

**ANALYSIS OF COLON CANCER CELLS THAT SURVIVE CHECKPOINT
ADAPTATION AFTER TREATMENT WITH A GENOTOXIC AGENT**

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Thesis Abstract

Most cancer treatments are genotoxic agents that target and damage DNA as part of their mechanism of action. Recently, it was discovered that cancer cells with damaged DNA can escape the DNA damage checkpoint and enter into mitosis, a process known as checkpoint adaptation. I used the human colon carcinoma cell line (HT-29) as a model to examine checkpoint adaptation in cells treated with camptothecin (CPT), a topoisomerase I inhibitor that damages DNA. Survival and clonogenic assays revealed that approximately between 1-3% of the cells that undergo checkpoint adaptation are able to survive CPT treatment. Immunofluorescence microscopy disclosed that approximately 90% of the surviving cells were negative for histone γ -H2AX, whereas, in parallel, the alkaline comet assay revealed that 73% of the cells displayed comets. Karyotype analysis showed survival cells had 35 chromosomes on average, whereas non-treated cells contained 65. FISH analysis revealed that survival cells appear to have major changes in chromosome structure because 45% of the chromosomes were missing telomeres and 28% of the telomeres were located in positions other than the tips of chromosomes. These findings provide valuable information about the integrity of the genome of cancer cells that survive checkpoint adaptation.

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List of Abbreviations

ATCC – American Type Culture Collection

ATM – Ataxia-telangiectasia mutated

ATR – Ataxia-telangiectasia mutated Rad 3-related

cm – Centimetre

CAS – Checkpoint adaptation survival

Cdk – Cyclin-dependent kinase

Chk – Checkpoint kinase

CLL – Chronic lymphocytic leukemia

CPT – Camptothecin

DAPI – 4', 6-diamidino-2-phenylindole

DMSO – Dimethyl sulfoxide

dH₂O – Distilled water

DNA – Deoxyribose nucleic acid

DSB – Double stranded break

EDTA – Ethylenediaminetetraacetic acid

EtBr – Ethidium bromide

EXO1 – Exonuclease 1

FISH – Fluorescence In *Situ* Hybridization

G0 phase – Gap 0 phase

G1 phase – Gap 1 phase

G2 phase – Gap 2 phase

HeLa – Henrietta Lacks cervical cancer cell line

HCC – Hepatocellular carcinoma

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HT-29 – Human colon carcinoma cell line

IC50 – Half maximal inhibitory concentration

KCl – Potassium chloride

KH₂PO₄ – Monopotassium phosphate

LMPA – Low melting point agarose

LOH – Loss of heterozygosity

M phase – Mitosis phase

MOLT4 – lymphocytic leukemia cells

MLL – Histone-lysine N-methyltransferase

mM – Millimolar

μL – Microlitre

nM – Nanomolar

NaCl – Sodium chloride

Na₂HPO₄ – Disodium phosphate

NaOH – Sodium hydroxide

NOTCH2 – Neurogenic locus notch homolog protein 2

PBS – Phosphate-buffered saline

PIKK – Phosphoinositide 3-kinase related kinase

Plk1 – Polo-like kinase 1

PNA – Peptide nucleic acid

RCF – Relative centrifugal force

RPMI – Roswell Park Memorial Institute medium

SNP – Single nucleotide polymorphism

S phase – Synthesis phase

SSC – Saline sodium citrate

Topo1 – Topoisomerase 1

Tris HCl – Trizma hydrochloride

U2OS – Human epithelial osteosarcoma cell line

Chapter 1

1. Literature Review

This thesis is about human cancer cells that survive checkpoint adaptation. To understand checkpoint adaptation, I will describe a cellular perspective of cancer, techniques that can be used to detect damaged DNA, and how cells respond to cytotoxic cancer drugs.

One of the features of cancer cells is that they contain many mutations in their genome (Kandoth *et al.*, 2013). Some of these mutations disrupt the normal biochemical pathways that control the process of the cell cycle. As a result, many types of cancer cells proliferate in a manner that is different from that of normal cells, leading to uncontrolled tumour growth. Another feature of cancer cells is genomic instability, which is believed to be in part linked to cell cycle deregulation (Hanahan and Weinberg, 2011). Understanding of the relationship between the cell cycle and genomic change in normal and cancerous cells will make it possible to one day exploit these differences and provide better treatments for cancer patients. In the following sections, I will describe the cell cycle, DNA damage pathways, chromothripsis, and the relationship between these processes, with the goal to understand better the role of checkpoint adaptation in human cancer cells. In the Results section that follows, I will describe experiments that we performed to answer questions about checkpoint adaptation and cancer cells that survive cytotoxic cancer treatments.

1.1 The Cell Cycle

The cell cycle is an orderly sequence of events, known as phases, in which a cell grows, replicates its DNA, and produces two genetically identical cells. Defects in cell cycle regulation may cause unscheduled proliferation and genomic instability, giving rise to cancer cells (Cerveira *et al.*, 2012). The four phases of the cell cycle are: G1 phase, S-phase, G2 phase, and M-phase (Figure 1) (Norbury and Nurse, 1992). At the end of mitosis the cell undergoes cytokinesis, which is the process that forms two cells, each with a complete genome. Of the four phases, the first three do not give rise to morphological changes that can be observed by light microscopy. By contrast, the M-phase produces a dramatic change in cell shape and chromosome condensation, which are readily observed by light microscopy. These features led to a historical classification of the cell cycle that is still used today: interphase (G1, S and G2 phases) and mitosis (M-phase). As will be shown, in our study of checkpoint adaptation, we used morphology changes to describe cells as interphasic or mitotic and prepare experiments based upon mitotic cells.

1.1.1 Interphase

Human cells, like most eukaryotic cells, begin the cell cycle in G1, which occupies the majority of their time in the cell cycle (Norbury and Nurse, 1992). During the G1 phase a cell grows by synthesis of bio-molecules, such as proteins and lipids (but not DNA), to almost double its original size (Vermeulen *et al.*, 2003). The synthesis of

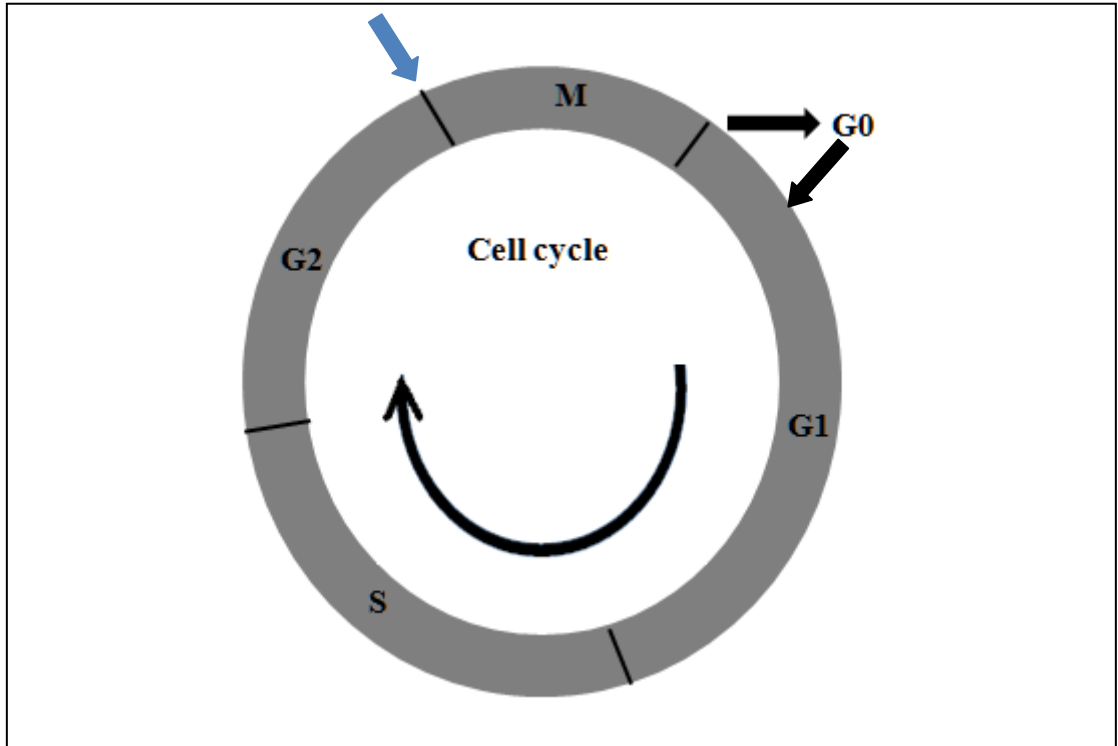


Figure 1. An overview of the cell cycle. The four phases of the cell cycle and their order are shown. The genome is duplicated during S-phase and segregated into two, separate copies during M-phase. At the end of M-phase, cells undergo cytokinesis to form two daughter cells (not shown) and exit the cell cycle as a G0 phase until the cell resumes proliferation. The transition from the G2-phase to M-phase is regulated by one of the DNA damage checkpoints (blue arrow). In the presence of damaged DNA, cells remain in the G2-phase.

DNA is specific to the S-phase of the cell cycle (the synthesis phase) (Cerveira *et al.*, 2012). During S-phase, the entire genome is copied once by template directed polymerization. After the two copies of DNA are synthesized, the cell exits the S-phase and enters the G2-phase. During this phase, the cell can grow, but it specifically prepares for M-phase by synthesising M-phase specific proteins. Although the distinctive biochemical features of the G1, S, and G2 phases are now well described, the morphology of the cell changes little during these phases. Typically for human epithelial cells, such as the ones we used in experiments in this thesis, interphase cells are adherent, flattened, and have visible nucleus.

1.1.2 M-Phase

The last phase of the cell cycle is the M-phase. In this phase, one complete copy of the genome is carefully segregated and distributed to opposite poles of a cell. This process is coordinated with cytokinesis to ensure that each daughter cell has one complete copy of the genome (Rieder and Khodjakov, 2003). Mitosis is characterized by several well defined processes: condensation of chromosomes into mitotic chromosomes; breakdown of the nuclear envelope; alignment of paired chromosomes; followed by separation of the sister chromatids to opposite poles of the cell; and re-formation of nuclei (Rieder and Khodjakov, 2003).

Cells in mitosis have a rounded morphology that distinguishes them from cells in interphase (Harris *et al.*, 1973). Cell rounding enables the cell to generate a mitotic

spindle, which is essential for a successful cell division (Lancaster *et al.*, 2013). As will be described later, the rounded morphology of mitotic cells was a characteristic that enabled me to examine checkpoint adaptation and cell survival.

Mitosis can also be characterized by several biochemical features in addition to changes in morphology. For example, mitotic cells have high levels of cyclin B1, Cdk1 activity, and high levels of phosphorylated ser10 (Pines and Hunter, 1989; Meijer *et al.*, 1989; Hendzel *et al.*, 1997). These features make it possible to identify and follow cells as they enter into mitosis, in both normal dividing cells (Juan *et al.*, 1998) and cells that contain damaged DNA (Syljuåsen *et al.*, 2006; Kubara *et al.*, 2012).

1.2 Cell Cycle Regulators

The transition between cell cycle phases is tightly regulated by cyclin dependent kinases (Cdk). The role of Cdks is to control cell cycle progression by phosphorylation of protein substrates on serine and threonine amino acids. The protein substrates have specific roles that contribute to the cellular events that occur during the cell cycle. For example, lamin B protein is phosphorylated by Cdk1 during mitosis, which causes nuclear envelope breakdown (Nigg, 1995). The correct timing of cell cycle phase changes is regulated by specific cyclin dependent protein kinase complexes. Cyclin dependent kinases are composed of two proteins: a catalytic subunit known as Cdk and a regulatory protein subunit known as cyclin (Kastan and Bartek, 2004). The cyclin proteins do not have enzymatic activity; however, they bind and activate the catalytic

subunits. At least 25 catalytic Cdks have been described, and each binds to at least one of a large family of cyclin binding partners, creating many possibilities for Cdk cyclin protein pairings (Bruyere and Meijer, 2013). The timing of the synthesis of specific cyclins is carefully regulated, which ensures that certain Cdk-cyclin complexes trigger different stages of cell cycle. For example, the Cdk4/cyclin D complex functions early in the G1 phase of the cell cycle in response to growth factors, whereas Cdk1/cyclin B complex enables cells to enter mitosis.

There are several biochemical steps required to activate Cdks in addition to cyclin synthesis, thus ensuring cell cycle fidelity. In one example, Cdk1 is phosphorylated on threonine 14 and tyrosine 15 by the cell cycle protein kinases Wee1 and Mik1 (Lundgren *et al.*, 1991). When phosphorylated on these amino acids, Cdk1 remains inactive even though it is bound to a cyclin. Cdk1 can be dephosphorylated at these sites by members of the Cdc25 protein phosphatases (Strausfeld *et al.*, 1991). This additional level of control enables cells to accumulate Cdk complexes and activate them in a short time. The cell is blocked in the G2 phase of the cell cycle if Cdk1 remains phosphorylated on tyrosine 15, which holds the enzyme in an inactive state regardless of cyclin B1 levels.

Eukaryotic cells (including human cells) have a second biochemical system that can regulate Cdk activity if the cell has damaged DNA (Smith *et al.*, 2010). Another protein kinase, Chk1 (Checkpoint kinase 1), can prevent the activation of Cdk1 by phosphorylating and promoting the degradation of Cdc25 phosphatases (Mailand *et al.*, 2000; Busino *et al.*, 2003; Sorensen *et al.*, 2003). Without Cdc25 phosphatases, cells

cannot dephosphorylate tyrosine 15 of the catalytic subunit of Cdk1, thus remain blocked in interphase, usually the G2-phase.

Overall, a series of protein phosphorylation and protein synthesis pathways converge to regulate cyclin dependent protein kinases. It appears that the complexity of these steps provides cells with opportunities to ensure that their genomes are accurately copied and distributed to daughter cells, while avoiding genome change. In my thesis, I focus on checkpoint adaptation, which is at the interface of cell cycle arrest by damaged DNA and entry into mitosis. Therefore, I will describe some of the key proteins that are required to study this event.

1.3 Cell Cycle Checkpoints

In addition to cell cycle regulators, appropriate progression through the cell cycle is tightly monitored by a series of checkpoints. Cell cycle checkpoints are biochemical pathways that detect physiological changes in cells. The cell must then respond to these changes before it can proceed into the next stage, for example, before it can exit G2 phase and enter mitosis (Hartwell and Weinert, 1989). There are at least three checkpoints in higher eukaryotic cells, each with distinct but sometimes overlapping roles. The first is the restriction point, which is a special case because it determines if a cell will enter the cell cycle or not. At the transition between the G1 and S phase, the restriction point is when cells are examined for normal growth patterns, potential space limitations and genome integrity (Weinert, 1998). The cell responds as if it makes a “decision” as to

whether the cell should divide, delay division or exit the cell cycle as a G0 phase. When conditions in the cells are right, the restriction point will be passed and the cell will be committed to enter the cell cycle and enter into the S phase (Figure 1) (Abraham, 2001).

1.3.1 The Spindle Assembly Checkpoint

The spindle assembly is a second checkpoint that monitors chromosome attachment and chromosome segregation during mitosis to ensure that the daughter cells receive a complete chromosome set (Rudner and Murray, 1996). Defects in spindle structure or failure of one or more chromosomes to align properly on the spindle are detected at the spindle assembly checkpoint, delaying mitosis usually until the defect is corrected (Gonzalez *et al.*, 2012). Malfunction in the spindle assembly checkpoint can lead to improper chromosome segregation, aneuploidy, cancer or even cell death (Abraham, 2001). Checkpoint adaptation occurs prior to the spindle checkpoint; therefore, despite the close temporal relationship between the two (a mitotic event), failure of this type of checkpoint is not directly related to checkpoint adaptation (On *et al.*, 2011). By contrast, it is believed that the DNA damage checkpoint may impact the mitotic checkpoint; however, this topic has yet to be adequately investigated (Andreassen *et al.*, 2003).

1.3.2 DNA Damage Checkpoints

Cells with damaged DNA initiate a biochemical pathway called the “DNA damage checkpoint”, which causes a delay in the cell cycle during S-phase or G2 phase to allow repair (Galgoczy and Toczyski, 2001; Weinert, 1998). It is believed that the role of DNA damage checkpoints is to prevent damaged DNA from being transmitted to daughter cells (Bartek and Lukas, 2007). The DNA damage checkpoint is composed of several overlapping checkpoint systems. An S-phase checkpoint provides continual monitoring of the DNA during DNA replication to ensure that blocked replication complexes or damaged DNA are repaired before replication (Palou *et al.*, 2010).

The successful completion of DNA replication is assessed in the G2 checkpoint. The G2 checkpoint enables cells to detect un-replicated DNA or DNA that may have been damaged by a variety of means such as genotoxic agents (Vermeulen *et al.*, 2003). The cell will delay its progression through the cell cycle at the G2 phase until errors are corrected.

1.3.2.1 G2 DNA Damage Checkpoint

The G2 DNA damage checkpoint inhibits the initiation of mitosis in cells that contain damaged DNA (Melo and Toczyski, 2002). By arresting the cell in the G2-phase, the cell can repair the damaged DNA prior to distributing it to daughter cells during mitosis. The G2 checkpoint protects multicellular organisms by acting as a major barrier to the propagation of genomic change in proliferating cells (Bartkova *et al.*, 2005). It is a

reasonable assumption that the inactivation or failure of the G2 checkpoint might then be a source of genetic change in cells (Syljuåsen, 2007).

