THE ROLE OF POLYMERASE η IN PROTECTING AGAINST GENOME INSTABILITY AND TELOMERE DEFECTS CAUSED BY THE GENERATION OF ENVIRONMENTALLY RELEVANT DNA LESIONS

by

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ABSTRACT

Telomeres, the protective caps at chromosome ends, shorten with age in most human cell types, but may be shortened prematurely by DNA damaging agents. Defective telomeres contribute to aging-related diseases and may give rise to genomic alterations implicated in carcinogenesis. Translesion DNA synthesis is a critical cellular mechanism that ensures progression of DNA replication forks, most notably, in the face of bulky DNA lesions. Numerous environmental exposures generate bulky lesions, such as ultraviolet (UV) light and hexavalent chromium (Cr(VI)). Translesion synthesis polymerase η's (polη) role in protecting against UV-induced lesions in the genome has been extensively documented, but its role at telomeres is unknown. Additionally, UV-induced lesions have been shown to form at telomeres. Chronic inhalation of Cr(VI) induces respiratory diseases associated with aging and telomere dysfunction, including pulmonary fibrosis and cancers, and our previous work established that Cr(VI) causes telomere damage. However, the mechanism(s) by which environmental genotoxicants promote telomere loss and defects is unknown. We investigated roles for polη in

preserving telomeres following acute physical UVC exposure and chronic chemical Cr(VI) exposure. Similar to its role in protecting against UV-induced DNA damage, we report that polη protects against cytotoxicity and DNA replication stress caused by Cr(VI). Our study supports a novel role for translesion DNA synthesis in preserving telomeres after UVC and Cr(VI) exposure and genotoxic stress. We uncover a mechanism by which environmental genotoxicants alter telomere integrity, and a fundamental cellular pathway that preserves telomere function in the face of genotoxic replication stress. Telomere alterations and dysfunction have been shown to impact human health. This research is significant and relevant to public health because knowledge gained will be useful for designing intervention therapies that preserve telomeres in human populations following exposure to environmental genotoxicants. The hope is that preventative measures will inhibit or delay diseases and pathologies related to telomere defects.

TABLE OF CONTENTS

LIST OF FIGURES

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This work is dedicated to my husband, Marco Varsalona, whose strength and support made it possible and worth it.

ABBREVIATIONS

6-4 PP	(6-4) pyrimidine-pyrimidone
8-oxoG	8-oxoguanine
AFM	atomic force microscopy
ALT	Alternative lengthening of telomeres
ATLD	Ataxia telangiectasia like disorder
ATR	Ataxia Telangiectasia and Rad3-related Protein
ATRIP	ATR interacting protein
ATM	Ataxia Telangiectasia Mutated
BLM	Bloom Syndrome protein
BS	Bloom syndrome
CAB-box	Cajal boby-box motif
Cdc	Cell-division cycle protein 13
CFS	Common fragile sites
CPD	cyclobutane pyrimidine dimer
Cr	chromium
CST	Cdc13, Stnl, Ten1
d-loop	displacement loop
DBDCI	5,5' –dibromo-4'4-dichloro-indigo
DDR	DNA damage response
DNA-PK$_{cs}$	DNA-dependent protein kinase, catalytic subunit
ds	double strand
DSB	double-strand breaks
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2´-deoxyuridine
EPA	US Environmental Protection Agency
HR	homologous recombination
IARC	International Agency for Research on Cancer
KD	knock down
MMR	mismatch repair
MRN	Mre11-Rad50-Nbs1
MutS/L/H	Mutator
NHEJ	non-homologous end joining
PCNA	proliferating cell nuclear antigen
PEL	permissible exposure limit
PIKK	phosphatidylinositol 3-kinase-related kinase
Polη	Polymerase η
POT1	Protection of Telomeres Protein 1
pRB	Retinoblastoma protein
RAD	RecA-like homolog

RAP1	Ras-related Protein 1
ROS	reactive oxygen species
RPA	Replication Protein A
RTEL	Regulator of Telomere Length
sh	short hairpin
SMARD	single molecule analysis of replicated DNA
ss	single strand
SSB	single strand breaks
SV	Shuttle vector
t-loop	telomeric loop
TERC	Telomere RNA Component
TERRA	Telomeric Repeat containing RNA
TERT	Telomere Reverse Transcriptase
TFIIH	Transcription factor II Human
TIN2	TRF1 Interacting Nuclear factor 2
TLS	translesion synthesis
TPP1	POT1-TIN2 Organizing Protein
TRF1	Telomeric Repeat binding Factor 1
TRF2	Telomeric Repeat binding Factor 2
UV	ultraviolet light
WRN	Werner syndrome protein
WS	Werner syndrome
Wt	Wild type
XPA	Xeroderma pigmentosum group A
XPC	Xeroderma pigmentosum group C
XPF	Xeroderma pigmentosum group F
XPG	Xeroderma pigmentosum group G
XPV	Xeroderma pigmentosum group Variant

1.0 INTRODUCTION

1.1 Telomeres

In 2009 the Nobel Prize for physiology and medicine was awarded to three scientists (Elizabeth Blackburn, Carol Greider, and Jack Szostak) for their research that uncovered the DNA sequence of telomeres, how telomeres protect the chromosome from degradation, and the enzyme telomerase that adds tandem TTAGGG nucleotides to the 3' end of telomeric DNA. The Nobel Prize represents recognition by the scientific and non-scientific community that telomeres have a critical role in aging and disease. Telomere structure and function are essential for maintaining genomic stability of each cell. Maintaining telomere integrity is fundamental to cell viability and a key determinant in the survival and health of the organism.

1.1.1 Telomere Structure and Function

Human telomeres cap chromosome ends with 5-15 kilobases (kb) of repetitive sequences (Fig. 1A). Telomere length is organism dependent. Laboratory mouse (*Mus musculus*) telomeres far exceed human telomeres in length, averaging 40-50 kb. These tandem repeats protect the genome from shearing and degradation (Friedberg et al., 2006). Mammalian telomeres consist of a 3' G-rich strand and a C-rich 5' strand. The

G-rich strand has six tandem repeats of 5' TTAGGG, while the C-rich strand compliments it with 5' CCCTAA sequences. Telomeres are duplex strands of DNA except for the end of the G-rich strand which terminates in a 150-200 nucleotide long single-strand overhang (McElligott and Wellinger, 1997). The G-rich overhang is capable of looping backwards onto itself to form a telomeric-loop (t-loop), invading the duplex DNA, and annealing to form a displacement-loop (d-loop). The t-loop and d-loop structures were observed in mouse models and human cells (Griffith et al., 1999) and may only be present on a small percentage of telomeres, yet they illustrate how telomeres provide protection against genome instability. The t-loop transforms the double-stranded genome ends from a structure that could otherwise be recognized as double-stranded breaks (DSB) (Griffith et al., 1999; Palm and de Lange, 2008) into a lasso-like structure that embeds the single strand overhang into the duplex DNA.

Telomeres function to preserve chromosome integrity and regulate the number of divisions a cell can undergo. On one hand, the presence of telomeric tandem repeats safeguards chromosomes from loss or degradation of genomic DNA sequences. On the other hand, small portions of telomeric DNA are lost with every cell cycle due to the inability to completely replicate the end of the chromosome (Wright and Shay, 2000). Shortening of the telomere eventually leads to a critically short telomere and to loss of the shelterin protein complex (a binding complex essential for telomere structure), and promotes a DNA damage response (DDR) at the telomere. Telomeres that are not protected by the shelterin complex become indistinguishable from DSBs, thereby signaling DNA repair pathways called homologous recombination (HR) or non-

homologous end joining (NHEJ) that can cause telomere end-to-end chromosome fusions (Palm and de Lange, 2008; Sabatier et al., 2005; Zou et al., 2009). Loss of telomeres and telomere defects can lead to irreversible cell arrest (called cell senescence) or apoptosis (Paeschke et al., 2010). Worth noting, telomeres also have secondary roles in the suppression of transcription for nearby genes (Gottschling et al., 1990), and they impact DNA replication origins (Ferguson and Fangman, 1992).

1.1.2 Shelterin Protein Complex

The duplex and the single stranded DNA (ssDNA) of telomeres are bound by a specific complex of six proteins called shelterin; TTAGGG- repeat binding factor 1 (TRF1), TRF2, protection of telomeres 1 (POT1), transcriptional repressor/activator protein (RAP1), TRF1 interacting protein 2 (TIN2), and POT1 and TIN2 organizing protein (TPP1) (Palm and de Lange, 2008; Xin et al., 2008) (Fig. 1B). Each protein has a unique role that contributes to the function of chromosomal end capping. TRF1 and TRF2 bind the duplex region of the telomere. TRF1 has been found to facilitate telomere replication possibly by mediating BLM and RTEL helicase recruitment (Martinez et al., 2009; Sfeir et al., 2009). TRF2 has roles in maintaining the 3' overhang (Zhu et al., 2003). POT1 binds the single-stranded overhang and regulates access of telomerase to the overhang (Lei et al., 2005). Conversely, TPP1 facilitates recruitment of telomerase to the 3' overhang (Abreu et al., 2010; Latrick and Cech, 2010; Zaug et al., 2010). TIN2 stabilizes the protein complex (Ye et al., 2004).

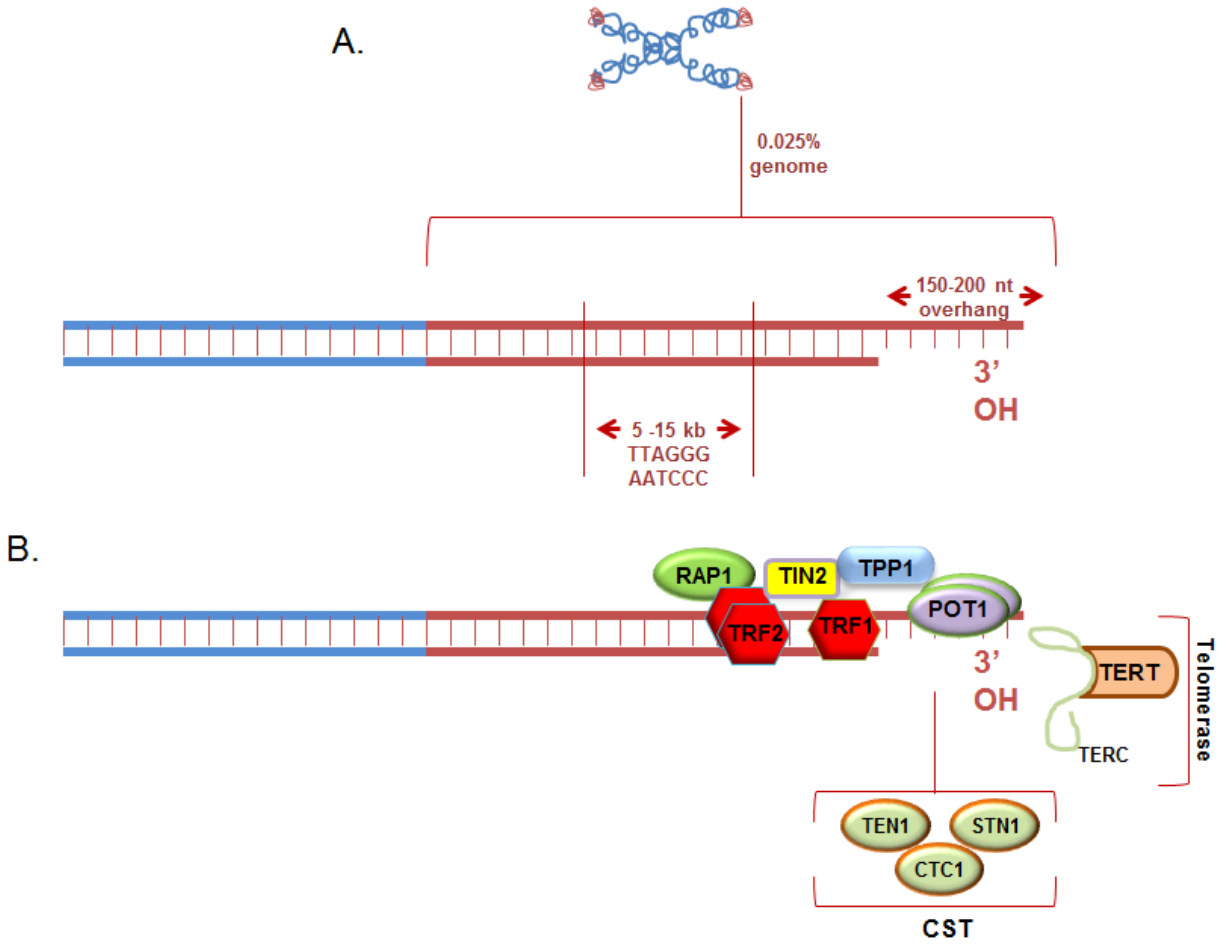
The combined functions of individual shelterin proteins protect telomeres from DNA damage repair proteins. POT1 association with the 3' overhang is essential for the inhibition of ataxia-telangiectasia mutated and Rad3-related (ATR) activation (Denchi and de Lange, 2007). In the absence of POT1, the 3' overhang is recognized as ssDNA which leads to replication protein A- (RPA) mediated recruitment of ATR. ATR activation promotes DNA damage checkpoint activation and causes cell cycle arrest (Cortez et al., 2001; Costanzo et al., 2003). Additionally, ataxia-telangiectasia mutated (ATM) is activated at DNA double strand breaks, but is inhibited at telomeres by the presence of TRF2 (Denchi and de Lange, 2007). Therefore, the dismantling of the shelterin complex from the telomere renders telomeres vulnerable to inappropriate processing by DNA repair mechanisms. Preventing ATR and ATM activation at the telomeres is necessary to protect telomeres from being recognized as DSBs that will result in the improper induction of mechanisms such as non-homologous end joining (NHEJ) that can cause telomere fusions.

1.1.3 Telomerase

Telomerase is a telomere binding ribonucleoprotein that has a role in telomere maintenance and cancer biology. The function of telomerase is conserved in most eukaryotes. The enzyme telomerase is a reverse transcriptase responsible for the elongation of the 3' single-stranded overhang of telomeric DNA (Fig. 1B). The structure of telomerase consists of two subunits; a non-coding RNA molecule, *hTERC*, that functions as the telomeric template during synthesis, and hTERT (telomerase reverse transcriptase) responsible for extending the telomere by reverse transcribing the

telomeric template. The hydroxyl group at the extreme end of the 3' overhang is recognized by telomerase and extends DNA in the 5'-to-3' direction. Telomerase RNA includes a CAB-box motif that facilitates binding with Cajal bodies, which enrich and facilitate telomerase association with the telomeres in S-phase (Cristofari et al., 2007; Jady et al., 2006; Tomlinson et al., 2006). Tcab1 operates at the CAB-box motif mediating telomerase recruitment to the 3' hydroxyl group of telomeric DNA (Venteicher et al., 2009).

While germ cells express telomerase, normal somatic and adult stem cells do not express enough telomerase to effectively maintain telomere length (Harley et al., 1990). Thus, each time the cell undergoes DNA synthesis in S-phase, telomeres lacking telomerase will shorten. Aging is linked to telomere degeneration since critically short telomeres promote cell senescence and genome instability (Blasco, 2005).



(A) Human telomeres contain duplex DNA and a ssDNA region called the 3' overhang. (B) Shelterin protein complex is comprised of six main proteins. TRF1 and TRF2 bind the telomeres at the double stranded region and POT1 binds the ssDNA overhang. Telomerase accesses telomeres through the 3' hydroxyl group on the end of the overhang. Telomerase constitutes to subunits, TERT and TERC.

Figure 1. Human telomere and human telomere binding proteins.

1.1.4 Telomere Replication

Replication at telomeres is unique in the genome since it involves replication of repetitive sequences, generation of the 3' overhang, and possible structure assembly and disassembly of loops. As a result, the telomere replication machinery requires the function of proteins in addition to the conventional replication machinery; BLM, WRN, and RTEL helicases, TRF1, and the CST complex (Crabbe et al., 2004; Ding et al., 2004; Price et al., 2010; Saharia et al., 2010; Sfeir et al., 2009). The helicase involvement during telomere replication is critical. They function to unfold ternary structures called G-quadruplexes (Vannier et al., 2012). These guanine tetrads form when replication takes place at single-stranded G-rich DNA by transiently folding upon themselves to form four stranded DNA molecules (Parkinson et al., 2002). Specialized helicases are also believed to be responsible for disassembly of the t-loop structure (Griffith et al., 1999).

At chromosome ends, telomeres cannot be replicated in their entirety due to a phenomenon known as the end replication problem (Olovnikov, 1971). Replicative DNA polymerases initiate DNA synthesis from an RNA primer on the template. These primers anneal to the template DNA sequences upstream of each initiation site. DNA replication is bidirectional but DNA polymerases are unidirectional and require a 3' hydroxyl group for nucleotide incorporation. Therefore, when replication proceeds on the leading strand in the 3' → 5' direction, DNA synthesis is continuous following extension from the 3' hydroxyl group of the RNA primer. However, when replication

occurs on the opposite lagging strand in the 5' → 3' direction, it requires synthesis of small discontinuous daughter strands, known as Okazaki fragments, from multiple RNA primers that are later joined by ligase. When the final RNA primer is removed from the extreme end of the lagging DNA strand, a 3' single strand overhang is left that cannot be duplicated. Since this extreme end cannot be replicated, approximately 50-200 telomere base pairs are lost with each round of DNA replication (Olovnikov, 1971).

The end replication problem causes telomere shortening, but it does function to efficiently restore the 3'overhang on the newly synthesized telomere formed from the lagging strand. The newly synthesized telomere deriving from the leading strand, on the other hand, requires a unique process to generate the 3'overhang. Studies have shown that telomere end resection begins by recognition of the telomere end as a double-strand break (DSB) by the MRN complex in humans (Longhese et al., 2010). Nucleases and helicases then cleave the 5' strand leaving a 3' single strand (Mimitou and Symington, 2009).

1.1.5 Telomere Aberrations

The progressive loss of telomeric repeats or shelterin proteins leads to defective telomeres. Chromosomal Telomere Fluorescent In Situ Hybridization (Telomere FISH) is a technique that has been instrumental in identifying several types of defective telomeres and in linking telomere aberrations to chromosome instability and tumorigenesis (Gollin, 2005; Murnane and Sabatier, 2004; Soler et al., 2005). Telomere loss or critically short telomeres are aberrations caused by cellular aging or by DNA

damage to telomeres. One model is that unrepaired DNA damage at telomeres could permanently block replication forks and cause collapse of the forks into a chromosomal break. Telomere doublets are characterized as two telomere foci arranged on one chromatid end. Although the mechanism that results in this phenotype is not clearly understood, doublets are associated with unresolved replication stress (Sfeir et al., 2009). They are hypothesized to arise from single stranded gaps in the telomeres or from fragmented telomeric DNA (Sfeir et al., 2009) representing more than one site of activated replication (origin firing). Telomere fusions are caused by inappropriate NHEJ at critically short telomeres (Maser and DePinho, 2004). In fact, TRF2 inhibition promoted NHEJ and resulted in telomere fusions and chromosome end-to-end fusions (Smogorzewska et al., 2002). Telomere fusions are observed as overlapping telomeric foci at the ends of either two chromosomes or two sister chromatids. Unlike telomere loss and telomere doublets that arise from replication stress in S-phase, telomere fusions are observed after mitosis.

1.1.6 Telomeres and Human Disease

Cell division in somatic cells decreases in frequency overtime and cells enter a state of replicative senescence (Chretien et al., 2008). Telomere shortening is the principle mechanism responsible for generating replicative senescent cells and can be prevented through the expression of telomerase (Bodnar et al., 1998). Critically short telomeres that are not rescued by telomerase or the alternative lengthening of telomeres pathway (ALT; an alternate telomere elongation pathway mediated through recombination) will ultimately activate the tumor suppressor proteins p53 or pRB, which then trigger the

induction of replicative senescence (Campisi and d'Adda di Fagagna, 2007; Feldser and Greider, 2007). In the absence of functional p53 or pRB, critically short telomeres promote genomic instability and may ultimately lead to apoptosis or malignant transformation (Hemann et al., 2001). While senescence contributes to aging-related diseases and premature aging disorders through the loss of regenerative capacity of degeneration in tissues, overriding senescence can lead to carcinogenesis (Campisi, 2001)

Several genetic disorders and diseases have been associated with defects in telomere maintenance or in essential telomere binding proteins. Accelerated telomere shortening due to mutations in telomerase or telomere associated genes leads to a spectrum of telomere shortening syndromes including dyskeratosis congenital, aplastic anemia, Hoyerall-Hreidarsson syndrome, pulmonary fibrosis and liver disease (reviewed in (Blasco, 2005)). BLM and WRN are RecQ helicases that have critical roles in telomere replication (Croteau et al., 2014). Bloom syndrome (BS) and Werner syndrome (WS), are caused by mutations in the genes that encode for helicases BLM and WRN, respectively, and both syndromes exhibit accelerated telomere loss. WS is characterized by premature aging (Gray et al., 1997) while BS is most notably characterized by short stature and a predisposition to a broad spectrum cancers (German, 1995). Ataxia telangiectasia (AT) leads to severe neurodegeneration and accelerated telomere shortening has been associated with the disease (Metcalfe et al., 1996). Seckel syndrome and ataxia telangiectasia like disorder (ATLD) have been

characterized with DNA repair signaling dysfunction that also includes dysfunctional telomere maintenance (Pennarun et al., 2010).

1.2 Replication Stress

Replication stress is defined as the slowing or delay of DNA replication fork advancement (Zeman and Cimprich, 2014). Many factors can obstruct the replication fork and cause stress. Typically, the DNA double helix continues to be unwound by replicative helicases while the replication machinery is inhibited at a physical obstruction on one strand, which then leads to an accumulation of ssDNA (Pacek and Walter, 2004). RPA binds ssDNA and prevents it from forming hairpins or other secondary structures (Wold, 1997), however, persistent RPA binding to ssDNA activates ATR kinase. ATR is a serine/threonine protein kinase in the phosphatidylinositol 3-kinase-related kinases (PIKKs) family. ATR, and its binding protein ATRIP (ATR interacting protein) have roles in DNA damage checkpoint activation (Abraham, 2001). ATR is one of the first proteins recruited to sites of replication stress in the DNA damage signaling cascade. ATR phosphorylates various proteins that function in the recovery of stressed DNA replication forks and is required for the G2 checkpoint activation (Cortez et al., 2001). The mechanism by which ATR arrests cell cycle progression is through inactivating Cdc2 (Shechter et al., 2004b) and Cdc7 (Costanzo et al., 2003); two S-phase kinases that are essential for replication origin firing. ATR inactivates these proteins by phosphorylating Chk1, which in turn phosphorylates Cdc25a and leads to the suppression of Cdc2 (Shechter et al., 2004a) and Cdc7 (Costanzo et al., 2003).

Once ATR and its downstream substrates have completed their functions and the replication stress has been overcome either through DNA repair mechanisms, fork rescue, or translesion synthesis, the replication fork can resume DNA synthesis (Petermann and Helleday, 2010).

