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INVESTIGATION OF THE RESPONSE AND REPAIR OF REPLICATION

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INVESTIGATION OF THE RESPONSE AND REPAIR OF REPLICATION STRESS-INDUCED DNA DAMAGE

by

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DEDICATION

"Cancer is the result of living…"- Dr. Siddhartha Mukherjee

My graduate work is in dedication to my friends and family who have and have had the displeasure of a cancer diagnosis. Many have overcome the disease due to perseverance, radiation and chemotherapy, but a few have succumbed. Though it is my hope that my work and the work of others will one day cure or even prevent cancer, I would like those that have and will face the diagnosis with the above quote in mind, "Cancer is the result of living."

ACKNOWLEDGEMENTS

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ABSTRACT

When a mammalian cell suffers DNA damage, DNA damage signaling responses and repair pathways are invoked. The phosphorylation of histone variant H2AX (γH2AX) and of replication protein A (pRPA) are two well-documented damage signals, marking double strand breaks and stalled replication forks, respectively. Inhibitors of thymidylate synthase (TS) and ribonucleotide reductase (RNR) are chemotherapeutics that act by depriving cells of the deoxynucleotides necessary for DNA synthesis, which causes damage. The response and repair pathways activated by these chemotherapeutics have been studied for a number of years but there remain unanswered questions as to how cancer cells perceive this damage, the kinases active in the response, and the promotion of damage repair. To investigate the damage response and the necessity of H2AX phosphorylation during TS inhibition, we utilized cell models in which H2AX has been knocked out genetically as well as a shRNA H2AX knockdown cell line. Cell survival assays and immunofluorescence for the homologous recombination (HR) protein RAD51 showed that H2AX mutation or deficiency do not affect cell sensitivity or HR damage response. However, significant differences in the kinetics of pRPA were noted: pRPA was seen as early as 4 h post TS inhibition in H2AX deficient cells compared to up to 24 h in H2AX proficient cells. The data suggests that H2AX signaling is not involved in the response to TS inhibition but may affect the repair pathway selection. A kinase known to be over-expressed in cancer and act in mitosis, polo-like kinase 1 (PLK1), has recently gained attention for its reported activity in S-phase stress. Therefore, we examined how

PLK1 inhibition during deoxynucleotide deprivation affected response and repair of DNA damage. Our research suggests that inhibition of PLK1 decreases pRPA during replication stress as well as affects the formation of RAD51 foci in response to damage. Overall, the data presented here re-enforce the notion that though replication stress inducers have been researched and used in chemotherapy for decades, aspects of the damage response are still unknown. As well, our data highlights the potential for new chemotherapeutic combinations of replication stress inducers with drugs that inhibit pRPA or PLK1 inhibitors.

PREFACE

The following pages represent my three years of research in graduate school. Chapter 1 is an introduction to familiarize the reader with the world of cancer cell replication, replication stress and how it is induced, and the DNA damage that occurs in response to replication stress. I've chosen to present Chapter 1 as a brief overview of the molecular events that occur during replication stress and DNA damage response. Chapters 2 and 3 are meant to be read as individual scientific papers that represent research on different proteins; however, both chapters focus on one overall hypothesis: Elevated replication stress in cancer cells is an important weakness of cancer cells that is therapeutically exploitable.

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List of Abbreviations

ATM ..... Ataxia-telangiectasia (mutated) kinase

ATR ..... Ataxia-telangiectasia and Rad3-related kinase

BSA ..... Bovine Serum Albumin

CHK1 ..... Checkpoint kinase 1

DMSO ..... Dimethyl Sulfoxide

DNA ..... Deoxyribose Nucleic Acid

DSB ..... Double Strand Break

dTMP ..... Thymidylate Monophosphate

dTTP ..... Thymidylate Triphosphate

dUMP ..... Deoxyuridine Monophosphate

EGF ..... Epidermal Growth Factor

EV ..... Empty Vector

FBS ..... Fetal Bovine Serum

FdUMP ..... 5-fluoro-2'-deoxyuridine monophosphate

FdUrd ..... 5-fluoro-2'-deoxyuridine

G1 ..... Gap 1phase of the cell cycle

G2 ..... Gap 2 phase of the cell cycle

H2AX ..... Histone Variant H2A

γH2AX ..... Phosphorylated H2AX

HCT116 ..... Colorectal Carcinoma Cells

HR ..... Homologous Recombination

HT29.....Colorectal Adenocarcinoma Cells

HU .....Hydroxyurea

IR..... Ionizing Radiation

KD .....Knock-down

M ..... Mitosis

MCF10A.....Non-Tumorigenic Breast Epithelial Cells

MEF/MEFs ..... Murine Embryonic Fibroblasts

MTHF .....Methyl-tetrahydrofolate

MTT.....3-[4, 5-dimethlthiazol-2-yl] 2, 5-diphenyltetrazolium bromide

Mut .....H2AX S139A Mutant

NHEJ .....Non Homologous End Joining

OR .....Origin of Replication

PBS.....Phosphate Buffered Saline

PLK1..... Polo-like kinase 1

PMX .....Pemetrexed

RNR.....Ribonucleotide Reductase

pRPA/pRPA2.....RPA Phosphorylated on the 32 kD Subunit

RPA/RPA2..... 32 kD subunit of Replication Protein A

RTX.....Raltitrexed

S139A.....Serine to Alanine Mutation at Position 139 on H2AX

S-phase..... Synthesis phase

ssDNA .....Single-Stranded DNA

TS ..... Thymidylate Synthase

WT.....Wild-Type

CHAPTER 1

INTRODUCTION

1.1 DNA REPLICATION IN CANCER CELLS

During DNA synthesis of both non- and cancerous cells, events are precisely orchestrated to replicate the DNA without error. A number of start points, termed origins of replication (OR), exist in the mammalian chromosome which are initiated for DNA synthesis (Burhans, 1990). As seen in Figure 1.1, replication progresses away from the OR in a bidirectional fashion, creating a two-forked replication bubble (Burhans, 1990). At each replication fork, the replication machinery acts to separate the DNA double helix and to copy both the leading and lagging strands in the 5' to 3' direction (Figure 1.2). Replication protein A (RPA) binds to the single-stranded DNA (ssDNA) to keep the strands from re-annealing to one another as the topoisomerase-helicase complex and polymerases act in concert to unwind the parental DNA and replicate both strands, respectively (Wold, 1997). The DNA polymerases epsilon and delta replicate the leading and lagging template strands, respectively, by recognizing the template base and pairing it with the complement nucleotide (Burgers, 1998), extending the newly synthesized DNA strands. If replication is blocked ahead of the fork or if the replication machinery becomes inhibited, DNA replication will not be able to continue.

Mammalian cells must accurately replicate DNA during the synthesis phase (S-phase) of the cell cycle to ultimately pass the genetic information from the parent cell to two identical daughter cells. Cellular proliferation in non-cancerous cells undergo a

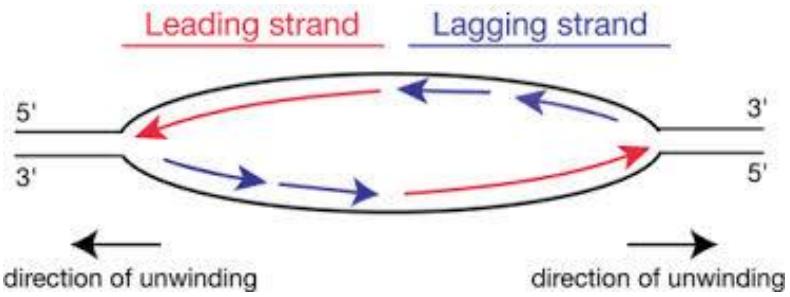


Figure 1.1. The Replication Bubble. Upon activation of an origin of replication (OR), replication progresses away from the OR, bidirectionally. Due to the directionality of DNA, each strand must be synthesized in the 5' to 3' direction, creating the necessity for a leading, continuously replicating strand (red) and a lagging, discontinuously replicating strand (blue). [Image produced by Dr. Aga Gambus, University of Birmingham, UK: http://www.birmingham.ac.uk/research/activity/mds/domains/Cancer/cancer-genetics-dna-damage/chromosomal-replication/index.aspx]

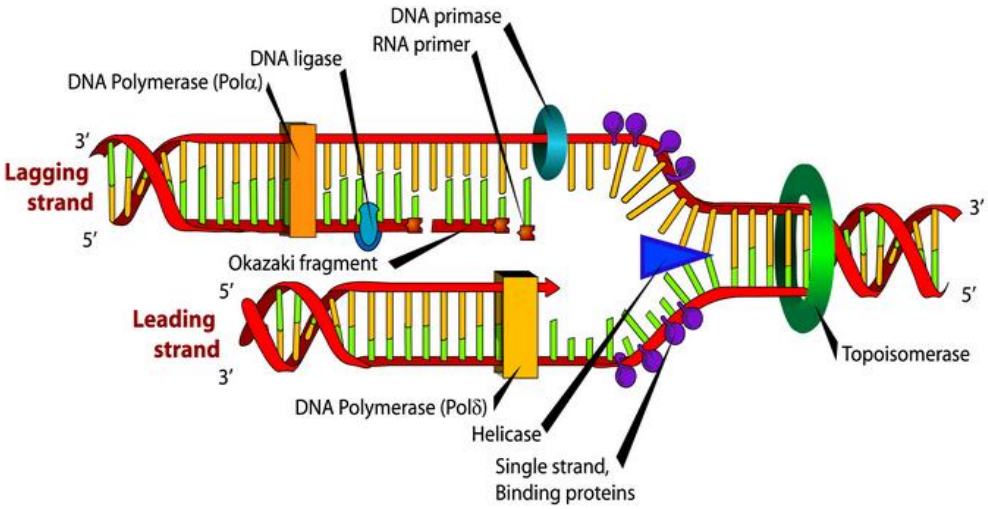


Figure 1.2. Machinery at the Replication Fork. The replication fork machinery, also termed the replisome, ensures the precise replication of both the leading and lagging strand template to duplicate DNA during S-phase. A few key proteins involved in replication are the DNA polymerases (yellow/orange rectangles), the helicase (blue triangle), the topoisomerase (green circle around DNA helix), and the single strand binding proteins (RPA; small purple circles). [Rouzer, C. A. (2011) VICB Communications, Vanderbilt Institute of Chemical Biology: http://www.vanderbilt.edu/vicb/DiscoveriesArchives/dnareplication_ipond.html]

stringent checks-and-balance system to ensure proper, precise replication of DNA and its segregation into the resultant daughter cells. These checks-and-balances, or cell cycle checkpoints, have been identified in all phases (Figure 1.3) and are necessary for error-free, faithful completion of the cell cycle to maintain genetic integrity. However, cancer

cells often acquire mutations in specific genes that control cell cycle checkpoints that alter or inhibit their activation and allow for numerous cell divisions, among other hallmarks of cancer (Hanahan and Weinberg, 2000). During replication of non-cancerous cells, the intra-S-phase checkpoint is activated when cells encounter DNA damage to ensure that the DNA is repaired and duplicated in its entirety without error (Willis and Rhind, 2009; Andrews and Clarke, 2005). The activation of this checkpoint can lead to the cells arrested in S-phase to allow for the repair of DNA damage (Abraham, 2001; Ye, 2003; Zhang, 2006). Cancer cells often escape the S-phase arrest due to the mutations in the intra-S-phase checkpoint, allowing for the accumulation of replication errors and further propagating mutated DNA (Willis and Rhind, 2009). Exploitation of the inherent mutations in the S-phase checkpoint may produce novel methods and targets to kill cancer cells.

