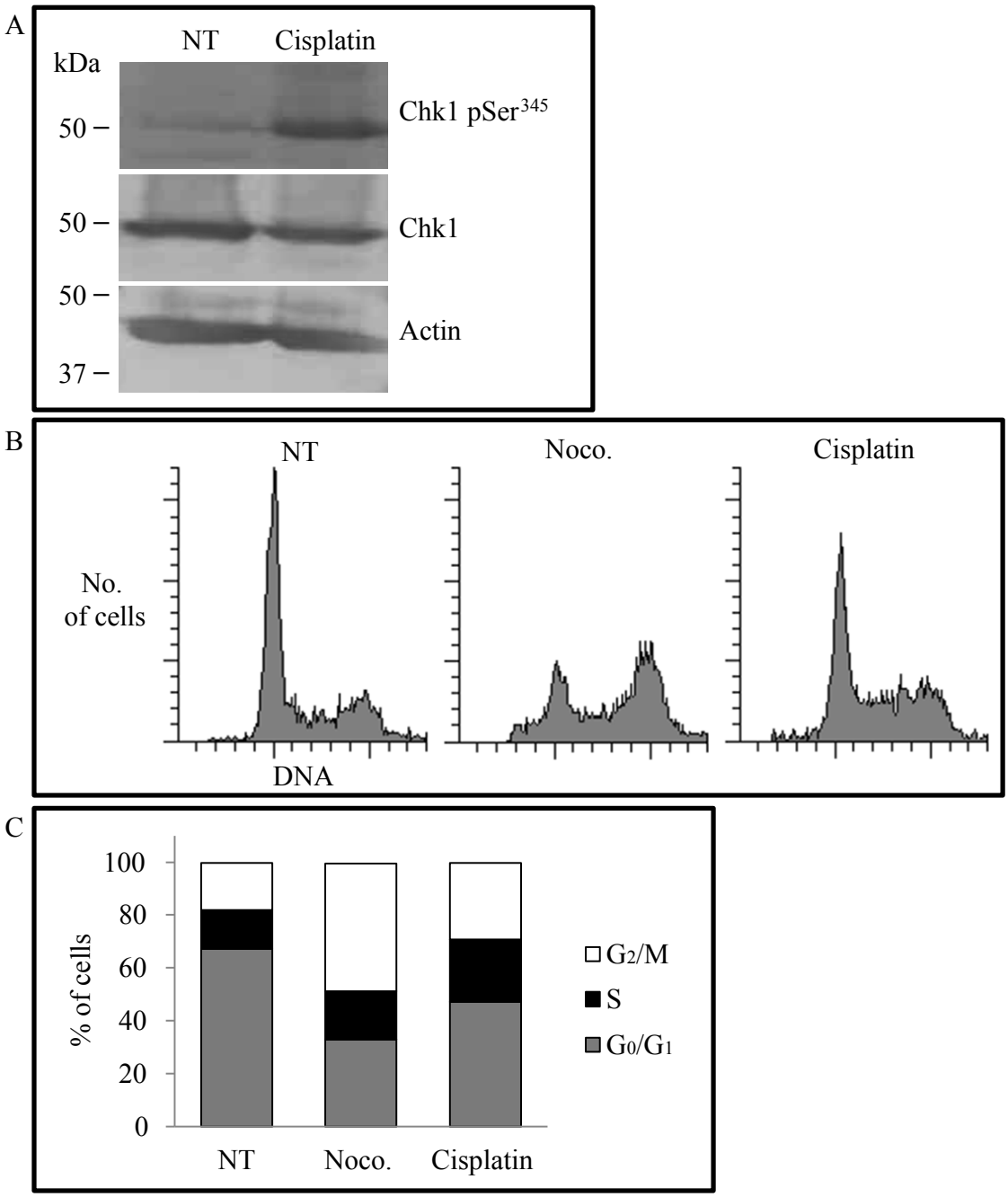


Figure 11. M059K cells engage the DNA damage checkpoint in response to treatment with cisplatin. Experiments were repeated at least twice.

A. Protein extracts were prepared from cells that were either non-treated or treated with 30 μM cisplatin for 24 h. Samples were processed by western blotting and antibodies against phospho-Ser^{345}-Chk1, Chk1, or actin.

B. Cells were non-treated (NT), treated with 200 ng/mL nocodazole (Noco.), or 30 μM cisplatin for 24 h and then analysed by flow cytometry. DNA content was determined with propidium iodine staining.

C. Mean percentage of cells in each phase of the cell cycle.

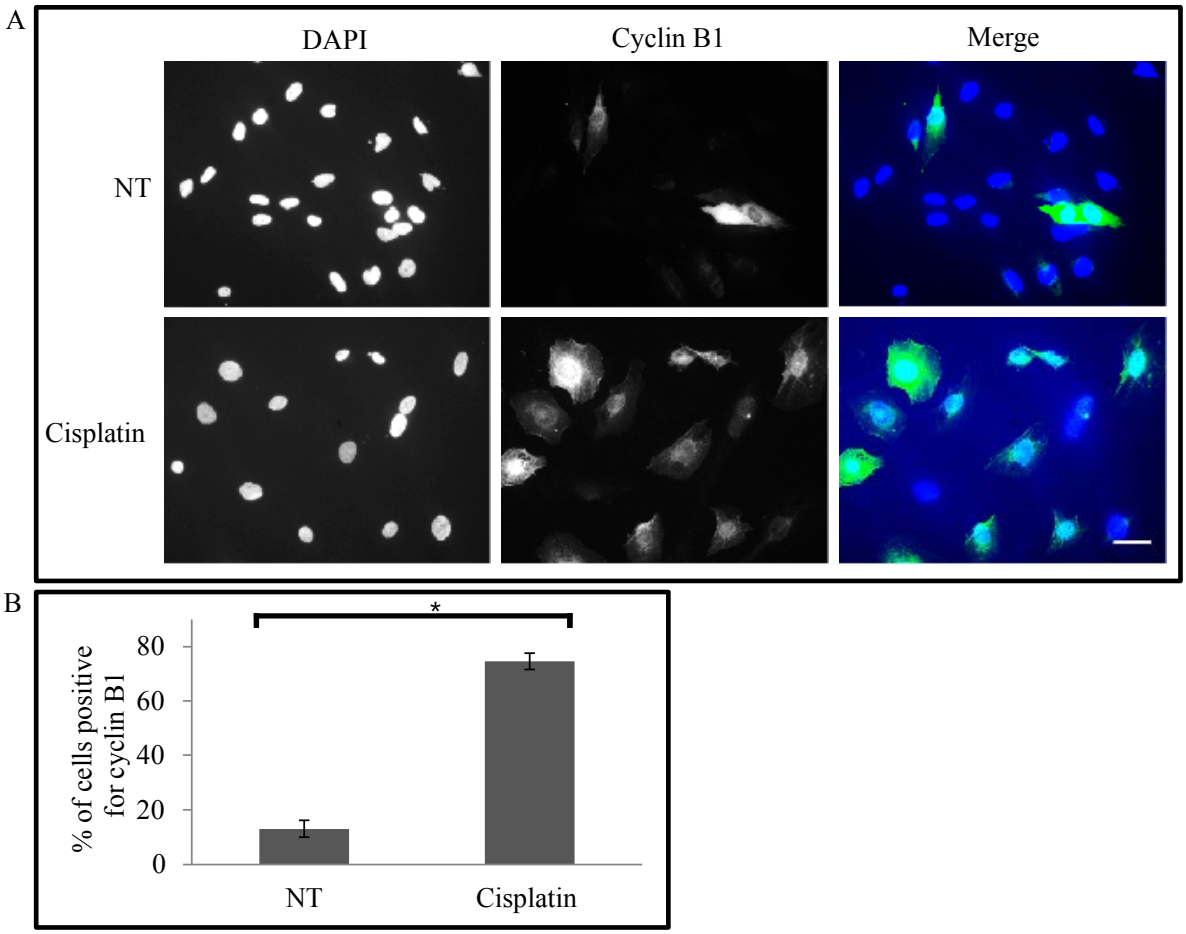


Figure 12. M059K cells treated with cisplatin accumulate cyclin B1 after 48 h. Cells were either non-treated or treated with 30 μM cisplatin for 48 h. At least 200 cells were counted and experiments were repeated twice.

A. Cells were stained for either DNA (top) or cyclin B1 protein (middle) and then observed by immunofluorescence microscopy. Merge of images is shown on bottom. Scale bar equals 50 μm.

B. The mean percentage of cells positive for cyclin B1 was calculated. Standard error of the means is shown. Asterisk shows signifcant difference. $t(1) = 6.31$, $p < 0.05$.