1.2.1 Consequences of Replication Stress

Although the cell has multiple mechanisms capable of restoring a stressed replication fork, there are circumstances that do not permit successful recovery. Examples of such circumstances include mutated or loss of DDR proteins, or failed DDR signaling or restart mechanisms. Regions on the genome that are difficult to replicate, such as common fragile sites (CFS) are more vulnerable to the consequences of replication stress (reviewed in (Debatisse et al., 2012)). Unresolved replication stress can lead to fork collapse and chromosome breaks, destabilization of the genome and can ultimately lead to disease (Friedberg et al., 2006). Unreplicated DNA inhibits separation of sister chromatids and creates fused chromatid bridges during anaphase (Mankouri et al., 2013). The tension of the fused chromatids will cause chromatid breaks through displacement of uneven chromosomal arms and will result in chromosomal rearrangements and deletions. It is believed that these abnormal structures are cleaved by nucleases in order to avoid more deleterious consequence of fused chromatids (Naim et al., 2013). Several diseases are linked to the inability, or reduced efficiency, to resolve replication stress. Mutations in the genes that encode for ATR or ATRIP cause in Seckel syndrome, which is characterized by growth retardation, dwarfism, microcephaly, and mental retardation (reviewed in (Zeman and Cimprich, 2014))

1.3 DNA Damage Response (DDR)

In the interest of genome integrity, the cell has evolved multiple mechanisms to recognize and repair damaged DNA. DNA damage ensues spontaneously from endogenous sources such as metabolic processes, and from exogenous genotoxic environmental exposures and medically-related treatments including chemotherapeutic agents. DNA damage response (DDR) mechanisms are designed to remove damaged regions of the genome or to mitigate the deleterious effects of these regions and restore correct DNA sequences or DNA structure (Friedberg et al., 2006). The mechanisms that have been identified can be classified according to their general function. Excision repair mechanisms are employed to remove chemically modified or incorrect bases or nucleotides and to restore correct DNA sequences. These mechanisms include base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Cells that experience breaking in the sugar-phosphate backbone that results in single strand breaks or DSBs, will induce homologous recombination repair mechanisms, or rejoining mechanisms, such as NHEJ (Friedberg et al., 2006). Finally, DNA damage tolerance mechanisms allow for the persistence of DNA lesions in the genome during replication that can then be repaired at a later time point. There are various identified tolerance mechanisms which include recombinational repair, replication fork regression, and translesion synthesis (TLS).

1.3.1 Basic Excision Repair (BER)

BER is believed to be the most utilized DNA repair mechanism by the cell and targets endogenous damage due to reactive oxygen species (ROS) or other metabolites (Friedberg et al., 2006). Enzymes called DNA glycosylases recognize and catalyze lesion-specific excision. An abasic (AP) site is generated and signals removal by AP endonucleases that incise or nick the dsDNA via hydrolysis. Hydrolysis takes places at the phosphodiester bond 5' to the AP site resulting in a 5' terminal deoxyribose-phosphate residue. DNA-deoxyribophosphodiesterase (dRpase) enzymes are activated to cleave the 5' residue paving the way for DNA synthesis to restore the correct nucleotides and DNA ligation to seal the nicks.

1.3.2 Nucleotide Excision Repair (NER)

NER is one of two mechanisms identified for the excision of UV-induced bulky DNA adducts; cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidones (6-4 PP) (Friedberg et al., 2006). The multi-step process begins by recognition of the helix distortion by XPC-RAD23B. Once this protein complex binds to the helix, another complex, TFIIH, XPA, RPA, and XPG, is signaled to the site in order to create a pre-incision structure. This complex of proteins unwinds 25-30 base pairs in the helix around the proximity of the lesion. XPG is then triggered to cleave the DNA at the 3' end of the damage site, while ERCC1-XPF incises at the 5' end. The cleaved fragment is then freed from the helical structure and a single-stranded gap is generated. Next,

the DNA polymerase holoenzyme synthesizes new DNA to close the gap and DNA ligase seals the strands together.

1.3.3 Mismatch Repair (MMR)

MMR is signaled into action upon the generation of DNA replication errors such as insertions, deletions, mis-incorporation of single bases, and small deletion loops (Kolodner and Marsischky, 1999). It also has a role in assisting HR repair to achieve an error-free repair mechanism due to its ability to proofread DNA synthesis. The MutSα complex (MSH2 and MSH6) is responsible for the recognition of mismatched base pairs, and can efficiently recognize even a single mismatch replication error (Acharya et al., 1996; Genschel et al., 1998). MMR has evolved the ability to distinguish the parent strands from the daughter strands during DNA synthesis. Studies have observed that nicks on the leading DNA strand may serve to provide the signal for the MMR proteins. The MutSβ complex (MSH2 an MSH3), then corrects the replication errors. Next, the MutLα complex (MLH1 and PMS2) is recruited to the MutSα complex and they jointly excise the region of ssDNA containing the mismatch (Kolodner and Marsischky, 1999). DNA polymerase performs new DNA synthesis and DNA ligase seals the helix.

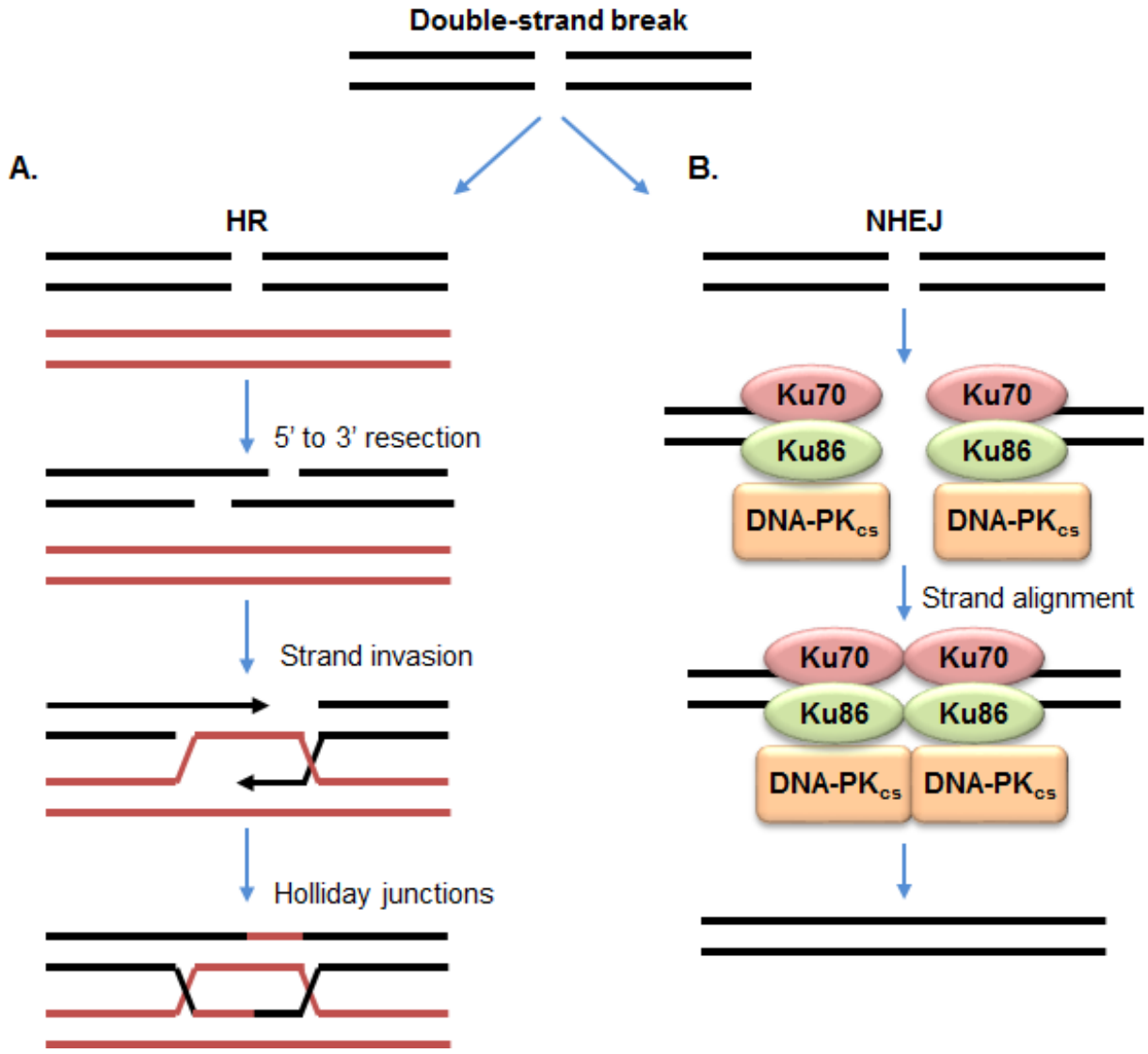
1.3.4 Homologous Recombination (HR)

HR is one of two distinct repair mechanisms that responds to DSBs. First, DNA at the broken ends is resected in a 5' → 3' direction (Fig. 2). Next, the single stranded 3' ends invade neighboring duplex DNA of a homologous sequence, present either in a sister

chromatid or a homologous chromosome, resulting in a displacement loop (D-loop). MMR proofreads the base pairing between the invading strand and the newly selected template sequence, and if significant differences are detected, the process is discontinued. If the sequences are complementary, then DNA polymerases extend the invading strand resulting in the formation of a Holliday junction. If strand invasion occurs from both DNA ends, then processing will lead to the formation of two Holliday junctions (reviewed in (Friedberg et al., 2006)). Repair is complete when the Holliday junctions are resolved.

1.3.5 Non-homologous End Joining (NHEJ)

NHEJ is another mechanism the cell uses to deal with DNA double strand breaks. The greatest difference between NHEJ and HR is that NHEJ does not rely on homologous sequences to repair the break. Therefore, HR is believed to be the more error-free mechanism of the two pathways. NHEJ is the more error-prone method since the process is tolerant of DNA deletions (Chu, 1997). The process begins with the Ku complex (Ku70 and Ku86) that binds the two broken ends of the duplex, and subsequently recruits DNA-PK$_{cs}$ kinase. The presence of Ku and DNA-PK$_{cs}$ on each end of the break leads to the alignment of the ends. DNA ligase IV then ligates the broken ends together (reviewed in (Bernstein et al., 2002)).



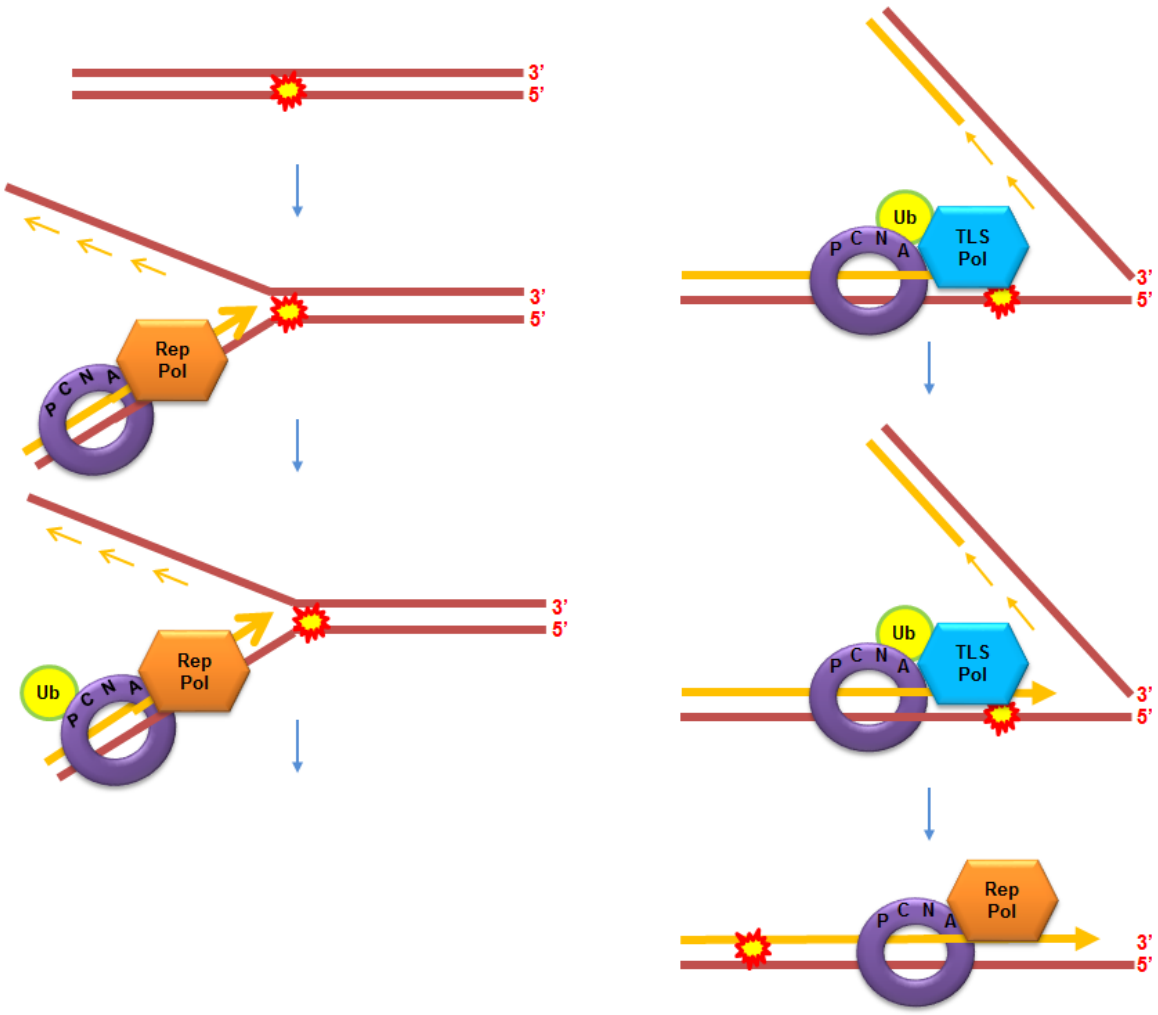
When DSB occurs at the DNA helix, the cell attempt to repair the damage. (A) An example of the major steps in the process of homologous recombination (HR). (B) An example of the major steps in the process of non-homologous end joining (NHEJ).

Figure 2. Two mechanisms for double-strand break repair.

1.3.6 Translesion Synthesis (TLS)

TLS is a DNA damage tolerance mechanism (Fig. 3). Most TLS polymerases are members of the Y family of DNA polymerases (Ohmori et al., 2001), which include Rev1, Polymerase κ, Polymerase η (polη), and Polymerase ι. Another important TLS polymerase is polymerase ζ, which is a member of the B family of polymerases. Different from the aforementioned mechanisms which function to repair damaged DNA, TLS bypasses the lesion, leaving it intact on the DNA helix (Friedberg et al., 2006). TLS spares the cell from more deleterious effects that can be caused by unresolved stalled replication forks. Persistent stalled replication forks lead to fork collapse, translocations, chromosome aberrations, and cell death. The precise mechanism of fork collapse into a chromosome break is unknown, but possibilities include loss of replisome components, nuclease digestion, or replication run-off (Zeman and Cimprich, 2014). The disadvantage of TLS compared to accurate DNA damage repair, is that TLS polymerases are generally more error-prone than replicative polymerases. TLS is often performed using mutagenic methods of base pair extension opposite the lesion. However, polη has efficiently evolved to function in the accurate bypass of UV-dimers (McCulloch et al., 2004). When the replication fork approaches an unrepaired lesion, the fork is blocked and unable to continue synthesizing DNA (Fig. 3). The processivity clamp proliferating cell nuclear antigen (PCNA) has a critical role in switching from the replicative polymerases to the TLS polymerases (Hoege et al., 2002). PCNA is monoubiquitinated by the catalytic activity of the Rad6-Rad18 complex, which initiates damage tolerance through TLS (Stelter and Ulrich, 2003). However, ubiquitination can

continue to polyubiquitination of PCNA thereby promoting a different tolerance pathway known as template-switching (Andersen et al., 2008). In the case of TLS, once the polymerases have been switched, the TLS polymerase will incorporate nucleotides opposite the lesion and continue DNA synthesis, thereby leaving the lesion intact. Next, PCNA again switches out the TLS polymerase and restores the replication polymerase to the fork. The replication machinery then continues synthesizing DNA.

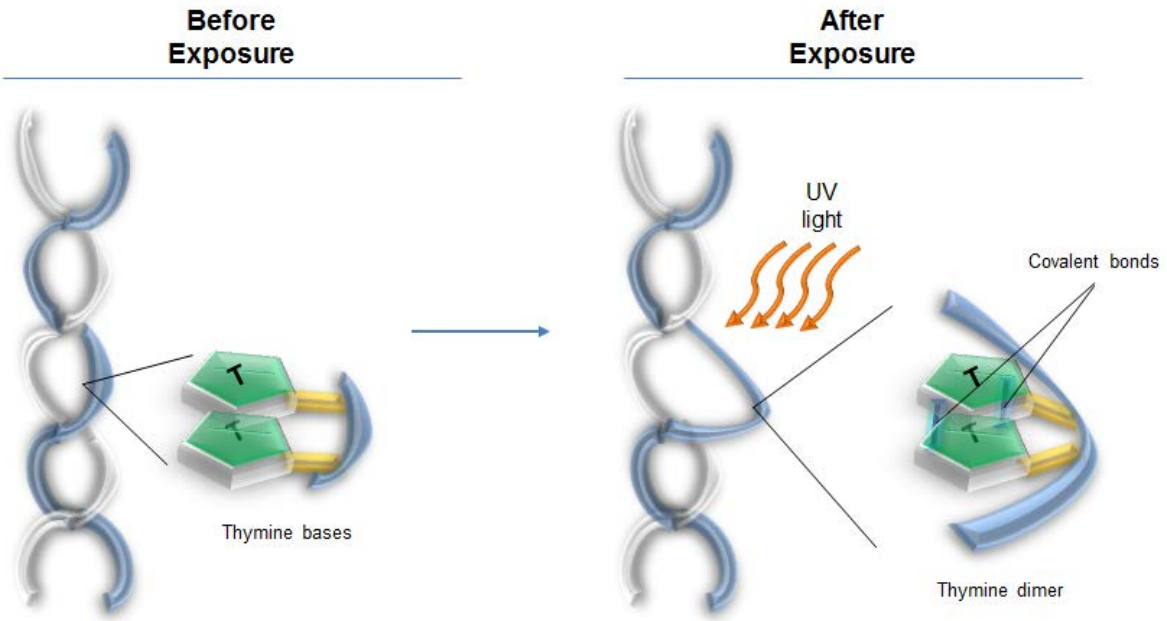


Translesion synthesis (TLS) is a mechanism by which DNA lesions are bypassed by a specialized polymerase in order to allow for continued progression of DNA replication fork. This processes is facilitated by the ubiquitination of PCNA, a DNA clamp that responsible for switching the replicative polymerase out and the TLS polymerase into position. The lesion remains unrepaired on the DNA after TLS

Figure 3. Translesion synthesis.

1.4 DNA Polymerase η (polη)

Polη and its yeast homolog Rad30 are highly conserved throughout eukaryotes. They are the most widely studied of the TLS polymerases. While TLS is often error-prone, polη efficiently bypasses *cis-syn* cyclobutane thymine dimers (McCulloch et al., 2004) (Fig. 4), accurately inserting adenines opposite the dimer and extending the primers a few nucleotides past the lesion (Masutani et al., 2000; McCulloch et al., 2004). Polη lacks this same efficiency with the other major UV-induced photoproduct, 6-4 PP. Pol η was observed to insert nucleotides opposite the thymines of 6-4 PPs *in vitro*, but was inefficient at bypassing these lesions (Masutani et al., 2000). Polη is homogenously distributed throughout the nucleus before activation but translocates in S-phase to sites of stalled replication forks in response to some genotoxic agents (Kannouche et al., 2001). Replication restart at stalled replication forks is believed to depend on polη (Lehmann, 2005).



Thymine dimer cartoon demonstrating a bulky lesion. UV irradiation generates two covalent bonds by reacting two adjacent thymines. Kinks form as a consequence of these bonds and distorts the helix.

Figure 4. Cyclobutane pyrimidine dimers after UV exposure.

1.4.1 Regulation of DNA Polymerase η

Despite polη's low fidelity of DNA synthesis on undamaged DNA templates, depletion of polη through the expression of targeted small interfering RNAs caused an increase in spontaneous DNA mutations in human cells that were not treated with any genotoxicants (Choi and Pfeifer, 2005). Mutations to *rad30* in *S. cerevisiae* did not alter the spontaneous mutation frequency compared to controls (McDonald et al., 1997; Roush et al., 1998). Furthermore, overexpressing polη did not alter mutagenesis rates in human cells, and insignificantly increased mutagenesis rates in *S. cerevisiae* (King et al., 2005; Pavlov et al., 2001). Collectively, these findings suggest that polη is tighly regulated in response to DNA damaged, and had limited access to undamaged DNA (Waters et al., 2009).

Ubiquitination affects polη is many important ways. First, polη interacts with the processivity clamp PCNA during TLS. This interaction takes place at the C-terminal PCNA-binding motif called the PNCA-interacting peptide (PIP) box (Kannouche et al., 2004) and is additionally mediated by polη's ubiquitin-binding zinc finger (UBZ) domain (Parker et al., 2007). Monoubiquitination of PCNA, strengthens the affinity between PCNA and polη. Although monoubiquitinated PCNA is not required for the recruitment of polη to stalled replication forks (Nikolaishvili-Feinberg et al., 2008), it is required for the accumulation of polη into nuclear foci (Plosky et al., 2006). Rad18, an E3 ubiquitin ligase, is believed to have a role in the recruitment of polη to stalled replication forks (Yuasa et al., 2006). As in the case of PCNA, polη accumulation into foci is dependent

on Rad18 (Yuasa et al., 2006). Additionally, Rad18 is involved is the monoubiquitination of PCNA. Lastly, there are studies that reported the monoubiquitination of polη via the UBZ domain (Bienko et al., 2005; Pabla et al., 2008), although the significance of this process is unclear.

1.4.2 Roles for DNA Polymerase η

Polη has also been studied in the context of other types of DNA damage and has been reported to successfully bypass a spectrum of DNA lesions. Polη bypasses 7,8-dihydro-8-oxoguanines rather accurately (Avkin and Livneh, 2002; Haracska et al., 2000b) and bypasses thymine glycols (Kusumoto et al., 2002), which provides evidence for a role in recovery from reactive oxygen species (ROS). This is significant as endogenous ROS is constantly generated during normal cellular functions. Polη has also been shown to function in the bypass of lesions formed from important environmental carcinogens such as (+)-*trans-anti*-benzo[*a*]pyrene-N^2-dG (Zhang et al., 2000), and O^6-methylguanine (Haracska et al., 2000a) and acetylaminofluorene-guanine adducts (Yuan et al., 2000). Polη also responds to adducts caused by chemotherapeutic agents. Polη deficient XPV cells are sensitive to cisplatin (Albertella et al., 2005; Chen et al., 2006) and oxaliplatin (Vaisman et al., 2000). While polη has a major role in bypass of a variety of genotoxic lesions, the enzyme is error-prone at regions of undamaged DNA (Matsuda et al., 2000).