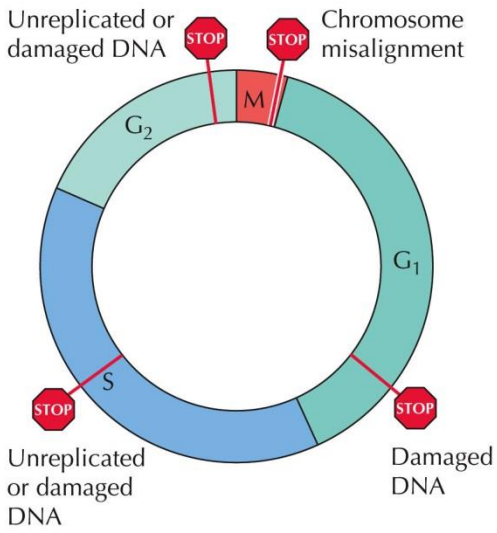


Figure 1.3. The Stages of the Cell Cycle and Its Checkpoints. The mammalian cell cycle consists of four stages: Gap1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). The 'stops' indicate the checkpoints within each stage of the cell cycle that regulate the progression of the cell through interphase (G1, S, G2) and into M, before the final stage of the cycle where the cellular material is divided into two cells, during cytokinesis. [The Cell, Fourth Edition, Figure 16.8]

1.2 DNA Replication Stress

DNA replication stress is defined as inefficient DNA replication that causes DNA replication forks to progress slowly or stall (Burhans and Weinberger, 2007). Replication stress is gaining acceptance as a fundamental cause of genome instability that drives tumorigenesis (Burrell, 2013; Barlow 2013). Replication stress is also a means by which several classes of anticancer chemotherapeutics act to kill cancer cells. Induction of replication stress during S-phase activates complex DNA damage response and repair cascades involving the replication stress sensor Ataxia-telangiectasia and Rad3-related (ATR) kinase and the phosphorylation of several downstream targets such as checkpoint kinase 1 (CHK1) (Zhou, 2000; Ward and Chen, 2001), as seen in Figure 1.4. There are two key signaling events that occur in response to DNA damage in S-phase: phosphorylation of histone variant H2AX (not shown in Figure 1.4) and phosphorylation of RPA (shown in Figure 1.4). H2AX phosphorylated at serine-139 (γH2AX) is well known to be an essential component of DNA double strand break (DSB) repair caused by ionizing radiation (Celeste, 2002; Xie, 2007). RPA is a heterotrimeric protein, and the 32kDa subunit (RPA2) becomes hyperphosphorylated (pRPA2) on its N-terminus in response to replication stress (Liu, 2006; Byun, 2005; Binz, 2004). Our lab has also shown that certain chemotherapeutics induce pRPA2 (Yang, 2008; Yang 2012). However, there remain important questions regarding exactly how cancer cells respond to the replication stress induced by a type of stress called deoxynucleotide deprivation.

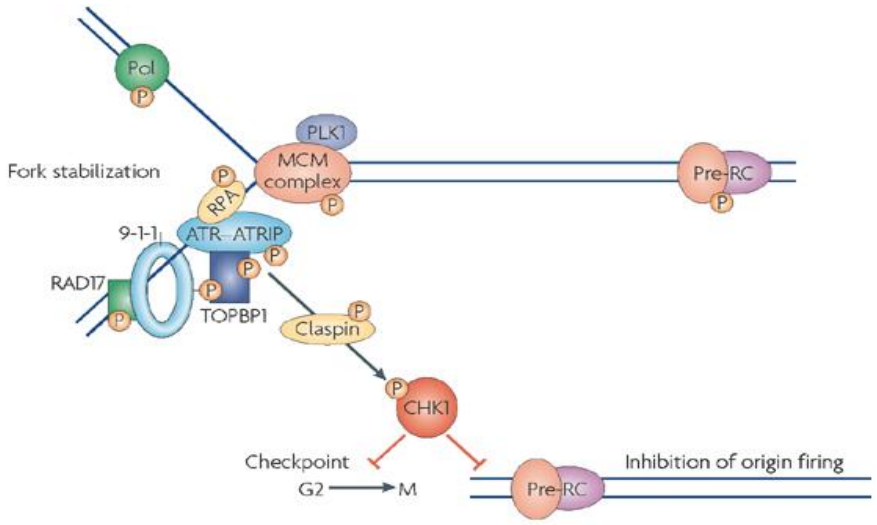


Figure 1.4. Replication Stress Response at the Replication Fork. When cells are stressed with chemotherapeutics that induce replication stress, several DNA damage response proteins are recruited to the replication fork to stabilize the fork and to activate repair of the damage. If the replication stress cannot be overcome, evidence suggests that stalled forks are collapsed and processed into DSBs. [Nature Reviews Molecular Cell Biology (2008) 9: 616-627.]

Polo-like kinase 1 (PLK1) is a serine/threonine protein kinase (shown in Figure 1.4), canonically a mitotic regulation protein, that is over-expressed in many cancers including breast, ovarian, pancreatic, prostate, and colorectal cancer (Takahashi, 2003; Weichert, 2004; Gray, 2004). Like the over-expression of the proto-oncogene HER2 (Ménard, 2000), the over expression of PLK1 qualifies it as oncogenic (Jang, 2006; Pellegrino, 2010). Inhibition of PLK1 by ATP-based inhibitor BI-2536 results in cell cycle arrest at pro-metaphase (Steegmaier, 2007). Several more recent studies have implicated oncogenic PLK1 as actively participating in overcoming replication stress (Trenz, 2008; Song, 2011; Song 2013; Yata, 2012). Also, it has been shown that PLK1 activity is suppressed in Ataxia-telangiectasia mutated (ATM) and ATR-activated cells (van Vugt, 2001) through the binding of the tumor suppressor BRCA1 to PLK1 (Zou, 2013). Conversely, in cells depleted of ATR and treated with replication stress inducers, PLK1 activity has been shown to suppress replication restart; inhibition of PLK1 in

combination with RNF4 inhibition allowed for limited replication and decreased DSB formation (Ragland, 2013). The current literature suggests an active role for oncogenic PLK1 in regulating replication progression during replication stress, involvement in replication fork collapse, and the activation of DSB repair mechanisms to promote resistance to replication stress, little of which is fully understood. Therefore, the role of PLK1 in response to replication stress caused by chemotherapeutic agents needs further investigation.

1.3 Deoxynucleotide Deprivation

Deoxynucleotides such as thymidylate monophosphate and cytidine monophosphate are the building blocks of DNA replication. When deoxynucleotide pools are imbalanced or decreased overall, replication forks cannot progress forward, among other serious consequences (James, 1997; Song, 2003; Chabosseau; 2011). Chemotherapeutics that induce replication stress by deoxynucleotide deprivation such as Hydroxyurea (HU) and fluoropyrimidines have been studied for fifty years or greater. HU inhibits the formation of deoxynucleotides cytidine, adenosine, and guanosine by binding to the enzyme ribonucleotide reductase (RNR) which quenches the free tyrosyl radical at the active site, inactivating RNR (Yarbro, 1992). The fluoropyrimidine 5-fluoro-2'-deoxyuridine (FdUrd) and some folate antimetabolites (or antifolates) inhibit the *de novo* synthesis of thymidylate (dTMP) by inhibiting the enzyme thymidylate synthase (TS). Specifically, FdUrd is phosphorylated to form FdUMP which binds to the deoxyuridine monophosphate (dUMP) site on TS, forming an irreversible covalent tertiary complex with methyl-tetrahydrofolate (MTHF) (Figure 1.5; Vazquez-Padua, 1989). Ultimately the *de novo* synthesis of dTTP decreases. Two antifolate-based

inhibitors, Pemetrexed (PMX) and Raltitrexed (RTX), inhibit TS as well as other enzymes involved in folate metabolism and the synthesis of deoxynucleotides (Adjei, 2004; Van Cutsem, 2002). Inhibition of RNR or TS causes depletion of the deoxynucleotide pools necessary for DNA synthesis. However, the precise mechanisms by which cancer cells respond to replication stress caused by deoxynucleotide deprivation are unclear.

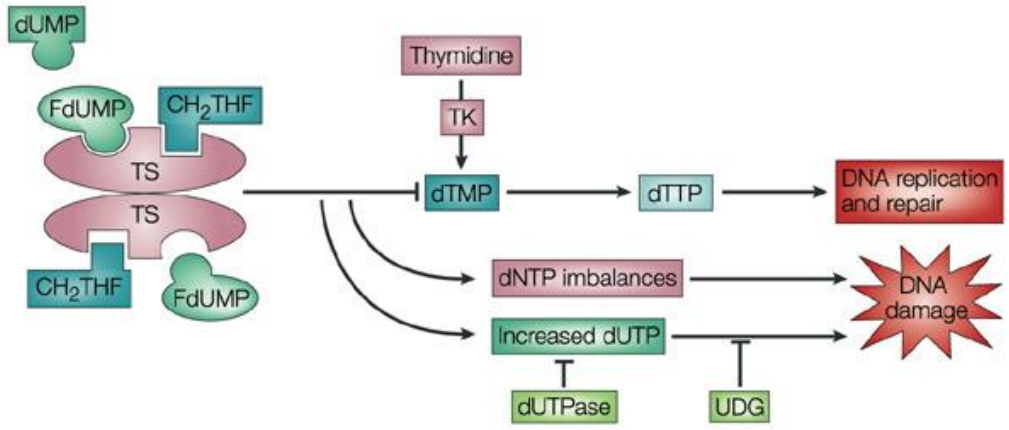


Figure 1.5. The Inhibition of Thymidylate Synthase by FdUrd. Once inside the cell, FdUrd is phosphorylated to the monophosphate FdUMP which then binds to TS, forming an irreversible covalent tertiary bond with methyl-tetrahydrofolate (CH_2THF), inhibiting the de novo synthesis of thymidylate. [Longley *et al*. (2003) 5-Fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer (3): 330-338.]

1.4 Damage Response

Both HU and TS inhibitors are known to induce γH2AX (Ward and Chen, 2001; Yang, 2008; Luo, 2008). However, recent observations have questioned the necessity of H2AX in the actual repair of some damage induced by chemotherapeutics, despite the robust induction of γH2AX by these agents (Revet, 2011; Cleaver, 2011). In other words, although some DNA damaging agents such as cisplatin induced γH2AX, H2AX deficient cells were not sensitized to cisplatin compared to cells expressing wild-type H2AX (Revet, 2011). Our lab and others have shown that RPA2 is hyperphosphorylated when

cells are treated with these drugs (Yang, 2008; Yang 2012). Though TS inhibitors have been studied, it remains unclear whether the stress caused by TS inhibitors is perceived by the cell to predominantly be a 'stalled replication fork (pRPA2)' or a 'DNA double strand break (γH2AX).'

Another important question is how the DNA repair machinery is engaged to repair the damage. The collapse of the stalled replication fork is becoming more widely recognized as an active event (Sirbu, 2011; Forment, 2011); the collapsed fork is then processed to form a DSB which can then be repaired by two pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). The selection and activation of the DSB repair pathway is largely variable with many proteins driving both pathways (Shrivastav, 2008); DSB repair pathway selection resulting from deoxynucleotide deprivation is unclear as well.