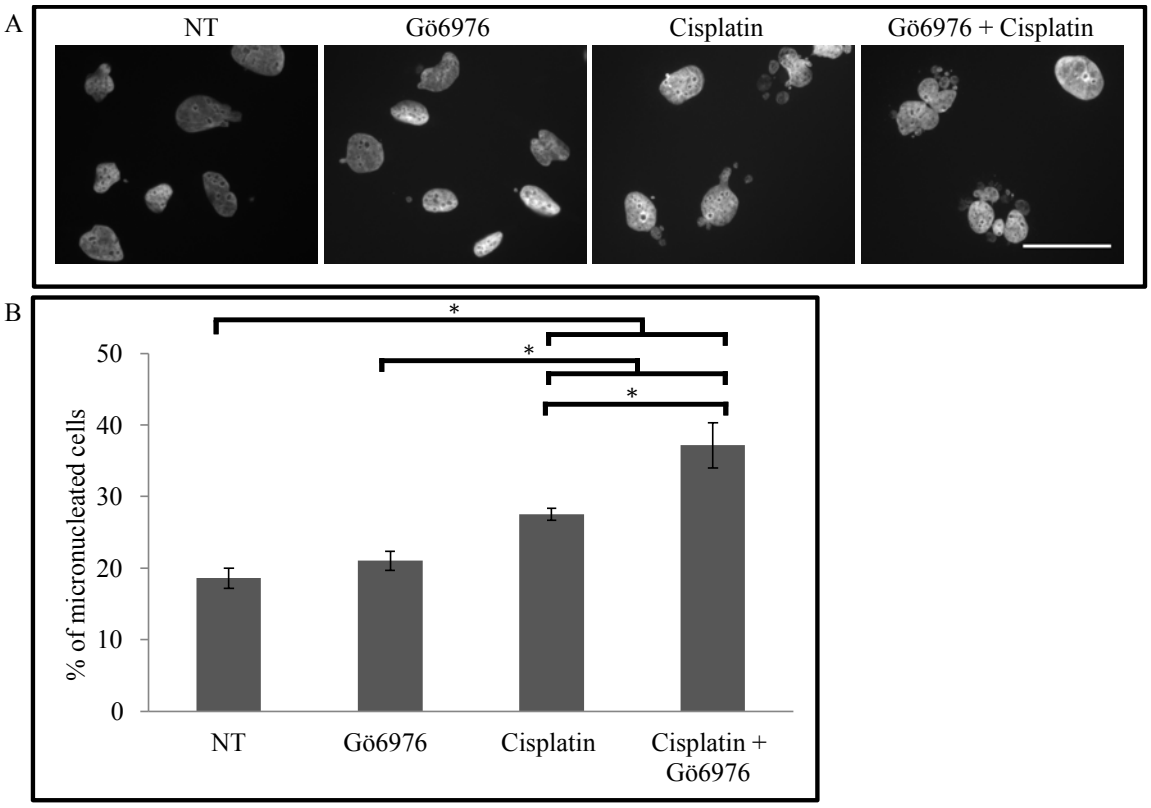


Figure 13. Inhibition of Chk1 increases the percentage of micronucleated M059K cells. Cells were non-treated, treated with 15 nM Gö6976 for 24 h, 30 μM cisplatin for 48 h, or 30 μM cisplatin for 24 h and then co-treated with 15 nM Gö6976 for an additional 24h. At least 200 cells were counted and experiments were repeated three times.

A. Cells were stained for DNA and then analysed by immunofluorescence microscopy. Scale bar equals 50 μm.

B. The mean percentage of micronucleated cells was calculated for each treatment and standard error of the means is shown. Asterisks show significant differences. $t(2) = 2.92$, $p < 0.05$.

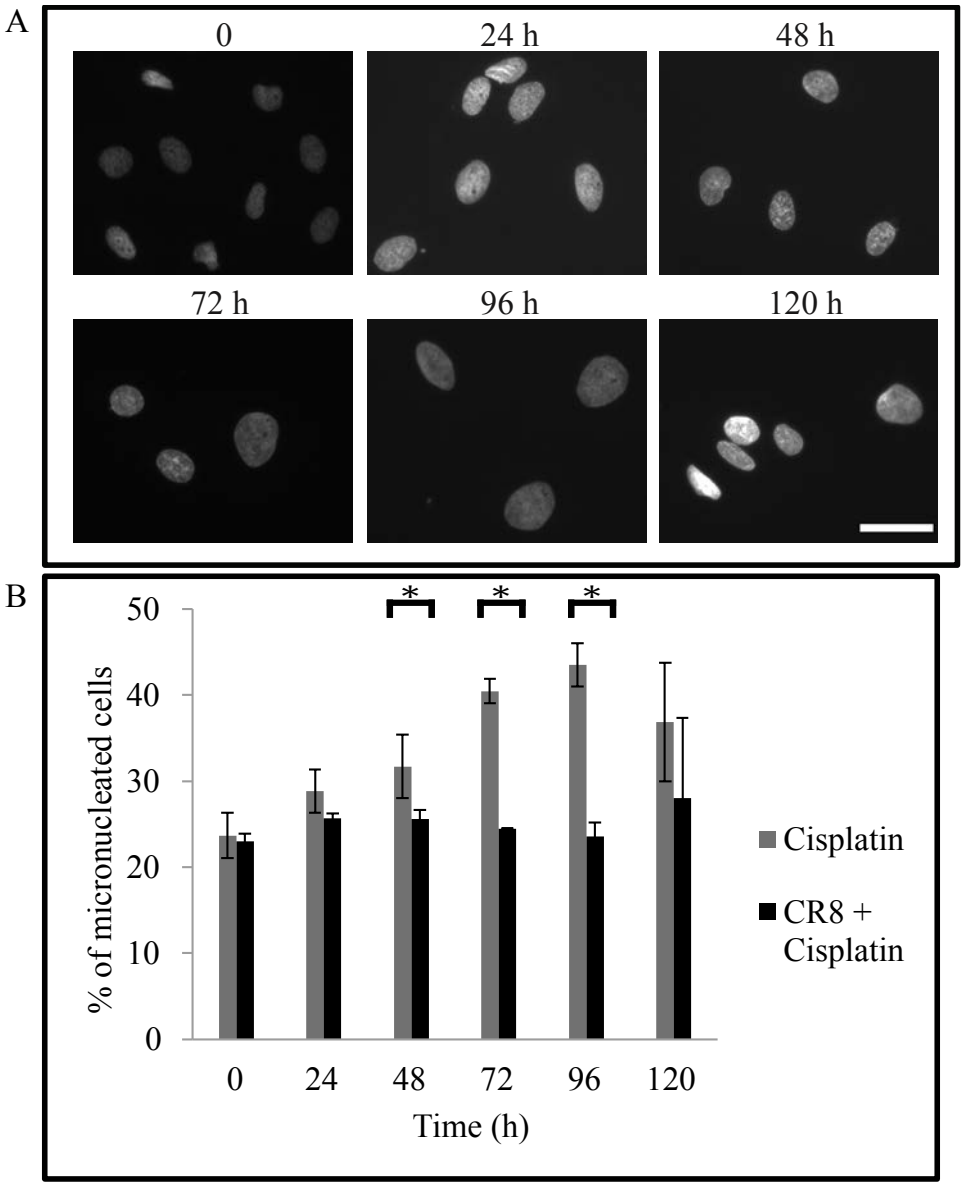


Figure 14. Co-treatment with cisplatin and a Cdk1 inhibitor (CR8) prevents new micronuclei from forming in M059K cells. Cells were non-treated, treated with 30 μM cisplatin, or co-treated with cisplatin and 500 nM CR8 for the indicated times. At least 200 cells were counted and experiments were repeated three times.

A. Cells co-treated with cisplatin and CR8 were fixed at the indicated times, stained for DNA, and observed by immunofluorescence microscopy. Scale bar equals 25 μm.

B. The mean percentage of micronucleated cells and standard error of the means is shown. Asterisks show significant differences. $t(2) = 2.92$, $p < 0.05$.