In addition to polη's function as a TLS polymerase, two additional roles have been reported. First, polη has been found to function in gene conversion events in

chicken cells (Kawamoto et al., 2005) and second, polη has been observed to perform DNA synthesis from the invading strand of D-loop structures (McIlwraith et al., 2005). Polη function at D-loop structures implies a role in recombination. However, cell lines from XPV patients lacking polη do not exhibit a defect in recombination. Moreover, sister chromatid exchanges, which result from recombination, were observed at higher frequencies in SV40-transformed XPV cells, arguing against a role for polη in promoting recombination (Cleaver et al., 1999).

1.4.3 Xeroderma Pigmentosum Group Variant

Xeroderma pigmentosum (XP) is an autosomal recessive genetic disorder with eight variations; XPA, XPB, XPC, XPD, XPE, XPF, XPG, and XPV. XP was first reported in 1874 by a professor of dermatology in Vienna named Moriz Kaposi (reviewed in ((DiGiovanna and Kraemer, 2012). However, it was not until 1968 when James Cleaver first characterized the disorder for the excision repair deficient forms of XP (Cleaver, 1968) and 1971 when Burk *et al.* described the TLS deficient form, XPV (XP Variant) (Burk et al., 1971). Finally, the gene mutated in XPV, POLH, was identified in 1999 (Johnson et al., 1999; Masutani et al., 1999). Non-melanoma skin cancers occur in XP patients 10,000-fold more frequently than the rest of the population, and XP patients show a 2,000-fold increase in melanomas (Kraemer et al., 1994). With the exception of XPV, this disorder derives from mutations in genes that encode for proteins that are critical for NER. Mutations in polη causes Xeroderma Pigmentosum group variant (XPV) but these patients are proficient in NER (Johnson et al., 1999; Masutani et al.,

1999). Patients are characterized by an increased mutation frequency and high rates of skin cancers due to UV exposure (Friedberg et al., 2006). Although NER is active in these patients, normal cells utilize both NER and TLS to efficiently recover from UV-induced lesions. In the absence of polη, the cell may use another TLS polymerase, such as polymerase ι, which is error-prone in the bypass of UV dimers leading to increased mutagenesis and carcinogenesis (Tissier et al., 2000).

1.5 Chromium

1.5.1 Chromium Overview

Chromium (Cr) is an abundant, naturally occurring, transition metal that can be found in various oxidation states in soil, water, plants, and animals (Barnhart, 1997; Vitale et al., 1997). The most common oxidation states are Cr(0), trivalent chromium (Cr(III)), and hexavalent chromium (Cr(VI)). Cr(0) is generally stable and is found in alloy metal mixes, such as stainless steel. However, industrial methods of processing these alloys under high temperatures oxidize Cr(0) to Cr(III) and Cr(VI). Millions of people globally are occupationally exposed to Cr or compounds comprised of Cr (Cancer, 1990; Registry, 1993). Industries involving the production and use of the man-made form of Cr, Cr(VI), include welding, chrome plating, chrome pigmenting, ferrochrome production, and leather tanning (Fishbein, 1981). Only Cr(VI) is biologically available and thus an environmental hazard that causes toxic effects. Cr(VI) is released into the air by the burning of fossil fuels and incineration of industrial and modern electronic

waste (ATSDR, 2005; Tsydenova and Bengtsson, 2011). 90,000,000 lbs of Cr(VI) are released annually into the environment in the US leading to atmospheric concentrations of 0.2 to 9 ng/m^3 in rural and residential areas (ATSDR, 2005). Non-occupational exposures to Cr(VI) result from landfills, toxic waste sites, and irresponsible chromate industrial contaminations (Reigistry, 2000).

1.5.2 Adverse Health Effects

The International Agency for Research on Cancer (IARC) categorizes Cr(VI) as a Group 1 human carcinogen (IARC, 1990). The US Environmental Protection Agency (EPA) classifies Cr(VI) as a Group A human carcinogen (EPA, 1984). Routes of exposure due to Cr(VI) are through inhalation, ingestion, and to a minimal degree, dermally. The respiratory tract and airway epithelium represent the primary locations of pathology upon inhalation exposure. Elimination of Cr(VI) accounts for less than 50% of the intake and it has been shown to bioaccumulate in the lung, liver, bladder, and bone (ATSDR, 2005). Health impairments include, pulmonary fibrosis, respiratory disease, and damage to the nasal epithelia (ATSDR, 2005). Indeed, potential carcinogenic outcomes result from long-term chronic inhalation exposures to the lung, and the degree of adverse health effects depends on the length and severity of the exposure (O'Brien et al., 2003). Epidemiological studies that were conducted by the EPA reported a 25% increased risk of dying from lung cancer for those people experiencing lifetime exposures to Cr(VI) under the permissible exposure limit (PEL) that was in place prior to 2006 (Gibb et al., 2000b; Park et al., 2004). Today the OSHA has implemented a new limit of 5 µg/m^2 of air over 8 h as a time-weighted average (OSHA, 2006).

Many studies have investigated the relationship between cumulative Cr(VI) exposure and lung cancer risk. Unfortunately, most of these studies are limited by insufficient controls such as inclusion of effects of tobacco smoke, or do not have sufficient follow-up periods to efficiently interpret the data. However, Gibb *et al.* examined lung cancer mortality in a large cohort of chromate production workers in Baltimore with an extended follow-up period of 26-32 years (Gibb et al., 2000b). The study included a retrospective assessment of Cr(VI) exposure and tobacco smoking in which they controlled for the effects of tobacco smoking using a predicted increased risk of lung cancer due to smoking. Based on this study, the National Institute for Occupational Safety and Health (NIOSH) reanalyzed the data to identify an exposure-response relationship (NIOSH, 2013). NIOSH identified an increased risk of lung cancer death for workers exposed to 1 µg Cr(VI)/m^3 (the previous NIOSH recommended exposure limit (REL)) over an occupational lifetime. Six lung cancer deaths per 1,000 workers were estimated at 1 µg Cr(VI)/m^3 and approximately one lung cancer death per 1,000 workers at 0.2 µg Cr(VI)/m^3 (NIOSH, 2013). Importantly, epidemiologic studies reported that chromeplating and stainless steel production employees developed nasal ulcerations and/or septal perforations and transient reductions in lung function at Cr(VI) concentrations ranging from 2 µg/m^3 to 20 µg/m^3 (NIOSH, 2013). The study conducted on the chromate production plant in Baltimore, reported that 60% of the cohort was diagnosed with irritated nasal septum or ulcerated nasal septum at 20-28 µg Cr(VI)/m^3 on average within one month of occupational exposure (Gibb et al., 2000a).

The experimental Cr(VI) concentrations used in the present research, with the exception of the mutagenesis study (Appendix), were based on concentrations that did not induce detectable cell death during the exposure times, and concentrations that caused increases in telomere aberrations without changes in cytotoxicity in previous studies (Liu et al., 2010; Nemec and Barchowsky, 2009). These concentrations are estimated to be significantly lower than the reported Cr(VI) needed to cause irritated or ulcerated nasal septum in the Baltimore study after one month of occupational exposure, 20 μg Cr(VI)/m^3 (Gibb et al., 2000a) (Fig. 5). The mutagenesis experiments involved exposing shuttle vector plasmids directly to Cr(VI) *in vitro* prior to replicating these vectors in human cells. Therefore, significantly higher concentrations were used to generate a higher density of adducts within the reporter gene of the shuttle vector construct. The concentrations we chose were based on previous studies (Guttmann et al., 2008; Reynolds et al., 2007).

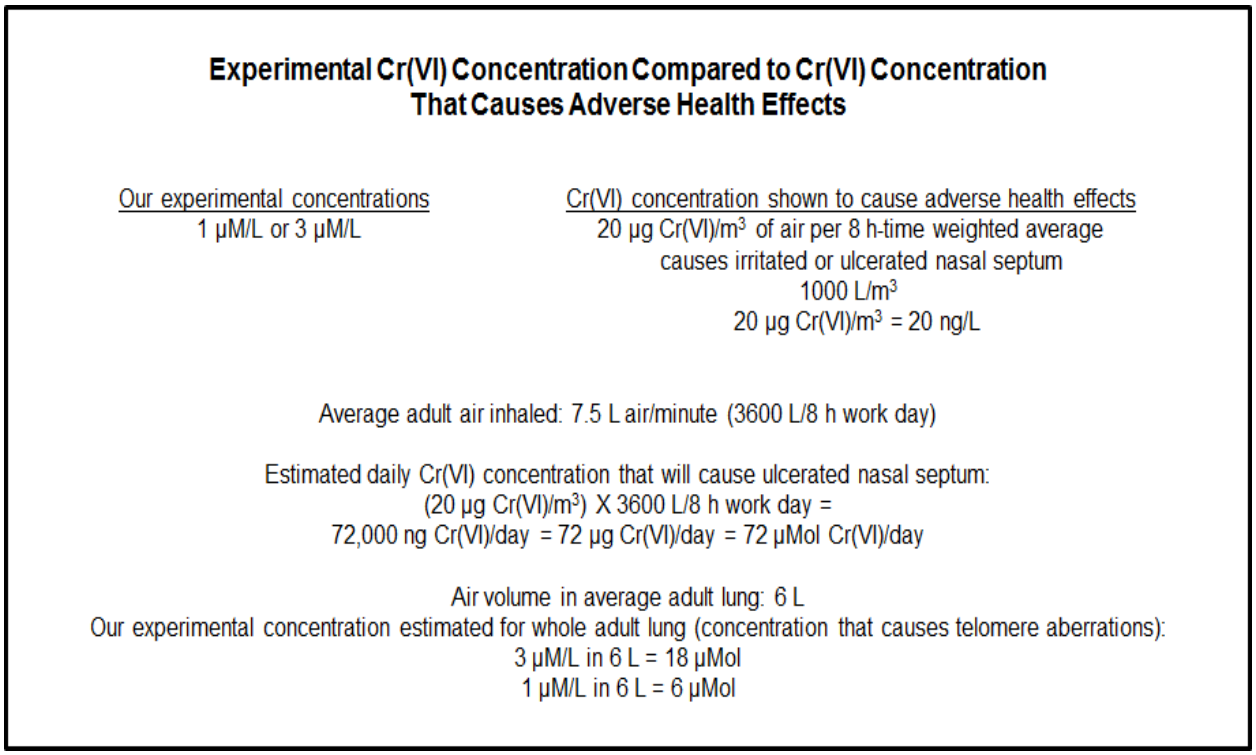
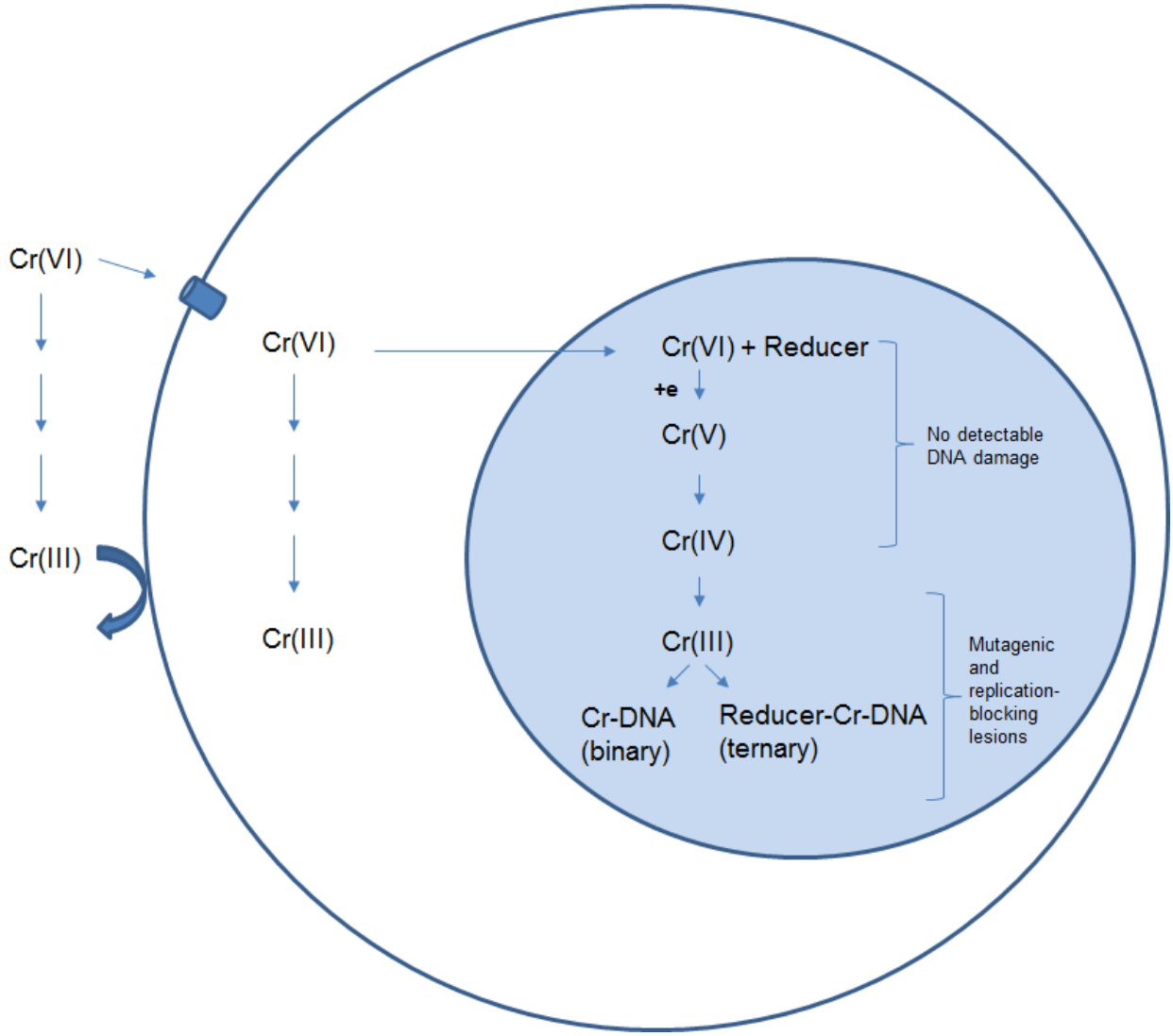


Figure 5. Experimental Cr(VI) concentration compared to Cr(VI) concentration that causes adverse health effects.

1.5.3 Chromium Metabolism

Chromate, the oxyanion of Cr(VI), is the most common form of Cr(VI). Chromate is different from Cr(III) in that it can pass through the cellular membrane by way of sulfate and phosphate anion channels due to structural similarities between Cr(VI) and these anions (Alcedo and Wetterhahn, 1990; O'Brien et al., 2003) (Fig. 6). Cr(VI) rapidly enters the cells where it can be reduced readily to a final biological oxidative state of Cr(III). Intracellular reduction occurs mainly through ascorbate (Standeven and Wetterhahn, 1991), likely due to its rate and efficiency of mediating reduction (DeLoughery et al., 2014; Quievryn et al., 2003). However, two thiols, glutathione and cysteine, can also reduce Cr(VI) (Quievryn et al., 2001; Standeven and Wetterhahn, 1991; Suzuki and Fukuda, 1990). Cr(III) is either generated through two-electron transfers via ascorbate or a one electron transfer in the case of thiol mediated reduction (Connett, 1984; Stearns and Wetterhahn, 1994). Cr(III) and the intermediate metabolites that form during reduction from Cr(VI) are biologically reactive with proteins and DNA molecules.



Cr(VI), unlike Cr(III), can pass through anion channels and enter the cell where it will be reduced to its final form, Cr(III). The intermediate metabolites do not cause DNA damage whereas Cr(III) adducts have been identified in the generation of mutagens and replication fork blocks.

Figure 6. Metabolism of Cr(VI) and formation of genotoxic lesions.

1.5.4 Chromium-Induced Lesions

Cr(III) and Cr(V), a transitory intermediate during reduction, are both genotoxic and form a spectrum of adducts with macromolecules (Cieslak-Golonka, 1992) and with DNA molecules (reviewed in (O'Brien et al., 2003; Zhitkovich, 2005)). Cr binds to DNA bases and the phosphodiester backbone either through covalent bonds or electrostatic interactions. 25% of Cr-DNA adducts are believed to be electrostatic (Quievryn et al., 2002), 40% of Cr-DNA bonds can be reversed through salt washes, and 20% of the bonds are removed through chelation (Snow and Xu, 1991) suggesting that the majority Cr adducts are robust covalent bonds. Kinetic characterization of Cr-DNA adduct formation was obtained by incubating Cr(III) or Cr(VI) in the presence of reducers, and showed that more than half of Cr-DNA bonds were formed within an hour at 37ºC (Quievryn et al., 2003; Snow and Xu, 1991). Cr(VI) reduction produces an array of lesions including Cr-DNA base or phosphate adducts, DNA strand breaks, oxidized bases, protein-Cr-DNA crosslinks, abasic sites, ascorbate-Cr-DNA adducts, and DNA-Cr-DNA interstrand crosslinks. Characterization of the genotoxicity is rather well-established, yet the ramification of such injury is poorly understood.

1.6 Ultraviolet Light (UV)

Natural UV rays come from solar light and are classified as UVA, UVB, and UVC. The wavelengths of all UV irradiation are shorter than visible light but longer than X-rays.

UVA ranges from 400-315 nm, UVB ranges from 315-280 nm, and UVC ranges from 280-100 nm. UVA and UVB are the two environmentally relevant forms since UVC does not reach the earth's surface but gets absorbed by the ozone and the atmosphere. Human skin that is exposed to the sun's rays responds by increasing the production of melanin, which is a protective pigment near the outer layers of the skin. However, intense acute exposure to UV results in cellular radiation damage that is manifested as a skin burn. Intense chronic exposure to UV can lead to melanoma and non-melanoma skin cancers (Gilchrest et al., 1999).

1.6.1 UV-Induced Lesions

The types of DNA damage induced by UV that contributes to the onset of skin cancer have been extensively documented in the literature. The effectiveness of UVC in generating DNA lesions has led to its widespread use for UV photoproduct research. Although UVB and UVA are less potent, they are more environmentally relevant than UVC (Kuluncsics et al., 1999). UV generates CPDs, 6-4 PP, single-strand breaks (SSB) and alkali-sensitive lesions (Peak et al., 1987). The production of singlet oxygen by UVA and UVB leads significant levels of reactive oxygen species (ROS) and 8-oxoguanine (8-oxoG) (Clingen et al., 1995; Douki et al., 1999). However, UVC does not produce singlet oxygen. CPDs are the most frequent UV-induced lesions (Yoon et al., 2000). CPDs are formed by covalent bonds between various adjacent bases (i.e., CC, TC, CT, or TT) (Fig. 4). Both CPDs and 6-4 PP are mutagenic if left unrepaired, and are either removed by NER or bypassed by TLS involving polη. 6-4

PPs, however, are repaired quicker than CPDs by NER (Friedberg et al., 2006; Lo et al., 2005). Polη is able to readily bypass the CPD thymine–thymine dimer (TT dimer) with high efficiency and moderate fidelity (McCulloch et al., 2004).

1.7 Statement of Problem and Hypothesis

Telomeres are 5-15 kilobases of duplex TTAGGG/CCCTAA repeats that create protective caps at chromosome ends. A recent study reported only five dysfunctional telomeres are required to trigger a cell to senescence (Kaul et al., 2012). Telomeres shorten with age due to cell division and oxidative DNA damage (Blackburn, 2000; von Zglinicki, 2002), and critically short telomeres contribute to a variety of aging-related diseases, cancers, genetic disorders and pulmonary diseases (Armanios and Blackburn, 2012; Calado and Young, 2009). Telomeres resemble common fragile sites in the genome, in that they are prone to replication fork stalling and sensitive to replication stress (Sfeir et al., 2009). Our previous work established that DNA replication stress induced by the man-made environmental pollutant, Cr(VI), causes telomere loss and aberrations (Liu et al., 2010). UV and Cr(VI) are two environmentally important genotoxic agents that result in the formation of DNA bulky lesions capable of impeding DNA replication and causing collapse of the replication fork into chromosomal breaks. Cells have a mechanism for bypassing replication blocking lesions called translesion synthesis (TLS). Studies in *S. cerevisiae* reported that TLS polymerase η accurately bypasses Cr(VI)-induced lesions (O'Brien et al., 2009). Polη also has an established role in the bypass of UV dimers (Masutani et al., 1999).

Very little is known regarding how genotoxic agents that induce replication-blocking lesions affect telomeres. Previous studies have shown that UV-induced lesions occur directly in the telomeres (Rochette and Brash, 2010). My first aim was to test the hypothesis that UV irradiation induces replication stress at telomeres and consequentially leads to telomere aberrations. My second aim was to test the hypothesis that TLS polη is required for telomere preservation after the induction of environmentally relevant bulky DNA lesions (UV photoproducts and Cr-DNA adducts).

XPV cell lines develop significantly more genomic mutations after UV exposure (McGregor et al., 1999), and XPV patients have considerably higher frequencies of skin cancer compared to the general population. Indeed, TLS proficiency is a critical cancer prevention mechanism (Kannouche et al., 2001). Polη's role in UV-dimer bypass has been shown to extend to other genotoxic lesions, which include those produced by chemotherapeutics. My third aim was to test the hypothesis that polη protects against global genome replication stress and mutagenesis in human cells induced by the environmental hazard Cr(VI).

1.8 Statement of Public Health Significance

Telomeres, the protective caps at chromosome ends, are essential for protecting the genome. Defective telomeres contribute to aging-related diseases and can cause genomic alterations that drive carcinogenesis. Translesion synthesis is a critical cellular mechanism that ensures progression of DNA replication forks, most notably, in the face

of bulky DNA lesions. Numerous environmental exposures generate bulky lesions, such as ultraviolet (UV) light and hexavalent chromium (Cr(VI)). Translesion synthesis polymerase η's (polη) role is well established in protecting against UV-induced lesions that lead to skin carcinomas and melanoma. Chronic inhalation of Cr(VI) induces respiratory diseases associated with aging and telomere dysfunction, including pulmonary fibrosis and cancers, and our previous work established that Cr(VI) causes telomere damage. The mechanisms by which environmental genotoxicants promote telomere loss and defects are largely unknown, as are the cellular pathways that preserve telomeres in the face of genotoxic stress. We investigated roles for polη in preserving telomeres following acute physical UVC exposure and chronic chemical Cr(VI) exposure. Similar to its role in protecting against UV-induced dimers, we report that polη protects against cytotoxicity and replication stress caused by Cr(VI). Our study supports a novel role for translesion DNA synthesis in preserving telomeres after UVC and Cr(VI) exposure and genotoxic stress. We uncover a mechanism by which environmental genotoxicants alter telomere integrity, and a fundamental cellular pathway that preserves telomere function in the face of genotoxic replication stress. Telomere alterations have been shown to impact human health. The public health significance is that knowledge gained from our research and findings may ultimately be used for designing preventative interventions that preserve healthy telomeres in human populations after exposure to environmental genotoxicants. The hope is that measures that preserve telomeres will inhibit or delay the onset of diseases and pathologies that are promoted by telomere defects.