The DNA damage checkpoint can stop the cell cycle to prevent damaged DNA from being transmitted to daughter cells (Kastan and Bartek, 2004). This process operates by a molecular system of proteins that act as sensors of damaged DNA and effectors to repair the damage or stop the cell cycle (Melo and Toczyski, 2002). The sensors of damaged DNA are members of the PI(3)kinase-like kinase family: ATM (ataxia-telangiectasia mutated); ATR (ATM- and Rad3-related); and DNA-dependent protein kinase (Smith *et al.*, 2010). These kinases interact with adaptors at sites of damaged DNA and phosphorylate other proteins that act as effectors of the DNA damage signal. For example, ATR phosphorylates checkpoint kinase 1 (Chk1) on serine 317 and serine 345, and ATM phosphorylates checkpoint kinase 2 (Chk2) on threonine 68. It is the type of DNA damage that determines which subset pathway will be activated. Breaks in double-stranded DNA activate ATM, whereas damage acquired during replication activates ATR (Liu *et al.*, 2000). However, ATR can also be activated after ATM, even when cells have one type of DNA damage, leading to crosstalk between these pathways (Jazayeri *et al.*, 2006).

The activation of the effector proteins has important consequences upon the cell cycle. One key protein, a multifunctional transcription factor, p53, becomes stabilized and directs the expression of p21, a protein that can inhibit Cdk2 complexes, thereby blocking cells in G1/S-phase of the cell cycle. In cancer cells, p53 is frequently inactivated by mutation (Weinstein *et al.*, 2013). The remaining functional effectors of

the DNA damage checkpoint are Chk1 and Chk2, which highlights their importance in cancer cells. Phosphorylation of Chk1 and Chk2 results in their activation and leads to the phosphorylation and inactivation of Cdc25 phosphatases. This is a point where the cell cycle and the DNA damage pathways meet. The reduction of Cdc25 phosphatase activity causes Cdk1/cyclin B to accumulate and remain inactive. Currently, Chk2 is believed to have a less important role relative to Chk1 in entry into mitosis (Morgan *et al.*, 2006).

Upon exposure to genotoxic agents, ATM and ATR also phosphorylate histone H2AX on serine-139 and convert it into histone γ -H2AX (Fernandez-Capetillo *et al.*, 2004). Histone H2AX phosphorylation occurs within minutes of a DNA damage event and is one of the key steps in the DNA damage response in human cells. The organization of histone γ -H2AX can take two forms in human cells with damaged DNA. In one case, histone γ -H2AX molecules are organized at sites of double stranded DNA breaks, where they recruit DNA repair and signaling molecules. These sites are visible as foci by use of specific antibodies to histone γ -H2AX and fluorescent microscopy, because the protein inserts at sites within two megabases of DNA from the break (Fernandez-Capetillo *et al.*, 2004). A second type of organization is diffuse pan-nuclear staining, in which histone γ -H2AX is found throughout the nucleus of a cell with damaged DNA. Although foci may form after short exposures to genotoxic agents, pan-nuclear staining occurs later on after exposure to some genotoxic agents, such as camptothecin (Furuta *et al.*, 2003), which reflect times observed in pharmacokinetic studies (Rivory *et al.*, 1997).

In the HT-29 cell model that I use, histone γ -H2AX organization occurs as pan nuclear staining in cells that treated with clinically relevant amounts of genotoxic drugs such as camptothecin, etoposide or alkylating agents (Leonce *et al.*, 2006; Cahuzac *et al.*, 2010). Cells that have cytotoxic amounts of damaged DNA contain histone γ -H2AX levels that are readily distinguished from cells in mitosis (Ferry *et al.*, 2011). Histone γ -H2AX foci are present in small numbers and low intensity in cells that are in mitosis; therefore, it is necessary to perform tests in parallel with non-treated mitotic cells to cells treated with genotoxic agents (McManus and Hendzel, 2005). Because of the rapid induction of histone γ -H2AX and its activation by nearly all genotoxic agents, histone γ -H2AX has become a standard marker for the presence of damaged DNA (Rogakou *et al.*, 1998; Fernandez-Capetillo *et al.*, 2004; Bonner *et al.*, 2008).

1.4 DNA Damaging Agents

The cells of our body are continuously challenged by agents and chemicals that damage DNA (Holland *et al.*, 2011). Some agents are from natural sources that we cannot avoid such as ultra violet energy from the sun (Holland *et al.*, 2011). Other genotoxic agents, such as camptothecin (CPT), are medically important, and used in cancer treatment. The majority of current cancer treatments are based upon genotoxic agents as a means to stop the growth of cancer cells (Siegel *et al.*, 2011).

In the experiments described in my thesis, we used CPT as the genotoxic agent because its mechanism of action is well characterized; it is also inexpensive and easily administered to cultured cells. CPT was first isolated by Wall *et al.* (1966) from extracts from a tree native to China called *Camptotheca acuminata*. CPT has been confirmed to be effective against an extensive range of tumors (Liu *et al.*, 2000). The pharmacokinetics of CPT has been investigated widely in humans to examine the genotoxic reaction in cancer cells (Pizzolato and Saltz, 2003). The primary cellular target of CPT is the enzyme topoisomerase I (Topo I) (Li *et al.*, 2006; Smith *et al.*, 1989). Topo I removes the DNA supercoil by cutting a single strand of the DNA double helix to relax the strand during transcription and DNA replication (Roca, 1995; Champoux, 2001). Once the DNA is relaxed, Topo-I religates the breaks by reversing its covalent binding (Pommier, 2006). Religation requires the 5'- hydroxyl group at the DNA end to be aligned with the tyrosine-DNA phosphodiester bond. CPT inhibits Topo I by blocking the rejoining step of the religation reaction of Topo I. CPT forms a ternary complex with topoisomerase I and single-stranded DNA (Hsiang *et al.*, 1989). This ternary complex stabilizes the covalent binding of Topo I to the 3' end of the broken DNA, which blocks DNA religation and generates irreversible double-strand breaks (Hsiang *et al.*, 1989; Liu *et al.*, 2000).

1.5 Outcomes of Initiating the G2 DNA Damage Checkpoint

The G2 DNA damage checkpoint is associated with biochemical pathways that delay or arrest the cell cycle progression or trigger cell death pathways, depending on the severity of the DNA damage (Niida and Nakanishi, 2006). There are three potential consequences of the G2 DNA damage checkpoint (Zhou and Elledge, 2000). The first is checkpoint recovery, in which the G2 DNA damage checkpoint delays the cell cycle progression, the damaged DNA is repaired, and the DNA damage checkpoint sensors become inactivated as the repair is completed. This inactivation enables cells to exit the G2 block and enter into mitosis (Clemenson and Marsolier-Kergoat, 2009). Another possible outcome is cell death, in which the cell is unable to repair the damaged DNA, so it initiates cell death via apoptosis, necrosis or by means such as the poorly characterized mitotic catastrophe (Castedo *et al.*, 2004; Vakifahmetoglu *et al.*, 2008). In either of these events, the cell does not distribute a damaged genome to the daughter cells. The third outcome of the G2 DNA damage checkpoint is termed as checkpoint adaptation (Toczyski *et al.*, 1997).

1.6 Checkpoint Adaptation

The process by which cells escape from the DNA damage checkpoint and enter into mitosis while DNA is still damaged is known as checkpoint adaptation (Clemenson and Marsolier-Kergoat, 2009). Checkpoint adaptation was originally detected in the yeast *Saccharomyces cerevisiae* (Sandell and Zakian, 1993). In this study, one telomere was

eliminated from the ends of dispensable yeast chromosomes by constructing experimental strains. The elimination of telomeres initially caused cell cycle arrest, induced by the DNA damage pathway. Nevertheless, many cells escaped the DNA damage checkpoint arrest without repairing the damaged chromosome. Eventually the cells with damaged chromosomes restarted the cell cycle, entered mitosis and made daughter cells. These cells had the ability to divide in spite of persistent DNA damage.

Checkpoint adaptation has also been observed in *Xenopus* egg extracts, which displayed a phenomenon equivalent to adaptation in yeast (Yoo *et al.*, 2004). In this study, the authors treated the *Xenopus* egg extracts with the DNA polymerase inhibitor, aphidicolin. They observed that after a prolonged G1/S-phase arrest, treated egg extracts were able to enter into a mitosis-equivalent state despite of the presence of incompletely replicated DNA. The illustration of checkpoint adaptation in yeast and *Xenopus* egg extracts raised the question of whether checkpoint adaptation could occur in human cells.

1.6.1 Checkpoint Adaptation in Human Cells

Checkpoint adaptation in human cells was first reported by testing osteosarcoma cells (U2OS) exposed to ionizing radiation (Syljuåsen *et al.*, 2006). Ionizing radiation generated DNA double-strand breaks, which activated the DNA damage checkpoint and caused a G2 arrest. Later on after exposure, however, the cells entered into mitosis while their DNA was still damaged. By using immunofluorescence microscopy, the research group showed that treated mitotic cells were positive for histone γ -H2AX staining which

means that the cells had damaged DNA. It was unclear, however, if checkpoint adaptation was a key event as they did not measure which proportion of the cell population entered into mitosis with damaged DNA. Syljuåsen *et al.* (2006) predicted that checkpoint adaptation can potentially cause genomic instability and lead to cancer development.

Since 2006, checkpoint adaptation has been described to occur in several other cancer cell lines, including HeLa, lymphocytic leukemia and colon carcinoma (Syljuåsen *et al.*, 2006; Cahuzac *et al.*, 2010; Rezacova *et al.*, 2011; Kubara *et al.*, 2012). In these experiments, the three criteria of checkpoint adaptation were examined: 1) a cell cycle arrest in G2 phase, 2) entry into mitosis by escaping the G2 arrest, and 3) damaged DNA in mitotic cells (Toczyski *et al.*, 1997). Lymphocytic leukemia cells (MOLT4) treated with fractionated radiation (small radiation doses delivered over several days instead of one large dose of radiation) arrested in S or G2 phases as determined by cell counting and flow cytometry (Rezacova *et al.*, 2011). MOLT4 cells were able to escape the arrest and enter into mitosis with double strand breaks (DSB) as detected by histone γ -H2AX immunofluorescence microscopy and by Western blotting of cyclin B protein.

Checkpoint adaptation has also been demonstrated in the human colon carcinoma cell line (HT-29) by Kubara *et al.* (2012). HT-29 cells treated with pharmacologically relevant concentrations of CPT underwent checkpoint adaptation as defined by the three criteria. A study on normal human fibroblastic cells demonstrated that checkpoint adaptation also occurs in non-cancerous human cells (Rezacova *et al.*, 2011). Similar to the MOLT4 cells described above, fibroblasts were treated with fractionated radiation to

create DNA double strand breaks. It was reported that this cell line underwent checkpoint adaptation, although there was no evidence of a delay in the cell cycle, activation of a checkpoint, or entry into mitosis. Damaged DNA was detected, however, by histone γ -H2AX immunofluorescence microscopy. Therefore, further studies are needed to determine if normal human fibroblastic cells undergo checkpoint adaptation.

It is likely that checkpoint adaptation also occurs in other cell types based upon descriptions of genotoxic treatments that cause cells to enter mitosis; however, few studies examine the three criteria required to meet the definition of checkpoint adaptation. In an example, a study on metastatic hepatocellular carcinoma (HCC) cells treated with cytotoxic concentrations of cisplatin entered mitosis as a result of changes in Cdc25A activity (Wang *et al.*, 2008). In support of the possibility that checkpoint adaptation is a common event, numerous studies of cells treated with genotoxic agents describe an increase in Cdk1 enzyme activity as a step in the toxicological cellular response. Knowing that Cdk1 is solely required for mitosis, it is possible that in some cases, authors are indirectly detecting cells that enter mitosis with damaged DNA (Golsteyn, 2005). These observations highlighted a need to understand the process of checkpoint adaptation in human cancer cells.

1.6.2 Consequences of Checkpoint Adaptation

A number of questions have arisen since the demonstration of checkpoint adaptation in human cells:

1. Does it happen in all human cancer cell lines, or is this specific to certain cell types?
2. Do cells survive checkpoint adaptation?
3. If cells survive, are they changed by this process?

The mechanism of checkpoint adaptation in which cells can enter into mitosis with damaged DNA and the biological significance of this phenomenon are largely unknown. Although the majority of cells that undergo checkpoint adaptation will die, it was predicted that some of them will survive and that these might have damaged DNA (Syljuåsen *et al.*, 2006; Kubara *et al.*, 2012). In addition, it is widely known that patients treated with genotoxic cancer drugs may acquire tumours that are genetically different from the primary tumour (Kastan and Bartek, 2004; Bartek and Lukas, 2007; Nakada *et al.*, 2006). It is possible that checkpoint adaptation might contribute to this phenomenon (Syljuåsen, 2007; Kubara *et al.*, 2012).

1.7 Colorectal Cancer

Colorectal cancer is the second most common cause of cancer death for males and the third most common cause of cancer death for females in Canada (Canadian Cancer Statistics, 2013). One in 13 Canadian males and 1 in 15 Canadian females are expected to be diagnosed with colorectal cancer during their lifetime. Both environmental factors such as diet and smoking, as well as genetic factors, play important role in the development of colon cancer. Colon cancer starts with small non-cancerous growths

called polyps that appeared in the colon. Colorectal cancer follows the multi-stage model, initially affecting normal mucosa and finally becoming an invasive adenocarcinoma (Mustafa *et al.*, 2013). This sequence of events happens as a result of the accumulation of genetic aberrations, which ultimately leads to dysfunctional changes in proliferation, differentiation and apoptosis. Over a period of several years, the rate of cell growth and apoptosis results in the development of cancerous polyps. A cancerous polyp grows rapidly inside, eventually breaking through the wall of the colon and spreading via lymph vessels and blood vessels to other parts of the body.

To study checkpoint adaptation, I needed to use a model that would represent features of the human disease while being suitable to experimental conditions in a laboratory. In this research, I have worked with the human colon adenocarcinoma cell line, known as the HT-29 cell line. The HT-29 cell model provides several advantages over the first cell model (U2OS) that was described by Syljuåsen *et al.* (2006). HT-29 cells are representative of human colon cancer, respond to camptothecin, and undergo a pronounced morphology change when entering mitosis. HT-29 cells were derived from a primary adenocarcinoma of the rectosigmoid colon. HT-29 is hypertriploid ($3n+$) and has accumulated numerous chromosomal structural aberrations (Kawai *et al.*, 2002). The typical chromosome number of the HT-29 cell line is 71 (Kondoh *et al.*, 1993; Guildbaud *et al.*, 2001). This cell line is widely used in the pharmaceutical industry to screen for new anti-colon cancer drugs.

1.7.1 Treatment for Colon Cancer

The methods for treating colon cancer patients are based upon the level to which the tumour has progressed in the tissue, which is called staging (Stein *et al.*, 2011). Some patients might need surgery to remove the diseased segment of the colon, followed by radiotherapy or chemotherapy. For stage 1, cancer that has not spread, surgery is the treatment of choice. Stage 1 colon cancer may be treated by removing the cancer tissue by performing a colonoscopy (Rex *et al.*, 2006). In fact, surgery may be the only treatment necessary for patient with stage 1 or 2 colon cancer. However, some patients with stage 2 cancer may elect to receive chemotherapy (Benson *et al.*, 2004). More extensive surgery is needed for stages 3 and 4 cancers to remove the part of the colon that is cancerous. Following surgery, almost all patients with stage 3 colon cancer receive adjuvant chemotherapy (such as topoisomerase I inhibitors and alkylating agents or irradiation) for 6 to 8 months (Schrag *et al.*, 2001; Watanabe *et al.*, 2001). Chemotherapy is also used to prolong survival in patients with stage 4 colon cancer. A variety of chemotherapeutic drugs have been tested and used in chemotherapy treatment for colon cancer. One of the most common and beneficial chemotherapeutic drugs used for colon cancer is camptothecin and its derivatives, as the overall drug toxicity is low, which allows for repeated courses of treatment (Giovanella *et al.*, 1989).