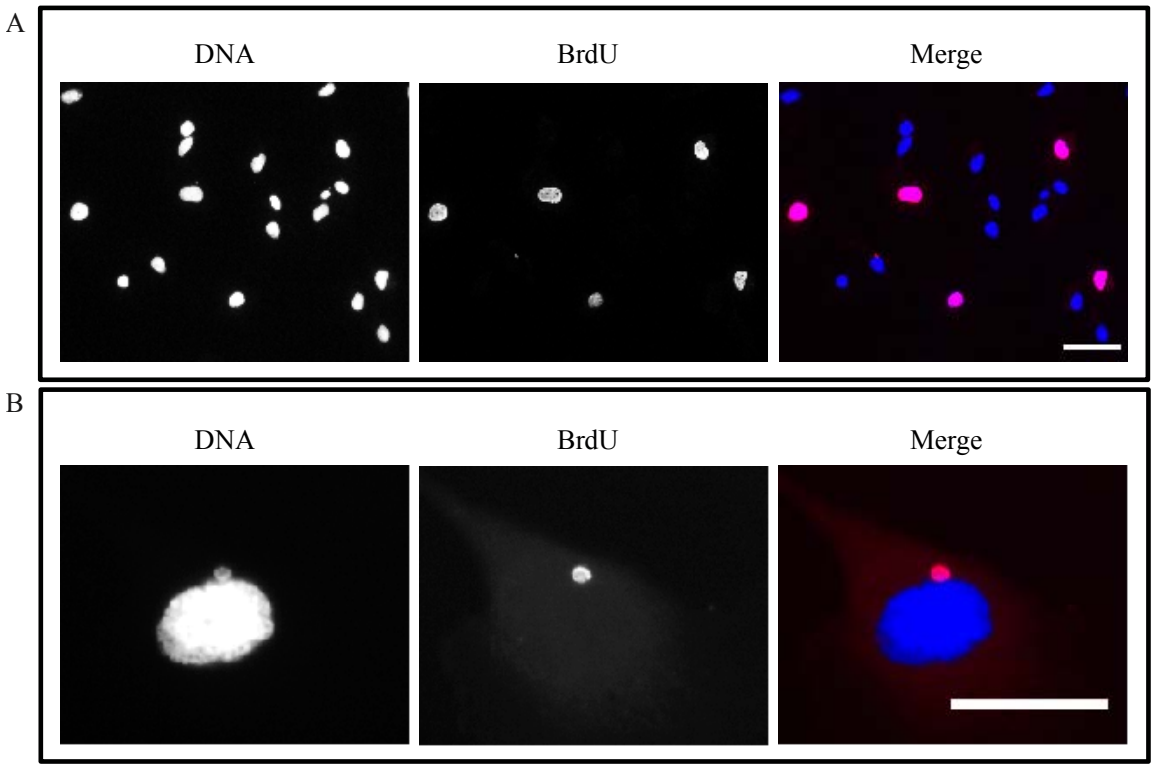


Figure 15. Micronucleated M059K cells show evidence of asynchronous DNA replication. Cells were treated with 30 μM BrdU reagent for 30 min. Experiments were repeated twice.

A. Cells were stained for both DNA (left) and BrdU (middle) and then observed by immunofluorescence microscopy. Merge of images is shown on right. Scale bar equals 50 μm. Images are representative of three experiments.

B. Micronucleated cell stained for DNA (left) and anti-BrdU (middle). Merge of images is shown on right. Scale bar equals 25 μm.

Table 3. Mean percentage of micronuclei that are incorporating BrdU in relation to the main nucleus. Standard error of the means is shown. At least 200 cells were counted and experiments were repeated twice.

BrdU	
Status	% of micronucleated cells
Synchronous	80 ± 7
Asynchronous	20 ± 7

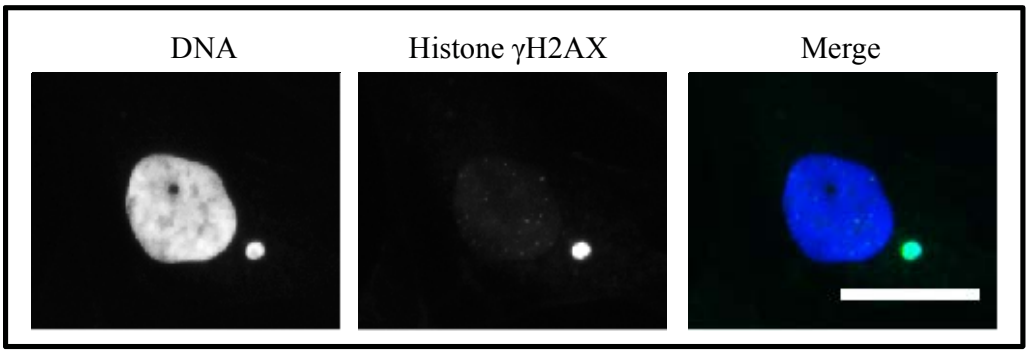


Figure 16. Micronuclei have damaged DNA.
M059K cells were stained for both DNA (left) and histone γH2AX (middle) and then observed by immunofluorescence microscopy. Merge of images is shown on the right. Scale bar equals 25 μm. Images are representative of three experiments.

Table 4. Mean percentage of micronuclei with damaged DNA. Standard error of the means is shown. At least 200 cells were counted and experiments were repeated twice.

Histone γH2AX	
Status	% of micronuclei
Positive	47 ± 9
Negative	53 ± 9

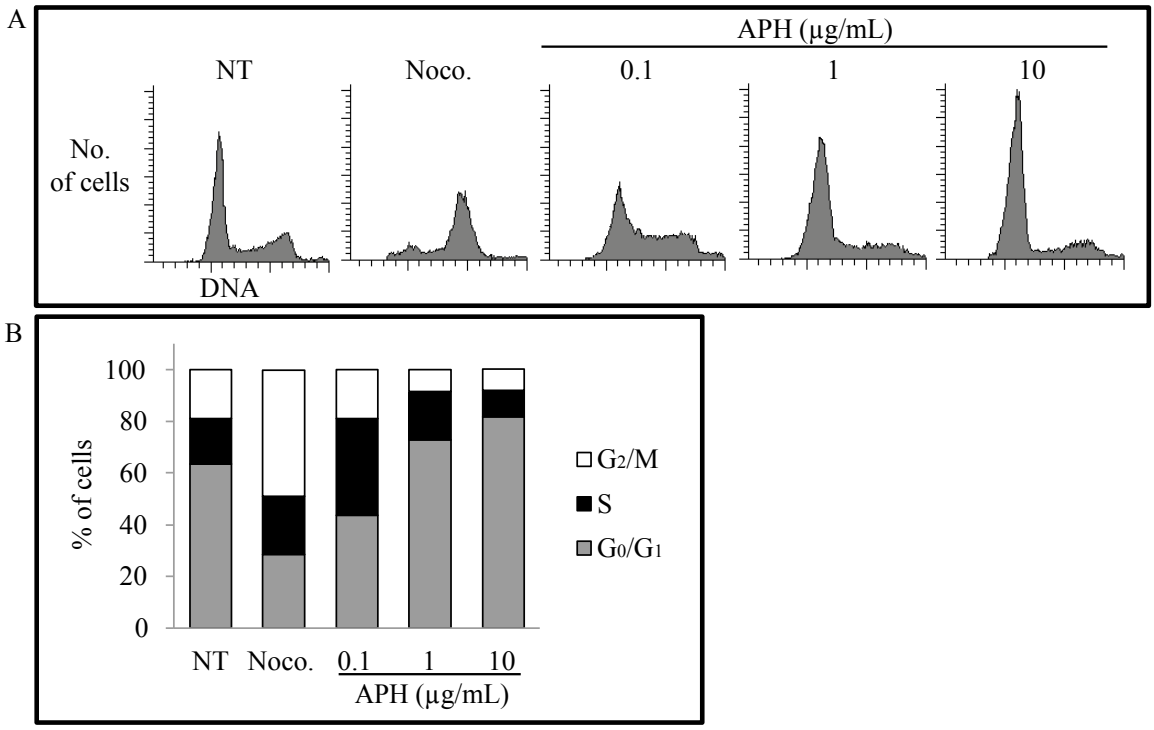


Figure 17. M059K cells treated with aphidicolin accumulate in G_1/G_0-phase. M059K cells were either non-treated, treated with 200 ng/mL nocodazole or with the indicated aphidicolin (APH) concentrations for 24 h. Experiments were repeated twice.
A. Samples were analysed by flow cytometry for DNA content. DNA content was determined by propidium iodine staining.
B. The mean percentage of cells in each phase following indicated treatments is shown. Experiments were performed twice.