2.0 POLYMERASE H SUPPRESSES TELOMERE DEFECTS INDUCED BY DNA DAMAGING AGENTS

2.1 Abstract

Telomeres at chromosome ends are normally masked from proteins that signal and repair DNA double strand breaks (DSBs). Bulky DNA lesions can cause DSBs if they block DNA replication, unless they are bypassed by translesion (TLS) DNA polymerases. Here we investigated roles for TLS polymerase η (polη) in preserving telomeres following acute physical UVC exposure and chronic chemical Cr(VI) exposure, which both induce blocking lesions. We report that polη protects against cytotoxicity and replication stress caused by Cr(VI), similar to UVC. Both exposures induce ATR kinase and polη accumulation into nuclear foci and localization to individual telomeres, consistent with replication fork stalling at DNA lesions. Polη deficient cells exhibited greater numbers of telomeres that co-localized with DSB response proteins after exposures. Furthermore, the genotoxic exposures induced telomere aberrations associated with failures in telomere replication that were suppressed by polη. We propose that polη's ability to bypass bulky DNA lesions at telomeres is critical for proper telomere replication following genotoxic exposures.

2.2 Introduction

Human telomeres are 5-15 kb of TTAGGG/CCCTAA tandem repeats at chromosome ends. The protein complex that binds telomeres, shelterin, functions with telomere structure to provide a protective cap to chromosome ends (reviewed in (Palm and de Lange, 2008)). Dysfunctional telomeres are recognized as a DNA double strand break (DSB), thereby signaling the recruitment of DNA damage signaling and repair proteins to the chromosome end (Takai et al., 2003). Accumulating evidence indicates that telomeres are hypersensitive to DNA replication stress induced either by polymerase inhibition with aphidicolin, oncogene expression or deficiencies in proteins that stabilize stalled replication forks including ATR kinase and specialized DNA helicases (Crabbe et al., 2004; McNees et al., 2010; Rizzo et al., 2009; Sfeir et al., 2009; Suram et al., 2012). These studies reveal that replication stress in cells leads to telomere aberrations that manifest on metaphase chromosomes as multi-telomeric signals at a chromatid end (doublet) or a telomere signal free end (telomere loss). Evidence indicates that stalled replication forks can collapse into DNA double strand breaks (DSB) (Zeman and Cimprich, 2014), which may be particularly detrimental at telomeres given that DSB repair pathways are normally suppressed by telomeric shelterin (Fumagalli et al., 2012; Sfeir and de Lange, 2012; Wang et al., 2004). Recent findings indicate that as few as five dysfunctional telomeres are enough to provoke cellular senescence (Kaul et al., 2012), demonstrating the importance of maintaining telomere integrity

Replication stress can also be induced at specific loci within the genome if the replication fork encounters a DNA lesions. Bulky lesions left unrepaired can block the

replication machinery and signal the recruitment of translesion (TLS) DNA polymerases. The TLS polymerase extends DNA synthesis across the lesion, and prevents replication fork demise, allowing the cell to complete genome replication so the lesion can be repaired at a later time (Reviewed in (Sale et al., 2012)). TLS is a DNA damage tolerance mechanism with the caveat that TLS may not always be error-free, and may introduce mutations. DNA polymerase η (polη) is distinguished for its efficiency in inserting correct nucleotides opposite UV-induced *cis-syn* cyclobutane pyrimidine dimers (CPD), the most frequent UV photoproducts (Brunk, 1973; Masutani et al., 2000; Masutani et al., 1999). Mutations in the *POLH* gene, which encodes polη, cause a rare autosomal recessive disorder called xeroderma pigmentosum group variant (XPV), characterized by sunlight sensitivity and a high incidence of UV-induced skin cancers (Masutani et al., 1999). Cells from XPV donors have normal nucleotide excision repair (NER) and can remove UV photoproducts, but exhibit increased UV-induced replication stress (Cleaver et al., 1979; Lehmann, 1979), mutagenesis (Wang et al., 2007), and chromatid breaks (Cordeiro-Stone et al., 2002). Homologous recombination (HR) serves as an alternative mechanism for bypassing DNA lesions or for repairing collapsed replication forks at blocking lesions (Alabert et al., 2009). However, numerous studies indicate that TRF2 and other shelterin factors repress HR repair proteins, protecting telomeres from aberrant processing or lengthening by the ALT pathway (reviewed in (Palm and de Lange, 2008)). Additionally, polη is required for successful replication at common fragile sites (CFS) (Bergoglio et al., 2013). Telomeres resemble CFS in that they are difficult to replicate and sensitive to aphidicolin (Sfeir et

al., 2009). However, roles for TLS polymerases in telomere preservation remain unexamined.

Previous studies show that telomeres are susceptible to genotoxic exposures that induce bulky lesions. Ultraviolet light causes bulky CPDs, which are either repaired by NER or bypassed by DNA polη if the lesion stalls replication at the fork. Telomere sequences contain hot spots for UV pyrimidine dimers on both the G-rich and C-rich strands (Kruk et al., 1995; Rochette and Brash, 2010). A recent study reported evidence that telomeres are deficient in CPD removal (Rochette and Brash, 2010). While UVB exposures of human cells did not alter mean telomere lengths (Rochette and Brash, 2010), the impact of UV on individual telomeres is unknown. Hexavalent chromium (Cr(VI)) is another environmental genotoxic agent that induces a spectrum of adducts including bulky lesions that are repaired by NER (Reynolds et al., 2004). Evidence indicates that Cr(VI) preferentially reacts with guanine runs (Arakawa et al., 2006), which predicts that telomeres are also susceptible to Cr(VI)-induced lesions. Consistent with this, we previously reported that Cr(VI)-induced replication stress causes telomere loss and aberrations (Liu et al., 2010). Furthermore, Cr(VI) exposure in *Saccharomyces cerevisiae* indicate that polη protects against Cr(VI)-induced mutagenesis (O'Brien et al., 2009).

In this study, we investigated a role for polη in the preserving telomeres following an acute physical (UVC) or chronic chemical (Cr(VI)) exposure that generates bulky DNA lesions in telomeric sequences. We demonstrate that replication stress is induced

at the telomeres following these exposures, which also triggered the accumulation of polη at telomeric regions. Furthermore, we demonstrate that these genotoxic exposures in cells lacking functional polη cause increased telomere aberrations associated with failures in telomere replication. Thus, we uncovered new evidence that a translesion DNA polymerase is necessary to defend telomeres against bulky DNA lesions.

2.3 Materials and Methods

2.3.1 Cell Culture and Exposures

SV40-transformed XP30RO human fibroblasts (XP-V)-pCDNA vector, and pCDNA-polη complemented were a generous gift from Alan Lehmann, University of Sussex. The XP30RO cells have a homozygous deletion near to the 5' end of the *polη* gene contributing to extensive truncation of the pol η protein (Masutani et al., 1999). U2OS cell lines expressing an EGFP-pol η construct were obtained by Fugene® HD Transfection Reagent according to the manufacturer's instructions. U2OS cells stably expressing GFP-ATR were a generous gift from Jiri Lukas. GFP-polη XPV cells were a gift from Alan Lehmann (Gohler et al., 2011). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (50 units/ml), and streptomycin (50 units/ml) in humidified chambers with 5% CO_2 and 20% O_2 at 37ºC.

Telomerase-expressing hTERT-GM02359 cells (a generous gift from Dr. Cordiero-Stone, University of North Carolina at Chapel Hill (Cordeiro-Stone et al., 2002), GM02359, and BJ cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and penicillin (50 units/ml), and streptomycin (50 units/ml) in humidified chambers with 5% CO_2 and 5% O_2 at 37ºC.

Cells were exposed to $K_2Cr_2O_7$ (Sigma-Aldrich, St. Louis, MO) as described previously (Liu et al., 2010), for 48 h at indicated concentrations. Cells were irradiated with 254 nm UVC light at 0, 5 and 10 J/m^2 UVC with a fluence of 1 J/m^2/s as measured with a UVX31 meter. Recovery was conducted in fresh Cr(VI)-free media at 37ºC for specified incubation times.

2.3.2 Cell Survival Assay

Cellular toxicity was determined by a cell counting assay using the Beckman Coulter™ Z1 Coulter® Particle Counter (aperture 100 µm). Cells were seeded at a density of 1 x 10^5 cells per dish in 35-mm culture dishes and incubated for 24 h. Cells were then exposed to either Cr(VI) for 48 h at various concentrations or to UVC at various doses as indicated and recovered for 6 h. Cells were then counted and subcultured at 4 x 10^4 cells per 10-cm culture dish. Following a seven day subculture in Cr(VI)-free media, cells were recounted.

2.3.3 Flow Cytometry

Cell cycle profiles were obtained using Click-iT® EdU Flow Cytometry Cell Proliferation Assay (Life Technologies™) according to manufacturer's instructions. Briefly, 2.5 x 10^5 cells were seeded in 10-cm culture dishes 24 h prior to exposures. Cells were exposed to either UVC or Cr(VI) as described and incubated with 10 µM Click-iT® EdU 1 h prior to harvest for each time point. Cells were harvested, counted, and then resuspended in 1% BSA (100 µl/1 X 10^6 cells). Next, cells were fixed and stored at 4ºC overnight in an

ice slurry. After cells were permeabilized and incubated with the reaction cocktail, they were stained with DAPI for DNA content. Detection of Click-iT® EdU performed by flow cytometry with BD FACSAria II.

2.3.4 Immunofluorescene-Fluorescence In Situ Hybridization (IF-FISH)

As previously described (Liu et al., 2010), IF-FISH was performed either immediately after Cr(VI) exposure or after 6 h recovery from UVC exposure. Cells were fixed in 2% paraformaldehyde for 15 min followed by permeabilization in 0.2% Triton X-100 for 10 min. Cells were then blocked in 1 mg/ml BSA, 3% FBS serum, 0.1% Triton X-100, 1mM EDTA (pH 8.0) in PBS for 1 h and immuno-stained with rabbit anti-GFP polyclonal antibody (1:400; GeneTex, Irvine, CA), anti-phospho-Histone H2AX (1:500, Millipore), or anti-53BP1 (1:500, Cell Signaling Technology). Next, cells were incubated with either Alexa 488-conjugated (Invitrogen, 1:500) goat anti-mouse secondary antibody or Cy5-conjugated goat anti-mouse (JIR laboratories, Inc., 1:400). Cells were fixed in 2% paraformaldehyde for 5 min and dehydrated in 70%, 95%, 100% ethanol for 5 min each. Samples were denatured for 10 min at 80ºC in hybridization solution (70% deionized formamide, 10% NEN blocking reagent [Roche], 0.1 M Tris-HCl [pH 7.4], $MgCl_2$ buffer [82 mM NaH_2PO_4, 9 mM citric acid, 20 mM $MgCl_2$], and 0.5 mg/ml Cy3-OO-$(CCCTAA)_3$ PNA probe (Panagene, South Korea)). Samples were hybridized for 2 h at room temperature and washed twice in 70% deionized formamide and 10 mM Tris-HCl [pH 7.4]. Samples were counterstained with DAPI and images were acquired with a Nikon A1 confocal microscope.

2.3.5 Chromosomal Telomere Fluorescent In Situ Hybridization (Telomere FISH)

Cells were seeded (3 x 10^5 for Cr(VI)-treated or 8 x 10^5 for UVC-treated) in 10-cm culture dishes 24 h before exposure. After exposures, cells were treated with 0.05 µg/ml colcemid (Invitrogen) for 8 h. As previously described (Liu et al., 2010), Telomere FISH was executed on metaphase spreads. Cells were harvested and incubated with 75 mM KCl hypotonic buffer for 12 min at 37ºC. Cells were then fixed and stored in 3:1 methanol/acetic acid. Cells were dropped onto slides and set overnight. Cells were then fixed in 4% formaldehyde for 2 min, washed in PBS and incubated with 0.1% pepsin in 0.01 N HCl for 10 min at 37ºC. Cells were fixed, washed, and then dehydrated in 70%, 90% and 100% of ethanol for 5 min. Samples were by air-dried and then denatured at 80ºC for 3 min in hybridization solution (see IF-FISH). Samples were hybridized for 2 h at room temperature, washed twice for 20 min each with wash solution I (70% deionized formamide, 10 mM Tris-HCl [pH 7.4], and 0.01% BSA) and three times 15 min each with wash solution II (100 mM Tris-HCl [pH 7.4], 66.7 mM NaCl, and 0.1% Tween 20). Finally, slides were stained with DAPI and mounted with coverslips.

A Nikon Ti90 epifluorescence microscope (Nikon Inc., NY) equipped with PlanApo 606/1.40 oil immersion objective was used to image metaphase chromosomes. Images were obtained and analyzed with NIS element advanced software using the same settings for set of cell lines in each experiment. A series of z-stacked images (0.15 mm steps) were acquired for the identification and examination of telomere signal free chromosome ends, doublets and aberrations for each metaphase.

2.3.6 Statistical Methods

OriginPro 8 software was employed for all statistical analyses. Two sample t-test for variance was used to determine significance of mean differences between two treatments or time points. One-way ANOVA followed by the Holm-Sidak test for means comparison test determined significance of differences among more than two treatments or time points. The statistically significant level was set at $p<0.05$.

2.4 Results

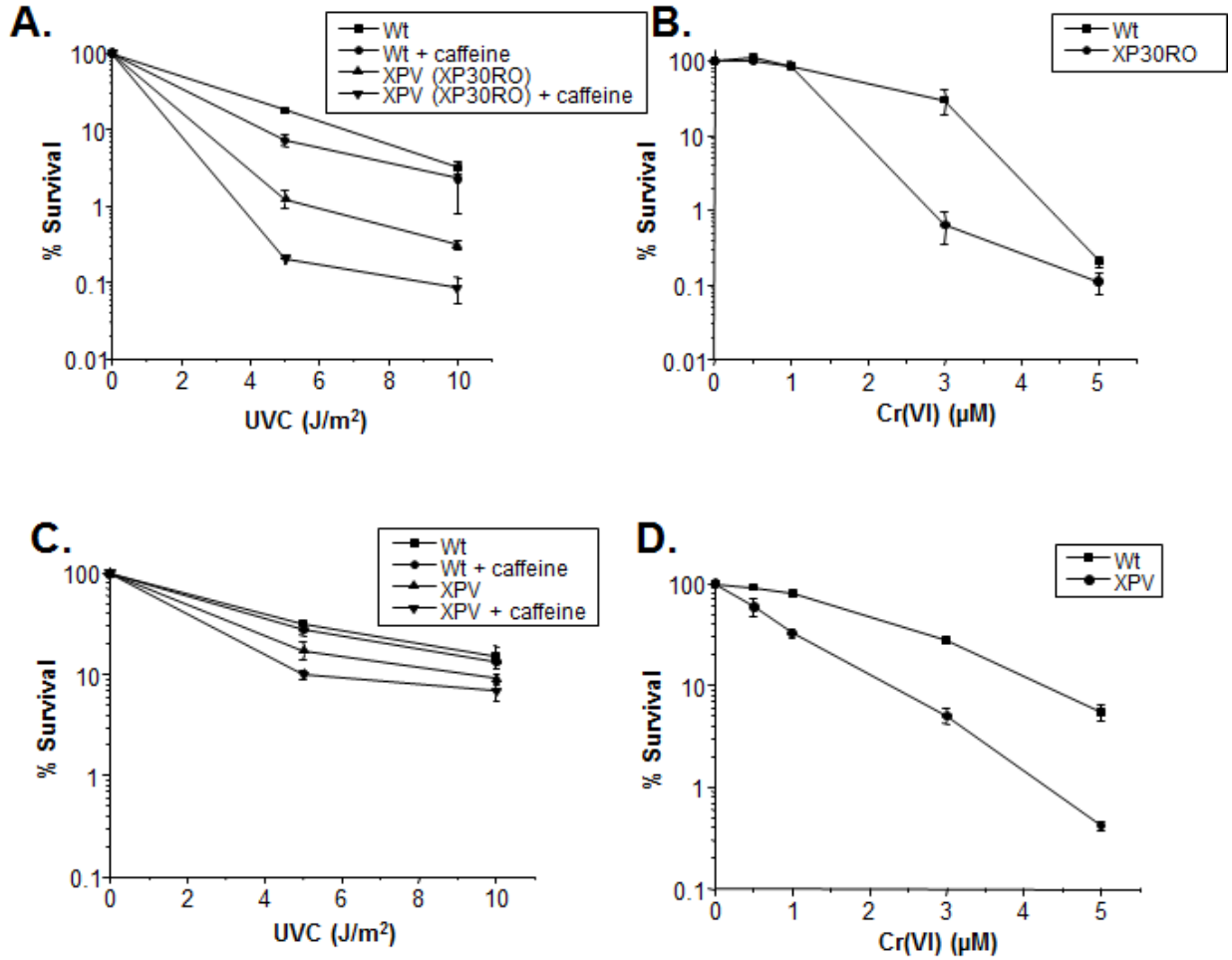
2.4.1 Polymerase η Deficiency Causes Increased Sensitivity to UVC and Cr(VI) Exposures

To test for a potential role for polη in preserving telomeres after genotoxic stress we chose to examine previously established and well characterized isogenic cell lines that are proficient or deficient for polη. SV40-transformed XP30RO human fibroblasts complemented with a polη expression vector (Wt) or vector alone (XPV) were generously provided by Dr. Alan Lehmann (University of Sussex). We first confirmed that XPV cells show increased sensitivity to UVC (Lehmann et al., 1975) (Fig. 7A). Following UVC exposures and 6 h recovery, the cells were sub-cultured and recovered for 8 days in fresh media, and then counted. Caffeine is used to override cellular checkpoints and is added to enhance UVC sensitivity of XPV cells but not Wt cells, as previously shown (Arlett et al., 1975). Polη deficient cells are also hypersensitive to

DNA replication stress induced by hydroxyurea and chemotherapeutic agents, including cisplatin and gemcitabine (Chen et al., 2006; de Feraudy et al., 2007; Lehmann et al., 1975). We and others showed that Cr(VI) exposure also causes replication stress and replication-dependent chromosome breaks (Bridgewater et al., 1994b; Ha et al., 2004; Liu et al., 2010; Zecevic et al., 2009). Therefore, we predicted that polη might similarly protect against Cr(VI)-induced cytotoxicity. Cells were exposed to various concentrations of Cr(VI) for 48 h, followed by recovery for 8 days in Cr(VI) free media. At 3 μM Cr(VI) exposure, XPV cells exhibited a dramatic increase in sensitivity, compared to Wt cells, as indicated by a 42-fold decrease in relative cell number (Fig. 7B). Similar results were obtained in primary cell lines from XPV patients (GM02359) compared to normal human fibroblasts (BJ) (Fig. 7C and D). We observed a 5.5-fold decrease in XPV cells after 3 μM Cr(VI) and a 13-fold decrease after 5 μM Cr(VI), compared to normal BJ cells. In general, the SV40-transformed cells exhibited greater sensitivity to UVC and Cr(VI) compared to the primary cells, likely due to SV40 large T antigen suppression of p53 protein, as described previously for UVC (Cleaver et al., 1999; Cordeiro-Stone et al., 2002). In conclusion, our results identify a novel role for polη in suppressing cytotoxicity following Cr(VI) exposure and suggest that polη TLS protects against Cr(VI)-induced replication stress, similar to its role following UVC exposures.

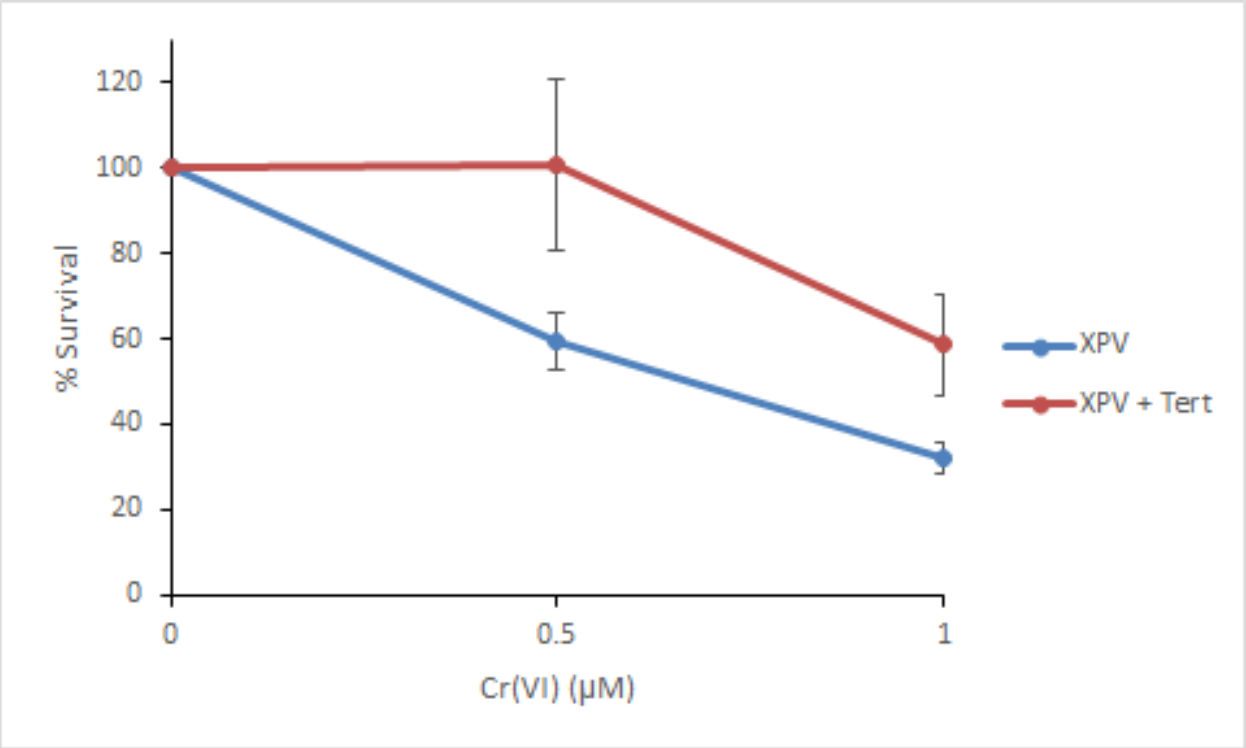
Finally, we asked whether telomerase would be protective against genotoxicity in the absence of polη. We obtained telomerase-expressing XPV cells (hTERT-GM02359) cells from Dr. Cordiero-Stone (University of North Carolina at Chapel Hill). We

observed that telomerase decreased cell sensitivity at 0.5 and 1 μM Cr(VI) concentrations compared to isogenic controls (Fig. 8). These data suggests that telomeres are involved in the increased sensitivity identified in polη deficient cells.



After indicated UVC irradiation and 6 h recovery, or Cr(VI) exposure for 48 h, cells were subcultured in medium (without Cr(VI)) for 8 days, and then counted using a Coulter counter. (A) UVC sensitivity of SV40 immortalized XP30RO-derived cells with vector alone or expressing polη (Wt) in the presence or absence of 0.38 mM caffeine. (B) Cr(VI) exposure sensitivity of SV40 immortalized XP30RO-derived cells with vector or expressing polη (Wt). (C) UVC sensitivity of XPV (GM02359) and BJ primary fibroblasts exposed with or without 0.38 mM caffeine. (D) Cr(VI) exposure sensitivity of XPV (GM02359) and BJ cells. Percent survival was determined by dividing the number of cells at each exposure by the number of cells in the untreated sample. Values represent the mean ± SE from two to five independent experiments for each survival assay.