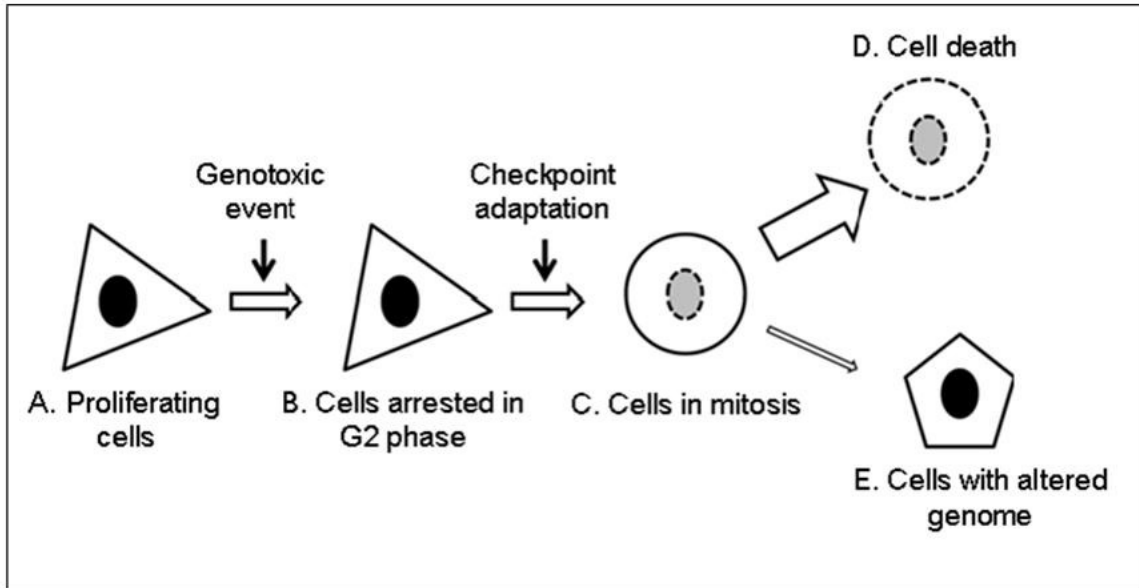


Figure 2. A model of the steps of checkpoint adaptation in human cancer cells treated with a cytotoxic concentration of genotoxic agents (from Kubara *et al.*, 2012, used with permission). Proliferating colon carcinoma cells arrest in the G2-phase of the cell cycle after experiencing a genotoxic event. By the process of checkpoint adaptation, cells enter mitosis. The majority of cells will die (large arrow), but a small number of cells will survive (small arrow), possibly with changes to their genome.

1.7.2 Checkpoint Adaptation in Colon Cancer

HT-29 cell model for checkpoint adaptation was first used by Kubara *et al.* (2012). This study demonstrated that HT-29 cells, following the treatment with 25 nM CPT, recruited histone γ -H2AX (indicating damaged DNA). Cells that have damaged DNA phosphorylate checkpoint kinase 1 (Chk1), which is an indicator of the activation of the DNA damage checkpoint. An analysis of treated cells by flow cytometry showed that cells arrested in the G2-phase of the cell cycle. Furthermore, Western blotting showed that cells contained high levels of cyclin B protein and high levels of tyrosine phosphorylated Cdk1. These data demonstrated that the cells had arrested in the cell cycle due to damaged DNA induced by CPT, the first criterion of the checkpoint adaptation process. By 48 hours after treatment, many HT-29 cells had entered mitosis. These cells had a rounded morphology that was distinct from cells in interphase, and presence of phospho-ser10 histone H3. Importantly, cells in mitosis retained intense histone γ -H2AX staining and had very poorly organized mitotic chromosome structures. Furthermore, it was reported that the cells that entered into mitosis with damaged DNA contained dephosphorylated Ser345-Chk1, indicating a relationship between Chk1 dephosphorylation and checkpoint adaptation. Finally, the ability of HT-29 cells to arrest in mitosis provided an experimental means to detect and collect cells that had undergone checkpoint adaptation (Gascoigne and Taylor, 2008).

1.8 Micronuclei

A micronucleus appears as a small membrane-bound nucleus that forms due to the improper incorporation of acentric fragments or chromosomes into daughter nuclei during cell division (Fenech *et al.*, 2011). Acentric chromosome fragments or whole chromosomes fail to be included in the daughter nuclei during mitosis due to improper attachment with the spindle during the segregation process. These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane that appears to be morphologically similar to nuclei, except for their smaller size.

The presence of micronuclei in cells is commonly used as evidence of mutations caused by damaged DNA (Hovhannisyanyan *et al.*, 2009). Genomic change acquired in micronuclei might contribute to cancer development (Crasta *et al.*, 2012). Micronuclei can also contribute to the formation of DNA breakage and chromothripsis (Crasta *et al.*, 2012). One feature of micronuclei is that they likely originate only from cells that have undergone mitosis (Luzhna *et al.*, 2013). Therefore, it is possible that a relationship exists between the presence of micronuclei and checkpoint adaptation. This notion is supported by the recent advancements in the study of chromosome damage, or chromothripsis.

1.9 Chromothripsis

The word chromothripsis is derived from the Greek terms *chromos* for color and later chromosome, and *thripsis* for shattering into pieces (Stephens *et al.*, 2011).

Chromothripsis is a phenomenon by which numerous chromosomal rearrangements occur in cells in a short time (Stephens *et al.*, 2011; Hübner *et al.*, 2009). The majority of chromothripsis events are believed to occur in one catastrophic event. The shattering event usually occurs when chromosomes condense into mitotic chromosomes. A chromosome or chromosomal region is broken into tens to hundreds of pieces and then rejoined by the DNA repair pathways in a patchwork of genomic fragments. These new chromosomes do not have the original order of the progenitor chromosome(s) (Stephens *et al.*, 2011; Forment *et al.*, 2012). The chromothripsis hypothesis and its implications for cancer development were first described by Stephens *et al.* (2011).

There are three distinguishing features of chromothripsis:

1. The presence of significant numbers of complex rearrangements in localized regions of one chromosome or chromosome arm;
2. Low copy number states (normally between one or two) of the genome throughout the rearranged region; and
3. The conservation of heterozygosity.

These characteristics have major significance for interpreting how and when chromothripsis originates. First, rearrangements in localized chromosomal regions suggest that the condensation of chromosomes is important for the occurrence of chromothripsis, such as during mitosis (Forment *et al.*, 2012). Next, the number of copy

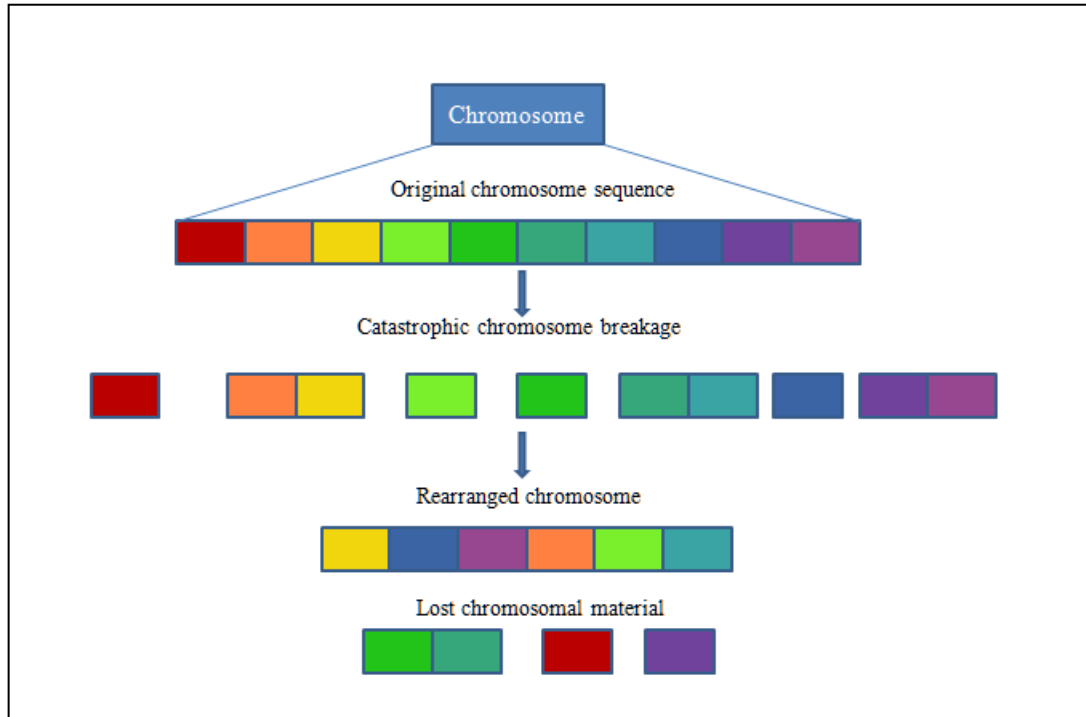


Figure 3. A simplified model for the chromothripsis. Chromothripsis can be generated by one catastrophic event in which the chromosome shatters into tens to hundreds of pieces. Rearrangements occur by rejoining the pieces together by the DNA repair pathways in a mosaic patchwork of genomic fragments, however some pieces are lost in the cell. These new chromosomes do not contain the complete, original order that was present in the progenitor chromosomes.

number states (normally between one or two, occasionally three) across the chromothriptic regions demonstrates that such rearrangements occur within a short time (Maher *et al.*, 2012; Forment *et al.*, 2012; Korbel and Campbell, 2013). If the rearrangements had occurred gradually over time, the copy number would eventually increase with the number of detected breakpoints, and this has not been observed (Forment *et al.*, 2012). Finally, the alternation of segments retaining heterozygosity with others presenting loss of heterozygosity (LOH) in chromothriptic regions suggests that the chromosomal rearrangements occur early in cancer cell development. Retention of heterozygosity in 'patches' throughout a chromothriptic region is difficult to explain by progressive rearrangement mechanisms, because once heterozygosity is lost, it cannot usually be regained. This adds support to the idea that chromothripsis generally occurs in one catastrophic event and is not an ongoing process. This view contrasts with that of accumulating simple mutations over time, as described earlier for colon cancer.

1.10 Overview of Thesis

Cancer patients are frequently treated with cytotoxic agents that ultimately target and damage DNA. Cancer cells with damaged DNA initiate a series of biochemical steps that enable them to detect the damaged DNA and respond to it. One of the late events in these steps is checkpoint adaptation, a process in which cells enter into mitosis despite the presence of damaged DNA. Checkpoint adaptation was first demonstrated in human cancer cells in 2006, following treatment with radiation (Syljuåsen *et al.*, 2006).

Although the majority of cells that undergo checkpoint adaptation die, a small percentage of them are able to survive and continue to divide with damaged DNA. The outcome for the cells that survive checkpoint adaptation is unknown.

In this thesis, I have examined HT-29 cells that survived checkpoint adaptation after treatment with CPT to provide insight into the possibility of genomic change caused by checkpoint adaptation. This will provide valuable information about the significance of checkpoint adaptation in HT-29 cells.

1.10.1 Hypothesis

Our hypothesis is that human colon cancer cells that survive checkpoint adaptation acquire changes in the organization of their genome. To test this hypothesis, I will perform experiments to achieve three research objectives.

1.10.2 Research Objectives

I will use the HT-29 cell culture model of checkpoint adaptation to do the following:

1. Assess the percentage of cells that survive checkpoint adaptation using defined conditions.

2. Examine the chromosomes of the survival cells before, during and after a genotoxic treatment.

3. Determine if cells that survive checkpoint adaptation contain damaged DNA.

By addressing these objectives, I intend to provide insight in the role of checkpoint adaptation in genomic change after genotoxic treatment.

Chapter 2

2. Materials and Methods

2.1 Cell Culture

The human cell line HT-29 was obtained from the American Type Culture Collection (ATCC). HT-29 cells were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% decompemented fetal calf serum (PAA Laboratories, Etobicoke, Ontario), 2 mM L-glutamine (Invitrogen), and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. Cells were grown at 37°C in 5% CO₂ and media were changed every second or third day. Typically, HT-29 cells were plated at 400,000 cells/25 cm² flask and cultivated for 48 hours prior to treatment. The compound camptothecin (CPT) (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 μM and stored at -20°C until further use. In experiments where we used non-treated cells, cultures were treated with an equivalent amount of DMSO to control for the solvent used to carry CPT.

2.2 Mechanical Shake-off

HT-29 cells were plated at 400,000 cells/25 cm² flask and cultivated for 48 hours prior to treatment with compounds. We incubated these cells with a pharmacologically

relevant and cytotoxic concentration of CPT (25 nM). This concentration is higher than the IC₅₀ of CPT for HT-29 and within the range of serum levels of treated patients (Rivory et al., 1997). At the desired time after treatment, culture medium was replaced with sterile phosphate buffered saline (PBS 1X; 137 mM sodium chloride (NaCl) (BDH Chemicals), 2.7 mM potassium chloride (KCl) (BDH Chemicals), 12 mM disodium phosphate (Na₂HPO₄) (Sigma-Aldrich), 1.8 mM monopotassium phosphate (KH₂PO₄) (Millipore), and distilled H₂O) to rinse the flask. PBS was removed by aspiration to eliminate the dead cells in suspension. A small volume of RPMI (40 μL/cm²) was added in the flask and the flask was tapped on all sides with medium force until rounded cells were released.

2.3 Light Microscopy

Images were taken with an Infinity 1.5 camera powered by Infinity Capture (Lumenera Corporation) software on an Olympus IX41 inverted microscope. Live cells were detected by dye exclusion with Trypan Blue 0.4% solution (Amresco) (a 1:1 ratio with a sample of cells from culture and Trypan Blue solution) to determine cell viability. Dead cells appeared blue and viable cells appeared clear with this dye when observed under by microscopy at 200 x magnification. The percentage of live cells was quantified within a population using a hemocytometer.

2.4 Survival Assay

HT-29 cells were plated at 400,000 cells/25 cm² flask and cultivated for 48 hours prior to treatment with compounds. At desired times after treatment, culture medium was replaced with a small volume of RPMI (40 μL/cm²), and flasks were tapped on all sides with medium force until rounded cells were released. Collected rounded cells were counted using a hemocytometer and cell viability was confirmed by vital dye exclusion. Cells were re-plated in a 25 cm² flask and placed in the incubator at 37°C until they formed sufficiently large colonies (approximately 10 to 12 days). At desired times, cells were collected by trypsinization. Cells were counted again using a hemocytometer to determine the number of cells surviving.

The number of surviving cells was estimated using the following formula: $N_0 = 10^{\{\log NT - (\log 2 / tg) T\}}$, where NT = number of cells collected at the end of the survival assay, N_0 = number of cells that were able to proliferate (i.e., survival cells), tg = generation time in hours (for HT-29 cells, tg = 31 hours), and T = time of culture in hours. Then, the percentage of survival cells was counted using the following formula: $P = (N_0 / N) \times 100$, where N = number of cells placed in the flask at the beginning of the survival assay (Leibovitz and Mazur, 1977). Experiments were repeated at least three times.

2.5 Clonogenic Assay

The method of clonogenic assay was performed as described by Franken *et al.* (2006) with some modifications. HT-29 cells were plated at 400,000 cells/25 cm² flask and cultivated for 48 hours prior to treatment with compounds. At the desired time after treatment, culture medium was replaced with a small volume of RPMI (40 μL/cm²), and cells were collected by mechanical shake-off. Collected rounded cells were counted using a hemocytometer.

A suspension of 10⁴ cells/ml was prepared and either 5x10³, 1x10⁴, or 2x10⁴ cells were cultivated in duplicate wells in 6-well plate for 14 days. The colonies typically contain at least 50 cells to represent viable cells. At desired times, cells were fixed and stained using a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 30 minutes at room temperature. Cells were rinsed with water. The plate with cell colonies was left to dry in normal air at room temperature. Non-treated cells were also analyzed separately for plating efficiency by clonogenic assay. For plating efficiency, a suspension of non-treated HT-29 cells at 2.0x10³ cells/ml was prepared and 50, 100, or 200 cells were cultivated in duplicate wells for 14 days. Colonies were counted using ImageJ software to determine the number of cells surviving.

The plating efficiency (PE) of the non-treated cells was determined by the following formula: PE = (number of colonies formed/ number of cells seeded) x 100%. The surviving fraction (SF) was counted using the following formula: SF= number of colonies formed after treatment/ (number of cells seeded x PE). Experiments were repeated at least twice.

2.6 Immunofluorescence Microscopy

Cells were plated on glass coverslips for 48 hours before treatment. Cells collected by mechanical shake-off were attached to glass coverslips coated with poly-L-lysine (Invitrogen). At desired times cells were fixed in 3% formaldehyde for 20 minutes at room temperature and permeabilized for 5 minutes in 0.2% Triton X-100. Cells were incubated with anti-histone γ -H2AX (1:300, No. 05-636, Millipore). The secondary antibody Alexa488 (1:400, Molecular Probes/Invitrogen) for anti-histone γ -H2AX was added for 2 hours. Nuclei were stained with 300 nM 4'6-diamidino-2-phenylindole (DAPI) in PBS for 15 minutes prior to mounting. Cells were observed on a Zeiss microscope operated by Axiovision 3.1 software. Images were collected by Zeiss MR camera within the linear dynamic range. Images were prepared for presentation using identical parameters with Adobe Photoshop CS3 10.0 software. Experiments were repeated at least three times. On average, 250 cells were analyzed per experimental group per replicate.

2.7 Alkaline Comet Assay

Cells were plated at 350,000 cells/ 25 cm² flask for 48 hours and were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% decompemented fetal calf serum (PAA Laboratories, Etobicoke, Ontario), 2 mM L-glutamine (Invitrogen), and 10

mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4 before treatment. Cells were grown at 37°C in 5% CO₂. Rounded mitotic cells were collected by mechanical shake-off and were used in comet assay analysis for DNA damage. Survival cells were also analyzed separately for DNA damage using the comet assay. These cells were compared to those that are not treated with CPT.