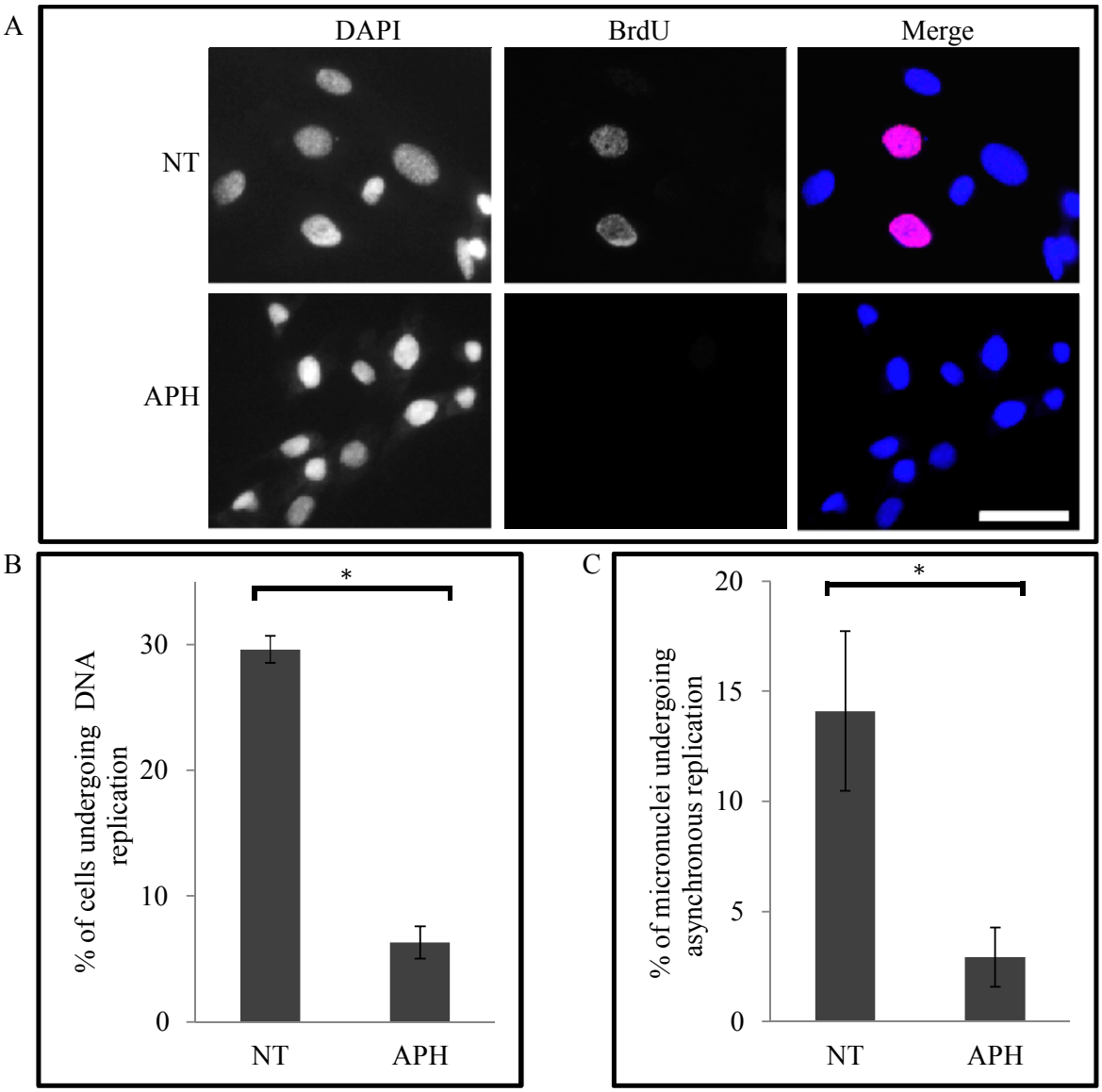


Figure 18. DNA replication is blocked in cells treated with aphidicolin. Cells were either non-treated or treated with 10 µg/mL of aphidicolin. At least 200 cells were counted, and experiments were repeated twice.

A. Cells were treated with 30 µM BrdU for 30 min, stained for either DNA (left) or BrdU (middle), and then observed by immunofluorescence microscopy. Merge of images is shown on the right. Scale bar equals 50 µm.

B. Mean percentage of cells undergoing replication. Standard error of the means is shown. Asterisks show significant differences. $t(1) = 6.31$, $p < 0.05$.

C. Mean precentage of micronucleated cells undergoing asynchonous replication. Standard error of the means is shown. Asterisks show significant differences. $t(1) = 6.31$, $p < 0.05$.

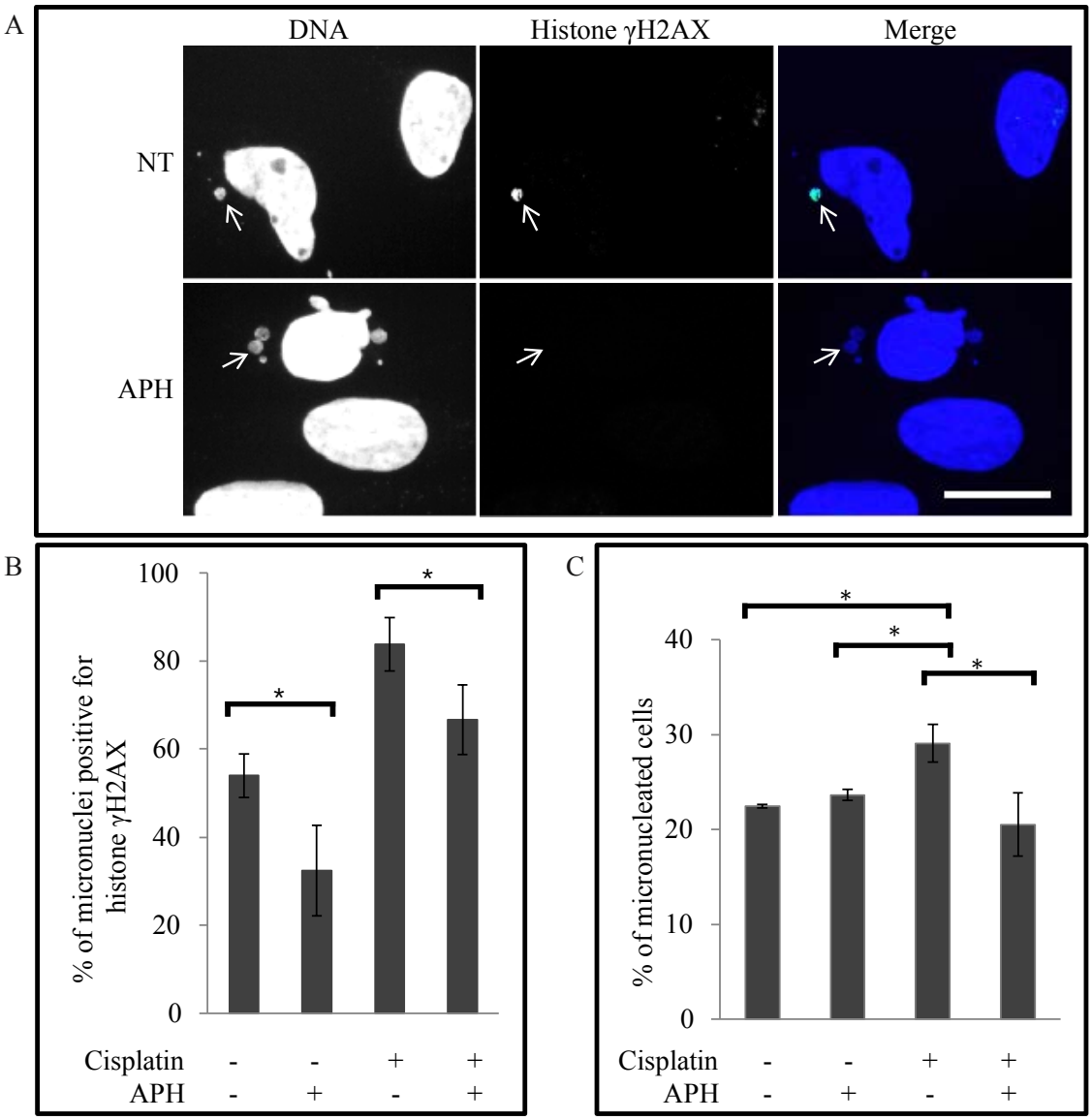


Figure 19. Treatment with aphidicolin results in fewer micronucleated M059K cells and fewer micronuclei that are positive for histone γH2AX. Cells were non-treated, treated with 10 μg/mL aphidicolin (APH) for 24 h, 30 μM cisplatin for 48 h, or treated with cisplatin for 24 h and then co-treated with APH for an additional 24 h. At least 200 micronucleated cells were counted and experiments were repeated twice.

A. Cells were stained for either DNA (left column) or histone γH2AX (middle). Merge of images is shown on right column. Cells were observed by immunofluorescence microscopy. Scale bar equals 25 μm.

B. Mean percentage of micronuclei positive for histone γH2AX. Standard error of means is shown. Asterisks show significant differences. $t(2) = 2.92$, $p < 0.05$.

C. Mean percentage of micronucleated cells.

Standard error of means is shown. Asterisks show significant differences. $t(2) = 2.92$, $p < 0.05$.