Lastly, what signaling kinases are involved in driving resolution of the damage? Well-known are the damage response kinases ATM and ATR which initiate the DNA damage response and repair cascades. Still, the current literature suggests an active role for the oncogenic PLK1 kinase in replication fork progression, fork collapse, and the activation of DSB repair mechanisms (Trenz, 2008; Song, 2011; Yata, 2012; Ragland 2013). Specifically, PLK1 binds to phospho-Mcm2 when ATR and CHK1 are activated by replication stress (reviewed in Song, 2012). PLK1 then phosphorylates Claspin, a necessary interacting protein for CHK1 and ATR, promoting Claspin degradation and downstream HR events (Trenz, 2008; Yata, 2012). Though data is compiling that PLK1 is necessary for replication progression during replication stress (Ragland, 2013), the phospho-substrates of the PLK1 kinase during replication stress are still poorly defined.

Chemotherapy-induced replication stress and the subsequent DNA damage response is enigmatic in nature. Better understanding how cells respond to this damage and the exploitation of the genomic instability inherent in all cancer cells will lead to more efficient chemotherapies. It was therefore decided to exploit cell culture models in which H2AX was mutated, disrupted, or deleted to test the importance of H2AX signaling in response to TS inhibition. As well, we investigated the role of oncogenic PLK1 activity in replication stress induced by deoxynucleotide deprivation using well-characterized human colorectal cancer cell lines.

CHAPTER 2

H2AX AND ITS PHOSPHORYLATION ARE DISPENSABLE IN THE CELLULAR RESPONSE TO REPLICATION STRESS

2.1 INTRODUCTION

DNA replication is a precisely timed and executed event that occurs once in every S-phase of the cell cycle to duplicate the genetic material, which is eventually separated into two daughter cells during mitosis (Figure 1.3). Disruption of the S-phase to activate the intra-S-phase checkpoint has been the objective of several classes of anticancer chemotherapeutics to kill cancer cells (Karnani and Dutta, 2011). Specific inhibition of enzymes such as ribonucleotide reductase (RNR) or thymidylate synthase (TS) by chemotherapeutics causes deoxynucleotide deprivation, which induces replication stress. The cellular response to replication stress involves a cascade of DNA damage response and repair proteins acting in concert to detect and attempt repair of the damage.

S-phase DNA damage activates the replication stress sensor Ataxia-telangiectasia and Rad3-related (ATR) kinase which phosphorylates several downstream targets (Flynn and Zou, 2011). Two of these downstream targets are well-documented signals of DNA damage in S-phase: the hyperphosphorylation of the single-stranded binding protein, replication protein A on the 32 kDA subunit (pRPA2) and the phosphorylation of histone variant H2AX (γH2AX) (Ward and Chen, 2001; Binz, 2004; Liu, 2006; Yang, 2008). RPA2 hyperphosphorylation is recognized as the marker for stalled replication forks (Liu,

2006; Binz, 2004). γH2AX is traditionally recognized as a biomarker for DNA DSB repair induced by ionizing radiation (Celeste, 2002; Xie, 2007). Inductions of both pRPA2 and γH2AX in response to TS inhibitors have been reported in the literature (Ward and Chen, 2001; Yang, 2008; Luo, 2008; Yang 2012). This induction of γH2AX by TS inhibition suggests that deoxynucleotide deprivation causes DSBs. However, the necessity of H2AX phosphorylation in signaling and repair of some DNA damage has been questioned, despite the robust induction of γH2AX by certain chemotherapeutics (Cleaver, 2011; Revet, 2011). For instance, Revet *et al.* 2011 reported that though the DNA damaging agent cisplatin induced γH2AX, H2AX deficient cells were not sensitized to the drug compared to cells expressing wild-type H2AX. We then questioned the importance of H2AX signaling in response to TS inhibition.

How cancer cells perceive damage signals from replication stress induced deoxynucleotide deprivation is largely unknown. Our lab has endeavored to resolve whether this damage is perceived as a 'stalled replication fork' (pRPA2) or a 'DNA DSB' (γH2AX) as well as the necessity of H2AX signaling in response to TS inhibitors. Here we have utilized the TS inhibitor 5-fluoro-2'-deoxyuridine (FdUrd) to induce replication stress to study the early cellular responses and the necessity of H2AX phosphorylation in responding to replication stress in cell models with wild-type or altered H2AX status. Our data suggests that H2AX and its phosphorylation are not involved in the repair response to TS inhibition.

2.2 MATERIALS AND METHODS

To examine the effect of H2AX signaling in response to TS inhibition, we utilized three models that have reduced or eliminated H2AX expression. First, murine embryonic fibroblasts (MEFs) were generated by others, in which the *H2AX* gene was disrupted to generate *H2AX*$^{-/-}$ cells (Celeste, 2002). From these *H2A* $^{-/-}$ MEFs, stable sublines were generated which contain an empty vector (EV, H2AX null), or add-back sublines containing a vector expressing full-length, wild-type H2AX (WT), or a serine to alanine (S139A) H2AX mutant (Mut) incapable of phosphorylation, which were obtained from Dr. James Cleaver at UCSF (Revet, 2011). It is important to note that cells containing WT or Mut H2AX were tagged by the FLAG protein, which increases the total kilo-Dalton weight of the protein. The next model was the human breast epithelial cell line MCF10A and a subline in which H2AX was eliminated by gene targeting (*H2AX*$^{-/-}$). In addition, we used the colorectal carcinoma cell line HCT116 in which H2AX was silenced by shRNA, reducing expression of H2AX by 70-80% as measured by Western blotting (Figure A.1). This cell line was developed by the Shtutman Lab.

All cell lines were grown in a 37°C incubator at 5% CO_2 and maintained in media specific to each cell line. The MCF10A cells were grown in DMEM-HAM's medium with 5% horse serum, 10 μg/mL human insulin, 0.5 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor (EGF), and 100 ng/mL cholera toxin. Freeze-thaw cycles were carefully noted for the EGF due to the sensitivity of the MCF10A cells to changes in the EGF. MEF cells were maintained in MEM/EBSS medium with 10% Fetal Bovine Serum (FBS), 25 μg/mL Blasticidin, 10 units/mL Pen/Strep, and supplemented with 100 nM Folinic Acid. HCT116 cells were maintained in RPMI 1640 medium with 10% heat-

inactivated FBS, 1% Pen/Strep, 2 μg/mL Puromycin and supplemented with 100 nM Folinic Acid.

To induce replication stress via deoxynucleotide deprivation, we utilized known TS and RNR inhibitors such as 5-fluoro-2'-deoxyuridine, hydroxyurea (HU), pemetrexed (PMX), and raltitrexed (RTX). Specifically the antimetabolite 5-fluoro-2'-deoxyuridine (FdUrd) was utilized. Briefly, FdUrd is converted in the cell to 5-fluoro-2'-deoxyuridine monophosphate (FdUMP), which then covalently binds to and inhibits TS [reviewed in Touroutoglou and Pazdur, 1996].

To measure the toxicity of the TS inhibition, the MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) assay was utilized. Specifically, 2000 cells each were plated into the wells of a 96-well plate and allowed to attach overnight in a 37°C incubator at 5% CO_2. Cells were then treated for 24 h with drug-containing medium which was then removed and replaced with drug-free media and replaced in the incubator for at least 3 days or until the control wells were confluent. Each plate was then processed as follows: MTT was added to each cell-containing well and incubated for 5 h, plates were spun down for 5 min at 1100 rpm, media was aspirated and dissolved in 200 μL of dimethyl sulfoxide (DMSO), and the absorbance of each well was measured at 595 nm. Data replicates were averaged and plotted as the cell survival versus drug concentration using Excel. Each data point is representative of 2 or 3 independent experiments, as noted in the figure legend.

To measure the kinetics of H2AX and RPA phosphorylation in cells that are deficient in H2AX, MCF10A and MEF cells were plated into 100 mm dishes, grown to ~60-70% confluence, and then treated at specific time-points (0, 4, 6, 8, 12, and 24 h)

with FdUrd. Cell extracts were then collected using two cell fraction kits: NE-PER® Nuclear and Cytoplasmic Extraction Reagents and Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific). Protein extracts were then separated on 12% acrylamide denaturing gels, transferred to PVDF membranes, and probed with specific antibodies: H2AX (Cell Signaling), γH2AX (Trevigen), RPA2 (Abcam), pRPA2 (Bethyl Laboratories), and β-actin (Abcam). Western blots shown are representative results of 2 independent experiments.

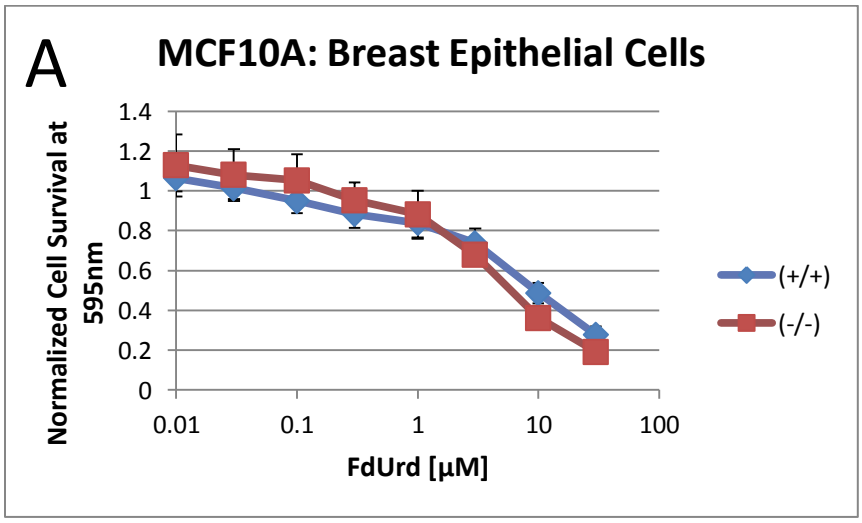
To examine the formation of RAD51 foci, MEF cells were plated at 50,000 cells/well onto coverslips in a 6-well plate and treated for 24 h with 10 μM FdUrd. Cells were then proceeded as follows: fixed with 4% formaldehyde in PBS, permeabilized with 0.3% Triton x-100/0.01 M Glycine in PBS, blocked for 1 h with 1% bovine serum albumin (BSA) in PBS, probed overnight at 4°C with anti-RAD51 antibody (Santa Cruz), and probed with secondary Texas Red antibody (Invitrogen) for 1 hour at room temperature. Coverslips were then removed and mounted using Prolong Gold Anti-fade with DAPI and sealed with Permount. Cells were imaged using an Olympus LX81 at 100x and images processed using MetaMorph software.

2.3 Results and Discussion

Using the three cell lines described, we endeavored to examine whether cells that have reduced or eliminated H2AX expression were more sensitive to TS inhibition compared to the wild-type (WT) expressing cells. All cell lines were treated for 24 h with FdUrd, followed by three days of recovery in drug-free media. As evident by Figure 2.1 A, H2AX deficiency in non-tumorigenic breast epithelial cells (MCF10A) does not increase sensitivity to treatment with FdUrd. Similarly, Figure 2.1 B shows that H2AX

mutation of serine-139 to a non-phosphorylatable alanine (S139A) mutant or deficiency in the gene does not impart sensitivity in mouse embryonic fibroblasts (MEFs). At concentrations greater than 1 μM of FdUrd, the H2AX-null (EV) and H2AX S139A mutant (Mut) cells may be slightly more resistant than the WT expressing cells. This resistance may be attributed to the lack of S139 phosphorylation which acts as a binding site for DNA damage sensing proteins (Singh, 2012). Finally, reduced expression of H2AX by shRNA in colorectal carcinoma HCT116 cells (Figure 2.1 C) did not increase sensitivity to TS inhibition. Similar toxicity profiles were obtained when the cells were treated with the alternative chemotherapeutics Pemetrexed (PMX) and Raltitrexed (RTX) that induce deoxynucleotide deprivation (Figure A.2 and A.3).