The method of alkaline comet assay was performed as described by Tice and Vasquez (1999) with some modifications. Partly frosted microscope slides were coated with 1% regular agarose (Bio Basic Inc) and allowed to dry for 2 to 3 minutes at room temperature. For next layer, approximately 40,000 cells were mixed with 2% low melting point agarose (LMPA; MJS BioLynx) and placed onto a pre-coated slide and immediately covered with a coverslip for 10 to 15 minutes. The coverslip was then removed from the initial agarose layer. Once set, 1% regular agarose was placed as a final layer on top of the cell-LMPA mix layer and again covered immediately with a coverslip. The final coverslip was removed once the gel had set. At this point the remainder of the assay was performed using indirect light.

Next, the slides were placed in the freshly made lysis solution (2.5 M sodium chloride (NaCl) (BDH Chemicals), 100 mM EDTA (pH 8.0) (BDH Chemicals), 1% sarkosyl (Sigma-Aldrich), 10 mM Tris-HCl (pH 8.0) (EMD), 10% DMSO (Bio Basic Inc), 10% Triton X-100 (EMD)) on a bed of ice for a minimum of 1 hour. After 1 hour, the slides were washed with ice-cold PBS for 15 minutes. Next, the slides were placed gently into a horizontal electrophoresis tank filled with cold alkali buffer (0.3 M sodium hydroxide (NaOH) (BDH Chemicals) and 1 mM EDTA) for 30 minutes to allow the

DNA to denature in the alkali buffer. The pH of the buffer was >13. The electrophoresis was performed at 22 V (500 mA) for 30 minutes. Next, the slides were removed and rinsed by placing them in ice-cold neutralising buffer (0.5 M Tris-HCl (pH 7.5) (EMD)) for 10 minutes followed by ice-cold PBS for 15 minutes. Ethidium bromide (0.1 mg/ml) (EtBr) (Sigma-Aldrich) stain was added to each slide to stain the DNA for 15 minutes and then washed with dH₂O. Stained DNA was analyzed by a Zeiss microscope operated by Axiovision 3.1 software. Images were collected with a Zeiss MR Camera within the linear dynamic range. DNA damage was measured using Comet Score software expressed as percent DNA in tail and Olive moment. Based on our experience and published literature, values higher than 20% DNA in the tail were considered as damaged cells (Olive *et al.*, 1990). Experiments were repeated at least three times. Typically, an average of 200 cells was analyzed per experimental group per replicate.

2.8 Karyotyping

The method for karyotyping was performed as described by Cassio (2006) with some modifications. Cells were plated at 350,000 cells/25 cm² flask for 48 hours and were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% decomplemented fetal calf serum (PAA Laboratories, Etobicoke, Ontario), 2 mM L-glutamine (Invitrogen), and 10 mM HEPES, pH 7.4 before treatment. Cells were grown at 37°C in 5% CO₂. Cells were treated with 25 nM CPT for 48 hours followed by overnight

treatment of KaryoMAX colcemid (10 µg/ml) (Cedarlane). Colcemid was used to increase the number of metaphase cells in a population. Next, freshly prepared hypotonic solution (0.075 M KCl (BDH Chemicals) and distilled H₂O) was added to the cells for 20 minutes to make nuclei swell osmotically. Then, cells were fixed by adding 3:1 methanol (Fisher Scientific) and acetic acid (BDH Chemicals) solution and incubated for 30 minutes with agitation followed by 5 minutes centrifugation at 220 rcf. Cells were re-suspended in 0.5 ml fixative. A drop of the cell suspension was placed on a cold, clean glass slide and blown hard to disperse the chromosomes. The slides were air dried at room temperature and stained with 300 nM DAPI (Life Technologies). Slides were analyzed by a Zeiss microscope operated by Axiovision 3.1 software. Images were collected by Zeiss MR Camera within the linear dynamic range. Survival cells were also analyzed for genomic change by performing karyotyping. These cells were compared to those that are not treated with CPT. Experiments were repeated at least three times. Typically, an average of 50 cells was analyzed per experimental group per replicate.

2.9 Fluorescence *In Situ* Hybridization (FISH) Analysis With PNA Probes

The method for Fluorescence *In Situ* Hybridization (FISH) was modified from the protocol supplied by PNA Bio Inc. Slides were prepared as described above in the Karyotyping section (Section 2.8). Samples were fixed using formaldehyde fixing solution (4% formaldehyde (Ted Pella) in PBS) for 2 minutes followed by RNase solution (100 µg/ml RNase A (Sigma-Aldrich) in PBS) and incubated for 20 minutes at 37°C.

Mixture of PNA probes (FAM labeled PNA TelC probe (CCCTAACCCCTAACCCCTAA); Alexa488 labeled PNA TelC probe (A488-OO-CCCTAACCCCTAACCCCTAA); and Cy3 labeled PNA centromere probe (ATTCGTTGGAAACGGGA) (PNA Bio)) and hybridization buffer (20 mM Tris, pH 7.4, 60% formamide (Sigma-Aldrich), 0.1 µg/ml UltraPure salmon sperm DNA solution (Life Technologies), and distilled water, final pH 7.4) was added to the slide followed by incubation at 85°C for 10 minutes. Next, the slide was immersed in a wash solution (2X SSC (distilled water, NaCl (BDH chemicals), sodium citrate (Sigma-Aldrich), HCl (Fisher Scientific) and 0.1% Tween-20 (Sigma-Aldrich)) at 55-60°C for 10 minutes. The slide was stained with 300 nM DAPI (Life Technologies) solution for 10 minutes followed by two washes with 1x SSC and 2x SSC. Chromosomes were analyzed by a Zeiss microscope operated by Axiovision 3.1 software. Images were collected by Zeiss MR camera within the linear dynamic range. Survival cells were also analyzed separately for genomic rearrangement by performing FISH analysis. These chromosomes were compared to those that were not treated with CPT. Experiments were repeated at least three times. Typically, an average of 50 cells was analyzed per experimental group per replicate.

2.10 Statistical Analysis

Statistical analysis was performed by the Student's t-test. Data are presented as mean \pm standard deviation (SD). A probability level of $P < 0.05$ was considered significant.

Chapter 3

3. Results

3.1 HT-29 Cells Treated with Camptothecin (CPT) Undergo Checkpoint Adaptation

Previously, Kubara *et al.* (2012) showed that HT-29 cells undergo checkpoint adaptation when treated with pharmacologically relevant concentrations of CPT. In this process, cells with damaged DNA become arrested in the G2 phase of the cell cycle. However, nearly all cells escape the DNA damage checkpoint and enter mitosis. These cells fulfil the three features that define checkpoint adaptation: an arrest in the cell cycle due to damaged DNA, overcoming this arrest, and then entering into mitosis despite the presence of damaged DNA (Toczyski *et al.*, 1997). The first step in my thesis was to set up this assay of checkpoint adaptation so that I could collect mitotic cells. These cells would allow me to test the hypothesis that cells which survive checkpoint adaptation will have changes in their genome. To address this hypothesis, I first attempted to answer this question: What is the percentage of cells that survive checkpoint adaptation?

At 24 hours after CPT treatment, nearly all cells were flat and strongly adherent. Rounded cells, which were weakly adherent, were present by 48 hours of CPT treatment (Figure 4A). Rounded morphology is one of the defining features of mitotic cells, especially those that have undergone checkpoint adaptation. These rounded mitotic cells were separated by mechanical shake-off (Figure 4C) from the flattened interphase

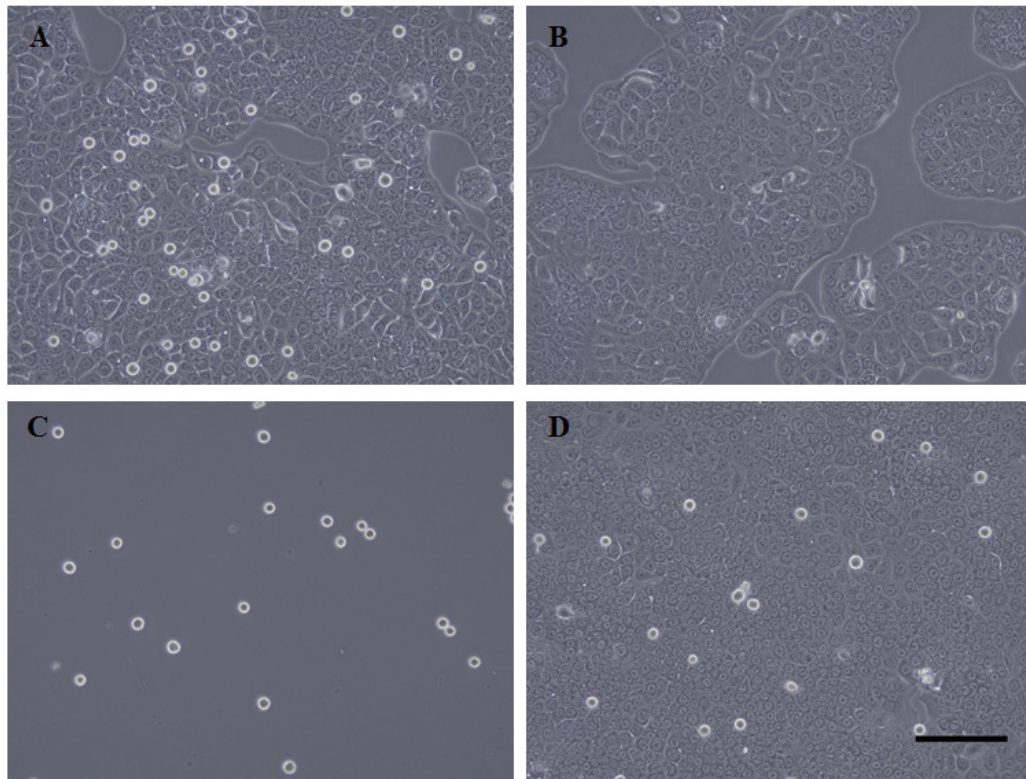


Figure 4. HT-29 cells treated with camptothecin (CPT) undergo checkpoint adaptation.

HT-29 cells entered mitosis by 48 hours after treatment with CPT. We used mechanical shake-off to isolate cells that have undergone checkpoint adaptation (mitotic cells) from interphase cells. Images were collected by light microscopy.

A. Cells treated with 25 nM CPT for 48 hours.

B. Cells remaining on culture dish immediately after mechanical shake-off at 48 hours.

C. Mitotic cells collected by mechanical shake-off.

D. The culture (B) 2 hours after performing a mechanical shake-off in which new mitotic cells (rounded cells) appear.

Scale bar = 100 μm .

cells. The vital dye exclusion technique was used to test if the CPT treated, rounded cells were alive or not. In this assay, live cells are able to exclude a vital dye, trypan blue. We confirmed that 95% of the HT-29 rounded mitotic cells were alive, which was consistent with previous results from my laboratory (Kubara *et al.*, 2012). The remaining adherent cells were re-cultivated (Figure 4B). We selected a time of two hours at which we observed by light microscopy that new rounded cells appeared from the flattened cells indicating confirming that checkpoint adaptation is a continuous process (Figure 4D). The result of this experiment confirmed that HT-29 cells enter into mitosis following 48 hours of 25 nM CPT treatment. By establishing this experimental system we would be able to further investigate the fate of human cancer cells that undergo checkpoint adaptation.

3.2 Cells that Undergo Checkpoint Adaptation are Able to Survive

The next step was to determine if the rounded mitotic HT-29 cells were able to survive and continue to divide. If the cells were able to survive, it would be possible that they have changed their genome because they originated from a population of cells with damaged DNA. We have used several approaches to determine the number of cells that survive checkpoint adaptation: the clonogenic assay and cell counting at various times (survival assay).

We investigated the number of cells that survive checkpoint adaptation by the clonogenic assay. Mitotic cells were first collected from non-treated HT-29 cells to measure the plating efficiency of the cell line. This efficiency value was needed to calculate the number of cells surviving under standard culture conditions, without treatment. For this purpose, 50, 100, or 200 non-treated HT-29 cells were cultivated in duplicate cultures in a 6 well plate (Figure 5). After calculation, we found that the plating efficiency of the HT-29 cells was 53%, consistent with that of previously published results (Ware *et al.*, 2007).

In preliminary experiments we learned that we needed to inoculate the cultures with many more cells when using the treated sample to accommodate the cell mortality from treatment, which was coherent with the goal of finding survivor cells. Mitotic cells were collected from the 48 hour CPT treated culture, and approximately either 5×10^3 , 1×10^4 , or 2×10^4 cells were cultivated in duplicate wells in a 6-well plate (Figure 6). The colonies of at least 50 cells were considered viable. Colonies were counted using ImageJ software and then calculated to determine the number of cells surviving. The clonogenic

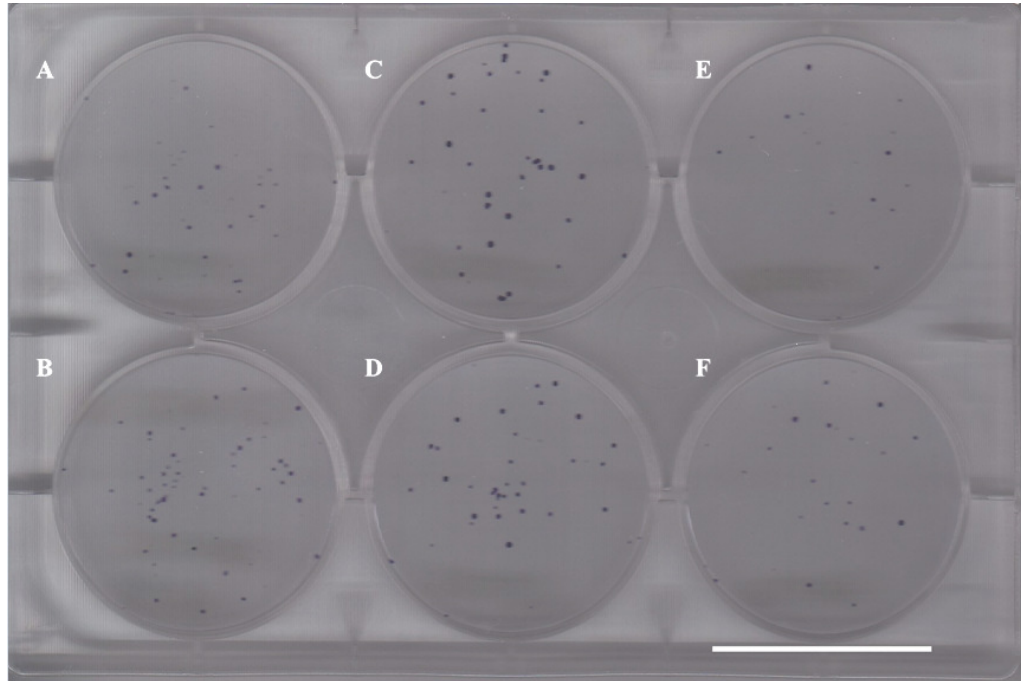


Figure 5. The clonogenic assay was performed on non-treated HT-29 colon cancer cells to measure plating efficiency. An image of a 6 well plate after staining with crystal violet is shown.

A and B. 200 non-treated HT-29 cells were cultivated.

C and D. 100 non-treated HT-29 cells were cultivated.

E and F. 50 non-treated HT-29 cells were cultivated.

Scale bar = 3.5 cm.

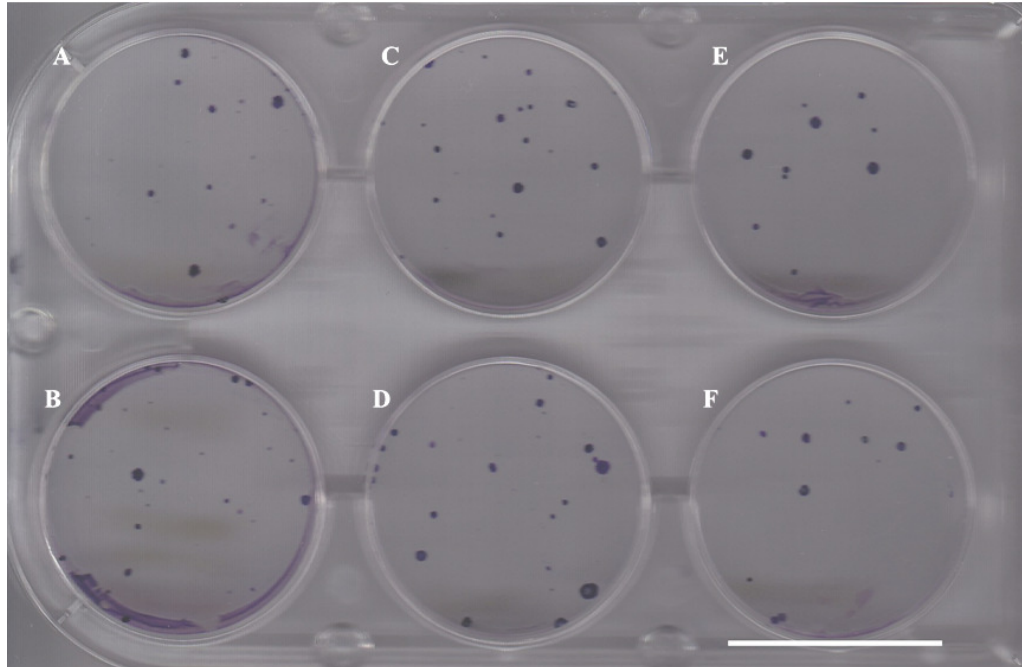


Figure 6. The clonogenic assay was performed on CPT treated, mitotic HT-29 cells to quantify the number of cells that survive checkpoint adaptation. An image of a 6 well plate after staining with crystal violet is shown.