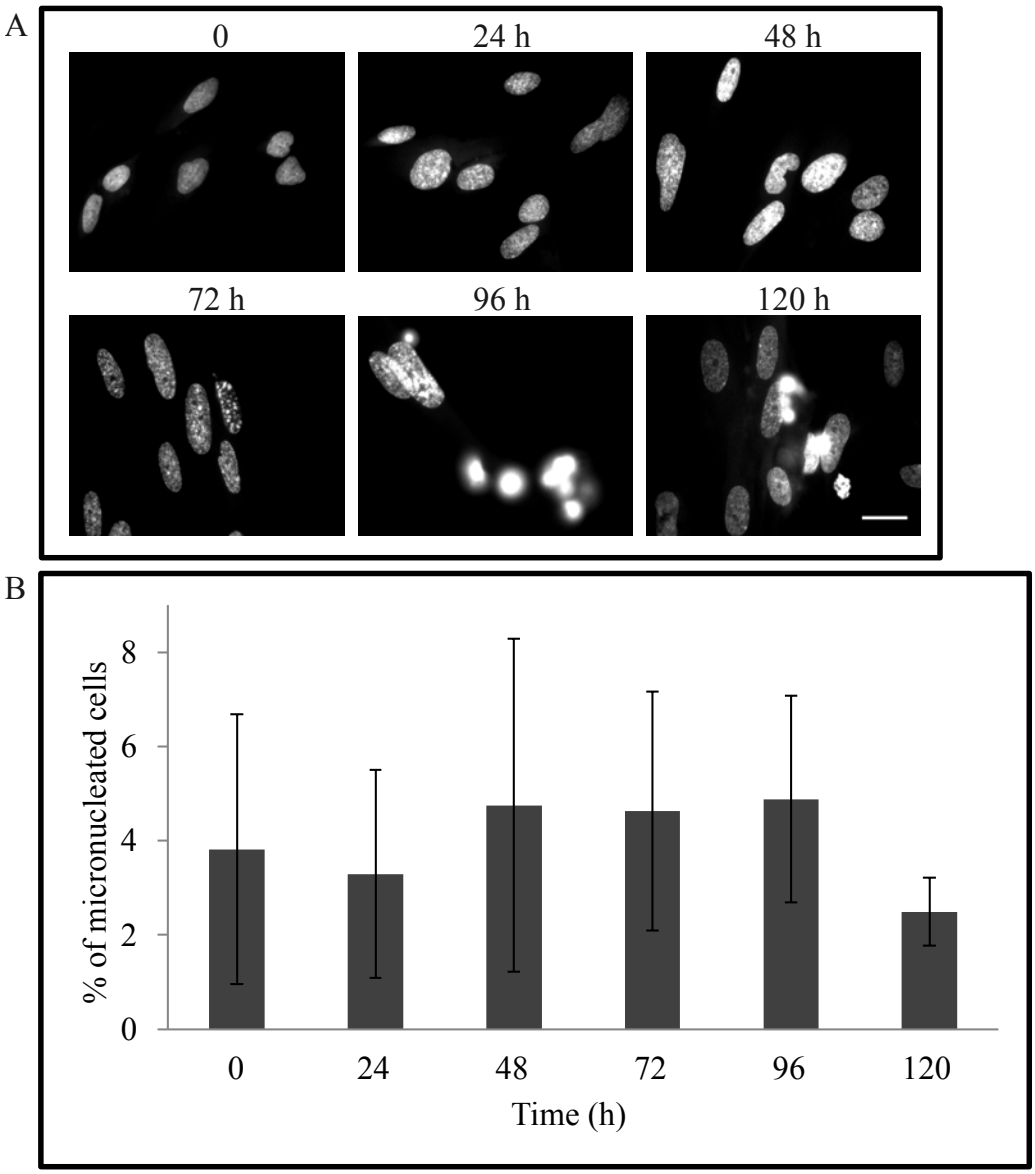


Figure 20. Treatment of WI-38 cells with cisplatin does not increase the percentage of micronucleated cells. At least 200 cells were counted and experiments were repeated twice.

A. Cells were treated with 30 μM cisplatin for up to 120 h and then fixed at indicated times, stained for DNA, and observed by immunofluorescence microscopy. Scale bar equals 25 μm.

B. The mean percentage of micronucleated cells was determined and compared over time. Standard error of means is shown.

Experiments were repeated twice.

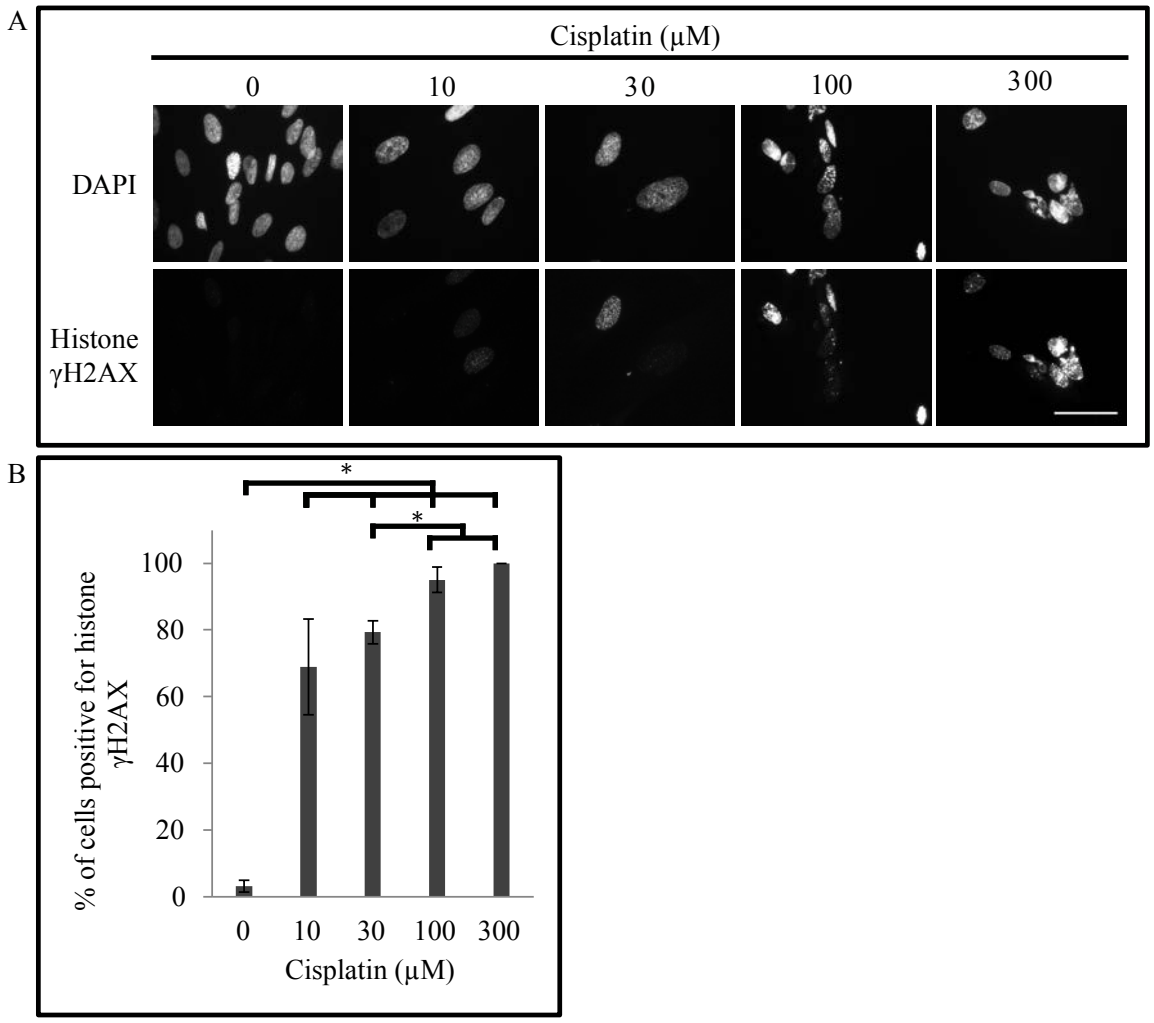


Figure 21. WI-38 cells signal damaged DNA in a dose-dependent response following treatment with cisplatin. At least 200 cells were counted and experiments were repeated twice.

A. Cells were treated with increasing concentrations of cisplatin for 48 h and then stained for either DNA (top) or histone γH2AX (bottom). Cells were observed by immunofluoroscence microscopy. Scale bar equals 50 μm.

B. The mean percentage of cells positive for histone γH2AX was calculated at each cisplatin concentration and standard error of means is shown. Asterisks show significant differences. $t(1) = 6.31$, $p < 0.05$.