Figure 7. Analysis of the sensitivity of polymerase η deficient cells to UV and Cr(VI).



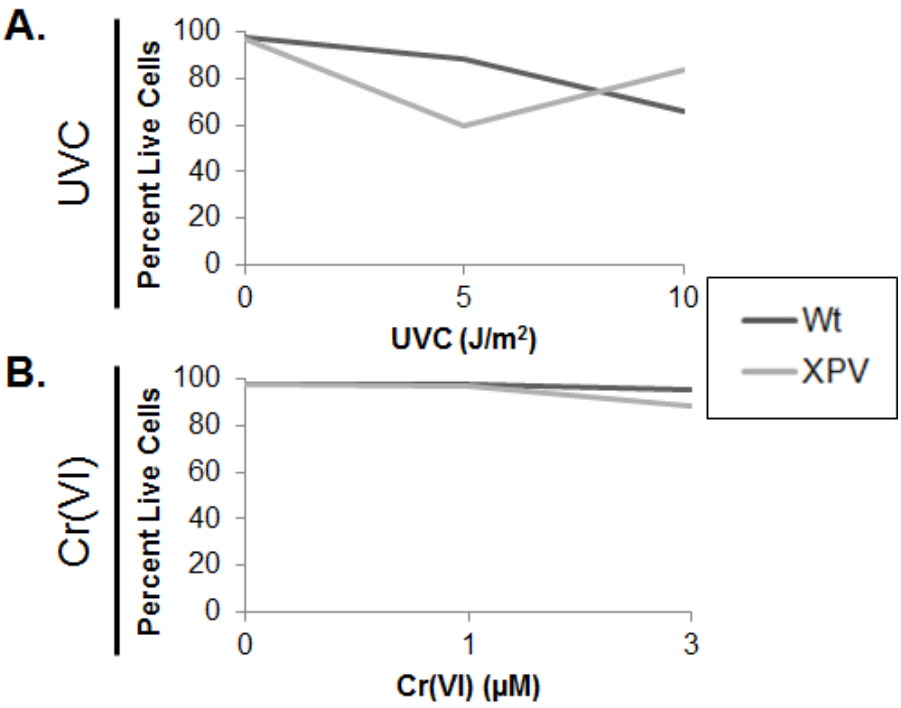
After indicated Cr(VI) exposure for 48 h, XPV and telomerase-expressing XPV (XPV+Tert) cells were subcultured in Cr(VI)-free medium for 8 days, and then counted using a Coulter counter. Percent survival was determined by dividing the number of cells at each exposure by the number of cells in the untreated sample. Values represent the mean ± SE from two to two independent experiments.

Figure 8. Analysis of the sensitivity of XPV + Tert cells to Cr(VI).

2.4.2 Polymerase η Deficient Cells Show Delayed Recovery from Genotoxic-Induced Inhibition of DNA Replication

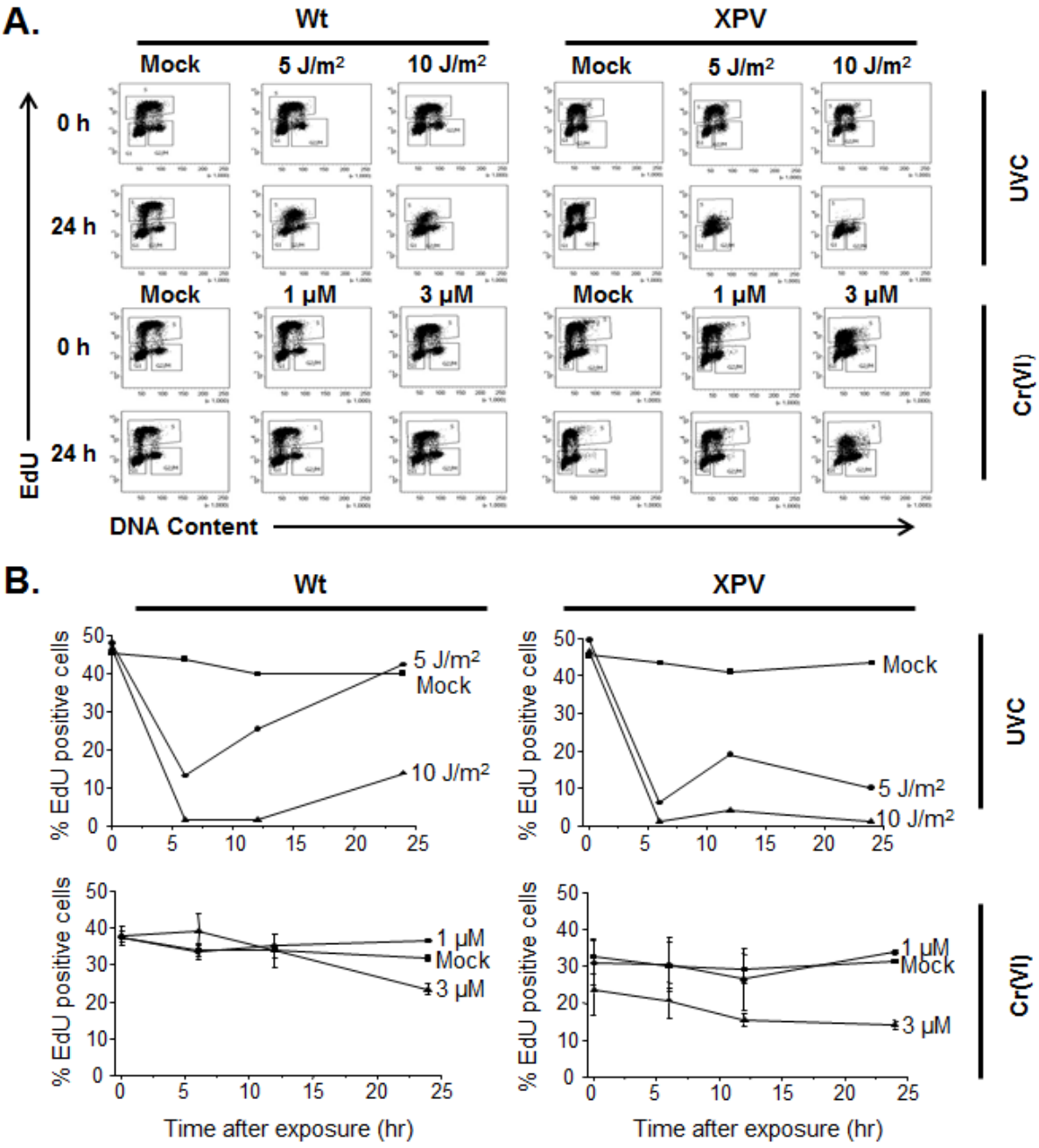
It is well established that polη deficient cells exhibit a longer UV-induced S-phase delay compared to normal cells, due to polη's essential role in normal resumption of DNA replication following UV exposure (Cordeiro-Stone et al., 2002; Lehmann et al., 1975). Next, we examined cell cycle profiles to confirm that polη complementation of XP30RO protects against UV-induced replication stress, and to test whether polη also suppresses Cr(VI)-induced replication stress. We expected XPV cells would show a reduced fraction of DNA replicating cells compared to Wt cells following recovery from UVC and Cr(VI) exposures. We obtained cell cycle profiles by FACs analysis of DNA content and identified cells undergoing DNA replication by EdU pulse labeling prior to harvesting at each recovery time point. To ensure data collection was from live cells, we simultaneously stained cells with LIVE/DEAD® Fixable dyes to eliminate any dead cells (Fig. 9). After 5 and 10 J/m^2 UVC exposures, both polη proficient and deficient cells show a reduction in the fraction of EdU positive cells at 6 hours recovery (Fig. 10). By 24 hours recovery, Wt cells exposed to 5 J/m^2 showed complete recovery of EdU positive cells to pre-exposure levels, while those exposed to 10 J/m^2 had increased but not yet fully recovered. In contrast, for XPV, both UVC exposures induced a greater reduction in the fraction of EdU positive cells, compared to Wt cells, at 12 and 24 hours recovery. Our results confirm that polη is essential for normal recovery of DNA replication and cell cycle progression after UVC exposure, consistent with previous studies (Lehmann et al., 1975) (Fig. 10).

Cr(VI) was shown to inhibit DNA replication and cause cell cycle arrest during exposure (Ha et al., 2004; Wakeman and Xu, 2006). To test if polη has a role in recovery from Cr(VI)-induced replication stress, we examined the fraction of cells replicating DNA at various time points following 48 hours of low levels of Cr(VI). Wt cells exposed to 1 μM Cr(VI) exhibited a similar fraction of EdU positive cells compared to untreated cells. However, following 3 μM Cr(VI) exposure, these cells show a reduction in EdU positive cells by 12 hours recovery progressing to greater reduction by 24 hours. XPV cells exposed to 1 μM Cr(VI) showed a slight reduction in EdU positive cells at 12 hours post-exposure, but recovered to pre-exposure levels by 24 hours recovery. XPV cells exposed to 3 μM Cr(VI) showed fewer EdU positive cells at 0 hour recovery compared to untreated cells, and did not recover by 24 hours post-exposure. In summary, we observed a greater reduction in cells replicating DNA following low levels of Cr(VI) exposure in the absence of polη. This suggests that translesion synthesis, as with UV lesions, is important in replication recovery from Cr(VI)-induced DNA lesions.



Cell viability was evaluated of SV40 immortalized XP30RO-derived cells expressing polη (Wt) or vector alone (XPV) prior to cell cycle profiling with LIVE/DEAD® Fixable Dead Cell Staining Kit. Cells were (A) irradiated with 0, 5, or 10 J/m^2 UVC or (B) exposed to 0, 1, or 3 µM Cr(VI) for 48 h and then recovery in fresh media for 48 h.

Figure 9. Viability test of Wt and XPV cells after UVC exposure or Cr(VI) exposure.


A.
Wt
XPV
Mock
5 J/m2
10 J/m2
Mock
5 J/m2
10 J/m2
0 h
24 h
UVC
Mock
1 μM
3 μM
Mock
1 μM
3 μM
0 h
24 h
Cr(VI)
EdU
DNA Content
B.
Wt
XPV
% EdU positive cells
5 J/m2
Mock
10 J/m2
Mock
5 J/m2
10 J/m2
UVC
1 μM
Mock
3 μM
1 μM
Mock
3 μM
Cr(VI)
Time after exposure (hr)
Time after exposure (hr)

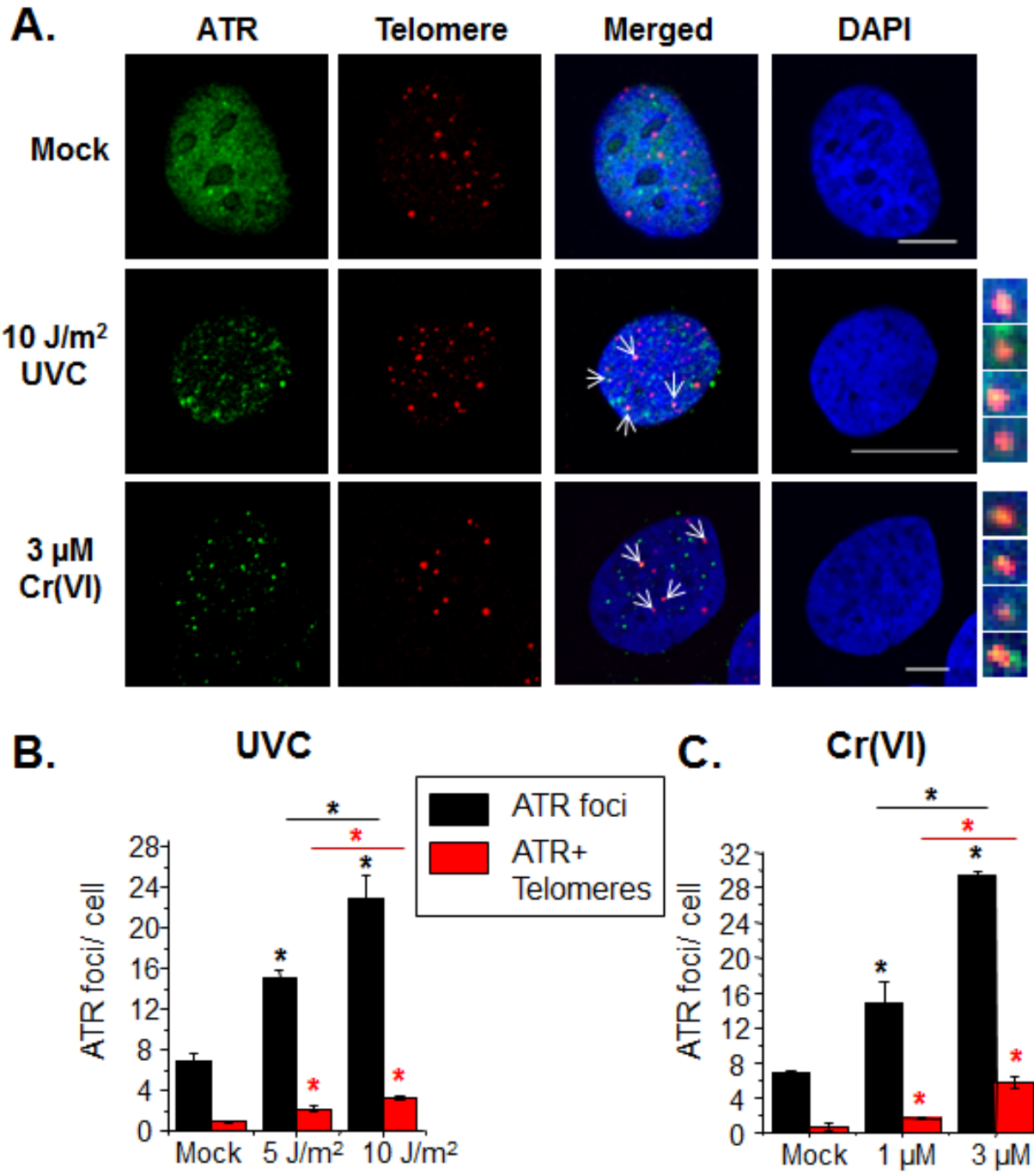
Cell cycle profiles of SV40 immortalized XP30RO-derived cells expressing polη (Wt) or vector alone (XPV). Cells were irradiated with 0, 5, or 10J/m^2 UVC or exposed to 0, 1, or 3 μM Cr(VI) for 48 h and then recovery in fresh media. Cells were labeled with EdU 1 h prior to harvesting at the various recovery time points and analyzed by flow cytometry. (A) Dot plots of G_1, S, G_2/M phases of the cell cycle show DNA content on the *x* axis and EdU incorporation on the *y* axis. (B) Quantitative analysis of percentage of cells actively incorporating EdU at the indicated recovery time points. Values for Cr(VI) represent the mean ± SE from two independent experiments.

Figure 10. Cell cycle profiles of Wt and XPV cells after UVC exposure or Cr(VI) exposure.

2.4.3 UVC and Cr(VI) Exposures Induce ATR Localization to Telomeres

Having confirmed that UVC and Cr(VI) impact DNA replication, we next asked whether these exposures cause replication stress at telomeres. Slowed or arrested cell cycle progression is characteristic of cell cycle checkpoint activation (reviewed in ref. (Abraham, 2001)). Ataxia telangiectasia and Rad3-related kinase protein (ATR) activation represents one of the initial signals for S-phase checkpoint activation. ATR is activated by RPA-bound single-stranded DNA (ssDNA) at sites of polymerase stalling (Zou and Elledge, 2003). Previous reports indicate that ATR is activated following UVC or Cr(VI) exposures (Despras et al., 2010; Wakeman and Xu, 2006), and that ATR is required for telomere maintenance (McNees et al., 2010; Pennarun et al., 2010). Since ATR localization to stressed replication forks is well established (Barr et al., 2003), we reasoned that ATR co-localization with telomeric DNA would serve as an indicator of replication stress at telomeres. For this we used the IF-FISH assay to stain telomeric DNA in U2OS cells that stably express eGFP-ATR (provided by Dr. Jiri Lukas, University of Copenhagen). Cells irradiated with UVC were recovered for 6 hours before processing and imaging by confocal microscopy. UVC exposures induced a dose-dependent increase in ATR foci formation (Fig. 11). The average ATR foci per cell increased two- and three-fold after 5 J/m^2 and 10 J/m^2 UVC, respectively, compared to mock exposure (Fig. 11B). An average of two to three ATR foci co-localized with telomeric DNA after UVC.

We then examined whether ATR formed foci after Cr(VI) treatment. Similar to results after UVC, cells treated with Cr(VI) for 48 hours showed concentration-dependent increases in the amount of ATR foci (Fig. 11C). We observed a two-fold or greater than four-fold increase in ATR foci per cell after 1µM or 3 µM Cr(VI), respectively, compared to mock exposures. On average, one or two ATR foci localized to telomeres after 1µM Cr(VI), while greater than four ATR foci co-localized to telomeres after 3 µM Cr(VI). Taken together, these results provide evidence that both UVC and Cr(VI) exposures induce replication stress at telomeric regions.


A.
ATR
Telomere
Merged
DAPI
Mock
10 J/m² UVC
3 µM Cr(VI)
B.
UVC
ATR foci
ATR+ Telomeres
ATR foci/ cell
Mock
5 J/m²
10 J/m²
C.
Cr(VI)
ATR foci/ cell
Mock
1 µM
3 µM

(A) Confocal images of EGFP-ATR U2OS cells exposed to UVC and recovered for six hours or exposed to Cr(VI) for 48 hours. Cells were analyzed via IF-FISH of ATR (green) and telomere (red) co-localization (yellow). Average ATR foci and co-localized ATR and telomere foci per cell after indicated UVC dose (B) or Cr(VI) concentration (C). The data represent mean ± SE from two experiments and approximately 50 interphase cells. Bars with a symbol of * indicates a significant difference compared to mock exposure and between the various exposures, black bars refer to ATR foci, red bars refer to ATR + Telomere foci ($p<0.05$). Bars, 10 µM.

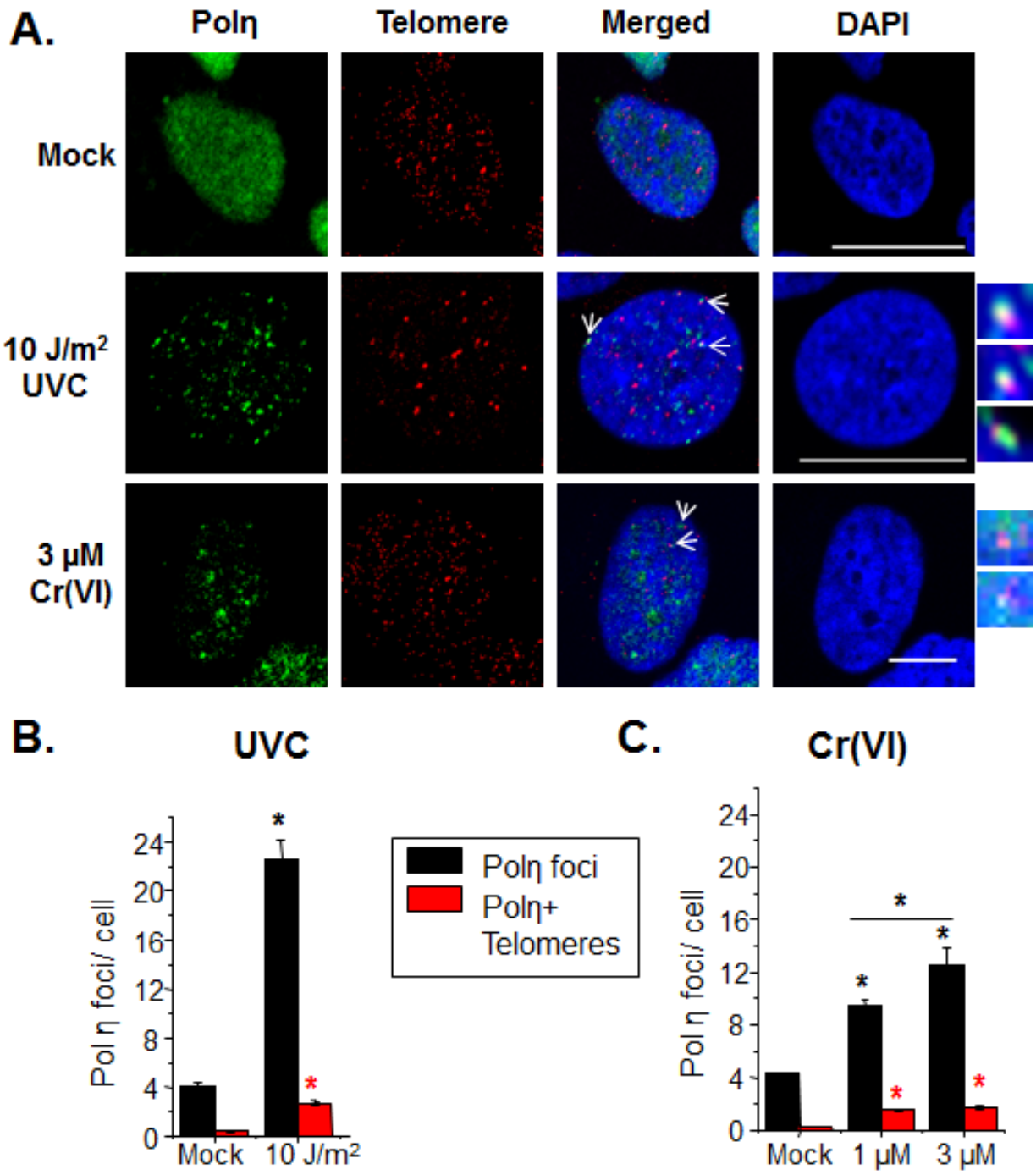
Figure 11. UVC and Cr(VI) induce replication stress at telomeres.

2.4.4 UVC and Cr(VI) Induce Polymerase η Foci Formation and Localization to Telomeres

Polymerase η accumulates in nuclear foci after UVC irradiation at sites of unrepaired DNA lesions and stalled replication forks (Kannouche et al., 2001). To study the localization of polη to telomeres after UVC or Cr(VI) treatment, we used SV40-transformed XP30RO cells that stably express eGFP-Polη (a gift from Dr. Alan Lehmann, University of Sussex (Kannouche et al., 2001) and IF-FISH (Fig. 12). Cells were exposed to 0 (mock) or 10 J/m^2 UVC and incubated for 6 hours before being processed for IF-FISH. In agreement with previous studies, we confirm that UVC increases polη foci formation, and observed a five-fold increase in polη foci per cell compared to mock treatment (Fig. 12B). After 10J/m^2 UVC, an average of two polη foci co-localized to telomeric regions per cell. We obtained similar results for UVC-induced polη localization to telomeres in telomerase negative human U2OS cells (Fig. 13). This represents the first report of polη localization to telomeres.