Cells were cultivated by a dilution series in a six-well plate:

A and B. 2×10^4 CPT treated HT-29 cells were cultivated.

C and D. 1×10^4 CPT treated HT-29 cells were cultivated.

E and F. 5×10^3 CPT treated HT-29 cells were cultivated.

Scale bar = 3.5 cm.

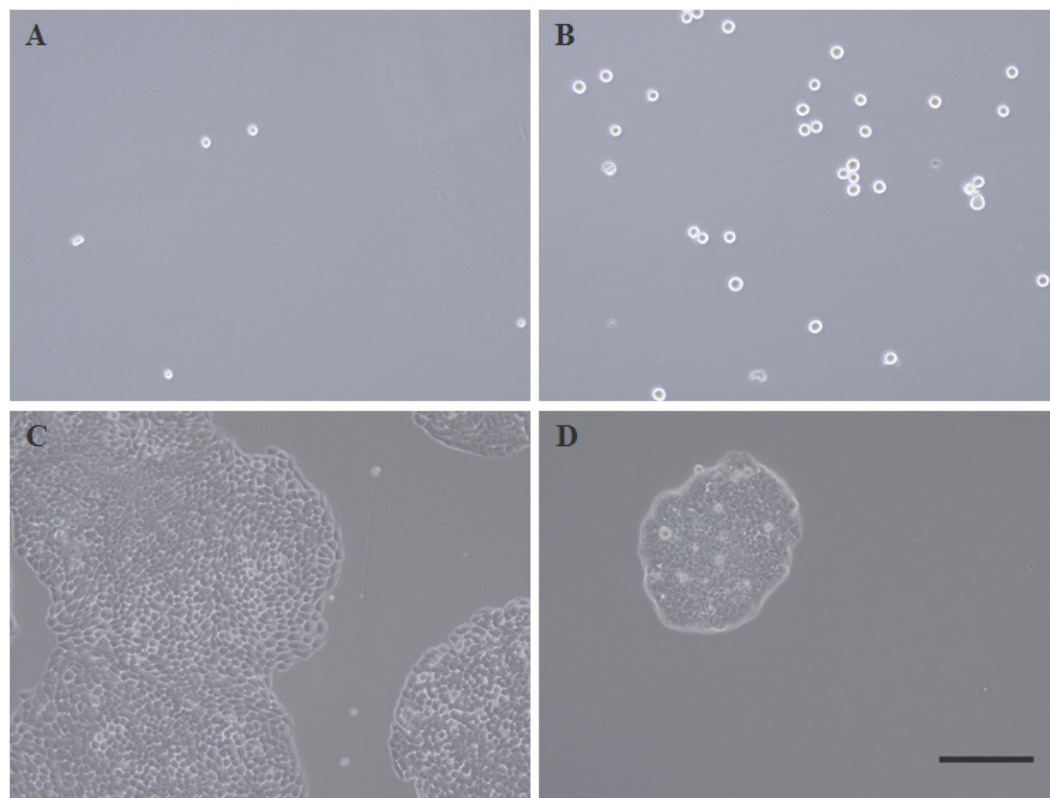


Figure 7. Images taken by light microscopy of non-treated and CPT treated HT-29 cells collected by mechanical shake-off and re-cultivated. Cells counts were performed to estimate the number of cells that survive CPT treatment.

- A.** HT-29 non-treated, mitotic cells after mechanical shake-off.
 - B.** Mitotic cells collected by mechanical shake-off after treatment with 25 nM CPT for 48 hours.
 - C.** Non-treated cells, 12 days after mechanical shake-off.
 - D.** CPT treated cells 12 days after mechanical shake-off (CAS cells).
- Scale bar = 100 μm .

assay revealed that 1 +/- 0.3% of the CPT treated, mitotic cells retained the capacity to produce colonies.

We then approached the cell counting experiment by a second method using direct counting with a hemocytometer. In this approach, HT-29 cells were either treated with 25 nM CPT or were untreated, as described in the previous section. At 48 hours, rounded cells were collected by mechanical shake-off, counted, and re-plated as new cultures and then counted again at 12 days post re-plating. Images of cells collected by mechanical shake-off and of cultures that arose from these two populations were taken (Figure 7A and B), revealing mitotic cells from both non-treated and treated populations. By 12 days, the non-treated cells culture contained many large groups of cells (Figure 7C) whereas the CPT treated mitotic cells produced few groups that colonized the culture dish (Figure 7D). At 12 days both the non-treated and treated cells were collected by trypsinization and counted using a hemocytometer. We found that on average, in three experiments, 3 +/- 0.9% ($n = 1.8 \times 10^5$) of the cells that underwent checkpoint adaptation were able to survive and proliferate. Thus, the two approaches, clonogenic assay and direct counting, gave similar results in that a small percentage of cells that enter checkpoint adaptation can survive. From now on, we will call the survival cells CAS cells (Checkpoint Adaptation Survival cells). These results indicated that we could now examine CAS cells for differences in their genome relative to non-treated cells.

3.3 Ninety Percent of the CAS HT-29 Cells are Negative for Histone γ -H2AX

Having established that 1-3% of HT-29 cells that undergo checkpoint adaptation are able to survive, we wanted to know if there were changes in their genome relative to non-treated cells. This question was important because the agent that we used to induce checkpoint adaptation was the genotoxic compound CPT (Goldwasser *et al.*, 1995). Therefore, we used several approaches to test cells for damaged DNA. In the first approach, we used immunofluorescence microscopy to look for histone γ -H2AX, a protein that is used by cells to signal and repair damaged DNA.

For this experiment we set up three populations of HT-29 cells:

1. Non-treated cells as a negative control,
2. Cells treated with 25 nM CPT for 48 hours,
3. CAS cells, collected at 10 days post mechanical shake-off.

Cells were grown on coverslips under the above conditions, then fixed and stained with DAPI to mark DNA, and stained with histone γ -H2AX antibodies to mark damaged DNA if present. Images were collected with CCD camera set so that signals were within the linear range of detection (Cahuzac *et al.*, 2010). We found that 3 +/- 0.7% (n = 750) of the non-treated HT-29 cells displayed histone γ -H2AX signals, as expected, (Figure 8D) even though several hundred nuclei were inspected by DAPI staining (Figure 8A). By contrast, HT-29 cells treated with CPT for 48 hours showed distinctive large nuclei (Figure 8B) by DAPI staining, and 94 +/- 2.1% (n = 750), $P < 0.05$ (where $P = 0.04$) of CPT treated cells displayed histone γ -H2AX pan-nuclear staining, with many foci, typical for this treatment in HT-29 cells (Figure 8E) (Furuta *et al.*, 2003), as previously reported

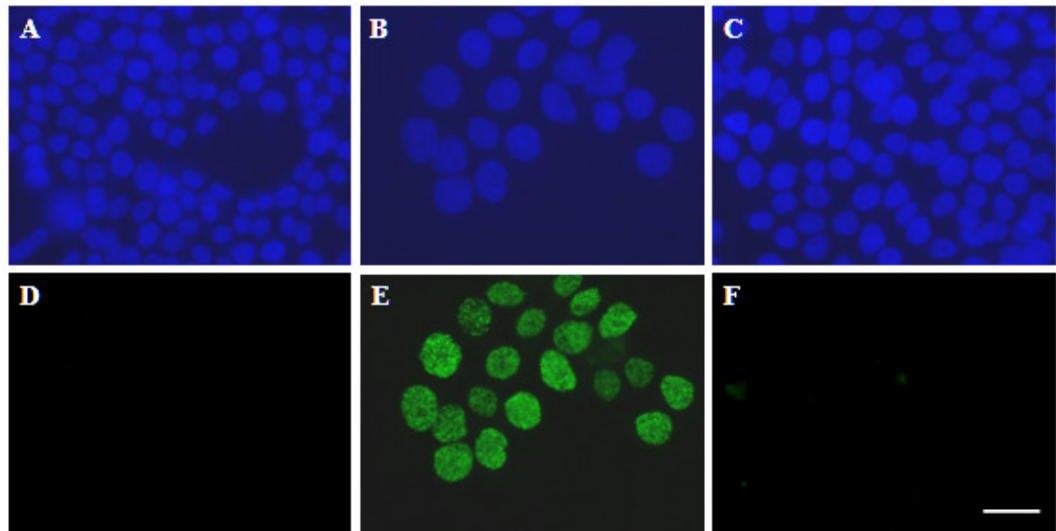


Figure 8. Analysis of non-treated and CAS cells by immunofluorescence microscopy for histone γ -H2AX staining.

HT-29 cells were either non-treated or treated with CPT. After 48 hours, cells were collected by mechanical shake-off and then re-plated, and analyzed by damaged DNA by immunofluorescence microscopy.

A and D. Non-treated HT-29 cells

B and E. Cells treated with 25 nM CPT for 48 hours.

C and F. CAS cells collected by mechanical shake-off and cultivated for 12 days.

Cells were stained with DAPI (top) or histone γ -H2AX antibodies (bottom). Scale bar = 25 μ m.

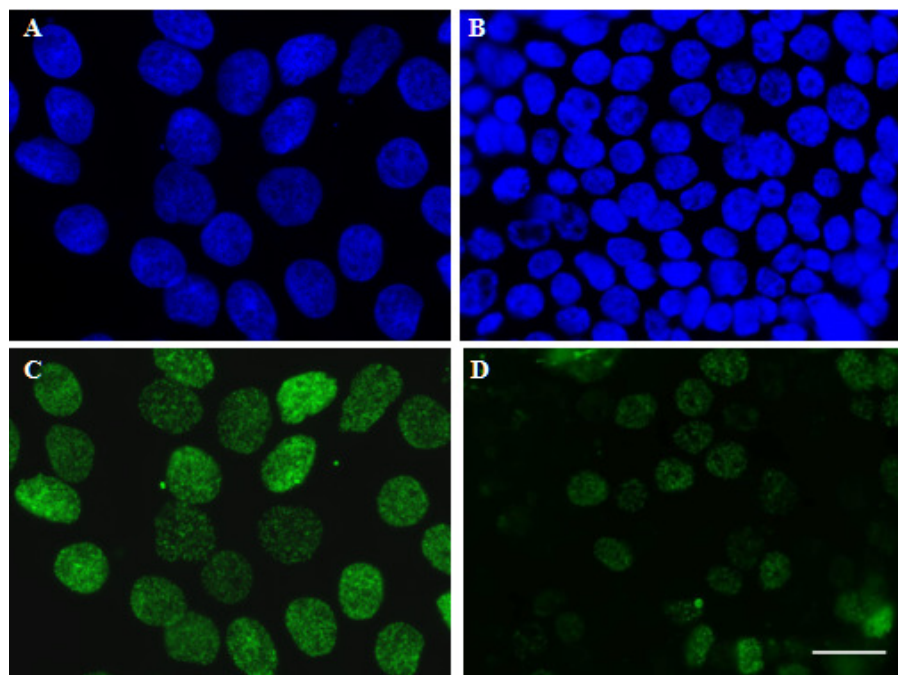


Figure 9. Analysis of non-treated and CAS cells by immunofluorescence microscopy for histone γ -H2AX staining after a second treatment with CPT.

A and C. CPT treated cells at 48 hours post-treatment (positive control).

B and D. CAS cells were retreated with CPT (25 nM) for 48 hours.

Cells were stained with DAPI (top), histone γ -H2AX antibodies (bottom). Scale bar = 25 μ m.

(Kubara *et al.*, 2012). However, immunofluorescence microscopy revealed that the nuclei of CAS cells were the size of the nuclei of non-treated cells (Figure 8C), and few of the nuclei of CAS cells were positive for histone γ -H2AX. This experiment, which was repeated three times, indicated that approximately 90 +/- 1.5% (n = 750), $P < 0.05$ (where $P = 0.01$) of the CAS cells no longer signaled damaged DNA (compared to the cells treated with CPT for 48 hours), although they originated from a population of cells with damaged DNA (Figure 8F).

Given that the CAS cells originated from CPT treated cells, we decided to ask if they were capable of signalling DNA damage, i.e. does the histone γ -H2AX pathway still function? To test this, we re-treated the CAS cells with 25 nM CPT for 48 hours then fixed and stained them for histone γ -H2AX to detect damaged DNA by microscopy. Upon re-treatment with CPT, an average of 45 +/- 4% (n = 750), of the CAS cells were histone γ -H2AX positive (Figure 9D), whereas control HT-29 cells were 94 +/- 2.1% (n = 750) (Figure 9C) positive after treatment (the difference is statistically significant at $P < 0.05$, where $P = 0.03$). These results provided our first evidence that some CAS cells might have a different genome from the original HT-29 population.

3.4 CAS Cells have Damaged DNA as Demonstrated by the Alkaline Comet Assay

I used the alkaline comet assay to measure damaged DNA. A comet assay is based on the principle that short fragments of DNA will migrate faster than the long fragments in an electric field (Collins *et al.*, 1997). In cells, undamaged DNA is intact and condensed in the nucleus; however, when damaged, the DNA breaks into pieces. The comet assay has the potential to measure damaged DNA in individual cells. In my project, the alkaline comet assay provided an opportunity to measure damaged DNA in cells that had undergone checkpoint adaptation and to complement studies in which the histone γ -H2AX signal was used to identify cells with damaged DNA. By a preliminary test, I provided an illustration of alkaline comet assay on CPT treated HT-29 cells (Figure 10). The appearance of the damaged DNA on the image created a comet like shape (Figure 10C). A bright and elongated tail in the gel was caused the presence of DNA fragments and represented intense DNA damage. The Comet Score software enabled one to quantify the overall signal strength and the relative amount of the fluorescence of the DNA strands (Figure 10B and D). A high signal indicated more damaged DNA as they move through the electric field (Figure 10C and D). No comet could be measured in non-treated cells as no fluorescence signal was detected outside of the nucleus (Figure 10A and B).

We explored the status of the DNA of the CAS cells by the alkaline comet assay because it was perplexing that 45% of the CAS cells no longer signaled damaged DNA after re-treatment with 25 nM CPT. For this experiment, we set up six populations of HT-29 cells:

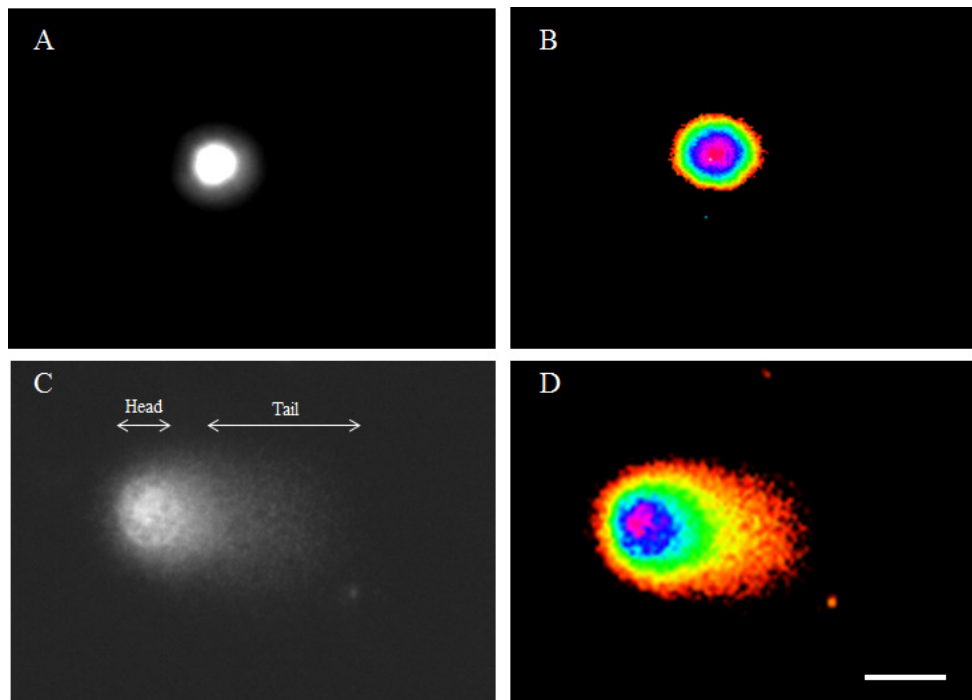


Figure 10. Levels of DNA damage after exposure to CPT evaluated by alkaline comet assay. HT-29 cells were either not treated (top row) or treated with 25 nM CPT for 48 hours (bottom row). Cells were collected and then processed by the alkaline comet assay to score them for damaged DNA using fluorescence microscopy and comet analysis software.

- A.** Fluorescence microscopy image of cells without CPT treatment (9% DNA in the tail, Olive moment = 2.3).
- B.** Image A after processing by the Comet Score software. The colour codes represent different amounts of fluorescence intensity from purple (highest) to red (lowest).
- C.** Fluorescence microscopy image of cells treated with 25 nM CPT for 48 hours (50% DNA in the tail, Olive moment = 44.3).
- D.** Image C after processing by the Comet Score software. The colour codes represent different amounts of fluorescence intensity from purple (highest) to red (lowest). Scale bar = 100 μm .