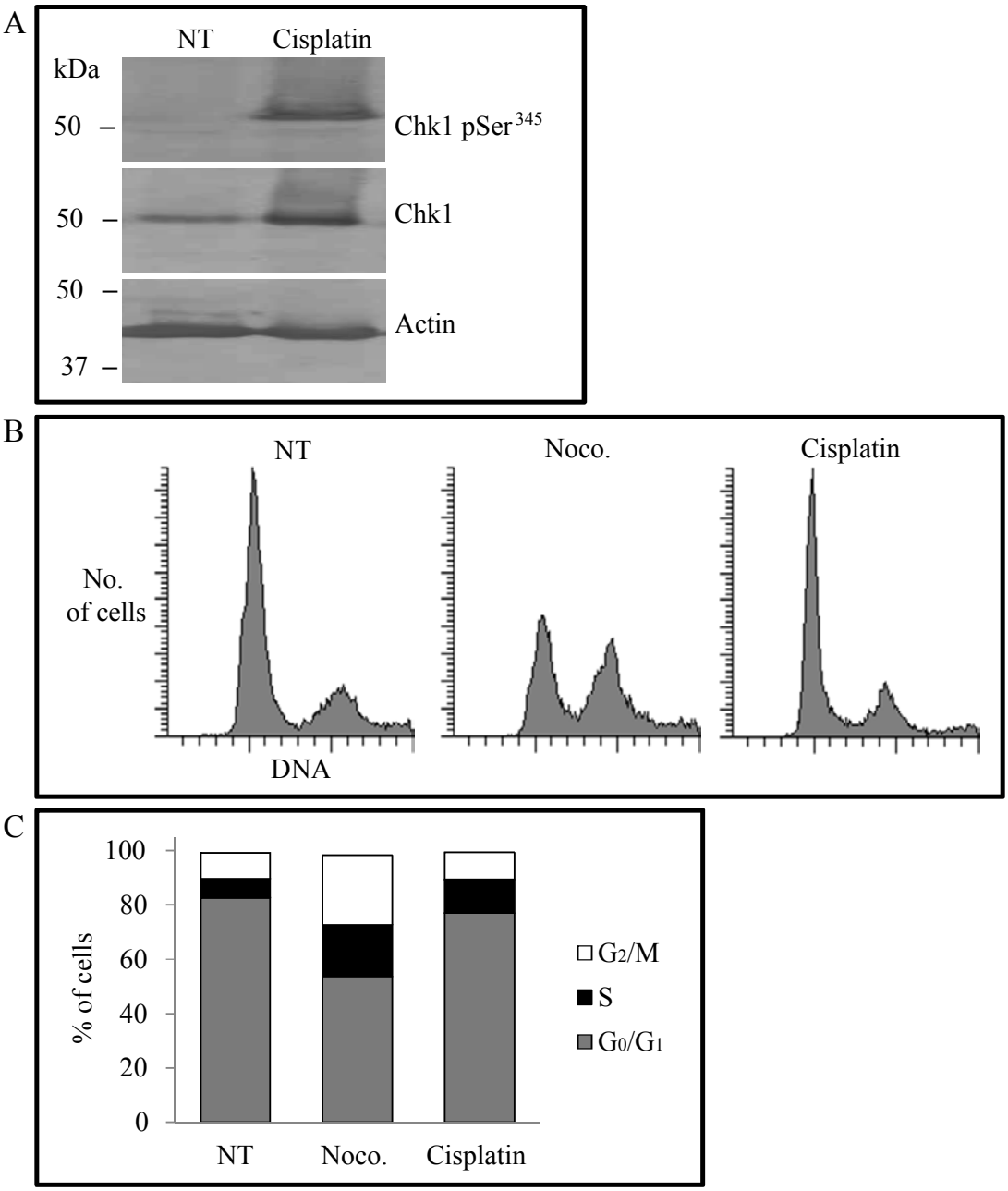


Figure 22. WI-38 cells activate Chk1 in response to treatment with cisplatin. Experiments were repeated twice.

A. Protein extracts were prepared from cells that were either non-treated or treated with 30 μM cisplatin for 24 h. Samples were processed by western blotting and antibodies against either phospho-Ser^{345} Chk1, Chk1, or actin.

B. Cells were either non-treated, treated with 200 ng/mL nocodazole, or 30 μM cisplatin for 24 h and then analysed by flow cytometry. DNA content was determined with propidium iodine staining.

C. Mean percentage of cells in each phase of the cell cycle.

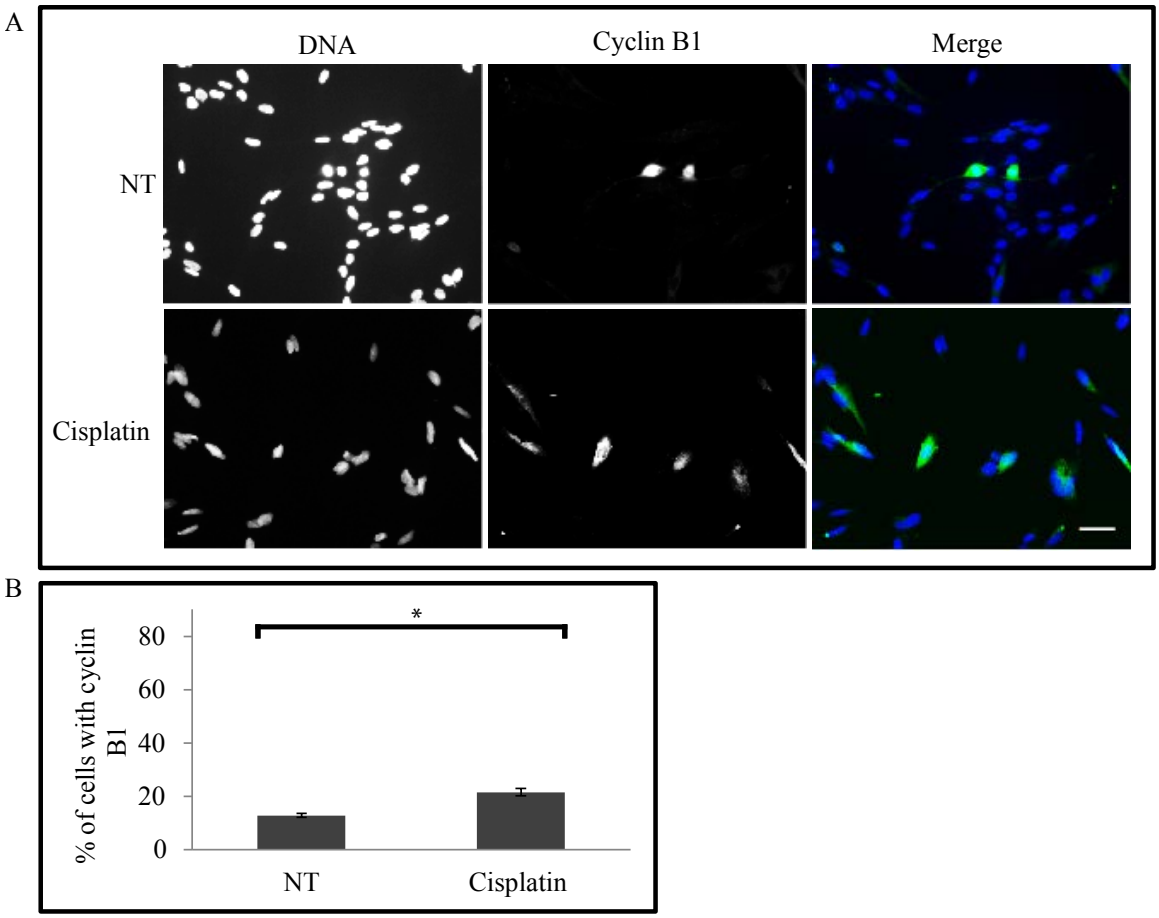


Figure 23. Cells treated with cisplatin accumulate little cyclin B1 after 48 h. WI-38 cells were either non-treated or treated with 30 μM cisplatin for 48 h. At least 200 cells were counted and experiments were repeated twice.

A. Cells were stained for either DNA (top) or cyclin B1 protein (middle) and then observed by immunofluorescence microscopy. Merge of images is shown on the bottom row. Scale bar equals 50 μm.

B. The mean percentage of cells positive for cyclin B1 protein was calculated. Standard error of means is shown. Asterisk shows significant differences. (1) = 6.31, $p < 0.05$.

Chapter 5 - Discussion

In 2008, The Cancer Genome Atlas (TCGA) sequenced tumour genomes from 206 glioblastoma patients and reported that every genome had a different nucleotide substitutions, gene amplifications, deletions, and chromosomal rearrangements. In a follow-up study, chromosome aneuploidy (abnormal chromosome number), was also found in the tumour genomes of another 128 glioblastoma patients [108]. This profound genomic diversity within each tumour may be the reason why glioblastoma treatments are rarely effective; only 3-5% of patients survive more than three years after treatment with radiation and/or chemotherapy [106]. To improve patient outcomes, it is necessary to understand how tumour genomes acquire these changes.