We then examined the ability of MEF H2AX S139A mutant or deficient cells to form RAD51 foci, indicative of homologous recombination (HR). Figure 2.2 shows representative images of MEF sub-lines WT, EV, and Mut exposed to 10 μM FdUrd for 24 h and probed for the HR protein RAD51. The yellow arrows point to RAD51 foci that



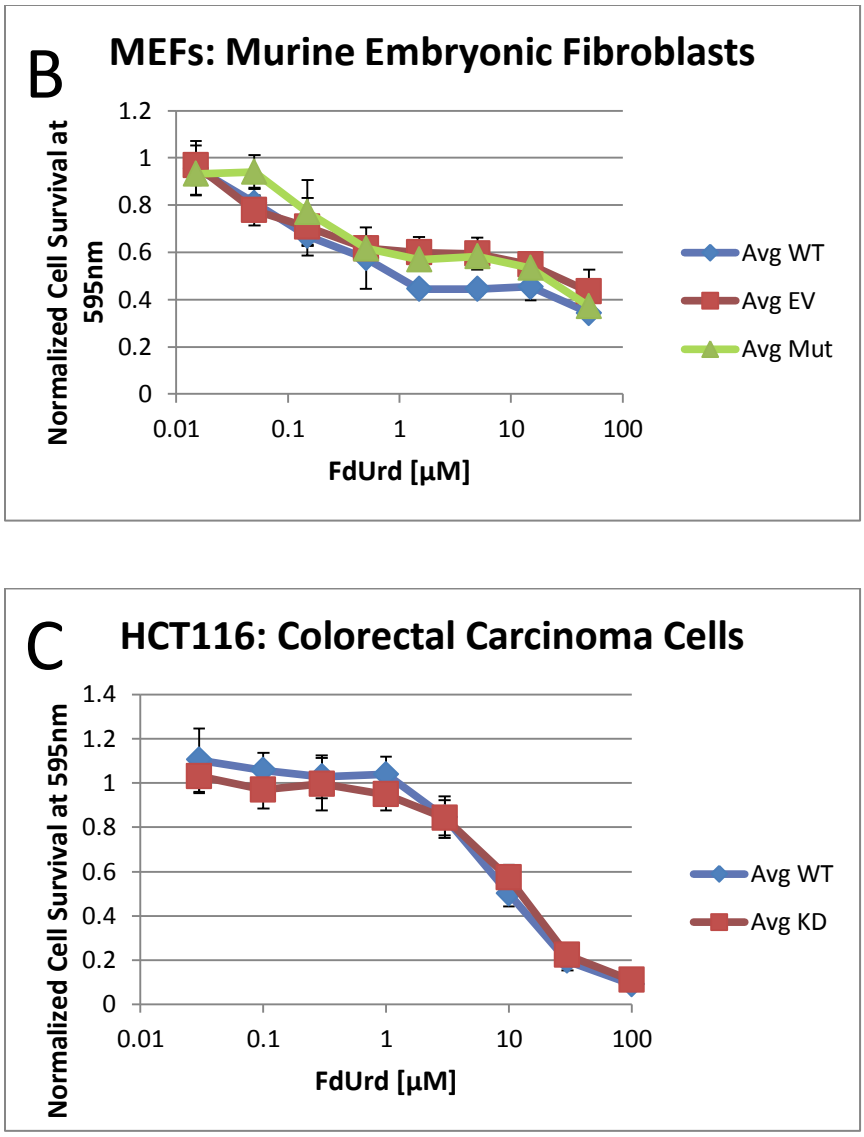


Figure 2.1. FdUrd-Treated Cell Survival Curves. **A)** Non-tumorigenic breast epithelial MCF10A that are H2AX proficient (+/+) or deficient (-/-). **B)** Murine embryonic fibroblasts with wild-type (WT), S139A mutant (Mut), or H2AX null (EV). **C)** Colorectal carcinoma HCT116 cells with WT or reduced (KD) H2AX expression. Cells were treated for 24 h with FdUrd concentrations in Folinic Acid-containing media. Blue lines with diamond data points represent cells with wild-type H2AX, red lines with square data points represents cells with decreased expression of H2AX or are H2AX-/-, and green lines with triangle data points are cells expressing the S139A H2AX mutant. Each data point represents n=2 in MEFs and HCT116 or n=3 in MCF10A.

have formed in the MEF sub-lines, indicating that S139A mutation or deficiency in H2AX do not impair foci formation (overlay images, Figure A.4). Results from the cell survival assays and immunofluorescence images suggest that H2AX signaling is not involved in the repair responses to deoxynucleotide deprivation caused by TS inhibition

that promote repair and survival. This data is consistent with other published data (Celeste, 2002; Revet, 2012).

In our investigation of H2AX and its role in replication stress induced by deoxynucleotide depriviation, we initially focused on longer time-points (24 h) of drug treatment. Singh *et al.* 2012 showed that as early as 15 minutes of ionizing radiation (IR), there is a strong induction of γH2AX in their cell model; however, little is known about the early S-phase DNA damage signaling events that occur as a result of TS inhibition.

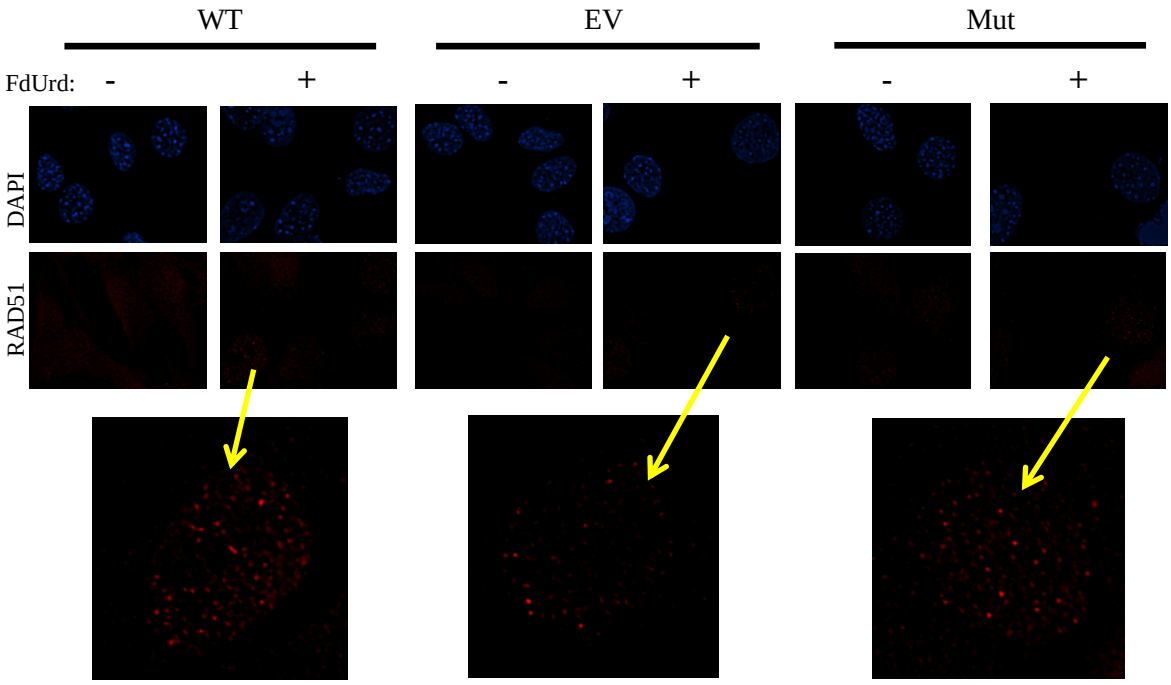
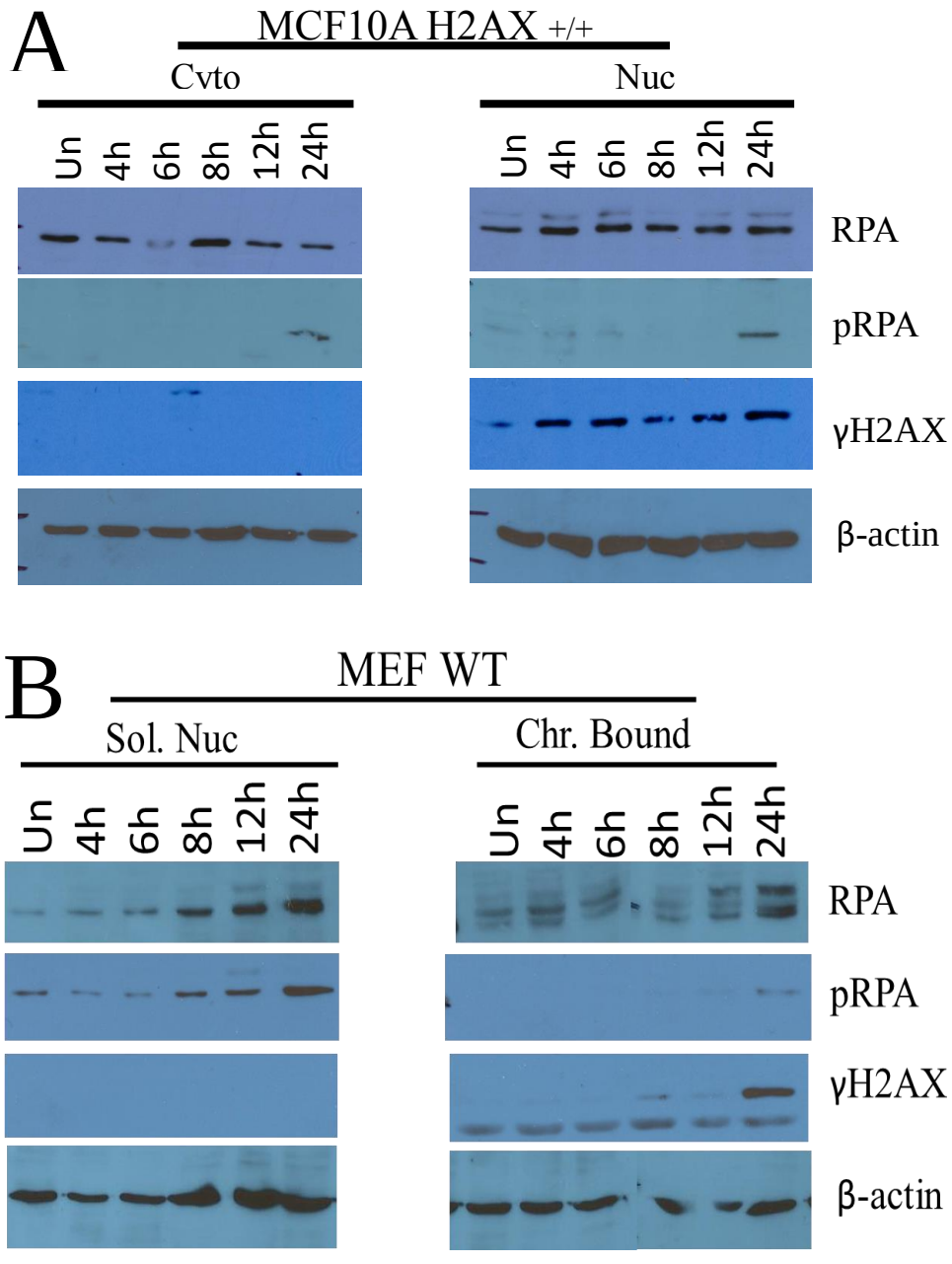


Figure 2.2. RAD51 Foci Form in H2AX mutant and null MEFs. Wild-type H2AX (WT), H2AX-null (EV), and H2AX S139A mutant (Mut) MEF sub-lines were treated for 24 h with 10 μM FdUrd and then probed for the HR protein RAD51. Yellow arrows point to RAD51 foci in the enlarged images of the treated cells. Blue: DAPI stained DNA; Red: RAD51. All images taken at 100x on the Olympus LX81 microscope and processed using the MetaMorph software.