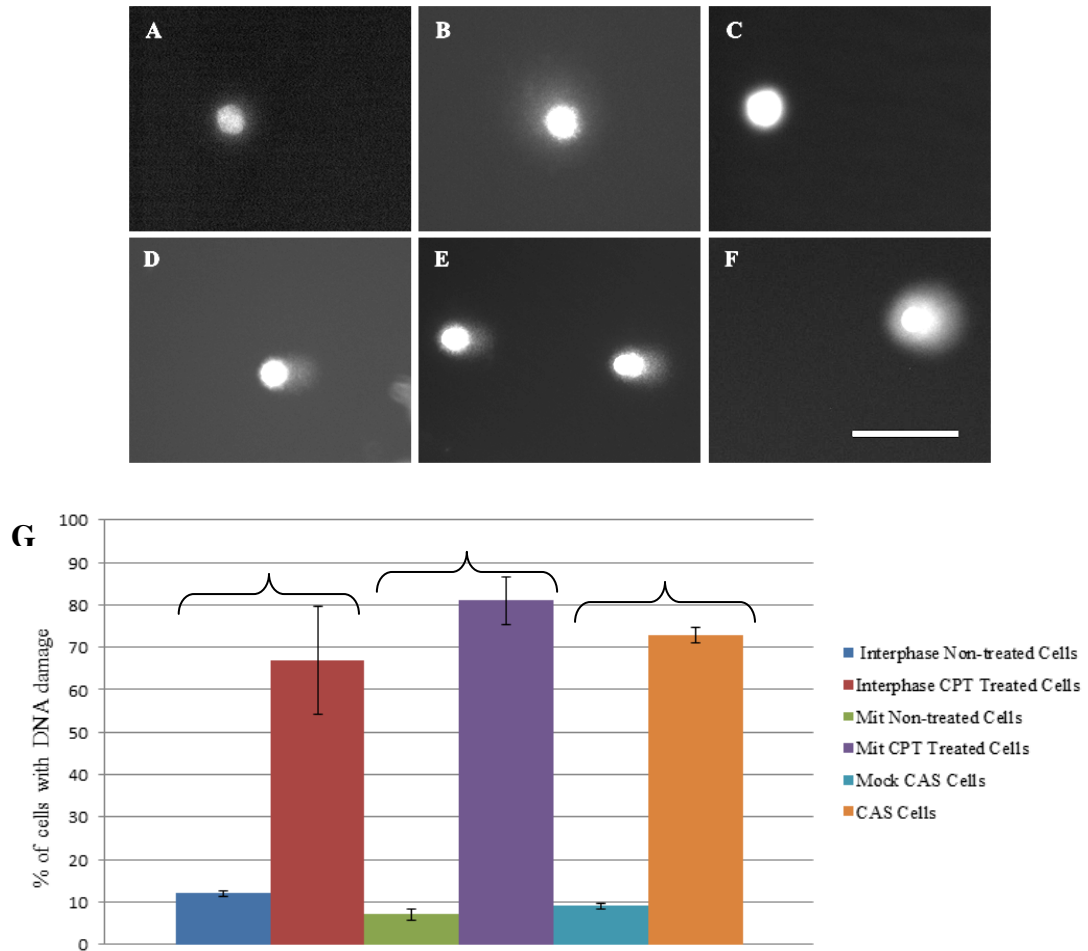


Figure 11. Analysis of the DNA of HT-29 cells with and without CPT treatment and in CAS cells by the alkaline comet assay. HT-29 cells either non-treated or treated with CPT were collected by mechanical shake-off at 48 hours post treatment. Cells were then re-plated and cultivated for 10 days. At each treatment, cells were prepared for analysis by the alkaline comet assay. Image of samples from the alkaline comet assay were taken by fluorescence microscopy. This experiment was performed three times.

A. Non-treated interphase cells.

B. Non-treated mitotic cells collected by mechanical shake-off.

C. Mock CAS cells.

D. Interphase cells treated 48 hours with 25 nM CPT.

E. Mitotic cells treated 48 hours with 25 nM CPT collected by mechanical shake-off.

F. CAS cells collected at 10 days. Scale bar = 100 μ m.

G. An average of 200 cells was analyzed per experimental group per replicate. The results were statistically significant at $P < 0.05$ using Student's t-test between each paired group of non-treated and CPT treated.

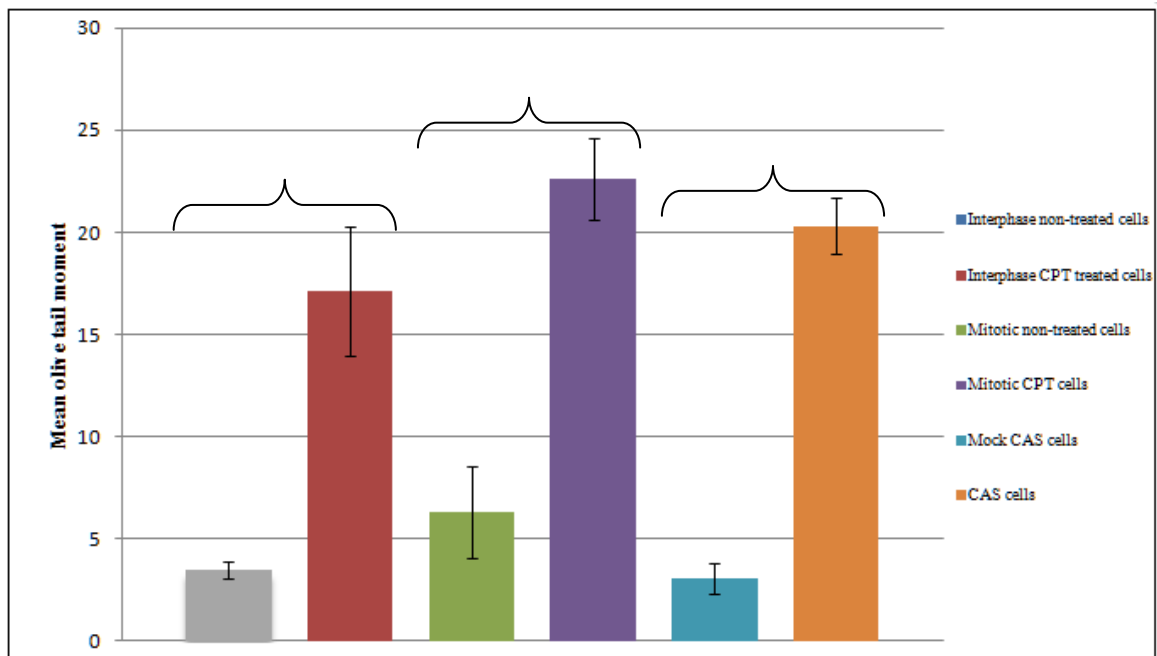


Figure 12. Olive tail moment determined by the alkaline comet assay of HT-29 cells either non-treated or treated with CPT and collected under various conditions. This experiment was performed three times. An average of 200 cells was analyzed per experimental group per replicate. The error bars show standard deviation. The results were statistically significant at $P < 0.05$ using Student's t-test between each paired group of non-treated and CPT treated.

1. Non-treated interphase cells,
2. Non-treated mitotic cells,
3. Non-treated mock CAS cells,
4. 25 nM CPT treated interphase cells for 48 hours,
5. 25 nM CPT treated mitotic cells for 48 hours, and
6. CAS cells collected at 10 days after mechanical shake-off.

The alkaline comet assay was performed to quantify the DNA damage with the percentage DNA in the tail and Olive moment (fraction of DNA in tail together with length of comet). The absence of a comet tail in the non-treated cells of each type (interphase, mitotic, and mock survival) (Figure 11A, B and C) confirmed that they contained little damaged DNA, as expected. By contrast, 68 +/- 8.3% (n = 600; $P = 0.003$) of the interphase and 82 +/- 4.7% (n = 600; $P = 0.0001$) of the mitotic HT-29 cells treated with CPT for 48 hours displayed comets indicating that this population had damaged DNA (Figure 11D and E). Strikingly, the alkaline comet assay revealed that 73 +/- 2.5% (n = 600; $P = 0.001$) of the CAS cells displayed comets (Figure 11F), suggesting that their genome contained damaged DNA. An increase in Olive moment also indicated a higher amount of DNA damage in CPT treated interphase cells and mitotic cells, and CAS cells at 17.2 +/- 3.2 (n = 600; $P = 0.006$), 22.6 +/- 3.1 (n = 600; $P = 0.007$), and 20.3 +/- 1.3 (n = 600; $P = 0.002$) respectively, compared to the same non-treated cells, which had an Olive moment of 3.5 +/- 0.4, 6.3 +/- 5.8, and 3.0 +/- 0.7 (n = 600) respectively (Figure 12). The results were statistically significant at $P < 0.05$ using Student's t-test between each paired group of non-treated and CPT treated cells. This result contrasted

with that of the staining with histone γ -H2AX antibodies, which had indicated that 90% of the culture did not signal damaged DNA. This result indicates that CAS cells contain damaged DNA and somehow are able to either tolerate it, or modify the pathways that signal it.

3.5 Karyotype Analysis Reveals a Shattered Chromosome Phenotype

We found that a small percentage of CPT treated cells were able to survive checkpoint adaptation to become CAS cells. These cells showed a mixed response to subsequent CPT treatment as measured by histone γ -H2AX staining and a positive score in the alkaline comet assay. It is likely that they have changes in the organization of their genome and might be genetically different from parent cells prior to treatment.

We then examined the nuclei of CAS cells by several different approaches to determine if changes had occurred during the process of checkpoint adaptation and survival. For this experiment, HT-29 cells were treated with 25 nM CPT for 48 hours. Following treatment, mitotic cells were collected by mechanical shake-off and cultivated for 10 days. We followed the same procedure for the non-treated culture. We first examined CAS cells by light microscopy for the formation of micronuclei. Micronuclei are used as evidence of chromosome and/or genomic changes (Nakada *et al.*, 2006, Nitiss, 2009; Luzhna *et al.*, 2013). In this case, we defined cells as being positive for micronuclei when they had more than one nuclear structure. We observed that 2 +/- 0.6%, of the CAS cells, when collected at 10-12 days, had micronuclei, whereas 1 +/- 0.1%

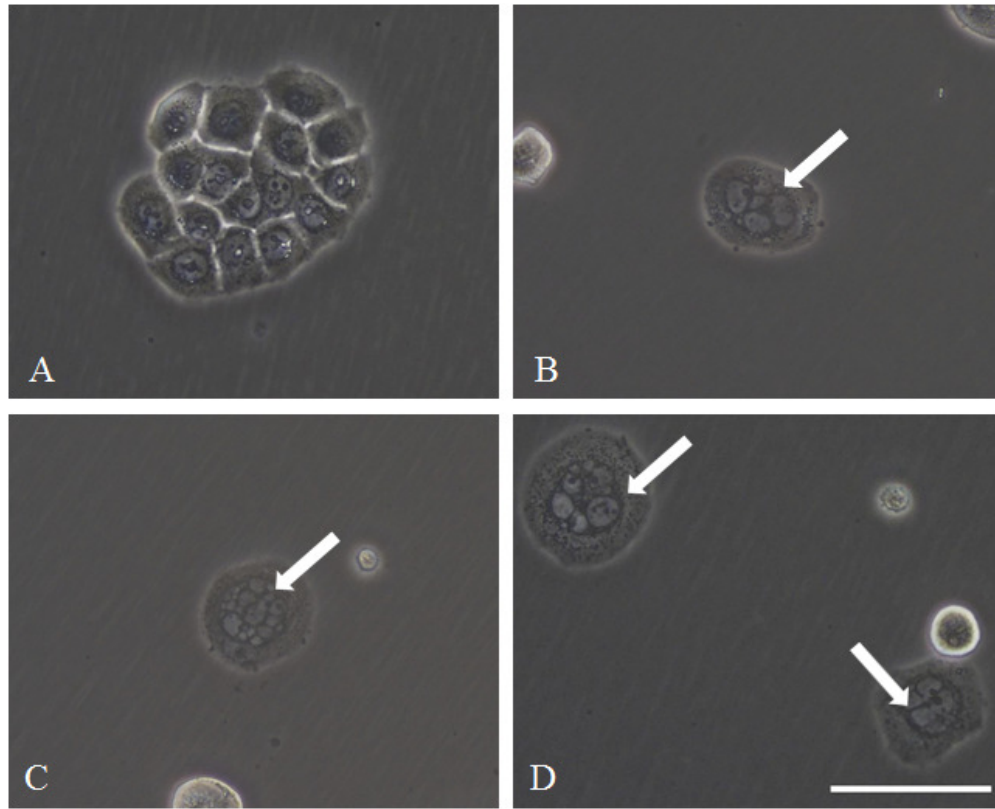


Figure 13. Cells that survive mitosis following treatment with CPT acquire micronuclei.

A. Non-treated cells 6 days after mechanical shake-off.

B, C and D. Mitotic cells collected by mechanical shake-off after treatment with 25 nM CPT for 48 hours and cultivated for 6 days. Arrows indicate nuclei. Scale bar = 100 μm .

micronuclei was observed in non-treated, parental cells (Figure 13). Although these differences in micronuclei were not significant, they led to new studies about the presence of micronuclei at earlier times, which are ongoing in the laboratory.

We decided to examine chromosome structure and chromosome number by karyotype analysis. To compare the karyotype of cells that had undergone checkpoint adaptation to those of non-treated cells, we prepared four populations of HT-29 cells:

1. Non-treated cells,
2. Non-treated mock CAS cells,
3. Cell treated with 25 nM CPT for 48 hours,
4. CAS cells, collected at 10 days.

Cells from each population were collected and chromosome were isolated and observed by microscopy. Analysis of the non-treated HT-29 cells revealed that they contained whole chromosomes with a characteristic shape, as expected (Figure 14A). By contrast, analysis of the cells that were undergoing checkpoint adaptation at 48 hours post CPT treatment contained tens to hundreds of chromosome pieces, as if the chromosomes had been shattered (Figure 14C). Images of the CAS cells, which had been treated with 25 nM CPT and then cultivated for 10 to 12 days, revealed that they contained chromosomes that were largely intact, but appeared to be shorter and wider (Figure 14D) than those of non-treated HT-29 mock CAS cells (Figure 14B). We found that CAS cells contained approximately 35 ± 13 ($n = 150$) chromosomes on average whereas non-treated cells contained 65 ± 8 ($n = 150$) chromosomes. These differences were statistically significant at $P < 0.05$ (where $P = 0.01$) using Student's t-test. The typical

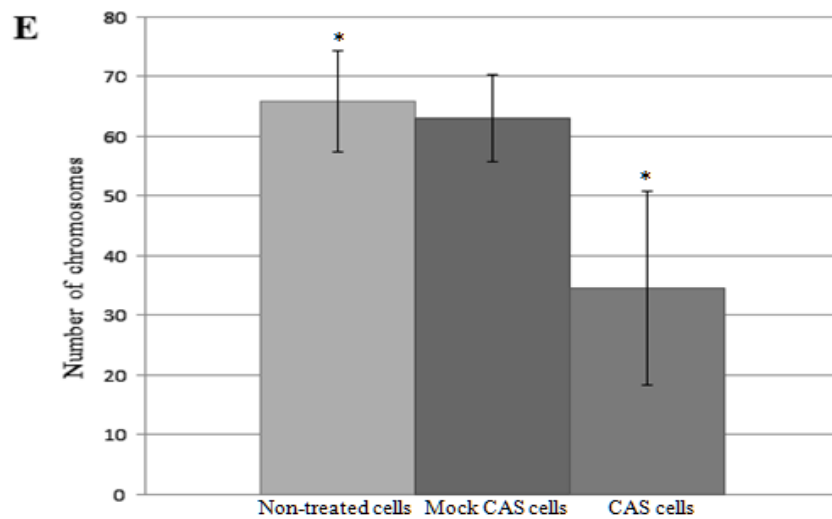
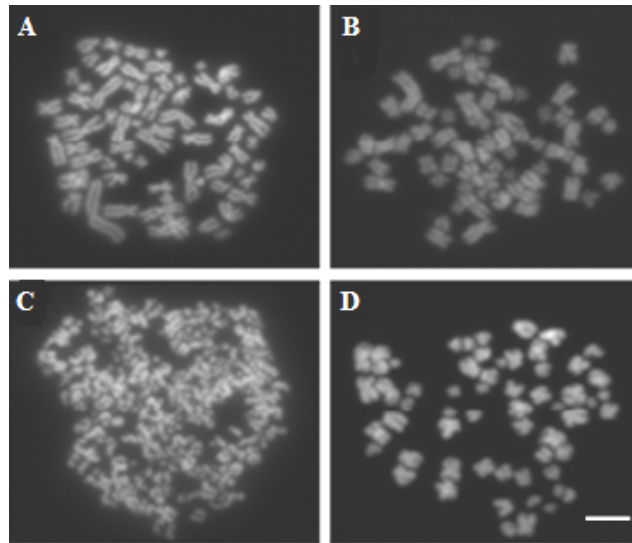


Figure 14. Karyotype analysis of CPT treated HT-29 cells reveals a shattered chromosome phenotype and changes in chromosome numbers.