One type of genomic change that is frequently found in cancer cells, including glioblastoma cells, is micronuclei. I investigated the relationship between the formation of micronuclei and checkpoint adaptation in glioblastoma cells. Checkpoint adaptation, or mitosis with damaged DNA, has been suggested as a mechanism of micronuclei formation [19], but it has not been previously investigated. I found that glial cancer cells (M059K) acquire additional micronuclei after genotoxic treatment with cisplatin. They do so after undergoing checkpoint adaptation. Independently of a genotoxic treatment, some micronuclei acquire damaged DNA. The damaged DNA acquired by these micronuclei was linked to DNA replication [61]. This result is particularly important because it suggests that the formation of a micronucleus leads to a process of further continuous genomic change, which may account for the genomic diversity reported in glioblastoma genomes. I was unable to detect the formation of micronuclei in normal

lung fibroblasts (WI-38) treated with cisplatin. Consistent with this observation, WI-38 cells treated with cisplatin did not undergo checkpoint adaptation. These findings suggest that checkpoint adaptation might have a determinant role in how cells respond to events that damage their genome. The results from our investigation provide insights into the complex problems of genomic change in cancer cells.

5.1. Percentages of micronuclei and nucleoplasmic bridges vary in human cancer cells

To study genomic change, I first needed a reliable cellular model to investigate the formation of micronuclei. The high number of micronuclei in M059K cells makes them a convenient model because one has sufficient numbers to test observations with statistical significance. Other cancer cell lines, such as HT-29 (colon) or U2OS (osteosarcoma), which are used in the laboratory, also harbour micronuclei, but only 2-5% of cells have them. I detected that 25% ± 1% of M059K cells contained at least one micronucleus in addition to the main nucleus (Figure 4). Some previous studies have reported lower percentages (1-7%) of micronuclei in these cells [116, 125]. However, these studies only used one staining method in their counting procedures. I confirmed the presence of micronuclei with additional counting methods: lamin A/C, histone γH2AX, and BrdU. Lymphocytes isolated from thyroid cancer patients have been reported to have similar percentages of cells with micronuclei (27%) [146]. The high percentage of micronuclei in M059K cells, and their capacity to undergo checkpoint adaptation [3] makes them a convenient model to study the relationship between micronuclei and checkpoint adaptation.

Consistent with our observations, the American Tissue Cell Culture has reported that M059K cells do not contain a stable number of chromosomes [115]. Typically, M059K cells have 65 to 79 chromosomes and at least 22% of cells exhibit polyploidy. Gurung *et al.* reported that non-treated M059K cells can have more than 87 chromosomes and have several complex chromosomal rearrangements, including reciprocal and non-reciprocal translocations [114]. An abnormal chromosome number is common in cells with micronuclei because micronuclei are often caused by unequal separation of chromosomes [61, 71]. Other genomic changes including reciprocal and non-reciprocal translocations have been reported to occur to chromosomes inside a micronucleus [61]. The presence of micronuclei helps to explain the variation in the number of chromosomes and the genomic rearrangements described in these cells.

5.2. Characterization of genotoxic molecules

Having selected M059K cells to study micronuclei, I then needed a genotoxic agent with which to induce checkpoint adaptation. I required a genotoxic agent that has an IC_{50} concentration to cultured cells that was similar to that used in clinical trials, and a difference in cell viability between early (48 h) and late (120 h) times after treatment [19]. I found that 30 μM cisplatin met these conditions. By choosing these criteria, I maintained a critical link between our experimental results and pharmacological results in treated patients [19, 130]. Cisplatin induced a cytotoxic response in both M059K cells and WI-38 cells (Figure 6E and F), at a concentration that was within the treatment range received by patients (12-40 μM) [19, 134-136]. Concentrations that exceed 40 μM are reported to cause liver and kidney failure, hearing loss, pancytopenia (reduction of blood

cells), and death in human patients [134] and are therefore not pharmacologically relevant. Importantly, 80% of M059K cells and 90% of WI-38 cells that were alive at 48 h had died after 120 h treatment with 30 μM cisplatin. The delay in the reduced cell viability between 48 h and 120 h is typical of cells that undergo checkpoint adaptation prior to cell death [3]. If the concentration of cisplatin was too high (100-300 μM), cells would die during the first 48 h and if the concentration was too low (0.3-3 μM) cisplatin was not effective in reducing cell viability. Therefore, I chose to treat cells with 30 μM cisplatin because they exhibited the ideal cytotoxic response to study checkpoint adaptation.

5.3. M059K cells treated with cisplatin form micronuclei

Having characterized our experimental model, I then tested if M059K cells formed new micronuclei or nucleoplasmic bridges (NPBs) following cisplatin treatment (Figure 7). I observed three changes in cells: (1) an increase in cells with micronuclei over 96 h from 24% ± 1% to 48% ± 7%; (2) an increase in the number of micronuclei per cell with an increase in treatment time; and (3) an increase in NPBs from 1% to 3% after 120 h. These observations are consistent with other studies that have reported that cisplatin also induces micronuclei formation in cancerous epithelial cells (HeLa and HT1080) [68, 147] and skin fibroblast cells [64].

There are conflicting reports on whether micronuclei are retained by cells. Some suggest that micronuclei are either degraded or exported out of the cells before the next mitosis cycle [63, 78, 148]. Others have reported that micronuclei are stably maintained by cells throughout interphase, and are sometimes reincorporated back into the main

nucleus in a subsequent mitosis [61]. I observed that 47% of cells still had micronuclei 8 to 10 days post treatment, which suggests that micronuclei are retained (Figure 8). Our findings are consistent with observations made by Crasta *et al.*, who reported that 97% of the micronuclei in U2OS cells were maintained throughout interphase, and that 84% of micronucleated cells that underwent mitosis gave rise to daughter cells with micronuclei [61].

5.4. Micronuclei arise in M059K cells that undergo checkpoint adaptation

Having confirmed that M059K cells treated with cisplatin formed micronuclei, I then investigated whether checkpoint adaptation was required for this process since both involve mitosis. The relationship between micronuclei and mitosis is well known. Recall that micronuclei originate during anaphase from lagging whole chromosomes [61, 62] or acentric chromosome fragments [63] that fail to be incorporated into daughter nuclei during telophase. This process can occur in mitotic cells that have unrepaired (or incorrectly repaired) DNA breaks [64, 65], dysfunctional mitotic spindles complexes or microtubules [66-68], defects in kinetochore proteins or assembly [69-71], and/or a defective mitotic checkpoint [71].

Cisplatin both damages DNA and triggers the synthesis of extra mitotic spindles, which can produce lagging chromosomes. Recall that 33% ± 1% of M059K cells contained six or more micronuclei after treatment with cisplatin for 120 h (Figure 7C). This response is typical of cells that have undergone a multipolar mitosis [67, 68]. Maskey *et al.* reported that HeLa cells treated with cisplatin synthesize additional spindle

complexes that then pull chromosomes to more than two poles during anaphase [68]. As a result of this process cells formed multiple micronuclei.

Although a multipolar mitosis can explain how a cell with multiple micronuclei formed, it does not account for cells that have only one or two micronuclei. However, if I consider that cisplatin treated cells have damaged DNA while in mitosis, I are able to better explain how fewer micronuclei arise. Jirsova *et al.* reported that DNA breaks induced by cisplatin caused chromosome segregation errors in mitosis and gave rise to micronuclei that contained chromosome fragments in skin fibroblasts [64]. Damaged DNA induced by cisplatin is also reported to trigger mitotic death in HT1080 cells [68] and ovarian cells (SKOV-3) [149]. These findings confirmed that cells treated with cisplatin can enter mitosis with damaged DNA.

One pathway that allows entry into mitosis with damaged DNA is checkpoint adaptation. Checkpoint adaptation is characterized by three steps: (1) DNA damaged induces cell cycle arrest in G_2-phase; (2) overcoming this arrest; (3) and entry into mitosis with damaged DNA [87]. Two key enzymes required for this process are Chk1 and Cdk1, which upon activation, control the arrest in G_2-phase and entry into mitosis respectively [3]. Checkpoint adaptation has been previously observed in M059K cells treated with CPT [3], however, its relationship to genomic change and micronuclei had yet to be investigated.

In our experiments, M059K cells treated with cisplatin demonstrated the key steps of cells preparing to undergo checkpoint adaptation: they signalled DNA damage by histone γH2AX (Figure 10), they activated Chk1 (Figure 11), they arrested in S-phase and G_2/M-phase, and the majority of cells expressed cyclin B1 (Figure 12). With this

information I could then modify the key enzymes that control checkpoint adaptation by the use of chemical inhibitors, and examine the effects upon micronuclei formation.

If checkpoint adaptation and micronuclei were linked, I would predict that the inhibition of Chk1 would cause more cells to enter mitosis with damaged DNA and generate additional micronuclei. I tested if M059K cells with damaged DNA would form more micronuclei in the presence of the Chk1 inhibitor Gö6976 [140]. I observed that the percentage of micronuclei in cells co-treated with Gö6976 and cisplatin was 37% ± 3% (Figure 13). By comparison, the percentage of cells with micronuclei after treatment with cisplatin alone was 27% ± 1%. This finding suggested that when Chk1 is active, cells do not enter mitosis with damaged DNA and thus do not form micronuclei. Our observations are consistent with those of Petsalaki *et al.* who reported that Chk1-deficient human colon carcinoma cells (BE) form additional micronuclei and NPBs after treatment with etoposide [150]. Our results are also consistent with those of Bartkova *et al.* who reported that when the DNA damage checkpoints are active, including Chk1, tumour growth is suppressed, but when the DNA damage checkpoints are inhibited, cell proliferation occurs and tumour size increases [151].