Using the H2AX wild-type and deficient MCF10A and MEF cells, we endeavored to examine the early DNA damage response to TS inhibition using the replication stress marker pRPA. Specifically, cells were treated for 4, 6, 8, 12, or 24 h with FdUrd, collected, and processed using protein extraction kits. Figure 2.3 A and B shows the

MCF10A and MEF H2AX proficient cells, respectively, that have been probed for total RPA, γH2AX, and pRPA. Induction of γH2AX occurs at different time-points: early, at 4 h, in MCF10A and late, at 24 h, in the MEFs. Phosphorylation of RPA, however, occurs at 24 h of FdUrd treatment in both H2AX proficient lines. In Figure 2.3 C and D, H2AX



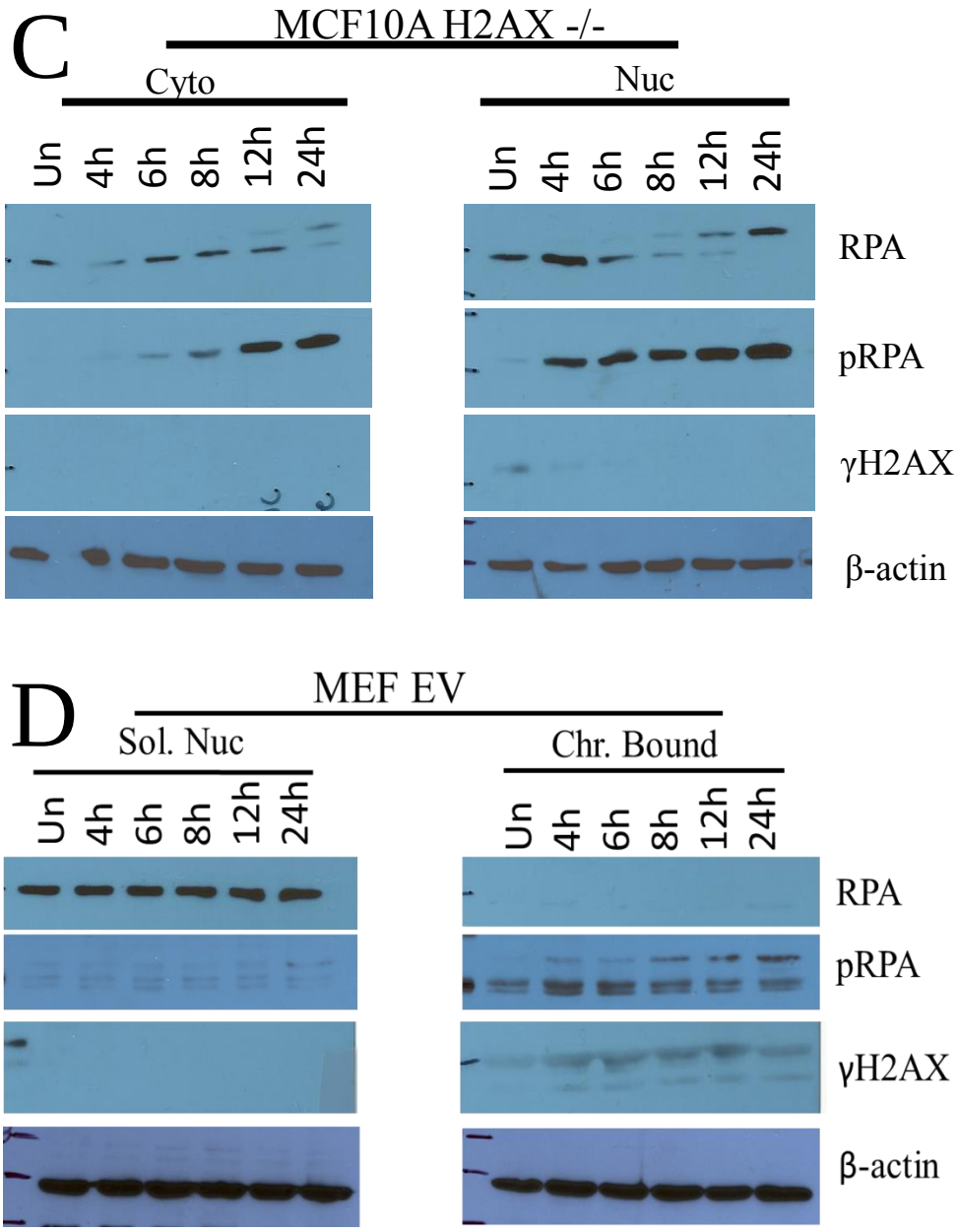


Figure 2.3. MCF10A and MEFs Respond to Replication Stress. H2AX deficient cells exhibit faster RPA phosphorylation kinetics than H2AX proficient cells. **A**) and **C**) MCF10A H2AX proficient (+/+) and deficient (-/-) cells, respectively, were treated with 10 μM FdUrd and the cytoplasmic (Cyto) and nuclear (Nuc) protein extracts were collected. **B**) and **D**) MEF H2AX WT and EV cells, respectively, were treated with 1 μM FdUrd and a subcellular fractionation kit was used to collect the soluble nuclear (Sol. Nuc) and the chromatin bound (Chr. Bound) extracts. Proteins probed: replication protein A (RPA), phosphorylated RPA (pRPA), γH2AX, and β-actin for the loading control.

deficient MCF10A and MEF cells do not have γH2AX bands, as H2AX is not expressed in these lines. But, in both H2AX deficient cell lines, the phosphorylation of RPA occurs

at time-points as early as 4 h. This data indicates that significant differences in the kinetics of RPA phosphorylation occur based on H2AX status: H2AX proficient cells undergo a H2AX dependent signaling pathway which leads to a delayed RPA phosphorylation and H2AX deficient cells undergo a H2AX independent signaling pathway, causing an early phosphorylation of RPA. Although the survival data suggest that H2AX deficiency does not sensitize cells to FdUrd or inhibit the formation of RAD51 foci, the alteration in pRPA suggestion signaling pathway choice is affected.

Overall, our data indicates that H2AX is not involved in the DNA damage response initiated by deoxynucleotide deprivation that promotes repair and survival. In three separate cell lines, we have shown that sensitivity to FdUrd is not altered in H2AX reduced, mutated, or deficient lines when compared to the wild-type H2AX expressing cells. Similarly, RAD51 foci formation was unaffected by altered H2AX status. This suggests that the damage response to deoxynucleotide deprivation from TS inhibition is more 'stalled replication fork' like as opposed to a "DSB". The finding that the RPA phosphorylation kinetics change based on H2AX status is a potential point of therapeutic exploitation. Combination treatments with TS inhibitors and drugs that disrupt pRPA kinetics of cancer cells may have therapeutic value.

CHAPTER 3

ONCOGENIC PLK1 ACTIVITY DURING REPLICATION STRESS PROMOTES THE SIGNALING AND REPAIR OF DNA DAMAGE

3.1 INTRODUCTION

The search for druggable targets that are over-expressed in cancer cells but not in normal tissue is one of the many goals of cancer research. One such target, polo-like kinase 1 (PLK1), has been researched for a number of years, and inhibitors of this protein are currently in clinical trials (Yim, 2013). PLK1 is a serine/threonine protein kinase that preferentially phosphorylates phospho-proteins, regulates mitotic progression, and is over-expressed in many cancers including breast, ovarian, pancreatic, prostate, and colorectal cancer (Takahashi, 2003; Weichert, 2004; Gray, Jr, 2004). Over-expression qualifies PLK1 as an oncogene and a potential chemotherapy target. One of the PLK1 inhibitors currently in clinical trials is the ATP-based inhibitor BI-2536, which results cellular arrest early in mitosis, specifically in pro-metaphase (Steegmaier, 2007). Though PLK1 expression is at its highest in mitosis (Golsteyn, 1994), several recent studies have shown that oncogenic PLK1 is an active participant in S-phase when replication is stressed (Trenz, 2008; Song, 2011; Yata, 2012; Song, 2013, Ragland, 2013).

Replication stress is a means by which several classes of anticancer chemotherapeutics act to kill cancer cells, such as the ribonucleotide reductase (RNR) inhibitor Hydroxyurea (HU). Inhibition of RNR by HU treatment decreases the production of deoxynucleotides necessary for DNA synthesis (Yarbro, 1992), thus

causing an S-phase arrest. Induction of replication stress begins with the increase of single-stranded DNA (ssDNA) which is then bound by replication protein A (RPA). RPA is then phosphorylated (pRPA) by the replication stress sensor Ataxia-telangiectasia and Rad3-related (ATR) kinase, in turn activating complex DNA damage response and repair cascades (Zhou, 2000), which includes the phosphorylation of CHK1 (Ward and Chen, 2001). Evidence is gathering that suggests that oncogenic PLK1 plays a dynamic role in how cancer cells respond to damage and overcome replication stress. Oncogenic PLK1 is suppressed by the activation Ataxia-telangiectasia mutated (ATM) kinase and ATR during replication stress (van Vugt, 2001; Zou, 2013). However, oncogenic PLK1 inhibits ATR signaling by the phosphorylation of Claspin, a necessary interacting protein for ATR and CHK1, promoting Claspin degradation and downstream homologous recombination (HR) events (Trenz, 2008; Yata, 2012).

Questions still remain of how oncogenic PLK1 activity in replication stress induced by deoxynucleotide deprivation promotes cell survival and whether other phospho-substrates exist for the kinase. We utilized the colorectal carcinoma cell lines HT29 and HCT116, replication stress inducer HU, and PLK1 inhibitor BI-2536 to further explore these questions. Our data shows that PLK1 inhibition during replication stress decreases RPA phosphorylation, suggesting that phosphorylated RPA may be a substrate of PLK1. We also provide evidence that the HR protein RAD51 and its foci formation are affected when PLK1 is inhibited during replication stress, in agreement with other published data (Yata, 2012).

3.2 Materials and Methods

To study the importance of oncogenic PLK1 activity during replication stress induced by deoxynucleotide deprivation, we utilized two well-characterized cell lines: colorectal carcinoma HCT116 and colorectal adenocarcinoma HT29. HCT116 cells were grown in McCoy's 5A media, 10% FBS, and 1% Pen/Strep; HT29 cells were grown in Low Glucose DMEM, 10% FBS, and 1% Pen/Strep. Both cell lines were incubated in a 37°C incubator at 5% CO_2. Hydroxyurea (HU) was used to inhibit RNR, resulting in replication stress. HU prevents the formation of deoxynucleotides by binding to the enzyme RNR, quenching the free tyrosyl radical at the active site, therefore inactivating RNR (Yarbro, 1992).