A. Non-treated cells.

B. Mock CAS cells.

C. Cells treated with 25 nM CPT for 48 hours.

D. CAS cells. Scale bar = 5 μ m.

E. A representation of the average number of chromosomes from three experiments in non-treated cells, HT-29 mock CAS cells, or CAS cells, respectively. An average of 50 cells was analyzed per experimental group per replicate. The error bars show standard deviation. * indicates significance based on Student's t-test ($P < 0.05$).

chromosome number of HT-29 cell line is 71 (Kondoh *et al.*, 1993). The number of chromosomes in CAS cells ranged from 18 to 51, whereas the number of chromosomes from the non-treated HT-29 cells ranged from 58 to 74. These data indicated that the number of chromosomes per CAS cell was variable within the survivor population. It provided evidence that CAS cells have major changes in the organization of their genome and that the genomes varied between survivor cells.

3.6 FISH Analysis Reveals Genomic Rearrangement within CAS Cells

To understand better the level of genomic change in HT-29 CAS cells, we performed Fluorescence *In Situ* Hybridization (FISH) analysis. FISH analysis can provide information about the presence or absence of structural elements that are required for chromosomes, such as telomeres or centromeres. Absence or misplacement of telomeres or centromeres in a chromosome would suggest that cells have a rearranged genome. To analyze CAS cells by FISH, we prepared three populations of HT-29 cells:

1. Non-treated cells,
2. Cells treated with 25 nM CPT for 48 hours and collected by mechanical shake-off, and
3. CAS cells, collected at 10 days.

FISH analysis of the non-treated HT-29 cells revealed that their chromosomes had a characteristic shape by DAPI staining (Figure 15A), and almost all the chromosomes contain centromeres (Figure 15B) and telomeres (Figure 15C) at the correct locations. In

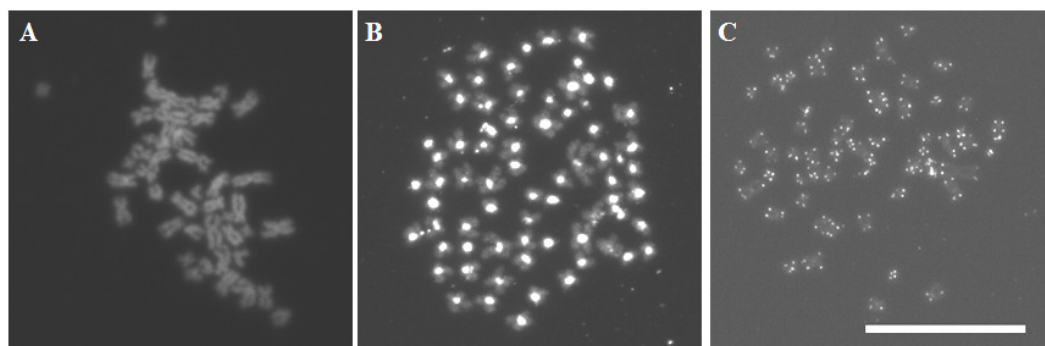


Figure 15. Chromosomes of non-treated HT-29 cells analyzed by FISH.

A. Non-treated cells stained with DAPI to mark whole chromosomes.

B. Non-treated cells labeled with the Cy3 centromere probe followed by staining with DAPI.

C. Non-treated cells labeled with the A488 PNA TelC telomere probe followed by staining with DAPI.

Scale bar = 25 μm .

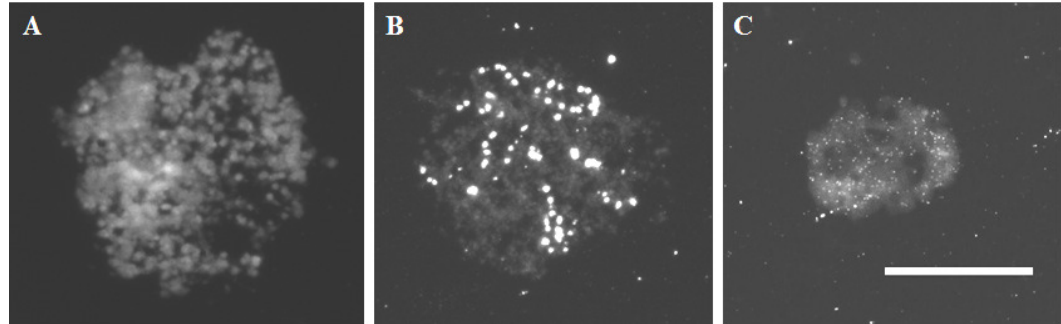


Figure 16. Chromosomes of HT-29 cells treated with CPT for 48 hours analyzed by FISH.

A. Cells were treated for 48 hours with CPT, collected by mechanical shake-off then stained with DAPI to mark the chromosome.

B. Cells were treated for 48 hours with CPT, collected by mechanical shake-off then labeled with Cy3 centromere probe followed by staining with DAPI.

C. Cells were treated for 48 hours with CPT, collected by mechanical shake-off then labeled with A488 PNA TelC telomere probe followed by staining with DAPI.

Scale bar = 25 μ m.

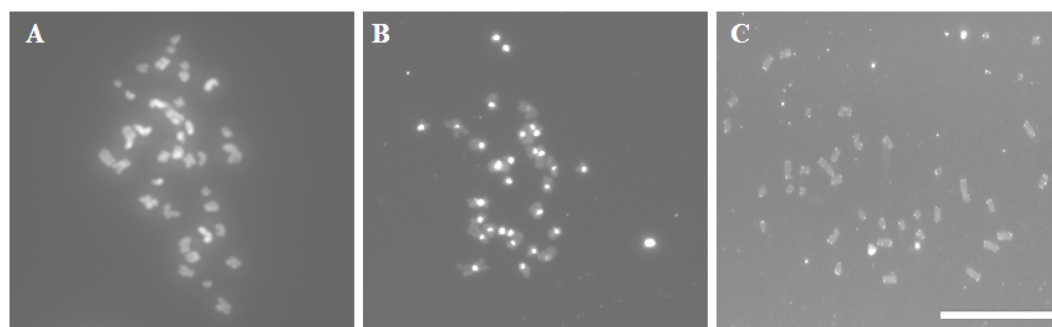


Figure 17. Chromosomes of HT-29 CAS cells collected at 10 days after treatment with CPT and analyzed by FISH.

A. CAS cells stained with DAPI to mark whole chromosomes.

B. CAS cells labeled with Cy3 centromere probe followed by staining with DAPI.

C. CAS cells labeled with A488 PNA TelC telomere probe followed by staining with DAPI.

Scale bar = 25 μ m.

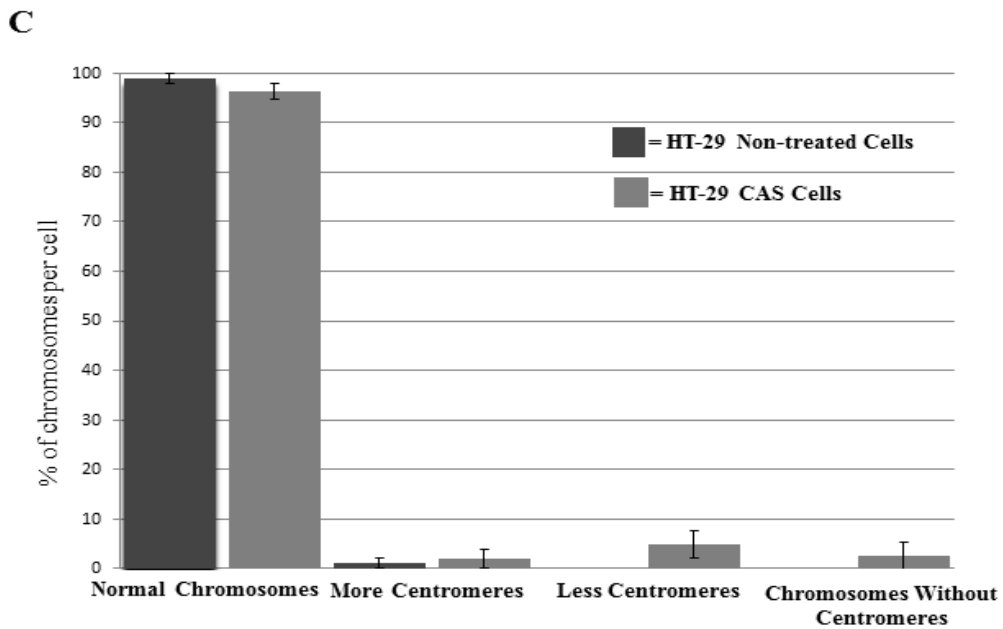
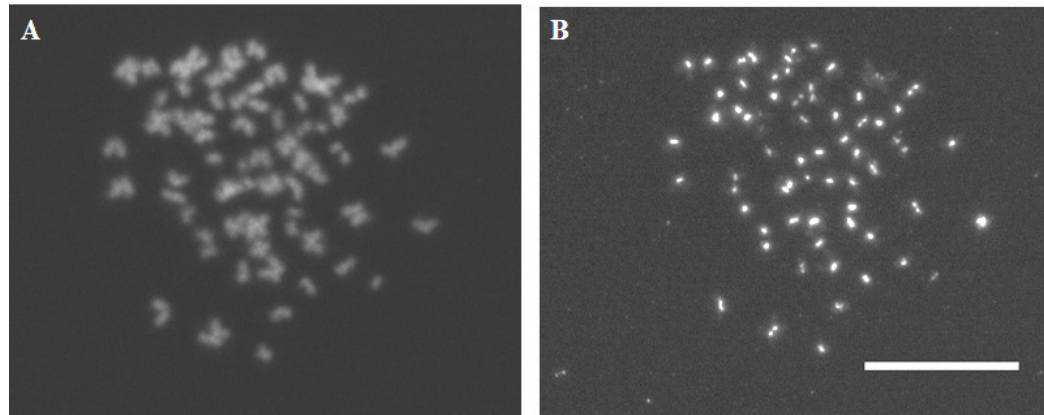


Figure 18. Comparison of the number of centromeres of non-treated HT-29 cells with CAS cells.

A and B. CAS cells were stained with DAPI (A) and labeled with the Cy3 centromere probe (B). Images were collected by fluorescence microscopy. Scale bar = 15 μ m.

C. The number of centromere signals by FISH analysis is represented as a percentage of signals from non-treated cells (Normal chromosomes). Black columns are counts from non-treated cells and grey columns are counts from CAS cells. The counts were grouped into four categories: normal chromosomes (signals within a chromosome), more centromeres (those with more than one centromere signal per chromosome), less centromeres (those with less than one centromere per chromosome in a chromatid), Chromosomes without centromeres. The error bars show standard deviation.

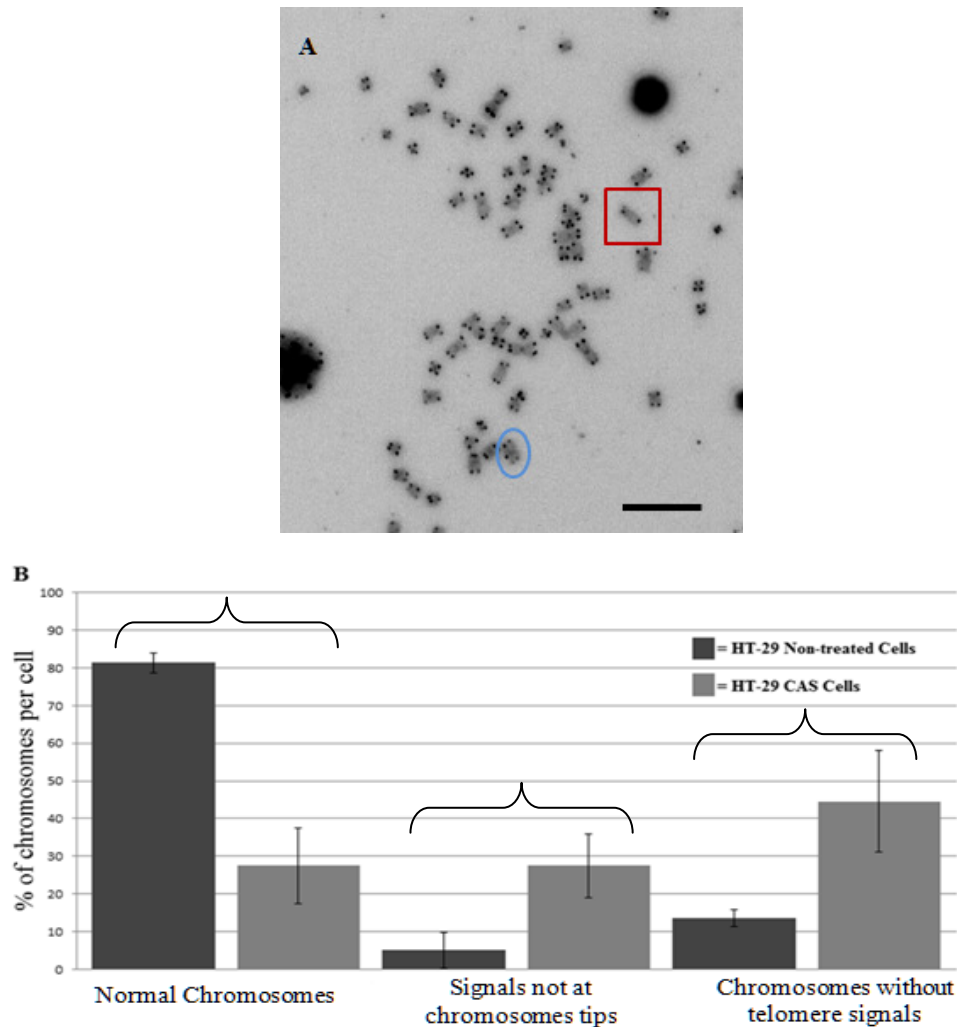


Figure 19. Comparative analysis of telomeres of non-treated HT-29 cells and HT-29 CAS cells.

A. CAS cells were labeled with A488 PNA TelC telomere probe and stained with DAPI. The square highlights a chromosome that is missing telomeres and the circle represents a chromosome in which the telomere is located a region other than the chromosome tip position. The image is shown as a negative format. Scale bar = 15 μ m.

B. The number of telomere signals from FISH analysis is represented as a percentage of signals from non-treated cells. Black columns are counts from non-treated cells and grey columns are counts from CAS cells. The counts were grouped into three categories: normal chromosomes (signals at tips of chromosomes), signals not at chromosome tips, chromosomes without telomere signals. The results were statistically significant at $P < 0.05$ using Student's t-test between each paired group of non-treated and CAS cells.

contrast, mitotic cells collected at 48 hours after CPT treatment showed a shattered chromosome pattern that was reminiscent of chromothripsis (Figure 16A). These cells displayed centromeres (Figure 16B) and telomeres (Figure 16C), however, it was not possible to describe their position relative to chromosomes because of the shattered structures. We found that mitotic cells collected at 48 hours after CPT treatment contained an average of 62 ± 6 ($n = 150$) centromeres, which was consistent with our values from karyotyping. By contrast there were 43 ± 7 ($n = 150$) centromeres on average, in the CAS cells. These differences were not statistically significant that support our observation from karyotype analysis in which the number of chromosomes in the CAS cells ranged from 18 to 51. Almost $96 \pm 3\%$ ($n = 150$) of the chromosomes in the CAS cells contained centromeres (Figure 17B and Figure 18B), as percentage that was not statistically different from the non-treated cells.

Analysis of telomeres in CAS cells, however, revealed a different pattern. At least one out of four telomeres was missing in about $45 \pm 7\%$ ($n = 150$; $P = 0.02$) of the CAS cells (Figure 17C and Figure 19A). Moreover, $28 \pm 6.5\%$ ($n = 150$; $P = 0.01$) of the telomeres in the CAS cells were not located at the ends of the chromosomes (Figure 19A). These results were statistically significant at $P < 0.05$ using Student's t-test between each paired group of non-treated and CAS cells. In addition to numerical changes in chromosome number in CAS cells, there were changes in telomere number or in detection of telomeres. Cells in mitosis with damaged DNA, however, contain telomere and centromere signals, but it was not possible to determine how they were organized amongst the pieces of DNA in a shattered phenotype. These observations

support that there are genomic rearrangements in the CAS cells that survive checkpoint adaptation.

Chapter 4

4. Discussion

In this thesis, I described experiments in which we analyzed the DNA of human cancer cells that have undergone checkpoint adaptation. These experiments are important because they address a key question in cancer cell biology: how might the genome of cancer cells change? To study this question, I first had to learn one experimental model of checkpoint adaptation which was recently published by my laboratory (Kubara *et al.*, 2012). By using our model, I was able to collect cells that survive checkpoint adaptation. We have examined these cells by techniques including immunofluorescence microscopy, the alkaline comet assay, karyotyping, and FISH analysis. What becomes of the cells that survive checkpoint adaptation has not been described previously. We now provide preliminary evidence that the genome may be changed in cells that appear to survive checkpoint adaptation.