I predicted that inhibiting Cdk1 would prevent cells with damaged DNA from entering into mitosis and thus stop the formation of new micronuclei. I then tested if M059K cells with damaged DNA formed new micronuclei in the presence of the Cdk1 inhibitor CR8 [13]. CR8 is an ATP competitor that blocks the active site of Cdk1 and related Cdks [12, 13]. CR8 has been shown to block mitosis in HT-29 cells, thereby preventing checkpoint adaptation [3, 10]. I co-treated cells with CR8 and cisplatin (Figure 14) and observed that the proportion of micronucleated cells did not change

compared to non-treated cells. Our findings are consistent with those of Kubara *et al.* who reported that in the presence of CR8, HT-29 cells that have damaged DNA do not undergo mitosis as measured by cell rounding and the phosphorylation of histone H3 on serine 10 [3]. I now provide evidence that CR8 also stops the formation of micronuclei by preventing checkpoint adaptation.

5.5. Micronuclei cause additional damage to DNA

While studying the relationship between micronuclei and checkpoint adaptation, I observed that nearly half of the micronuclei had damaged DNA in the absence of genotoxic treatment. Crasta *et al.* reported that the DNA in a micronucleus undergoes aberrant DNA replication which damages DNA in two steps [61]. First, DNA breaks are introduced during DNA replication but are not repaired. Second, DNA replication often occurs in late G_2-phase and if not completed prior to mitosis, chromosomes prematurely condense and fragment into several pieces. Importantly, micronuclei that undergo this process can be identified by the presence of damaged DNA and by DNA replication that occurs asynchronously to the main nucleus.

In our experiments, the micronuclei in M059K cells demonstrated several signs of aberrant DNA replication: 20% ± 7% signalled that they were undergoing asynchronous DNA replication (Figure 15; Table 3) as measured by the incorporation of the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU) and 47% ± 9% signalled damaged DNA by histone γH2AX (Figure 16; Table 4). Our observations are consistent with those of Crasta *et al.* who reported that 30% of micronuclei in epithelial cells (RPE-1) have asynchronous BrdU signals and 60% of micronuclei have histone γH2AX [61]. With this

information I could then inhibit the DNA polymerases required for replication by the use of chemical inhibitors, and examine the effects upon histone γH2AX signalling.

The results from the histone γH2AX signals suggested that micronuclei acquire damaged DNA following asynchronous DNA replication. This observation led me to predict that the inhibition of DNA polymerase α would reduce that amount of histone γH2AX observed. I tested this prediction by treating M059K cells with aphidicolin (APH). APH is a deoxycytidine triphosphate (dCTP) competitor that blocks the active site of DNA polymerase α [152]. It has been shown to inhibit DNA replication in M059K cells [153]. I observed that in the presence of APH, histone γH2AX signals declined from 54% ± 5% to 32% ± 10% (Figure 19). Our findings are consistent with that of Crasta *et al.* who reported that blocking DNA replication with excess thymidine also reduced histone γH2AX in the micronuclei of RPE-1 and U2OS cells [61]. This finding is particularly important because it suggests that the formation of micronuclei after checkpoint adaptation leads to a process of continuous genomic change, which may account for the genomic diversity in glioblastomas.

5.6. Micronuclei are rare in normal cells

I observed that M059K cells treated with cisplatin undergo checkpoint adaptation and form micronuclei, and those micronuclei continue to damage their DNA as a result of aberrant DNA replication. I then investigated whether normal cells undergo checkpoint adaptation or form micronuclei.

To study micronuclei formation in normal cells, I needed a cell line that had a similar morphology to M059K cells. Since culturing normal neural cells is difficult, I

instead selected WI-38 cells because they are also fibroblasts. I observed that 2% ± 2% of WI-38 cells had micronuclei (Figure 5), which was 12.5 times less than the percentage observed in M059K cells. Previous studies have reported similar percentages (1%) of micronuclei in WI-38 cells, which confirmed our counting methods [126].

Consistent with our observations WI-38 cells are reported to have a stable chromosome number consisting of 23 chromosome pairs [126, 154]. Buganim *et al.* analyzed the chromosomes in these cells and reported that there were no visible chromosomal translocations [154], which are observed in M059K cells with micronuclei [114].

5.7. WI-38 cells do not form new micronuclei

Having established that micronuclei were 12 times rarer in WI-38 cells than in M059K cells, I then tested if new micronuclei would form following treatment with cisplatin, as was observed in M059K cells. I treated cells with cisplatin for 120 h, but did not observe significant changes in the percentage of micronuclei (Figure 20). Our observations are consistent with those of Fink et al. who reported that micronuclei do not form in WI-38 cells after treatment with the genotoxic agent neocarzinostatin [126]. Micronuclei also do not form in normal skin fibroblasts (WS1) that are exposed to the genotoxic agent hydroxyurea [128].

5.8. WI-38 cells do not undergo checkpoint adaptation

Since I did not observe new micronuclei in WI-38 cells treated with cisplatin, I tested whether checkpoint adaptation even occurred in these cells. Previous studies have

reported that normal lymphocytes and skin fibroblasts might undergo checkpoint adaptation [94, 155]; however, these studies did not apply an analysis of the three steps required for checkpoint adaptation: a DNA damage induced cell cycle arrest in G_2-phase, overcoming this arrest, and resuming mitosis with damaged DNA.

In our experiments, WI-38 cells treated with cisplatin signalled damaged DNA by histone γH2AX and activated Chk1 protein as was observed in M059K cells, but they did not arrest in G_2-phase and most did not have cyclin B1. These data suggested that WI-38 cells may not be capable of undergoing checkpoint adaptation, which may explain why there was not an increase in cells with micronuclei.

5.9. Defects in DNA repair and cell-cycle checkpoint proteins may promote checkpoint adaptation

WI-38 cells responded differently to cisplatin compared to M059K cells. M059K cells treated with cisplatin undergo checkpoint adaptation and then form new micronuclei. By contrast, I could not detect checkpoint adaptation or new micronuclei in WI-38 cells. The reasons why checkpoint adaptation did not occur in WI-38 cells are not known. I do know that cancer cell lines are typically deficient in one or more of the proteins associated with either cycle checkpoints or DNA repair [19]. These deficiencies, which are not present in normal cells, may allow cancer cells to undergo checkpoint adaptation and form micronuclei in response to genotoxic treatment.

Interestingly, depletion of either 53PB or Ku70, which are proteins involved in double-strand break repair, have been reported to induce micronuclei formation in WI-38

cells treated with neocarzinostatin [126]. It is possible that these same deletions may be one way to allow checkpoint adaptation to occur in these cells.

Alternatively, checkpoint adaptation may occur in WI-38 cells if tumour suppressor p53 is depleted. p53 has roles in initiating the G_1/S DNA damage checkpoint, triggering cell death through apoptosis, and signalling repair of double-stranded DNA breaks [17, 27-29]. In at least 80% of cancer cell lines, p53 is reported to be non-functional [17]. M059K cells have a mutated non-functional version of p53 [143, 145, 156], whereas WI-38 cells have a functional protein p53 [126]. Fink *et al.* reported that the depletion of p53 in WI-38 cells increased the percentage of micronuclei in cells by at least six fold [126]. Depletion of p53 is also reported to be necessary for the formation of micronuclei in other normal cells (RPE-1 and WS1) [61, 128] with damaged DNA. It is therefore possible that the loss of p53 may be another way to induce checkpoint adaptation in WI-38 cells prior to the formation of micronuclei.

Chapter 6 - Thesis conclusions

The purpose of this thesis was to test if there was a relationship between genomic change and checkpoint adaptation. I observed that M059K cells treated with cisplatin can acquire genomic change in a two-step process. First, cells with damaged DNA enter mitosis (checkpoint adaptation) and then produce daughter cells with micronuclei. If cells were prevented from entering mitosis with damaged DNA, as was observed by treatment with the Cdk1 inhibitor CR8, micronuclei do not form. By contrast to CR8 treatment, if cells were forced to enter mitosis with damaged DNA by treatment with the Chk1 inhibitor Gö6976, additional micronuclei formed.

Then I found that once micronuclei are formed, some of them are prone to additional DNA damage because they undergo aberrant DNA replication. In addition, micronuclei that form in cells are not degraded and/or lost during the cell cycle, which means there is potential for continuous genomic change. WI-38 cells, which are not cancerous, did not undergo checkpoint adaptation and did not form micronuclei. Since normal cells did not enter mitosis with damaged DNA they likely have mechanisms that protect them from acquiring genomic change.

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