To examine how oncogenic PLK1 and pRPA induction change during replication stress, HT29 and HCT116 were plated in 60 mm dishes and treated with HU for a total of 18 h. ATP-based inhibitor BI-2536 was then used to inhibit PLK1 activity (Steegmaier, 2007) at specific time-points during replication stress. More precisely, cells were treated with 5 mM HU to induce replication stress and during the HU treatment, BI-2536 was added to the media at a final concentration of 30 nM for 6 or 10 h of HU and BI-2536 co-treatment. It is important to note that HU was delivered first to ensure that the cells were arrested in S-phase before BI-2536 treatment allowing for the study of PLK1 inhibition specifically during replication stress. At the end of the drug treatment (18 h total HU with 6 or 10 h of BI-2536 co-treatment), cells were trypsinized and lysed using a whole cell lysis buffer that contained both protease and phosphatase inhibitors to maintain the protein integrity and phosphorylation, respectively. Extracts were then run on 12% non-denaturing acrylamide gels, transferred to PVDF membranes, and probed with specific

antibodies: PLK1 (Milllipore), pRPA2 (Bethyl Laboratories), and β-actin (Abcam). Western blots shown are representative images of 2 independent experiments. Band intensity measurements were then taken using ImageQuant software.

For immunofluorescence experiments, HT29 and HCT116 were plated at 50,000 cells/well onto coverslips in a 6-well plate and treated with HU and BI-2536 using the same co-treatment process that was used for Western blotting. At the end of the co-treatments, cells were then processed as follows: fixation with 4% formaldehyde in PBS, permeabilized with 0.3% Triton x-100/0.01 M Glycine in PBS, blocked for 1 h with 1% BSA in PBS, probed overnight at 4°C with anti-PLK1 and anti-pRPA2 or RAD51 (Santa Cruz) alone. After multiple wash steps with 1% BSA-PBS, the cells were probed for 1 hour at room temperature with secondary antibodies Texas Red goat anti-rabbit (Invitrogen) for pRPA2 or RAD51 and Fluorescin goat anti-mouse (Invitrogen) for PLK1. The coverslips were then stained with DAPI for 10 minutes and rinsed with PBS. Finally, the coverslips mounted using DABCO and sealed using clear nail polish. Cells were imaged using an Olympus LX81 at 100x and images processed using MetaMorph software. Slides were also viewed on a Nikon Eclipse E600 to quantitate the number of pRPA2 positive cells. For each treatment, 140-170 cells were scored, the percentage calculated, and plotted using Excel.

3.3 Results and Discussion

Oncogenic PLK1 is gaining recognition as an important kinase in the response to DNA damage. Here we have examined how this canonical mitotic regulation kinase behaves during deoxynucleotide deprivation and the consequences of inhibiting PLK1 during this type of replication stress. Figure 3.1 shows how PLK1 and pRPA in both

HT29 and HCT116 responds to HU or BI-2536 treatment alone and in combination. In the left panel, the PLK1 band in HT29 cells treated with 18 h HU only appears to decrease in comparison to the untreated cells. This finding is in agreement with the data published by Zou *et al*. (2013) who showed that in replication stress induced by HU treatment, BRCA1 is activated and binds to oncogenic PLK1, down-regulating its kinase activity, and decreasing the amount of unbound PLK1. It is important to note that in all HU and BI-2536 co-treatments, HU was delivered first to ensure that the cells were arrested in S-phase to allow study of the cellular response of PLK1 inhibition during replication stress. When both cell lines were co-treated with HU and BI-2536, PLK1 decreases relative to the untreated cells. We hypothesize that this band reduction in the

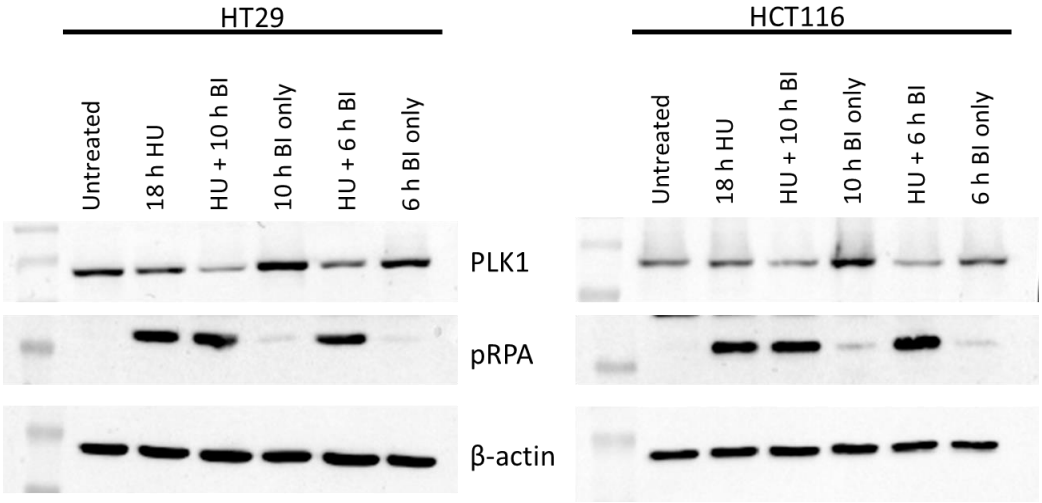


Figure 3.1. Colorectal Carcinoma Cell Lines Co-treated with HU and BI-2536. HT29 and HCT116 show reduced PLK1 expression in cells co-treated with HU and BI-2536. Both cell lines were treated with 5 mM HU or 30 nM BI-2536 alone or in combination. At the end of the treatment, whole cell extracts were collected for Western blotting. Proteins probed: PLK1, phosphorylated RPA (pRPA), and β-actin, used as a loading control. Images are representative of 2 independent experiments.

co-treated cells could result from the combination of BRCA1 and BI-2536 inhibition decreasing the amount of unbound PLK1. Cells that were treated with BI-2536 alone have stronger PLK1 banding than the untreated cells. We believe this is due to the cells

being arrested in mitosis—the consequence of BI-2536 treatment—where PLK1 levels are at their highest during the cell cycle (Steegmaier, 2007); similarly, there is a lack of pRPA in the BI-2536 only treated cells because RPA phosphorylation primarily takes place in S-phase, not mitosis.

RPA phosphorylation as a result of replication stress was also examined in the whole cell lysates from the HT29 and HCT116 cells. As expected, there is a strong induction of pRPA when cells are treated with HU alone. Upon examination of the band intensity (Figure 3.2), we found that in co-treated cells, the amount of RPA phosphorylation changes. Specifically, in HT29 extracts (Blue) treated with HU and BI-2536, pRPA band intensity decreases to roughly 40 fold change from 50 fold change in HU only treated extracts. Due to the decrease in phosphorylation that is seen in the co-

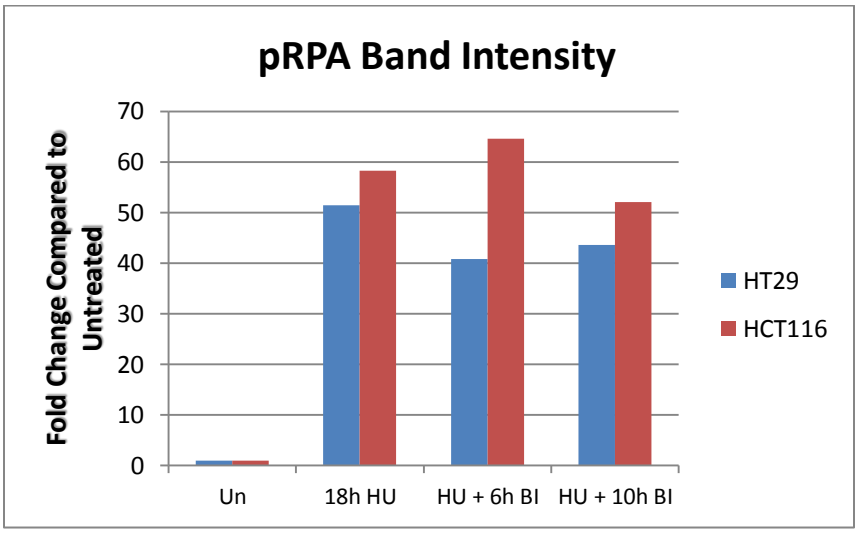


Figure 3.2. pRPA Band Intensity. pRPA band intensity of HT29 cells decreases in HU and BI-2536 co-treatments. RPA phosphorylation in HT29 (Blue) appears to decrease in HU and BI-2536 treated cells compared to HU alone. HCT116 (red) cell extracts do not behave similarly. Band intensity was first normalized to β-actin and then the fold change was taken in respect to the untreated control. Intensity measured using ImageQuant software.

treated extracts, we hypothesize that pRPA may be a target substrate for PLK1 phosphorylation and when oncogenic PLK1 is inhibited by BI-2536 during replication

stress, the amount of phosphorylated RPA decreases due to this inhibition. HCT116 extracts (Red) do not behave similarly to the HT29 in respect to the pRPA decrease in the co-treated extracts (Figure 3.2). It is well-documented that there are differences in the intra-S-phase checkpoint activation of the HT29 cells compared to HCT116 (Parsels, 2004), and that HCT116 cells are defective in a complex that detects and signals DNA damage (Takemura, 2006).

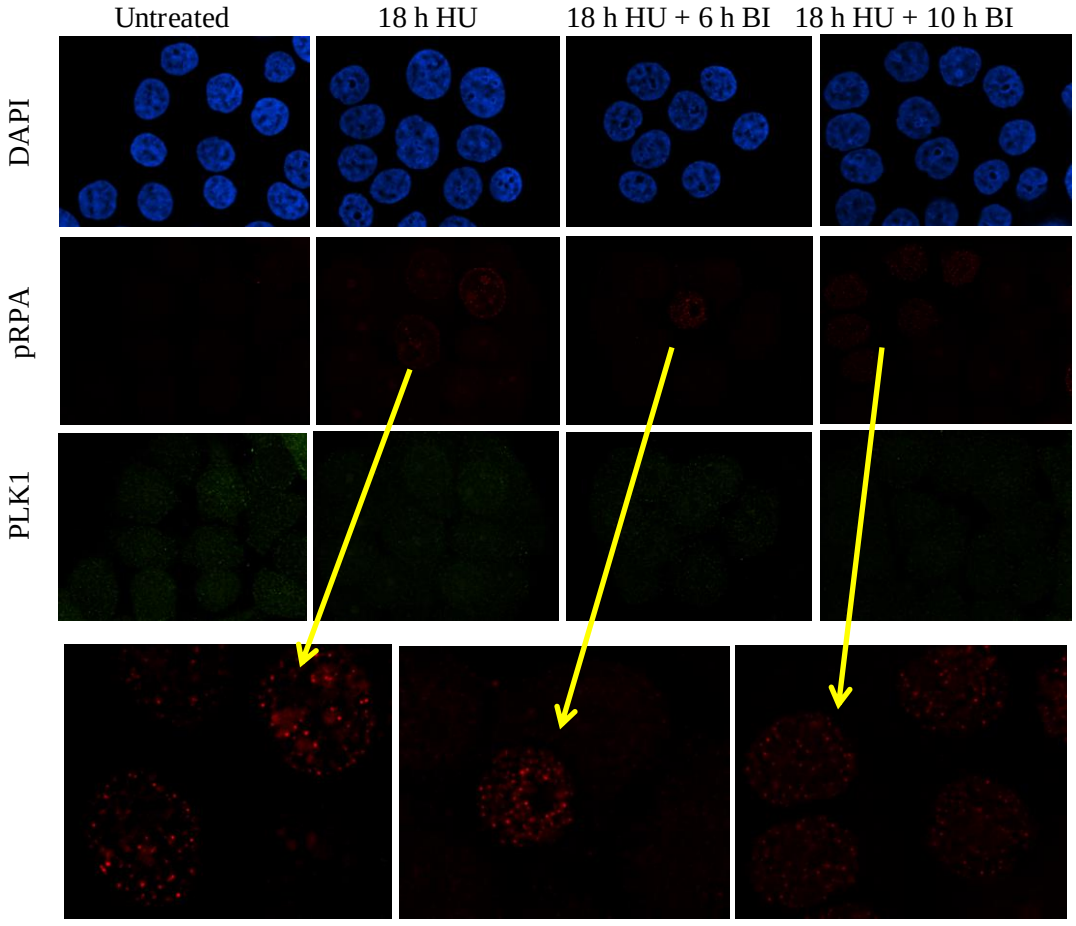


Figure 3.3. Immunofluorescence for pRPA and PLK1 in HT29. Oncogenic PLK1 does not form foci or localize in the nucleus in response to replication stress. pRPA foci size and number appear to decrease in co-treated cells. HT29 cells were treated with 5 mM HU alone or with 30 nM BI-2536 and probed for pRPA (Red) and PLK1 (Green) and the DNA stained with DAPI (Blue). All images taken at 100x on the Olympus LX81 microscope and processed using the MetaMorph software.