4.1 HT-29 Cells Treated With Camptothecin (CPT) Undergo Checkpoint Adaptation

Checkpoint adaptation is a process that occurs in several different types of cancer cells when they are treated with pharmacologically relevant concentrations of genotoxic agents (Syljuåsen *et al.*, 2006; Cahuzac *et al.*, 2010; Rezacova *et al.*, 2011; Kubara *et al.*, 2012). It is characterized by an arrest in the cell cycle, overcoming this arrest, and then

entering into mitosis despite the presence of damaged DNA (Toczyski *et al.*, 1997). We used the HT-29 cell line because these cells, when treated with 25 nM CPT, fulfil the three features that define checkpoint adaptation (Kubara *et al.*, 2012). Eventually, these treated cells escaped the G2 phase arrest and entered into mitosis by 48 hours even though they have damaged DNA. The advantage provided by the HT-29 cell line over the original U2OS cell model in which checkpoint adaptation was first identified in human cells (Syljuåsen *et al.*, 2006) is the pronounced rounded cell morphology when they are in mitosis. Furthermore, HT-29 cells are known to be arrested in mitosis following a treatment with antimitotic compounds rather than immediately being engaged in programmed cell death (Gascoigne and Taylor, 2008). Although we did not study mitotic arrest in this thesis, mitotic cells (and thus checkpoint adaptation) is easier to detect in HT-29 cells than other cell lines such as U2OS. Finally, the technique of mechanical shake-off (Terasima and Tolmach, 1963) permits one to isolate populations of cells undergoing checkpoint adaptation and re-cultivate them. These features enabled us to test the hypothesis that checkpoint adaptation may potentially promote genomic instability in cancer human cells.

We designed our checkpoint adaptation assay by drawing from parameters that are relevant to the human disease of colon cancer. For example, HT-29 cells are derived from human colon carcinoma tumor. Furthermore, CPT or its derivatives, are one of the treatment options for patients diagnosed with colon cancer (Pizzolato and Saltz, 2003). We chose a concentration of CPT that was both cytotoxic for HT-29 (Larsen *et al.*, 2001);

yet within the MTD (Maximum Tolerated Dose) range of serum levels of treated patients (Rivory *et al.*, 1997).

4.2 Some HT-29 Cells That Undergo Checkpoint Adaptation Are Able To Survive

Studies have established that most cells that undergo checkpoint adaptation eventually die (Syljuåsen *et al.*, 2006; Kubara *et al.*, 2012). However, it has been also suggested that checkpoint adaptation might be a cause of genomic change in cancer cells (Syljuåsen *et al.*, 2007; Bartek and Lukas, 2007). Inherent in this suggestion was the possibility that some cancer cells survive checkpoint adaptation. Therefore, we tested this by collecting CPT treated mitotic cells with sequential mechanical shake-off and re-cultivating them. We found that the majority of cells that underwent checkpoint adaptation had died - which was expected, as we were treating cells with a cytotoxic compound. Some cells, however, underwent checkpoint adaptation and survived, and could be cultured for at least 12 days in our experiments. We have used survival assay and clonogenic assay to determine the number of cells that survived checkpoint adaptation under our laboratory conditions of standard cell culture. The survival assay and clonogenic assay revealed that approximately 3% and 1% of the cells that undergo checkpoint adaptation are able to survive CPT treatment and proliferate (CAS cells). Although these percentages seem small, they might be biologically significant when one considers the number of cells that make up a tumor. We note, however, that we have collected cells after one “treatment” and detected survival cells; patients would likely

receive several cycles of treatment during an anti-cancer treatment regime (Pizzolato and Saltz, 2003).

With the knowledge that it is possible, under experimental conditions, to collect and cultivate cells that survive checkpoint adaptation, it then became possible to examine survivors (which we call CAS cells) for genomic change. We were motivated to examine this because previous results from my laboratory detected that cells undergoing checkpoint adaptation (i.e. mitotic) were positive for histone γ -H2AX. We have examined CAS cells using different techniques to find if genomic changes had occurred.

4.3 HT-29 CAS Cells Have Damaged DNA

In our study, we have verified that HT-29 cells, following the treatment with CPT, recruited histone γ -H2AX. Positive signals for histone γ -H2AX indicated that the cells have damaged DNA. By collecting CAS cells we found that 90% of them were negative for histone γ -H2AX staining. This result suggested that the CAS had likely repaired their genome following checkpoint adaptation.

To determine if the CAS cells were still capable of signalling histone γ -H2AX, we re-treated the CAS cells with CPT. Upon retreatment, an average of 45% of the CAS cells was positive for histone γ -H2AX. This result indicated some of the CAS cells lost the ability to display a DNA damage signal after being re-treated with CPT. We do not know at which point in the DNA damage pathway the ability to signal by histone γ -H2AX was lost. It might be caused by a mutation, for example in ATM or in histone γ -H2AX, or it

might be due to a reduced sensitivity to CPT. Nonetheless, one interpretation of this result is that the CAS population was composed of at least two cell types: those that can signal damaged DNA, and those that cannot when treated with a previously cytotoxic concentration of CPT. This experiment provided the first evidence that some of the CAS cells within the population might be different from parental cells.

Studies using histone γ -H2AX staining are widely used; however, this method is only able to detect a signal for damaged DNA and does not directly measure damaged DNA. In my research, we examined cells for damaged DNA by directly observing DNA using the alkaline comet assay. The alkaline comet assay provided an independent means to correlate the amount of damage with the capacity to undergo checkpoint adaptation and survive mitosis. Prior to my thesis work, a demonstration of damaged DNA in CAS cells at the level of individual cells had not been done. This study showed that 73% of the CAS cells displayed comets, whereas non-treated cells normally displayed only 9%. We used the criterion that a comet must have 20% or more of the total DNA outside of the nucleus, as previously published (Olive *et al.*, 1990). The presence of comets in CAS cells presented supportive evidence for our observation that a large number of CAS cells were not recognized by anti-histone γ -H2AX antibodies when re-treated with CPT. It seemed possible that a large number of CAS had damaged DNA but did not signal it.

In previous studies in other laboratories, there are examples in which histone γ -H2AX staining and the alkaline comet assay provide corroborative results, and there are examples of when they do not (as was our case with CAS cells). Burgess *et al.* (2006) used histone γ -H2AX staining and alkaline comet assay to evaluate the response of

neuroblastoma cells (SHSY-5Y cells) treated with low-dose ionizing radiation (IR). The results obtained from both techniques were consistent with each other. Similar results were found when HCT116 cells were treated with hedamycin, a DNA alkylating agent (Tu *et al.*, 2005). A positive correlation was identified between the results when using either the comet analysis or histone γ -H2AX fluorescence.

Although some laboratories found a close correlation between the result of immunofluorescence microscopy using histone γ -H2AX and the alkaline comet assay, it is not always the case. Yu *et al.* (2006) used the alkaline comet assay to analyze human amnion cells (FL cells) treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). They found that a higher percentage of cells scored positive with the alkaline comet assay than they did after being stained with histone γ -H2AX antibodies and immunofluorescence microscopy. Gallmeier *et al.* (2005) used histone γ -H2AX staining and alkaline comet assay on RKO colorectal cancer cells treated with norethindrone to test its genotoxicity. No correlation was identified between the results when comparing measurements of the tail moment or histone γ -H2AX fluorescence.

Although we favoured the notion that the histone γ -H2AX signals might be inactivated in some CAS cells, other explanations are possible. The alkaline comet assay can detect types of damaged DNA such as abasic nucleotides, or excision sites, which might not provide histone γ -H2AX signals (Wood *et al.*, 2010). If this type of damage is present in some CAS cells, it might also account for the difference in the results between immunofluorescence microscopy using histone γ -H2AX antibodies and comet assay.

More detailed studies are currently being investigated in my laboratory as a result of this finding.

4.4 CAS Cells and Micronuclei

A micronucleus appears as a small membrane-bound nucleus, in addition to a main nucleus, which forms due to improper incorporation of acentric fragments or chromosomes into daughter nuclei during cell division (Fenech *et al.*, 2011; Luzhna *et al.*, 2013). Micronuclei are commonly used as evidence of chromosome and/or genome mutations caused by damaged DNA (Hovhannisyan *et al.*, 2009). We detected micronuclei in 2% of the CAS cells after 10-12 days of culture. In comparison to non-treated HT-29 cells with 1% micronuclei, these two numbers were not significantly different. New data from the laboratory reveal that this number is much higher in samples of CAS cells taken at earlier times after checkpoint adaptation, which suggests a profound restructuring of the nucleus during the process of establishing a survival population. Genomic change acquired in micronuclei might contribute to cancer development, or in this case, contribute to change in cells that are already cancerous. Micronuclei can also contribute to the formation of DNA breakage and chromothripsis (Crasta *et al.*, 2012).

4.5 Chromothripsis in CAS Cells

In this thesis, HT-29 cells were studied to investigate if there was an evidence of genomic change in CAS cells. We observed pulverized, or highly broken, chromosomes by DAPI staining and microscopy in cells undergoing checkpoint adaptation (Figure 14C and Figure 16). We have performed karyotype analysis on HT-29 cells which provided the opportunity to examine the outcome of the cells that survived checkpoint adaptation for signs of genomic change. Results from the karyotype experiments revealed that we could create a mitotic event in which chromosomes shatter into tens to hundreds of pieces in HT-29 cells treated with CPT. These cells might be undergoing mitotic catastrophe, which results from abnormal mitosis associated with the formation of micronuclei (Swanson *et al.*, 1995; Ianzini and Mackey, 1997; Roninson *et al.*, 2001). Catastrophe usually occurs when chromosomes condense for mitotic cell division, in this instance 48 hours (Stephens *et al.*, 2011; Forment *et al.*, 2012). Later (in 10 to 12 days), the pulverized chromosomes appear to be rejoined in a mosaic patchwork of genomic fragments. At a superficial level this resembles chromothripsis, although we did not test this directly by DNA sequencing, as had been done in the original papers describing the phenomenon chromothripsis (Stephens *et al.*, 2011; Liu *et al.*, 2011).

Chromothripsis was also observed in the genome of a chronic lymphocytic leukaemia sample (CLL) (Forment *et al.*, 2012). Paired-end sequencing of the CLL sample revealed 42 chromosomal rearrangements on the long arm of chromosome 4. Even though the majority of rearrangements occurred in chromosome 4, other rearrangements were detected in chromosomes 1, 12 and 15. Additional studies of

chromosome structures in other tumour samples and cell lines revealed that chromothripsis is present in 25% of the bone cancers (osteosarcoma and chordoma) (Stephens *et al.*, 2011) and possibility in at least 2% - 3% of all human cancers, such as melanomas, sarcomas and gliomas, as well as colorectal, lung, oesophageal, renal, and thyroid cancers (Kloosterman *et al.*, 2011; Magrangeas *et al.*, 2011; Molenaar *et al.*, 2012).

The pathways in which the cells survive checkpoint adaptation with damaged DNA, such as rejoining the shattered chromosome, remain unknown. As we have observed, the cells were able to repair chromosomes in some manner by the time when we observed them at 10 to 12 days post-collection. However, there might be a limit to what can be repaired because, as we have seen, approximately 99% of the cells die. This suggests that the repair is not sufficient for all the cells to survive.

4.6 Mechanisms that Regulate Chromothripsis

The mechanisms that cause chromothripsis are not well understood. Under conditions that do not include cancer treatments, one possibility is that chromothripsis is initiated when one or more chromosomal regions encounter a localized, ionizing radiation particle during mitosis, which induces breaks in one or a few chromosomes (Stephens *et al.*, 2011). Another possibility is dysfunction of telomeres, which lead to further chromosome breaks and rearrangements such as fusion between sister chromatids, aneuploidy and polyploidy. A current hypothesis of a cause of chromothripsis is the

possibility that micronuclei contribute whole or partial chromosomes that shatter during mitosis (Crasta *et al.*, 2012). These pulverized chromosome segments can rearrange the genome if reassembled and subsequently reintegrated into the main nucleus of a daughter cell.

4.7 Chromothripsis in Colon Cancer

Chromothripsis might have a role in colon cancer. Wigard *et al.* (2013) described a pairwise genomic analysis of matched primary and metastatic colon cancer samples from four patients. They performed genome-wide mate-pair sequencing, single nucleotide polymorphism (SNP) array profiling, and exome sequencing to determine the chromosomal rearrangements that contribute to the formation of colon cancer. Cancer genes, such as NOTCH2, EXO1 and MLL, are affected by breakpoints of chromothripsis clusters and abnormal rearrangements, as demonstrated in their studies. They also confirmed mutations in 24 genes by sequencing the coding regions of a cancer exome in all colon tumor samples. This study concluded that large and small chromothripsis events occur in every colon tumor sample, and this event is one of the contributors to chromosomal rearrangements.

Karyotype analysis of CAS cells revealed that the average number of chromosomes present in each cell was 35 instead of 65 (Kondoh *et al.*, 1993; Guildbaud *et al.*, 2001). Strikingly, there was tremendous variation in this number, which suggested that the CAS cells in a population were not simply composed of one genomically

identical clone. The variation in chromosome number in the CAS cells led us to ask if the chromosomes in the CAS cells maintain all the morphological characteristics of an ideal chromosome such as telomeres and centromeres. Typically, telomeres are located at the ends of the linear chromosome, where they cap the chromosome and prevent fusions between the ends of different chromosomes (Bickmore, 2001). Centromeres are located on sister chromatids to provide a site for microtubule attachment (Bickmore, 2001). Fluorescence *In Situ* Hybridization (FISH) analysis provided a better understanding of genomic rearrangements of CAS cells by confirming the presence of telomeres and centromeres.

FISH analysis revealed that nearly all the chromosomes in CAS cells contained centromeres, as percentage that was not statistically different from the non-treated cells. However, we found that at least one out of four telomeres was absent in 45% of the CAS cells. Moreover, 28% of the telomeres in the CAS cells were observed to be located in positions other than the tips of chromosomes. The frequency of either missing or wrong positioning of the telomeres in the chromosomes in CAS cells suggests that genomic rearrangements have occurred. Furthermore, we also found variation with these types of changes, suggesting that we were observing cells of more than one origin, which is consistent with the possibility of generating genomic variation by checkpoint adaptation. One limitation of this research was the inability to show exact genomic rearrangement on the chromosomes. This would require other techniques such as chromosome painting analysis (Metzenberg, 2005) of the CAS cells or sequencing.

4.8 Genomic Rearrangements in CAS Cells

We propose three possibilities to explain the presence of genomic changes in CAS cells:

1. The CAS cell originated from a cell that was already present in the HT-29 population, it has the same genome as other cells, and it survived because it managed to avoid CPT treatment.
2. The CAS cell originated from a cell that was already present in the HT-29 population and it survived because it already had a genome that was different from the parental population.
3. The CAS cell acquired a different genome by undergoing checkpoint adaptation and repairing its shattered genome.

To address these possibilities, our experiments enabled us to examine the genome under several different conditions and by using several different techniques. In all cases, however, the technique of using mechanical shake-off helped us interpret the outcome. The ability of HT-29 cells to become rounded is one of the features that make them a valuable model to answer these types of questions.

By collecting cells that are rounded (that were undergoing checkpoint adaptation), we increased the likelihood that we are studying cells that have responded to treatment by a genotoxic agent. Therefore, we have reduced the possibility that we are studying cells that originate from prediction 1. It remains formally more difficult to eliminate the possibility that we are studying a cell from prediction 2. However, the results from karyotyping and from FISH are more in support of prediction 3 than that of prediction 2.

For example, the variation in the number of chromosomes in CAS cells suggests that a number of different cell types are present in the CAS population. If the CAS cells originated from one or two pre-existing cells, we would expect the chromosome number to show less variability. Overall, these data suggest that HT-29 cells do undergo checkpoint adaptation when exposed to CPT, and the cells that survive checkpoint adaptation have a rearranged genome. We are able to see genomic change in CAS cells at several levels:

1. Karyotype analysis reveals change of chromosome numbers.
2. Chromosome structural changes by FISH analysis
3. The inability of some of the CAS cells to detect damaged DNA (unable to signal histone γ -H2AX).

These findings provide valuable information about the integrity of the genome of the CAS cells and support the hypothesis that checkpoint adaptation might cause genomic change in genotoxic treated human cancer cells.

4.9 Thesis Conclusion

The purpose of this research project was to test the hypothesis that HT-29 cells that survive checkpoint adaptation have changes in their genome. To address this, I first established that some HT-29 cells can survive checkpoint adaptation.

1. Ninety percent of the HT-29 CAS cells were negative for histone γ -H2AX without further treatment; however, 73% of the CAS cells displayed comets, indicating that their genome was damaged. This phenomenon suggests that the genome in CAS cells might be unstable.
2. CAS cells contained an average of 35 chromosomes whereas non-treated cells contained 65. This provides evidence that the cells that survive checkpoint adaptation contain an altered genome.
3. FISH analysis revealed that CAS cells appear to have changes in the organization of their genome as 45% of the chromosomes were missing telomeres and 28% of the chromosomes had telomeres in the wrong position.

From this research we believe CAS cells might be a source of cells that become resistant and cause cancer relapse following treatment. If this is the case, an understanding of the mechanism of checkpoint adaptation and its outcomes might eventually lead to improvements in cancer treatments in the future. For example, by understanding this process we can search for treatments to maintain toxicity, while preventing further genomic change. One opportunity would be to explore if we can prevent checkpoint adaptation such as stopping mitosis with Cdk inhibitors (Bettayeb *et al.*, 2008). This would diminish the number of CAS cells. Based on our findings, more

studies are being conducted in the laboratory to examine genomic change in human cancer cells that survive checkpoint adaptation.

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