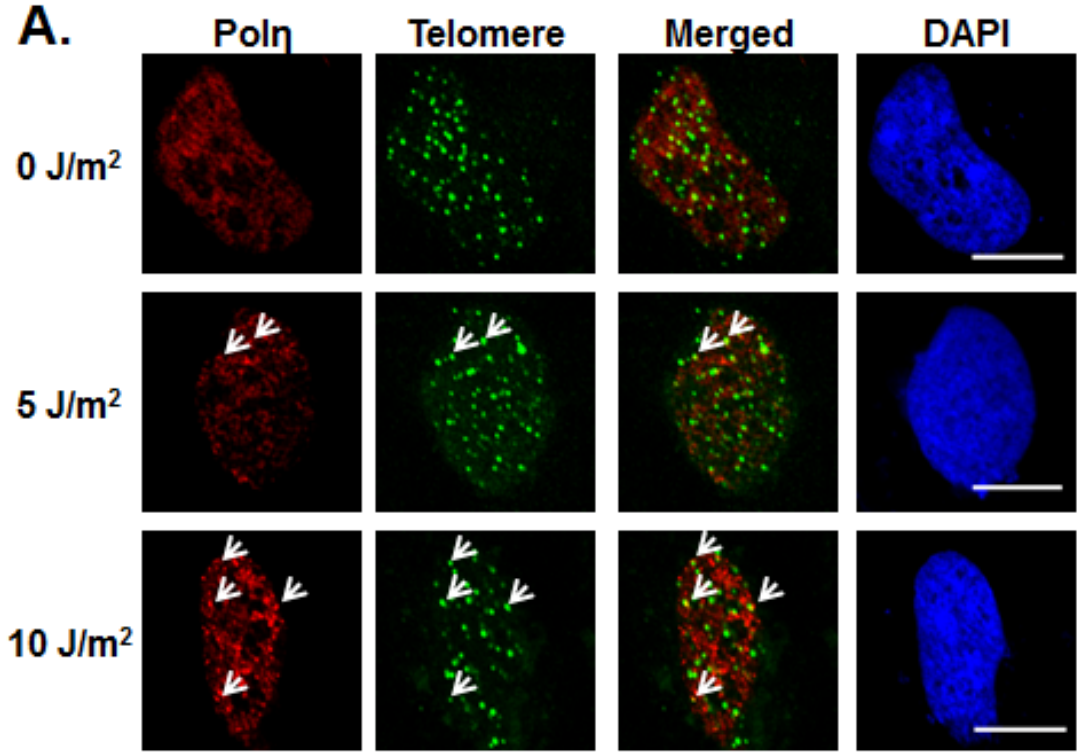
Cr(VI) exposures for 48 hours also induced a concentration-dependent increase in polη foci formation (Fig. 12C). We observed a two-fold or three-fold increase in polη foci per cell following 1 or 3 μM Cr(VI), respectively, compared to mock treatment. Cr(VI) exposures induced between one to two co-localized polη and telomere foci per cell. These results indicate that in addition to polη's established role in responding to UVC, polη responds to DNA lesions induced by low level Cr(VI) exposure. Moreover, these results demonstrate polη's ability to access telomeric DNA after both physical and

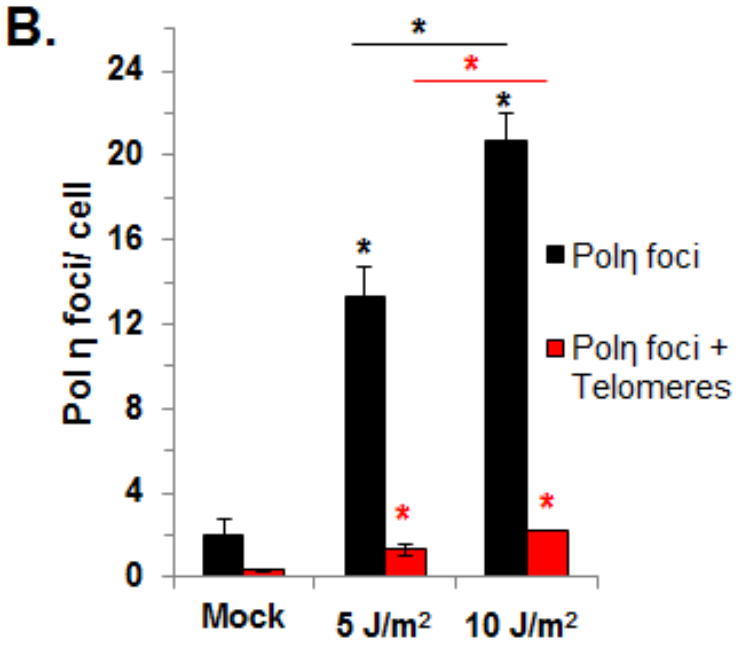
chemical genotoxic exposures, and suggest that polη responds to stalled replications forks at telomeres.


A.
Polη
Telomere
Merged
DAPI
Mock
10 J/m2
UVC
3 μM
Cr(VI)
B.
UVC
Pol η foci/ cell
Mock
10 J/m2
Polη foci
Polη+
Telomeres
C.
Cr(VI)
Pol η foci/ cell
Mock
1 μM
3 μM

(A) Confocal images of EGFP-Polη XP30RO cells exposed to UVC and recovered for 6 h or exposed to Cr(VI) for 48 h. Cells were analyzed via IF-FISH of polη (green) and telomere (red) co-localization (yellow). Average polη foci and co-localized polη and telomere foci per cell after indicated UVC dose (B) or Cr(VI) concentration (C). The data represent mean ± SE from two independent experiments and a minimum of 50 interphase cells. Bars with a symbol of * indicates a significant difference compared to mock exposure and between the different exposures ($p<0.05$). Bars, 10 μM.

Figure 12. UVC and Cr(VI) induce polymerase η localization to telomeres.


A.
Polη
Telomere
Merged
DAPI
0 J/m2
5 J/m2
10 J/m2


B.
Polη foci/ cell
24
20
16
12
8
4
0
Mock
5 J/m2
10 J/m2
Polη foci
Polη foci + Telomeres
*

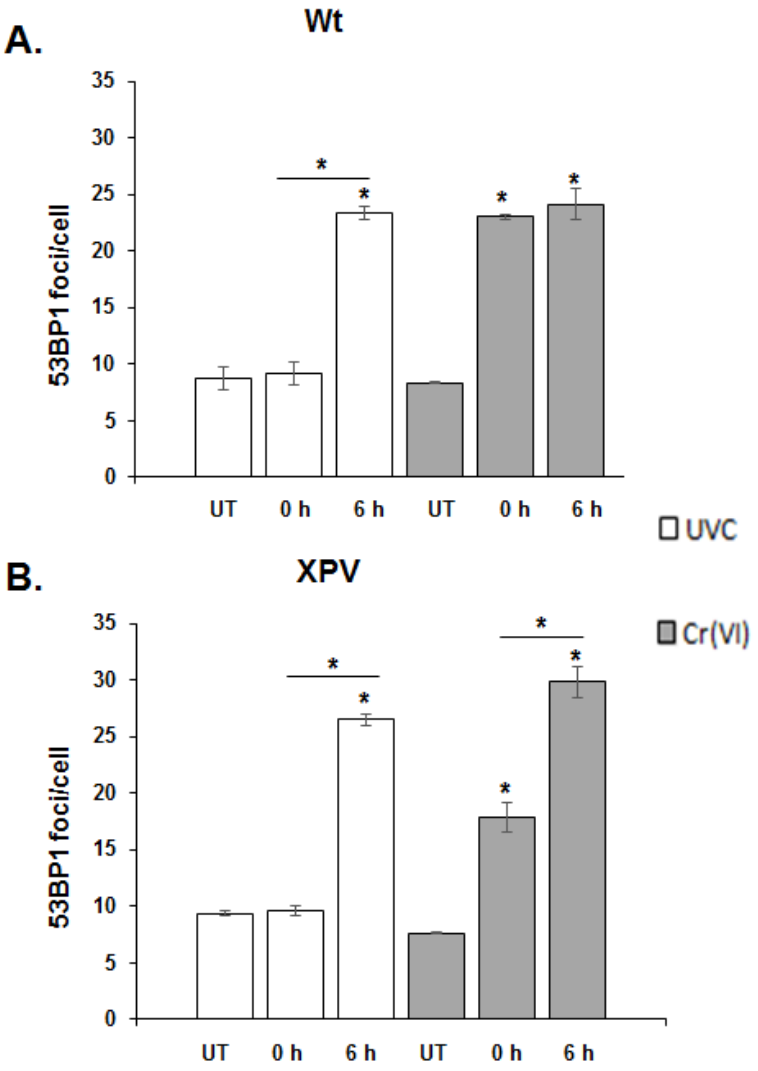
(A) Confocal images of EGFP-Polη U2OS cells exposed to UVC and recovered for 6 h. Cells were analyzed via IF-FISH of polη (red) and telomere (green) co-localization (yellow). (B) Average polη foci and co-localized polη and telomere foci per cell after indicated UVC dose. The data represent mean ± SE from two individual experiments and a minimum of 100 interphase cells. Bars with a symbol of * indicates a significant difference compared to mock exposure and between the different concentrations, black bars refer to Polη foci, red bars refer to Polη + Telomeres ($p<0.05$). Bars, 10 μM.

Figure 13. UVC induces polymerase η localization to telomeres in U2OS cells.

2.4.5 Polymerase η Suppresses DNA Damage Signaling at Telomeres

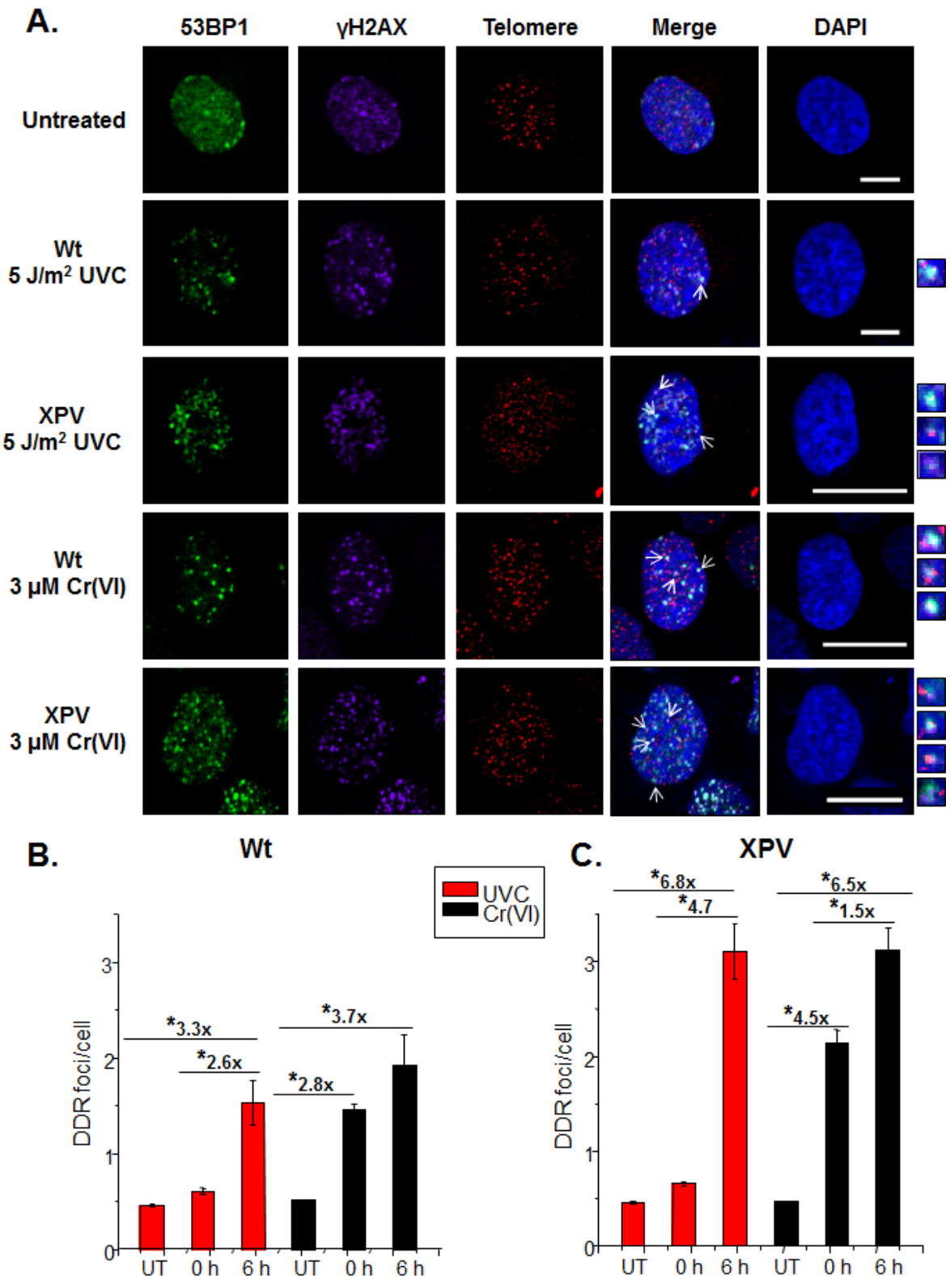
Stalled replication forks at blocking DNA lesions can collapse into a DNA double strand break. Previous studies show both UVC and Cr(VI) exposure induce chromosome breaks that depend on S-phase progression and genome replication (de Feraudy et al., 2010; Ha et al., 2004; Limoli et al., 2002; Liu et al., 2010; Squires et al., 2004; Zecevic et al., 2009). These studies show proteins that signal a DNA damage response (DDR) and DSBs, including phosphorylation of histone H2AX (γH2AX) and p53-binding protein 1 (53BP1), form foci after UVC and Cr(VI) exposures in a manner that requires S-phase progression. Since polη was shown to suppress γH2AX response after UVC exposures (de Feraudy et al., 2010; Limoli et al., 2002), we asked if polη also prevents DDR signaling at telomeres following the genotoxic exposures. Wt or XPV cells were exposed to 5 J/m^2 UVC and then fixed either 0 or six hours after recovery in fresh media. Six hours was selected based on evidence for S-phase checkpoint activation for both agents at this time point (Fig. 10). Given that γH2AX can also form at non-DSB sites (de Feraudy et al., 2010), we identified DDR positive telomeres as foci containing triple co-localized γH2AX, 53BP1 and telomeric DNA using the IF-FISH assay and confocal microscopy. We used an unbiased approach of including both small 53BP1 foci and large 53BP1 bodies (Lukas et al., 2011), but the majority was small foci (data not shown). Both exposures induced 53BP1 foci formation (Fig. 14). UVC did not induce a significant increase in DDR+ telomeres immediately following exposures for either cell line (Fig. 15). However, we observed a 3.3-fold increase in DDR+ telomeres at six hours recovery in Wt cells and a larger than 6.8-fold increase in XPV cells,

compared to untreated cells (Fig. 15). At the six hour recovery time point, cells lacking polη harbored a two-fold increase in DDR+ telomeres compared to Wt. Following 48 hour exposure to 3 μM Cr(VI), Wt cells showed a 2.8- and 3.7-fold increase in DDR+ telomeres at zero and six hours recovery, respectively, compared to untreated (Fig. 15). Moreover, XPV cells showed a 4.5-fold and 6.5-fold increase in DDR+ telomeres at 0 and six hours recovery, respectively, compared to untreated. The number of DDR+ telomeres was greater in XPV cells compared to Wt at both recovery time points. The difference in quantifiable sites of DNA damage at telomeres between cells proficient and deficient in polη provide further evidence for a fundamental role of TLS in protecting telomeres.



SV40 immortalized XP30RO-derived cells expressing polη (Wt) or vector alone (XPV) following irradiation with 5 J/m^2 UVC and recovered for six hours or exposure to 3 μM Cr(VI) for 48 h. Cells were analyzed via IF-FISH for 53BP1 at 0 or 6 hours recovery for average 53BP1 foci per cell after UVC (A) or Cr(VI) (B). Data represent averages from two individual experiments and a minimum of 50 cells. Bars with a symbol of * indicates a significant difference compared to mock exposure and between the two concentrations ($p<0.05$). Bars, 10 μM. Untreated, UT.

Figure 14. UVC and Cr(VI) induce 53BP1 foci formation.


A.
53BP1
γH2AX
Telomere
Merge
DAPI
Untreated
Wt
5 J/m2 UVC
XPV
5 J/m2 UVC
Wt
3 μM Cr(VI)
XPV
3 μM Cr(VI)
B.
Wt
UVC
Cr(VI)
DDR foci/cell
*3.3x
*2.6x
*3.7x
*2.8x
UT
0 h
6 h
C.
XPV
*6.8x
*4.7
*6.5x
*1.5x
*4.5x

SV40 immortalized XP30RO-derived cells expressing polη (Wt) or vector alone (XPV) following irradiation with 5 J/m^2 UVC or 3 µM Cr(VI) for 48 h. Cells were analyzed via IF-FISH of 53BP1 (green), γH2AX (magenta), and telomere (red) co-localization (white) at 0 or 6 hours recovery. (A) Confocal images of untreated Wt cells and Wt and XPV cells exposed to UVC or Cr(VI) and recovered for six h. Average DDR foci and telomere foci per cell after 0 or six h recovery from UVC (B) or Cr(VI) (C). Data represent averages from two independent experiments and a minimum of 50 cells. Bars with a symbol of * indicates a significant difference compared to mock exposure and between the various recovery time points ($p<0.05$). Bars, 10 µM. Untreated. UT.

Figure 15. UVC and Cr(VI) induce a DNA damage response (DDR) at telomeres.

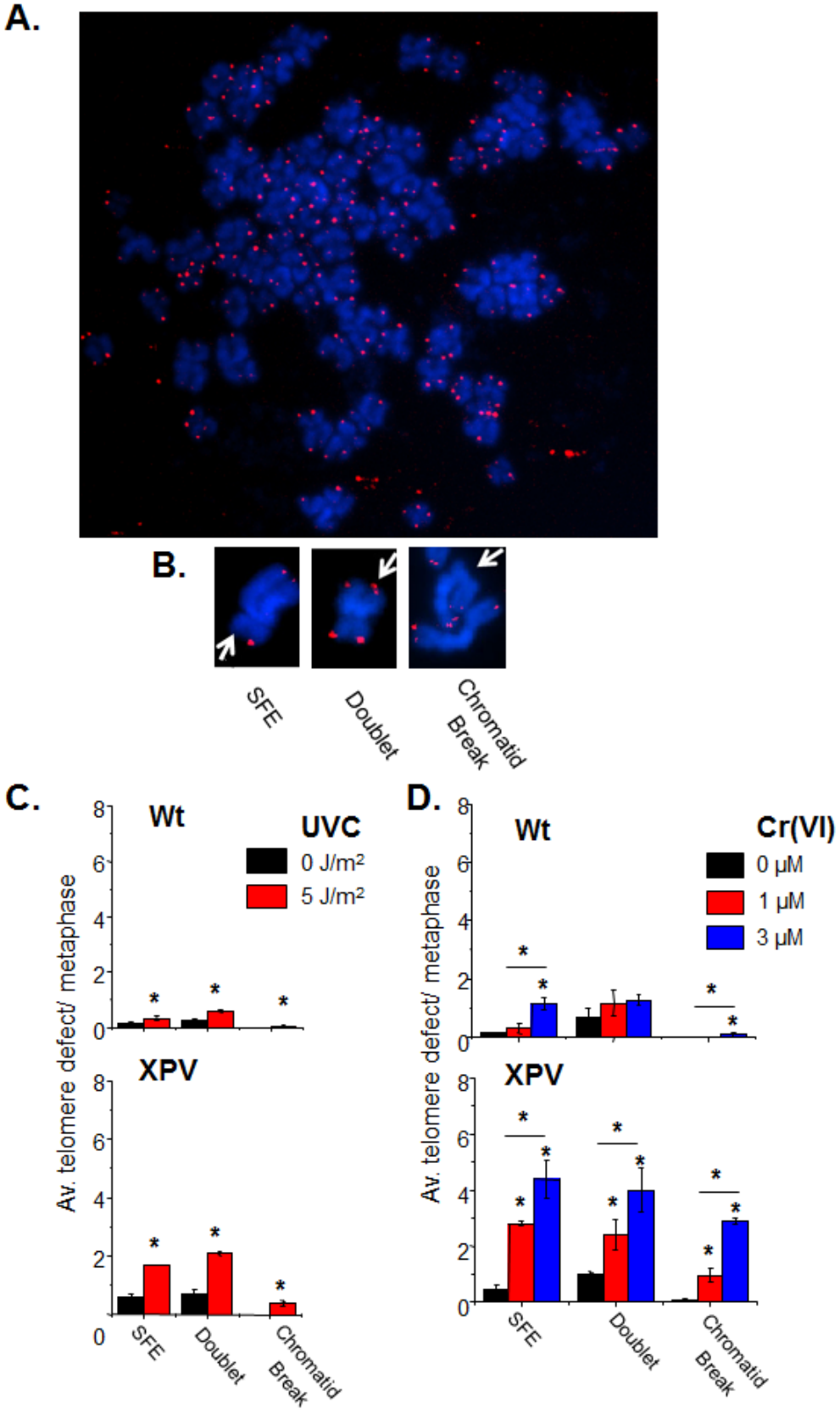
2.4.6 Polymerase η Protects Against UVC and Cr(VI) Induced Telomere Aberrations

Having established that polη localizes to telomeres and suppresses DDR signaling at telomeres after UVC and Cr(VI) treatments, we next asked whether polη functions in preserving telomere structure and integrity following genotoxic exposure. We previously reported that Cr(VI)-induced replication stress leads to telomere aberrations in human fibroblasts (Liu et al., 2010). While exposing human fibroblasts to UVB failed to alter mean telomere lengths, the impact on individual telomeres had not been examined (Rochette and Brash, 2010). To examine polη function in preserving telomeres structure after the exposures, we prepared and stained chromosome metaphase spreads for telomeres by fluorescent in situ hybridization (Telo-FISH) (Fig. 16A). Following six hours recovery from UVC exposure, cells were treated with colcemid for eight hours to arrest cells in metaphase. Since this time point coincides with active DNA synthesis in the cells that received 5 J/m^2 UVC, but not 10 J/m^2 (Fig. 10), we reasoned that only the lower dose would allow the cells to reach metaphase within the experimental time frame. Interestingly, the mock treated XPV cells exhibited 3.7-fold more signal free ends (SFEs) and 2-fold more telomere doublets, compared to mock Wt cells (Fig. 16). This may be related to polη roles in bypass of oxidative damage and/or fragile site stability (Bergoglio et al., 2013; Sale et al., 2012). UVC exposure of Wt cells induced a 2-fold increase in telomere aberrations, although averaging less than one aberration per metaphase for both telomere losses and doublets (Fig. 16C). However, XPV cells showed a significant increase in telomere losses and doublets (about 3-fold

each) after 5 J/m^2 when compared to untreated cells. Additionally, we observed UVC induces chromatid breaks in polη deficient cells consistent with previous reports (Cordeiro-Stone et al., 2002) and confirming polη's important role in chromosome stability. Similar results were obtained in primary skin fibroblasts, BJ and XPV (GM02359), exposed to 0 and 5 J/m^2 UVC (Fig. 17).