We then examined whether oncogenic PLK1 forms foci in response to deoxynucleotide deprivation and, if foci were present, if they co-localized with pRPA

foci. PLK1 (green) in HT29 (Figure 3.3) did not form foci when treated with HU and BI-2536 co-treatment or HU alone. When cells were treated with BI-2536 only, those cells arrested in mitosis had pan-nuclear PLK1 staining with localized intense staining at the ends of the condensed chromosomes (Figure A.5). We also have made the preliminary observation that cells co-treated with HU and BI-2536 have pRPA foci that appear both

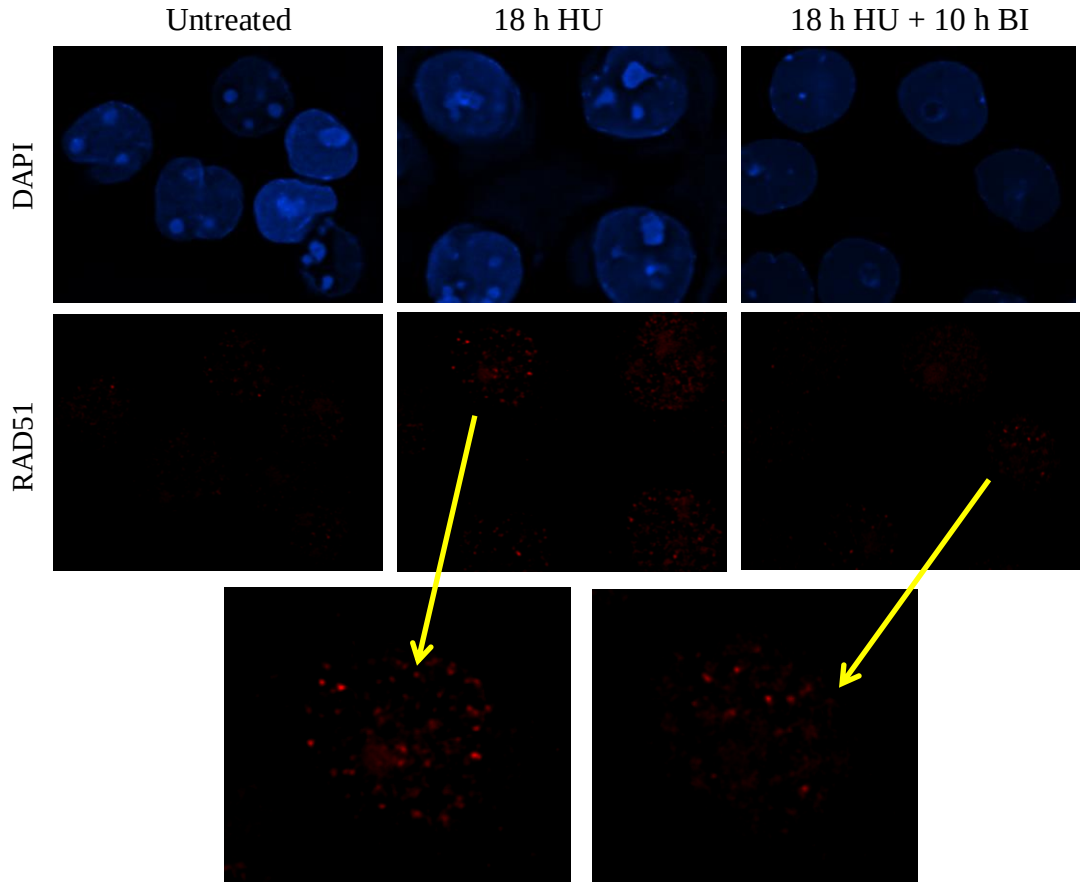


Figure 3.4. Immunofluorescence for RAD51 in HT29. RAD51 foci in HT29 decrease in size and number in HU and BI-2536 co-treatments. HT29 cells were treated with 5 mM HU and 30 nM BI-2536 and probed for RAD51. In the HU and 10 h BI-2536, RAD51 foci appear to be smaller than in the HU only treated cells. RAD51: Red, and DAPI-stained DNA: Blue. Images were taken on the Olympus LX81 microscope and processed with the MetaMorph software.

smaller and less numerous than cells treated with HU alone (Figure 3.3). The most noticeable difference in pRPA foci size and foci is between the HU alone and the Hu + 10 h BI-2536 (enlarged images, Figure 3.3). This observation strengthens our previous

hypothesis that oncogenic PLK1 further phosphorylates pRPA in response to replication stress and that the inhibition of PLK1 decreases RPA phosphorylation.

Recently, Yata *et al.* (2012) reported that RAD51 is phosphorylated *in vivo* by PLK1 in response to ionizing radiation and that this phosphorylation is necessary for DNA damage recognition and recruitment of RAD51 to the damage site. Therefore, we probed HT29 cells treated with HU alone and in combination with BI-2536 with RAD51 and studied the formation of RAD51 foci in response to our treatment scheme. Figure 3.4 shows RAD51 foci formation as a result of HU treatment as well as the BI-2536 co-treatments. The enlarged immunofluorescent images better show the foci in HU alone and HU in combination with 10 h of BI-2536. We noted that RAD51 foci appeared smaller and less numerous in the combination treated HT29 cells. This observation most likely results from the inhibition of PLK1 by BI-2536, which inhibits the phosphorylation of RAD51 and subsequent recruitment of RAD51 to the damage site caused by replication stress.

Our data strengthens the argument that oncogenic PLK1 is essential in the response of cancer cells to DNA damage (Trenz, 2008; Song, 2011; Yata, 2012; Ragland, 2013). More precisely, we have shown that PLK1 inhibition during replication stress induced by deoxynucleotide deprivation alters phosphorylation of RPA and potentially foci size and formation of both pRPA and RAD51. RPA phosphorylation is indicative of cellular replication stress and acts as a signal to arrest replication and repair damage. RAD51 foci formation indicates that DNA damage resulting in a DSB has occurred and the cell is attempting to repair the break by HR. Therefore, oncogenic PLK1 activity during replication stress promotes cell survival by maintaining the pRPA stress signal and

phosphorylating RAD51 to promote foci formation and DSB repair. These findings highlight the potential of replication stress inducers and PLK1 inhibitors as a combination treatment in cancer chemotherapy.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 CONCLUSIONS

Data presented here provides evidence that even though DNA replication and the response to DNA damage in cancer cells has been studied and exploited for chemotherapeutic treatment, there are still elements of the replication and damage response processes that are poorly understood. We have endeavored to specifically explore the DNA damage response that is activated in cancer cells treated with chemotherapeutics that induce replication stress as a result of deoxynucleotide deprivation. By depriving cancer cells of deoxynucleotides necessary for DNA synthesis, the cells can no longer complete replication, causing the cells to arrest and to initiate response and repair cascades to attempt repair of the damage. It is the response to the damage and repair pathway selection that can be targeted for disruption to promote cancer cell death.

Certain cellular responses such as the phosphorylation of histone variant H2AX occur in response to multiple forms of DNA damage (Celeste, 2002). Though the induction of γH2AX is strongly associated with DNA DSBs sustained from IR treatment, our lab and others have shown that treatment with TS inhibitors also induce γH2AX (Ward and Chen, 2001; Yang, 2008; Luo, 2008). TS inhibition has been shown to induce pRPA, which is a marker for stalled replication forks (Yang, 2008; Yang, 2012). The question then asked was whether the damage caused by TS inhibition is perceived by the

cell as a stalled replication fork (pRPA) or as a DSB (γH2AX). Using the TS inhibitor FdUrd to induce replication stress by deoxynucleotide deprivation, our data shows that H2AX is dispensable in the response to TS inhibition: H2AX mutation, reduction, or deficiency does not alter the sensitivity to FdUrd treatment or inhibit the formation of RAD51 foci. However, in two different cell lines, the kinetics of RPA phosphorylation was quickened in cells that lack H2AX, indicating that H2AX may play a role in repair pathway selection in response to deoxynucleotide deprivation.

When a protein is found to be over-expressed in cancer cells and sustains a critical cellular function, that protein is often examined for potential as drug target. For example, the human epidermal growth factor HER2 was found to be over-expressed in multiple forms of breast cancer and conveys a survival advantage by inhibiting apoptosis (Ménard, 2003). Oncogenic PLK1 is over-expressed in multiple forms of cancer and canonically functions to regulate mitosis. Several recent publications indicate an additional role for PLK1 in overcoming replication stress, including the phosphorylation of key proteins (Trenz, 2008; Song, 2011; Yata, 2012; Ragland, 2013). Thus, PLK1 is an ideal target for drug development due to its over-expression in cancer and multiple functions. Using the RNR inhibitor HU to induce replication stress and BI-2536 to inhibit PLK1, our data shows that this co-treatment reduces PLK1 expression, RPA phosphorylation is reduced and the formation of RAD51 foci appear to be reduced in size and number in comparison to HU only treated cells. Decreased RAD51 foci formation is in agreement with published data (Yata, 2012). The evidence showing RPA as a probable phospho-substrate of oncogenic PLK1 is a novel finding.

The research presented here enhances the knowledge of how cancer cells respond to replication stress induced by deoxynucleotide deprivation and the novel actions of well-studied proteins in response to this type of stress. TS and RNR inhibitors have been used in chemotherapy for a number of years but the finding that RPA phosphorylation kinetics are altered in response to H2AX status shows that there is still more to learn about the damage response to this class of drug. As well, the finding that pairing a drug that induces deoxynucleotide deprivation with a PLK1 inhibitor decreases damage response and repair signals indicate that combination treatments deserve more attention in both the laboratory and in clinical trials.

4.2 Future Directions

Several points have not yet been investigated in this project that would further clarify the response and repair pathways activated in response to deoxynucleotide deprivation. In the future, efforts will be focused primarily on oncogenic PLK1: discovery of phospho-substrates within DNA replication stress, how the inhibition of PLK1 during replication stress influences cell repair or death decisions and the markers thereof, and how changes in the expression level of PLK1 potentially alter the response of cancer cells to DNA damage. This route of research is advantageous due to the number of PLK1 inhibitors that are currently in clinical trials or in development. Knowledge of the molecular responses of cancer cells to oncogenic PLK1 inhibition in combination with replication stress could then be exploited clinically to develop new therapeutic combinations.