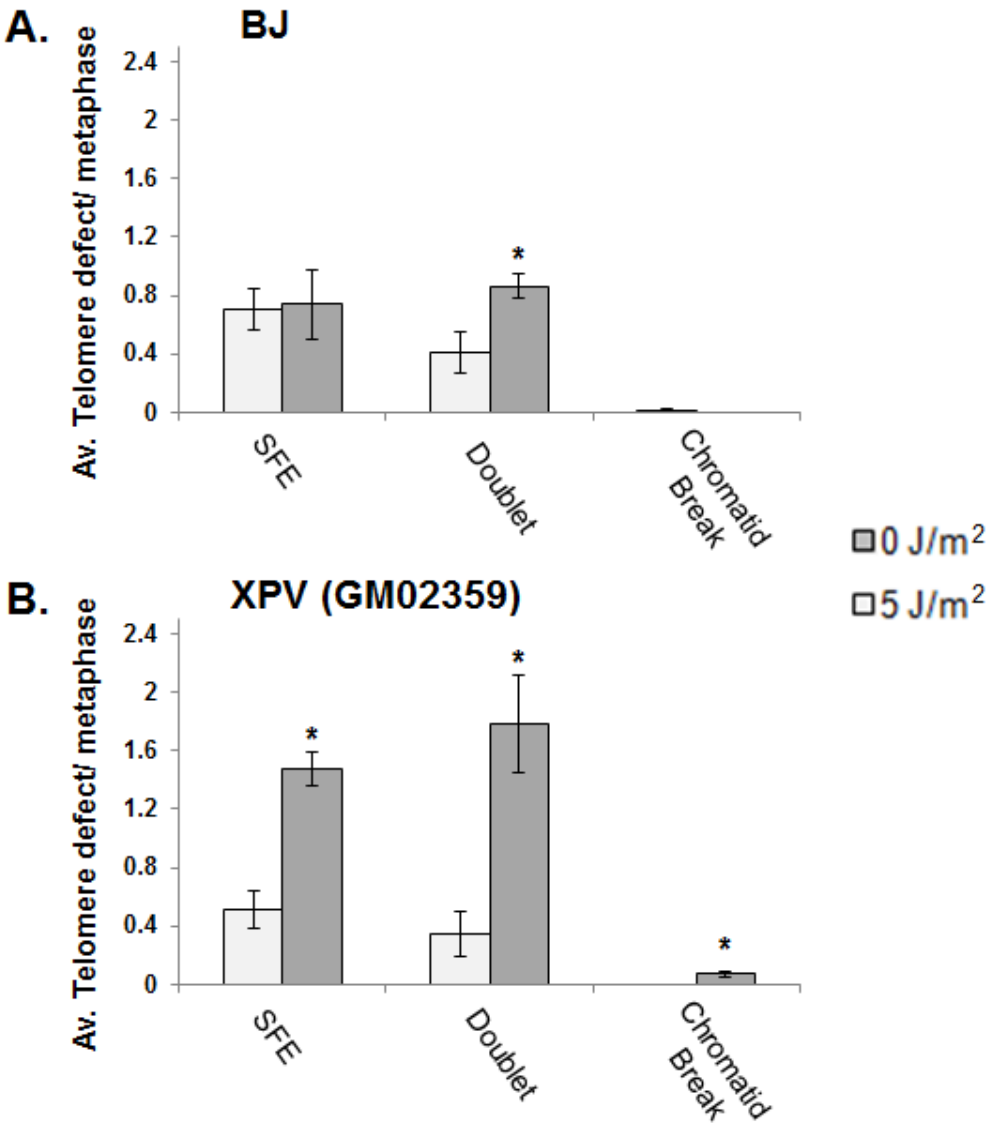
We examined telomeres following 1 and 3 μM Cr(VI) since exposures at these concentrations revealed cell cycle progression during the recovery period required to obtain metaphase cells for chromosomal analysis (Fig. 10). Colcemid was applied immediately following the 48 hours of Cr(VI) exposure. Similar to UVC, the mock treated XPV cells exhibited a higher level of telomere loss and telomere doublets, compared to Wt (Fig. 16D). Cr(VI) exposures induced a concentration dependent increase in both telomeres losses and doublets for the Wt cells, although the total aberrations per metaphase remain close to one. Strikingly, we observed a greater Cr(VI) induction of telomere aberrations for the XPV cells compared to Wt, in most cases. The 1 μM Cr(VI) exposure of XPV cells induced a six-fold increase in telomere losses and a 2.5-fold increase in doublets compared to mock. The 3 μM Cr(VI) caused a 9.5-fold increase in telomere losses and a 4-fold increase in doublets compared to mock. Notably, these two types of aberrations are associated with replication stress, while our findings of telomere chromosome or chromatid fusions were less than 0.05 and 0.25 in fifty metaphases analyzed for 5 J/m^2 UVC and 3 μM Cr(VI), respectively, in XPV cells (data not shown). Similar to UVC, we also observed that polη suppressed Cr(VI)-induced chromatid breaks illustrating a role for polη at non-telomeric regions

following Cr(VI) exposures as well (Fig. 16D). Our findings are a first account of a role for polη in preserving telomere integrity after relatively low levels of UVC or Cr(VI) exposure. The dramatic increase in replication-associated telomere aberrations in cells lacking functional polη compared to Wt cells suggests that polη is required for proper replication of telomeres following the induction of bulky DNA adducts.


A.
B.
SFE
Doublet
Chromatid Break
C.
Wt
UVC
0 J/m²
5 J/m²
XPV
Av. telomere defect/ metaphase
SFE
Doublet
Chromatid Break
D.
Wt
Cr(VI)
0 μM
1 μM
3 μM
XPV
Av. telomere defect/ metaphase
SFE
Doublet
Chromatid Break

(A) Representative metaphase of telomere FISH of untreated Wt cells. (B) Representative images of telomere aberrations and chromatid breaks. SFE, signal free end. (C) Average telomere defect per metaphase after 0 or 5 J/m^2 UVC irradiation, 6 h recovery, and 8 h colcemid or (D) 0, 1, or 3 μM Cr(VI) for 48 h and 8 h colcemid. Bars with * are significantly different ($p<0.05$). The data represent mean ± SE from two individual experiments with approximately 50 metaphases. Telomere signal free ends, SFE.

Figure 16. UVC and Cr(VI) induce telomere aberrations in XPV cells.



(A) BJ and (B) XPV (GM02359) primary fibroblasts exposed to 0 or 5 J/m^2 UVC, recovered for 6 h, and incubated for 10 h with colcemid. Analysis of telomere defects per metaphase was evaluated using telomere FISH. Bars with * are significantly different ($p<0.05$). The data represent mean ± SE from an average of two to three independent experiments and 75 metaphases. Telomere signal free ends, SFE.

Figure 17. UVC induces telomere aberrations in BJ and GM02359 cells.

2.5 Discussion

Our findings provide strong evidence that TLS polymerase η gains access to, and functions at telomeres, after the induction of bulky DNA lesions. We uncovered a novel role for polη in telomere preservation. Previous studies have shown that various DNA repair pathways are either reduced or suppressed at telomeres (Fumagalli et al., 2012; Palm and de Lange, 2008; Rochette and Brash, 2010). TLS represents a DNA damage tolerance pathway that does not repair the damage, but defends the genome against consequences of unrepaired DNA lesions. Polη's role in TLS prevents stalled replication forks from collapsing into DSBs through its ability to bypass DNA lesions during replication. Our data provide new evidence that telomeres rely on lesion bypass mechanisms for replication after genotoxic stress, consistent with reports that alternative mechanisms of fork recovery including HR and DSB repair are normally suppressed at telomeres (Fumagalli et al., 2012; Palm and de Lange, 2008).

2.5.1 Polη Roles after Cr(VI) Exposure

Polη's role in lesion bypass extends beyond UV-induced CPDs to roles in normal replication after hydroxyurea (de Feraudy et al., 2007) cisplatin, and gemcitabine (Chen et al., 2006). O'Brien et al. showed that polη mediated TLS prevents Cr(VI) induced mutations in *S. cerevisiae* (O'Brien et al., 2009). Here we report the first evidence that polη protects against Cr(VI) exposure in mammalian cells. Polη deficient human cells

exhibited hypersensitivity and increased chromatid breaks (Fig. 7 and 16). Furthermore, Cr(VI) induced polη foci formation similar to UVC (Fig. 12). These data suggest that polη functions to bypass Cr(VI) lesions during replication in a similar fashion as CPDs. UV photoproducts are bulky lesions that distort the double helix and stall replication forks (Brunk, 1973). Cr(VI) forms a spectrum of DNA lesions, most of which are bulky binary or ternary Cr-DNA adducts bound to the phosphodiester DNA backbone (Salnikow and Zhitkovich, 2008) which impede polymerase progression (Bridgewater et al., 1994b). Nucleotide excision repair (NER) removes photoproducts (Friedberg, 2001) and Cr-DNA adducts (Reynolds et al., 2004). Therefore, it is not surprising that TLS mechanisms operate at Cr-DNA adducts, similar to UV photoproducts to prevent replication fork collapse at unrepaired lesions.

Cell cycle analysis revealed that polη functions in normal recovery from Cr(VI)-induced replication inhibition (Fig. 10). Control experiments with UVC confirmed that cells lacking polη were delayed in S-phase, based on fewer cells synthesizing DNA during recovery compared to Wt cells (Fig. 10) (Cleaver et al., 1979; Gohler et al., 2011). The pattern of EdU positive cells differs for Cr(VI) compared to UVC (Fig. 10B), and we attribute this to an acute physical versus chronic chemical exposure for UVC and Cr(VI), respectively. At 0 hours recovery, XPV cells exposed to 3 μM Cr(VI) show reduced EdU positive cells, whereas reductions were not observed until 6 h recovery from UVC. S-phase checkpoint activation likely occurred during the 48 h Cr(VI) exposure, but would require time for replication forks to encounter UV lesions after the acute irradiation. We interpret the reduction in EdU positive XPV cells after Cr(VI) as a

Cr(VI)-induced S-phase delay, similar to UVC, because significant cell death did not occur during recovery (Fig. 9). Consistent with this, we observe cell proliferation in both Wt and XPV cells eight days post exposure (Fig. 7). However, the lack of full recovery by 24 hours following 3 μM Cr(VI) exposures of XPV cells suggests a fraction of these cells remain arrested.

2.5.2 Evidence that Bulky Lesions Induce Fork Stalling at Telomeres

UV irradiation and Cr(VI) exposure revealed concentration-dependent increases in ATR foci and polη per cell (Fig. 11). We propose these foci identify sites of replication stress at DNA lesions and contribute to signaling the S-phase checkpoint based on previous reports. Blocked replication forks produce ssDNA intermediates provoking RPA-mediated ATR recruitment during S-phase (Barr et al., 2003; Zou and Elledge, 2003), and ATR mediates S-phase checkpoint signaling in response to ssDNA intermediates (Zou and Elledge, 2003). S-phase checkpoint activation inhibits DNA synthesis as cell cycle progression pauses to repair the damage, which is consistent with the cell cycle profiles following UVC and Cr(VI) exposure (Fig. 10). The induction of both ATR and polη foci at telomeres in response to UVC and Cr(VI) exposures (Fig. 11 and 12) suggests that replication forks are stalled at telomeres due to unrepaired lesions blocking the forks. Previous studies show polη translocates to stalled replication forks and polη foci overlap with CPD antibody staining (Kannouche et al., 2001). Furthermore, CPDs were detected at telomeres following UVC exposures (Kruk et al., 1995; Rochette and Brash, 2010), our unpublished data). Although the foci counts are low per cell for both ATR and polη at telomeres, we believe they are significant.

Confocal microscopy displays one plane of focus of the nucleus where on average 20 telomere foci are visible in our images. Of these foci, about 14% co-localized with polη foci after 10 J/m^2 UVC and 9% co-localized with polη foci after 3 μM Cr(VI) (Fig. 12). Conversely, about 12% or 14% of polη foci localized to telomeres after 10J/m^2 UVC or 3 μM Cr(VI), respectively. Given that telomeres comprise less than 0.025% of the genome, we propose this represents a striking TLS response.

2.5.3 Polη Suppression of DDR at Telomeres after Bulky Lesion Production

Signaling of the DSB damage response (DDR) at telomeres may signify DSB formation and/or unprotected and dysfunctional telomeres. The DDR is normally suppressed at functioning telomeres, but is activated when telomeres are deprotected upon loss of structure or the shelterin complex (Takai et al., 2003). Unprotected telomeres are vulnerable to inappropriate DNA repair and chromosome fusions because they are physically similar to DSBs (Palm and de Lange, 2008). DDR also occurs when stalled forks collapse into DSBs (de Feraudy et al., 2010; Limoli et al., 2002), which may cause telomere loss based on reports that DSBs are not repaired at telomeres (Fumagalli et al., 2012). XPV cells show more cells with γH2AX foci after UV, hydroxyurea, and psoralens, and increased activation of ATR after UV (Bomgarden et al., 2006; de Feraudy et al., 2007; de Feraudy et al., 2010; Mogi et al., 2008). In agreement with these reports we found polη suppresses global DDR and decreased DDR at telomeres after UVC and Cr(VI) (Fig. 15). We propose the telomeric DDR arise from replication fork demise at telomeres because we and others showed γH2AX foci formation following UVC and Cr(VI) depend on S-phase progression (Ha et al., 2004; Limoli et al.,

2002; Liu et al., 2010; Squires et al., 2004; Zecevic et al., 2009). In contrast, DDR at telomeres due to shelterin loss does not depend on cell cycle (Konishi and de Lange, 2008), and the UVC and Cr(VI) lesions frequency is unlikely to be high enough to displace significant shelterin.

Consistent with previous reports (Harrigan et al., 2011; Lukas et al., 2011), we observed two types of 53BP1 formations; small foci or large bodies. Small foci are typically more abundant than the large bodies and were found to occur during S-phase (Harrigan et al., 2011). While our analyses included both variations of 53BP1 foci, we observed the vast majority were smaller foci rather than larger bodies. Both types of formations indiscriminately co-localized to γH2AX (data not shown). Moreover, the pattern of small 53BP1 foci we observed after UVC and Cr(VI) resembled those formed after aphidicolin treatment or loss of shelterin protein, which causes foci formation and fork stalling at telomeres (Sfeir et al., 2009).

2.5.4 Polη Suppression of Telomere Aberrations Caused by Bulky Lesion Production

Telomere losses and telomere doublets have been reported as consequences of replication fork stalling at telomeres (Sfeir et al., 2009; Suram et al., 2012). Telomere losses, or critically short telomeres, are proposed to arise from telomeric breaks that occur in response to collapsed forks (Crabbe et al., 2004). Doublets are also termed fragile telomeres because they arise upon cellular treatments that induce breaks at common fragile site sequences (McNees et al., 2010; Sfeir et al., 2009). The molecular

nature of telomere doublets remains unknown, but they are proposed to represent aberrantly condense chromatin due to regions of unreplicated ssDNA (Sfeir et al., 2009). We found the generation of replication blocking lesions also causes both forms of telomere aberrations and is significantly enhanced in cells lacking polη (Fig. 16). This suggests that lesion bypass by polη resolves replication blocks at telomeres, thereby suppressing breaks and accumulation of ssDNA or aberrant replication intermediates. Notably, the stabilization of blocking G-quadruplex structures at telomeres also induces both telomere loss and doublets (Rizzo et al., 2009). We see an average of two telomere losses and doublets per metaphase after UVC and 2-5 telomeres losses and doublets after Cr(VI) (Fig. 16). Several factors influence detections of telomere aberrations. 1) Measuring telomere aberrations on metaphase chromosomes requires cell cycle progression. Therefore, the aberrations in XPV cells may be underestimated due to the increased S-phase delay in polη deficient cells after genotoxic exposures (Fig. 10). 2) Both unrepaired replication forks and dysfunctional telomeres can activate p53-mediated G2 checkpoints and prevent progression to mitosis (Cordeiro-Stone et al., 2002; Thanasoula et al., 2012). Previous reports indicate that SV40-transformed XPV fibroblasts are more sensitive to UV and show more UV-induced sister chromatid breaks compared to primary cells due to large T-antigen suppression of p53 (Cordeiro-Stone et al., 2002). Consistent with this, we observed fewer UV-induced chromatid breaks in primary cells, and fewer UV-induced telomere loss in the primary BJ cells (Fig. 17). Since detection of telomere loss and doublets occurs when checkpoints fail to prevent cell progression to metaphase, they may be more apparent in p53 defective cells (Karlseder et al., 1999).

One possibility is that telomere aberrations result from global DNA synthesis inhibition due to signaling from stalled forks elsewhere in the genome, rather than due to stalled forks at telomeric DNA lesions. We do not favor this model for several reasons. First, low level chronic Cr(VI) exposures caused a modest decrease in cells replicating DNA compared to UVC (Fig. 10), yet Cr(VI) induces more telomere aberrations and DDR positive telomeres (Fig. 16 and 15, respectively). Second, we and others have demonstrated that UV photoproducts form at telomeres following UV irradiation (unpublished data) (Kruk et al., 1995; Rochette and Brash, 2010). Third, previous studies reported that UV irradiation with a porous filter resulted in ATR and polη staining only at sites of UV-induced lesions (Kannouche et al., 2001; Volker et al., 2001; Ward et al., 2004), suggesting that UVC irradiation does not induce replication stress at sites lacking DNA lesions. Finally, if the telomere aberrations are caused by global DNA replication inhibition and not lesions at the telomeres, then we would expect the level of UVC and Cr(VI)-induced aberrant telomeres to be higher and more similar to aphidicolin treatment after which every telomere is affected (Sfeir et al., 2009). If individual lesions are causing the replication stress that leads to telomere defects, then only those telomeres with a lesion should be affected, and we would not expect every telomere would harbor blocking lesions. Lesion generation is random and stochastic. Aphidicolin affects all replication forks because the DNA polymerase is inhibited. Importantly, some telomeres with a lesion might be bypassed by other than TLS polymerases, such as polymerase ι or polymerase ζ (Sale et al., 2012).

2.5.5 Roles for Polη in Preserving Telomeres in the Absence of Exogenous Damage

The telomere aberration analysis also revealed that untreated XPV cells show an increase in telomere losses and doublets compared to untreated Wt cells (Fig. 16). The difference for losses and doublets is significant for both the mock untreated samples in both the UVC and Cr(VI) experiments (Student's *t*-test, $p<0.05$). Other studies have demonstrated that telomere doublets and aberrations result from endogenous damage. Both telomere losses and doublets were reported in cells lacking glycosylases that remove 8-oxo-guanine and oxidized pyrimidines (Vallabhaneni et al., 2013; Wang et al., 2010) and that harbor unresolved G-quadruplexes (Rizzo et al., 2009). Previous studies report polη bypasses 8-oxoguanine and thymine glycol lesions (Chen et al., 2006) demonstrating the importance of polη in cells experiencing endogenous damage. Furthermore, polη deficient cells are hypersensitive to ligands that stabile G-quadruplex structures which can form in telomeric DNA (Betous et al., 2009). Combined with these previous studies, our work suggests that polη has a role at telomeres even at sites of endogenous lesions emphasizing polη as a requirement for telomere maintenance.

2.5.6 Biological Implications

Our study reveals the novel finding that polη protects against telomere defects after both an acute physical (UVC) and chronic chemical (Cr(VI)) exposure, and this role likely extends the induction of bulky lesions from other sources capable of causing replication stress (Chen et al., 2006). Based on reports that telomeres lack robust DNA

repair mechanisms compared to the rest of the genome (Fumagalli et al., 2012; Kruk et al., 1995; Palm and de Lange, 2008; Rochette and Brash, 2010), our data supports the model that telomeres, in particular, may rely heavily on TLS to avoid the consequences of replication fork collapse (i.e. DSB formation). Our data also uncover new evidence that UVC irradiation can induce telomere loss and fragility. This is significant in light of new studies that classify UV irradiation as an environmental geratogen based on evidence that UV exposure induces cell senescence in irradiated p16-reporter mice (Sorrentino et al., 2014). Our findings provide evidence that UV light and the consequent DNA photoproducts may promote senescence and aging in part by disrupting telomeres that harbor the lesions.

3.0 FINAL DISCUSSION

3.1 Summary of Findings

The research herein advances our knowledge of DNA damage response mechanisms at telomeres and DNA damage response mechanisms to an important environmentally hazardous metal, Cr(VI). We have made three principle and novel discoveries in the field. 1) UV irradiation induces telomere aberrations associated with replication stress. 2) Polη protects against Cr(VI)-induced cytotoxicity and replication stress. 3) Polη, in all probability through TLS, is required for proper replication of telomeres after the induction of bulky DNA lesions.

3.2 Cr(VI)-Induced Replication Stalling Lesions

Lesions induced by Cr(VI) include DNA adducts, DNA strand breaks, DNA-protein crosslinks, oxidized bases, abasic sites, and DNA intra- and interstrand crosslinks (O'Brien et al., 2003). Of these, interstrand crosslinks, oxidized bases, and Cr(III)-DNA adducts mediated by cysteine reduction have been implicated in arresting DNA replication polymerases (reviewed in (O'Brien et al., 2003)). Binary Cr(III)-DNA adducts to phosphates develop into ternary adducts, of which DNA crosslinks involving

glutathione, cysteine, or ascorbate were initially believed to be the most common until very recent findings (Quievryn et al., 2002; Zhitkovich et al., 2001). Cr-DNA crosslinks were shown to be mutagenic and inhibit replication of Cr-exposed shuttle vectors in human cells (Quievryn et al., 2003; Voitkun et al., 1998). Cr(VI) reactions with ascorbate and cysteine formed DNA interstrand crosslinks *in vitro* (Bridgewater et al., 1994a; Flores and Perez, 1999; Zhitkovich et al., 2000) but glutathione did not (O'Brien et al., 2001). Importantly, glutathione is the main reducer available for Cr(VI) metabolism in cell culture medium (Morse et al., 2013). In cell culture medium supplemented with ascorbate, 1-2% of ascorbate-chromium reactions resulted in interstrand crosslinks, which were insufficient to cause Cr(VI) hypersensitivity in cell lines deficient in crosslink repair, specifically cells lacking FANCD2 or XPF-ERCCI (Morse et al., 2013). Previous studies have identified a role for polη in response to interstrand crosslinks (Ho and Scharer, 2010; Mogi et al., 2008; Zheng et al., 2003). In these studies, polη was proposed to partner with NER or XPF-ERCCI to fill in the single strand gaps created by removal of the crosslink. Although recent data suggest interstrand crosslinks are unlikely to be a major contributor to Cr(VI)-induced genotoxicity, replication stalling caused by just a few crosslinks may nevertheless require polη for efficient recovery.

As in the case of UV dimer removal, NER was reported to mediate the efficient removal and rapid repair of Cr(III)-DNA adducts that modestly distort the DNA helix (Reynolds et al., 2004). This study also reported a remarkably fast repair rate of about 50,000 lesions/min/cell in human fibroblasts by NER, reflecting an adduct population of

20-25 Cr(III)-adducts/10^4 DNA base pairs (Reynolds et al., 2004). Moreover, they observed a 10-fold increase in mutagenesis in cells deficient in NER after Cr(VI) exposure. Indeed, no study to date has excluded Cr(III)-DNA adducts as possible replication blocking adducts. In fact, Zhitkovich *et al.* showed that Cr(VI) reduction by cysteine formed lesions on pSP189 shuttle-vector that interfered with replication in human fibroblasts (Zhitkovich et al., 2002). In this investigation, Cr(VI) generated 54% and 45% of binary and ternary DNA adducts respectively, accounting for the majority of Cr(VI)-induced lesions (Zhitkovich et al., 2002) and implicating these lesions as the most likely replication stalling adducts.