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APPENDIX A – SUPPLEMENTAL DATA

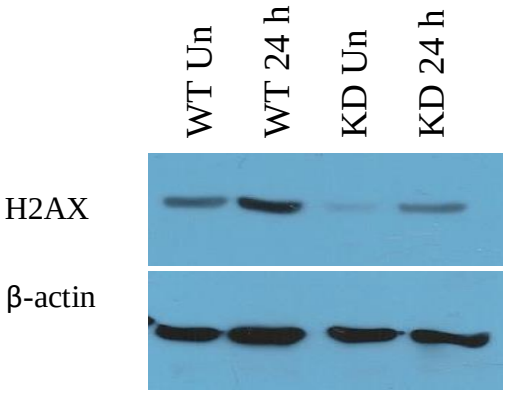
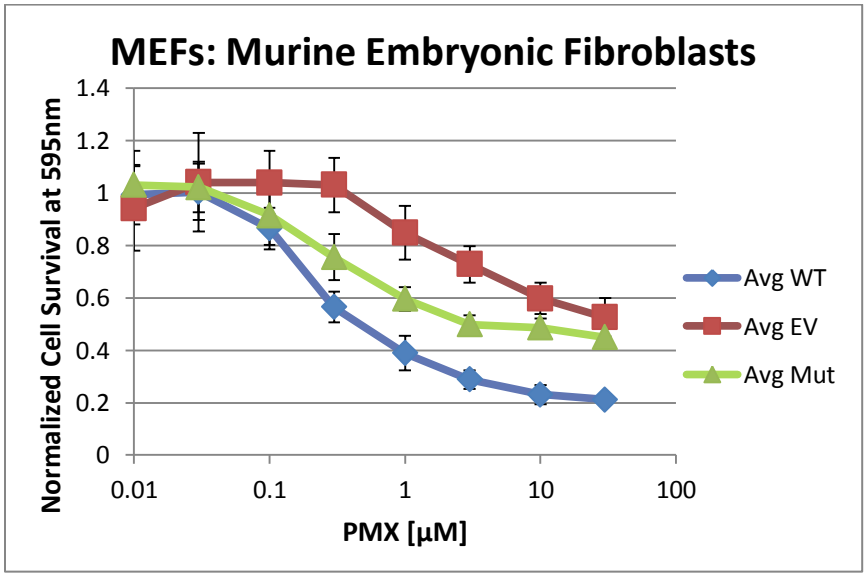


Figure A.1. H2AX knock-down in HCT116 by shRNA silencing. HCT116 H2AX-proficient (WT) and H2AX-reduced (KD) cells were treated for 24 h with 10 μM FdUrd and probed for H2AX. Comparing untreated (Un) WT and KD lanes, there is a reduction in total H2AX in the KD sub-line. We also noted that H2AX appears to be induced by FdUrd treatment. β-actin is shown as a loading control.



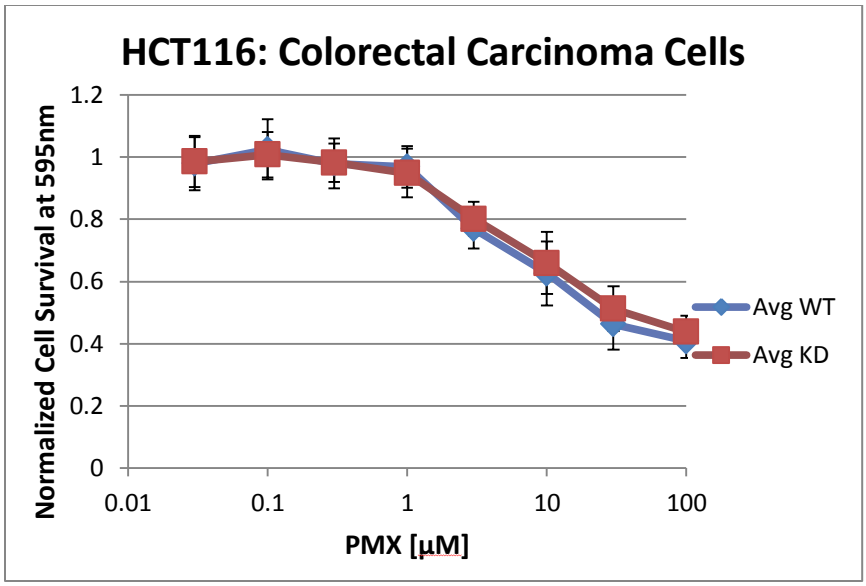
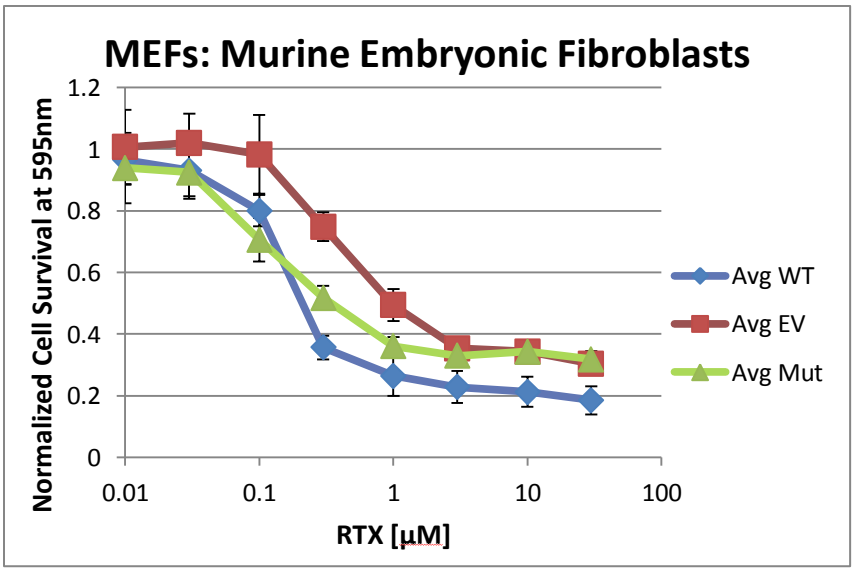


Figure A.2. Cell survival assays using alternative TS inhibitor Pemetrexed (PMX) that induces deoxynucleotide deprivation. MEF and HCT116 cells were treated for 24 h with PMX, followed by 3 days in drug-free media. The survival curves for the MEFs indicate that the response to TS inhibition by PMX may depend on H2AX; however, HCT116 cells are not affected by decreased H2AX expression. PMX has other targets other than TS, which may account for the differences in toxicity seen in the MEFs. N=3.



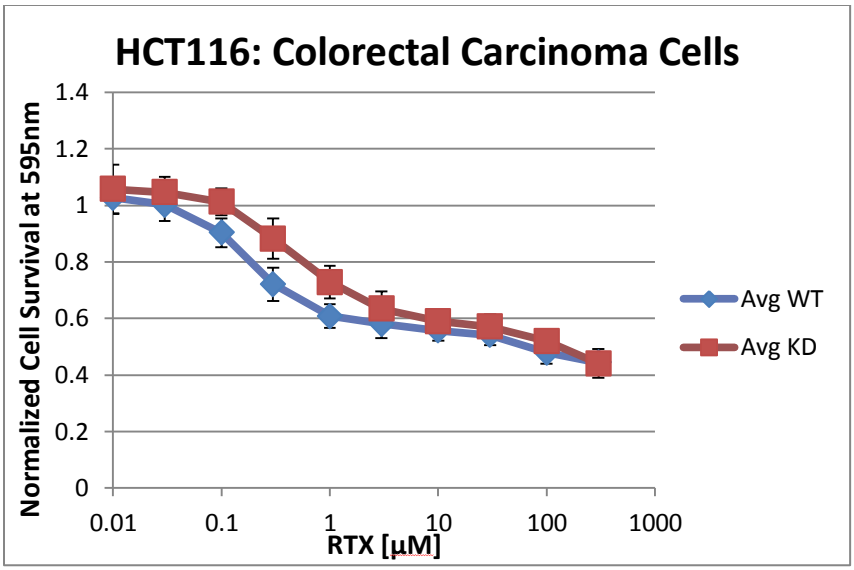


Figure A.3. Cell survival assays using alternative TS inhibitor Raltitrexed (RTX) that induces deoxynucleotide deprivation. MEF and HCT116 cells were treated for 24 h with RTX, followed by 3 days in drug-free media. The survival curves for the MEFs indicate that the response to TS inhibition by RTX may depend on H2AX; however, HCT116 cells are not affected by decreased H2AX expression. RTX has other targets other than TS, which may account for the differences in toxicity seen in the MEFs. N=3.

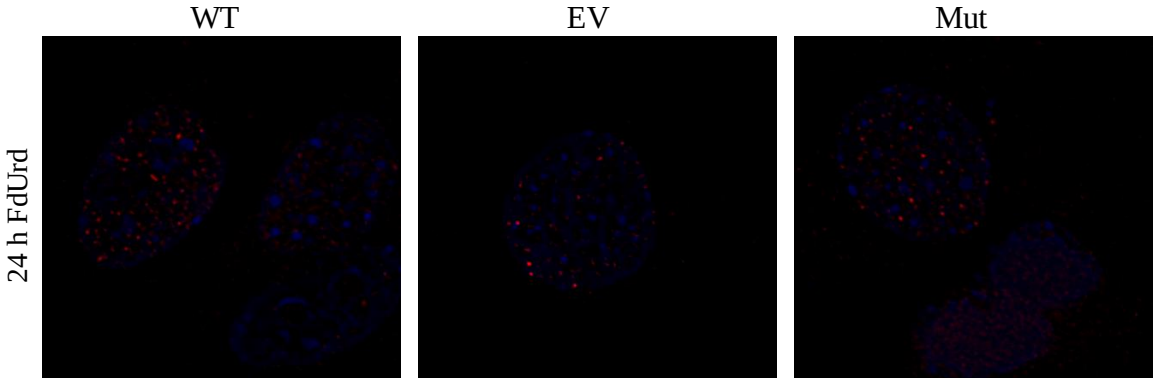


Figure A.4. Overlay of RAD51 and DAPI in MEFs. To show that the RAD51 foci that formed in response to 24 h of 10 µM FdUrd treatment were located in the nucleus, the images for RAD51 (Red) and the DAPI-stained DNA (Blue) were overlaid using the MetaMorph imaging software. As shown above, the RAD51 foci are located within the nucleus.

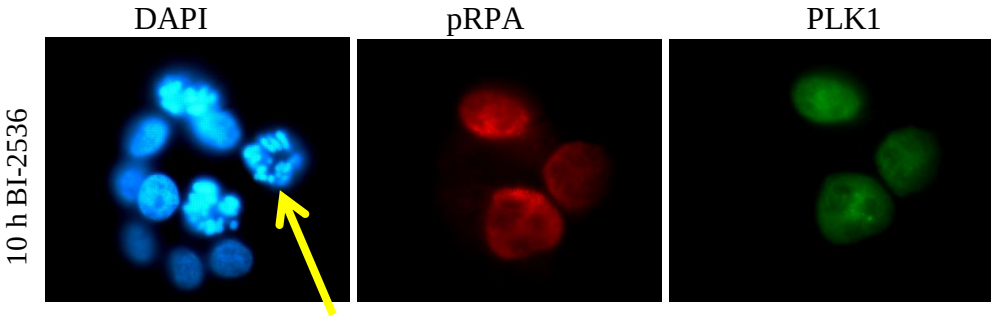


Figure A.5. Immunofluorescent staining of HT29 cells treated with BI-2536. HT29 cells were treated for 10 h with 30 nM BI-2536. Cells arrested in mitosis (yellow arrow) had condensed chromosomes, pan-nuclear staining of pRPA, and pan-nuclear staining of PLK1 with localization around the ends of the chromosomes. DNA: Blue, pRPA: Red, and PLK1: Green. Images taken at 100x on the Nikon Eclipse E600 microscope.