3.3 Theoretical Lesion Estimation at Telomeres

The estimation of binary and ternary Cr(III)-adducts cited above, 20-25 Cr(III)-adducts/10^4 DNA base pairs, is based on a 5 μM Cr(VI) exposure for three hours of human fibroblasts (IMR90 cells) (Reynolds et al., 2004). Although we used a slightly lower concentration, 3 μM Cr(VI), based on this study we can roughly estimate how many replication blocking lesions we would expect at the telomeres following Cr(VI) exposure. Since healthy human telomeres are estimated to be around 10 kb, we may expect every telomere to be affected, exhibiting between 20-25 Cr(III)-adducts per telomere. Normal interphase cells have 46 chromosomes and 92 telomeres. Consequently, we can predict that between 1840-2300 Cr(III)-adducts would form at telomeres. There are 92 sister chromatids in metaphase and thus 184 telomeres. In

theory, metaphase chromosome spreads could have between 3680-4600 Cr(III)-adducts at the telomeres.

UVC-induced dimers were reported to be about 1 CPD/1.3x10^5 basepairs/Jm^2 (Mitchell, 1988). If telomeres are on average 10 kb long, then in contrast to the Cr(VI) exposure, we would not expect that every telomere would harbor a UV-dimer after the 10 J/m^2 exposure study. Rather, we estimate about 1 CPD/13 telomeres. In interphase cells with 92 telomeres, we would expect about 7 total CPDs at telomeres per cell. At metaphase, with 184 telomeres we would expect about 14 total telomeric CPDs per cell.

Although the theoretical number of lesions is much closer to our study results for UVC than for Cr(VI), neither estimation corresponds to the number of telomeric sites of replication stress, telomeric polη foci, or telomere aberrations quantified in our study for either UVC or Cr(VI). We report 2-3 ATR foci at telomeres after UVC and 6-7 ATR foci at telomeres after Cr(VI) (Fig. 11). About 2-3 polη foci localize to telomeres following both UVC and Cr(VI) exposure (Fig. 12). About 2 signal free ends and 2 doublets were identified following UVC in XPV cells (Fig. 16C) and 2-5 signal free ends and doublets were detected following Cr(VI) exposure in XPV cells (Fig. 16D). There are several possible explanations for the lower levels of telomeric replication stress and aberrations compared to the estimated number of adducts at each telomere following the exposure. First, the amount of adducts reported for both UV and Cr(VI) exposure is based on data for genomic DNA. Telomeric DNA may be shielded by the shelterin complex which may offer some degree of protection against the formation of lesions. Second, our

microscopy confocal images represent a snapshot in time and only one focal plane of view (one z-slice is 300 nm at a pinhole of one airy unit). Third, multiple cellular events must coincide in order for a replication fork to stall or a telomere aberration to occur. Regarding polη foci formation, the replication fork must be present at the site of a lesion at the time the cell is processed for analysis. Fourth, alternative TLS polymerases may be recruited to some lesion sites, which could prevent persistent fork stalling and subsequent telomere aberrations. It is also worth noting that telomere aberrations on metaphase chromosomes can only be detected in those cells that bypass checkpoints and proceed through S-phase to metaphase. Finally, every lesion at the telomere may not lead to replication stress.

3.4 Werner Syndrome Protein Interaction with Polymerase η

Of noteworthy importance, Werner Syndrome Protein (WRN) has been implicated in mediating polη efficiency and fidelity of DNA synthesis (Maddukuri et al., 2012). TLS polymerases lack the proofreading ability that replicative polymerases possess which contribute to decreased fidelity during DNA synthesis by a TLS polymerase (Friedberg, 2005; Prakash et al., 2005). WRN is endowed with exonuclease activity capable of proofreading base pairs that polη incorporates opposite DNA lesions or on undamaged DNA templates. We previously reported that WRN contributes to the maintenance of telomere integrity after Cr(VI) exposure of human cells (Liu et al., 2010). If WRN suppresses the formation of telomere aberrations at sites of replication stress caused by Cr-DNA adducts, it is reasonable to predict that WRN may also function to suppress

deleterious events at UVC-induced CPDs or 6-4 PPs. Moreover, WRN may cooperate with polη to prevent replication fork stalling at telomeres. Taken together, these studies support the model that telomere loss is caused by failures in telomere replication.

3.5 Study Limitations and Future Directions

3.5.1 Evidence of Replication Stress

ATR is an important checkpoint protein kinase and has been shown to respond to a stalled replication fork, particularly at the single-stranded DNA intermediate (Abraham, 2001). Previous studies demonstrate that UV (Despras et al., 2010; Heffernan et al., 2002) and Cr(VI) (Wakeman and Xu, 2006) exposure activate ATR as part of the S-phase checkpoint response. We showed ATR foci formation after exposure to UVC and Cr(VI) in human U2OS cells (Fig. 11). Furthermore, we showed that ATR foci form at telomeres after these genotoxic agents. To our knowledge, this study represents the first demonstration of ATR foci formation at telomeres. However, while ATR foci formation suggest ATR activation, visibility of foci is not direct evidence of protein function or kinase activity. Indeed, a mutant of polη harboring a mutation in a phosphorylation site, S601, still formed visible foci, even though the protein was not fully functional (Gohler et al., 2011). On the contrary, a mutant form of Rad18 was observed function in DNA damage tolerance pathways in spite of its inability to form foci (Nakajima et al., 2006). Evidence of replication stress could be further strengthened in our study through protein analysis of downstream targets of ATR activation, namely

Chk1 phosphorylation. Chk1 is a well-established marker for checkpoint activation in response to replication stress (Smits, 2006).

As further evidence for out model that polη suppresses persistent replication fork stalling after UV and Cr(VI), we expect that ATR foci formation would be increased in polη deficient cells compared to wild type cells. To test this in future studies we could use isogenic XPV and Wt cells and immunofluorescene-fluorescence in situ hybridization (IF-FISH) analysis to stain for phosphorylated forms of ATR and Chk1 at telomeres following UVC or Cr(VI) exposure. Göhler *et al*. reported that ATR phosphorylated polη after UV exposure (Gohler et al., 2011), suggesting a direct interaction between ATR and polη. Protein-protein interactions could be examined through co-immunoprecipitation of polη and ATR after UVC and Cr(VI).

In the future, to further characterize protein interactions with telomeric DNA, we could use chromatin immuno-precipitation (ChIP). Mammalian telomeres are bound by the shelterin protein complex which functions to prevent telomeres from being recognized as DNA double strand breaks (de Lange, 2005). Replication fork stalling at telomeres in XPV and Wt cells could be examined directly by the incorporation of BrdU analogs to mark replicating DNA concurrently with ChIP (Rizzo et al., 2009). Briefly, UVC and Cr(VI) exposed cells synchronized at the G_1/S phase are incubated with BrdU prior to harvesting at various time points through cell cycle progression. DNA-bound proteins are cross-linked to the genome and chromatin is fragmented by sonication. The protein-bound telomeric DNA is isolated by precipitation with antibodies against

shelterin proteins, TRF1, TRF2, and POT1. Proteins are released by reversing the cross-links and the fraction of replicating telomeric DNA is evaluated by western blotting with an antibody that recognizes the BrdU analogs incorporated into the newly synthesized daughter strand. If replication forks are stalled at telomeres after UVC and Cr(VI) exposures in XPV cells, then we would expect fewer XPV cells would show BrdU incorporation in the telomeric DNA compared to Wt due to their reduced capacity to bypass blocking lesions. However, the low number of telomeric ATR foci per cell (Fig. 11); with the low abundance of telomeric DNA (less than 0.025% of the genome), suggest that this ChIP assay may not be sensitive enough to detect fork stalling at telomeres in our study. Furthermore, as noted earlier, the frequency of UV-induced lesions at telomeres is low, and suggests that not every telomere would harbor a replication-blocking lesion after UV.

3.5.2 Cr(VI)-Adducts and Replication Stress

The formation of UV-dimers at telomeres has been demonstrated previously (Kruk et al., 1995; Rochette and Brash, 2010). Cr(VI) on the other hand, is known to induce bulky lesions capable of stalling replication forks, but Cr(VI)-induced bulky lesion formation has not been investigated or measured at telomeres. Inductively-coupled plasma mass spectrometry (ICPMS) is a highly sensitive method to measure Cr-DNA binding and can be applied to Cr(VI) exposures in cell culture. Following Cr(VI) exposure, cells are washed and harvested. DNA is isolated and digested at subtelomeric regions, followed by purification of the telomeric fragments. ICPMS is then used to measure Cr-telomeric DNA adducts. If the Cr-telomeric DNA adducts are too

few and below the detection limit of ICPMS, ^{51}Cr-labeled Cr(III) can be converted to ^{51}Cr-labeled dichromate and exposed to cells in culture. Cells are then harvested, DNA is isolated and digested at subtelomeric regions to separate genomic from telomeric DNA. The DNA is then loaded onto an electrophoresis gel for detection of the radioactive isotope.

Further evidence of replication fork stalling at telomeric Cr-adducts could be obtained through the use of single molecule analysis of replicated DNA (SMARD). SMARD uses two halogenated nucleoside analogs that are incorporated into replicating DNA; one analog is incorporated first for about 2-4 hours followed by incorporation of the second analog for an additional 2-4 hours. DNA fibers are stretched and stained with fluorophore-conjugated antibodies against these analogs making them visible under a fluorescent microscope. SMARD can detect various replication events including progression, stalling, and termination by comparing the staining patterns of nucleotide analog 1 and analog 2. This assay can be specific to telomeric DNA fibers by using restriction enzyme digests to release subtelomeric and telomeric fragments, and by annealing PNA-probes that bind the telomeric and subtelomeric DNA sequences (Sfeir et al., 2009). Prior to SMARD, we would examine and compare the kinetics of Cr(VI) uptake in the XPV and control cells. The analysis of cellular Cr(VI) uptake involves a rather straightforward process of harvesting Cr(VI) treated cells in the presence of ethylenediaminetetraacetic acid (EDTA) and measuring Cr concentration by atomic absorption spectrometer, as described previously (Morse et al., 2013). Once the kinetics of Cr(VI) is known, Cr(VI)-treated and untreated XPV and control cells would be

processed for SMARD, and direct detection of replication patterns would be compared between the different conditions and cell types. Since the kinetics of Cr(VI) uptake is known and would be used to determine the length of Cr(VI) exposure, this experiment will provide evidence based on initial Cr(VI) genotoxic activity and identification of early products of Cr(VI) exposure as opposed to accumulated toxic cellular activity. Addressing initial Cr(VI) adduct formation will help determine whether the replication events were directly due to Cr(VI), rather than indirectly due to global Cr(VI)-induced cellular stress.

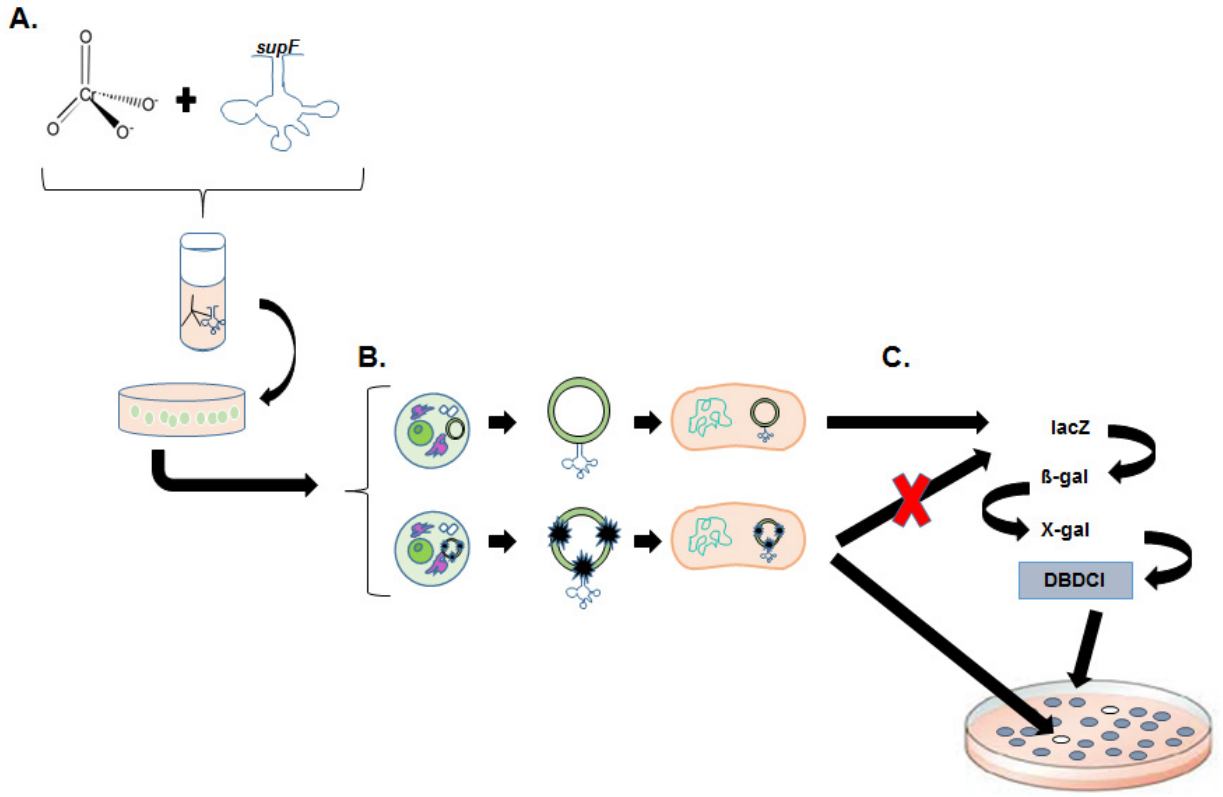
3.5.3 Polymerase η Functions at Telomeres after Exposure to Genotoxic Agents

We demonstrated that polη localizes to telomeres and we showed that telomere defects increase in the absence of polη following UV and Cr(VI) exposures, but further studies are needed to establish polη's precise role at telomeres. While mutagenesis assays have been done previously in polη deficient cells exposed to UV (Stary et al., 2003), mutagenesis rates at telomeric DNA is not known and mutagenesis rates after Cr(VI) exposure of mammalian cells is not known. Comparison of mutation frequencies between UVC- or Cr(VI)-treated and untreated shuttle vectors (SVs) containing telomeric DNA, replicated in either Wt or XPV cells would provide evidence regarding polη's role in suppressing the formation of mutations at telomeric and non-telomeric DNA sequences. Although SVs containing telomeric DNA are not equivalent to intact telomeres in structure or chromatin assembly, SVs are able to demonstrate polη's potential role in suppressing the formation of mutations in telomeric DNA sequences. We would expect that the replication of UVC exposed SVs in XPV cells would result in

more mutations, compared to Wt cells, at the SV telomeric and non-telomeric sequences, based on previous report for to genomic DNA (Stary et al., 2003). However, it is worth noting that the traditional SV mutagenesis assays only allow for the identification of mutations in a reporter gene. Therefore, the identification of telomeric DNA mutations would require DNA sequencing of all the SV that were replicated in, and isolated from, human cells. This could potentially be achieved using Next Generation Sequencing approaches. Specifically in the case of Cr(VI), if polη is accurately bypassing Cr(VI)-induced lesions, then Cr(VI) exposed SVs should show more mutations after replication in the XPV cells compared to the Wt cells.

Our studies indicate that polη forms foci after Cr(VI) exposure in human cells (Fig. 12), but whether polη accurately bypassing Cr(VI)-induced adducts in these cells is unknown. To address this, we began a study using the *supF* shuttle vector (SV) mutagenesis assay (Fig. 18). This assay uses the pSP189 SV with the tyrosine amber suppressor tRNA *supF* reporter gene. Our study design originally planned to compare SV containing six telomeric repeats that were either mock exposed or exposed to Cr(VI) *in vitro*. The telomeric repeats are adjacent to the *supF* reporter gene, therefore, while base substitutions within the telomeric repeats are not detectable, deletions and rearrangements within the telomeric repeats that also impact the reporter gene are detectable. However, our study was not completed and the colonies reflecting mutagenesis events were not sequenced. Therefore, specific types of mutations after Cr(VI) exposure in cells lacking functional polη that are normally detectable by this assay, are not reported. However, we obtained preliminary *supF* mutation frequencies

(see Appendix, Fig. 19). The supF tRNA inserts a tyrosine at UAG stop codon in a mutant *lacZ* gene and restores β-galactosidase activity in the MBM7070 bacteria strain. This protein converts X-gal to a blue color product called 5,5' –dibromo-4'4-dichloro-indigo (DBDCI). Wild type *supF* bacteria colonies are blue and mutant colonies are white in X-gal media. The SV contains an SV-40 origin of replication. First, we exposed the SV to 100 μM Cr(VI) and the dominant reducer of Cr(VI), ascorbate (Standeven and Wetterhahn, 1992), *in vitro* for 30 minutes. Cr(VI)-treated SVs were then transfected into human cells. U2OS cells stably expressing either a control shRNA against GFP (Control) or an shRNA targeted against polη (shPolη) (Appendix, Fig. 19B) were used to replicate the Cr(VI)-treated SV. Cells were allowed to replicate the SV for 48 h before isolating the SV and transfecting them into the *E. coli* reporter MBM7070 cells for the identification of colonies harboring inactivating supF SV mutations. We observed a 1.5 fold increase in Cr(VI)-treated SV mutation frequency in shPolη cells compared to control cells. Therefore, our preliminary data suggests that, similar to yeast, polη is able to accurately synthesize past Cr(VI)-induced adducts (Appendix, Fig. 19). Future studies will require comparisons of mutation frequencies for untreated SVs after replication in polη deficient cells compared to Wt cells, as well as comparisons at various Cr(VI) concentrations. However, previous reports have shown that untreated XPV cells do not exhibit a higher level of spontaneous genomic mutations compared to Wt cells (Stary et al., 2003).



(A) Shuttle vector (SV) plasmids harboring *supF*, a suppressor tyrosyl tRNA gene, are incubated with Cr(VI) and transfected in cells for replication. (B) SV replicates are isolated and transfected into bacteria engineered with *lacZ* amber mutations. (C) Cells that contain non-mutated plasmid generate blue colonies via the *lacZ* protein product, β-gal, on X-gal-treated agar plates. Cells that contain mutations on the plasmid generate white colonies because they lack the ability to suppress the mutation in *lacZ*.

Figure 18. Schematic of SupF shuttle vector mutagenesis assay.

3.5.4 Telomeres with Mutation Accumulation. What's Next?

If TLS polη functions at telomeres after Cr(VI)-induced bulky lesions and if TLS polη is error-prone, what are the consequences of mutation accumulation at telomeres. Whether NER can access the telomeres and repair damaged telomeric DNA is currently a topic of debate (Fumagalli et al., 2012; Kruk et al., 1995; Rochette and Brash, 2010). On-going studies in the Opresko Laboratory are currently addressing this question. Studies have shown that NER can remove lesions left behind following TLS. Telomeres are transcribed into long non-coding RNA, called TERRA (Telomeric Repeat containing RNA) (Feuerhahn et al., 2010). Examination of the consequences of mutant TERRA transcripts would shed light on the potential effects of the accumulation of mutations at telomeres.

3.5.5 Apoptosis, Senescence, or Carcinogenesis

We observed that cellular exposures to 10 J/m^2 of UVC and 3 μM Cr(VI) led to a decrease in the fraction of cells actively incorporating EdU up to 24 h of recovery after the exposures (Fig. 10). Our cytotoxicity study indicates that the majority of Wt and XPV cells are alive at 48 h recovery (Fig. 9). Yet whether these cells eventually recover, are permanently arrested and become senescent, or become unstable and transform into cancer cells is unknown. In human fibroblasts, critically short telomeres lead to cellular senescence (d'Adda di Fagagna et al., 2003). We report ATR foci

formation after UVC and Cr(VI) exposure (Fig. 11). However, ATR has a dual role in DNA repair and apoptosis (Bernstein et al., 2002). The pathway for DNA repair includes many proteins such as BRCA1, p53, hChk1, and causes cell cycle arrest. The pathway for apoptosis includes BRCA1 and p53 (reviewed in (Bernstein et al., 2002)). To investigate apoptotic, senescent, or carcinogenic endpoints, experiments would need to include time points beyond 24 h recovery after UVC and Cr(VI) exposure. Testing for endpoints further downstream of telomere aberrations would improve our understanding of the importance of TLS after genotoxic agents at telomeres.

3.6 BIOLOGICAL IMPLICATIONS

DNA damage tolerance is a basic biological function that ensures successful progression of stalled replication forks. However, DNA damage tolerance is not always error-free, due to the low fidelity of TLS polymerase. If replication blocking lesions are not repaired and are inaccurately bypassed by TLS polymerases, this can cause a mutation. Multiple studies have uncovered roles for TLS polη beyond the bypass of UV dimers (reviewed in Chapter 1), but not all report the efficient or accurate bypass that occurs between polη and CPDs. Indeed, polη has probably evolved a specialized role in CPD bypass because DNA damage from solar light is as ancient as DNA itself. The ability of polη to bypass other types of DNA damage, including Cr(VI)-induced lesions, is probably due to polη's relatively loose catalytic active site based on X-ray crystal structure data (Trincao et al., 2001). However, our study observed preliminary evidence that polη suppresses Cr(VI)-induced mutagenesis and that polη has a critical role in

preserving telomeres. Taken together with reports that polη suppresses Cr(VI)-induced mutagenesis in yeast, our studies emphasizes that polη TLS could contribute to the protection from human respiratory disease and cancer due to Cr(VI) exposure.

XP patients are characterized by an increase in genomic mutations and high rates of skin cancers due to UV exposure (Friedberg et al., 2006). Although NER is active in XPV patients, normal cells utilize both NER and TLS to efficiently recover from UV-induced lesions. As discussed previously, polη is highly regulated and not believed to typically function at undamaged DNA except during somatic hypermutation (Waters et al., 2009). We have identified an increased incidence of telomere aberrations in XPV patient cells after UVC and Cr(VI) exposures, compared to normal cells. Telomere aberrations can lead to cellular senescence or cancer. XPV patients show an increased incidence in skin cancer after UV exposure and are characterized by premature aging of the skin after sunlight exposure. Therefore, the identification of telomere defects could serve as potential biomarkers of pre-malignant or malignant skin cancer lesions after UV exposures in XPV patients. Beyond XPV patients, the early identification of telomere aberrations after genotoxic exposures could be useful in detecting deficiencies in DNA damage response mechanisms, or in detecting pre-malignant skin lesions since telomere alterations can drive carcinogenesis. The manifestation of telomere aberrations in XPV cells as a result of two ubiquitous environmental genotoxicants, UVC or Cr(VI), illustrates the special value of polη's role in preserving chromosomal integrity. Polη not only ensures replication fork progression, it creates another opportunity for the cell to properly repair the DNA damage following replication. Polη works to preserve

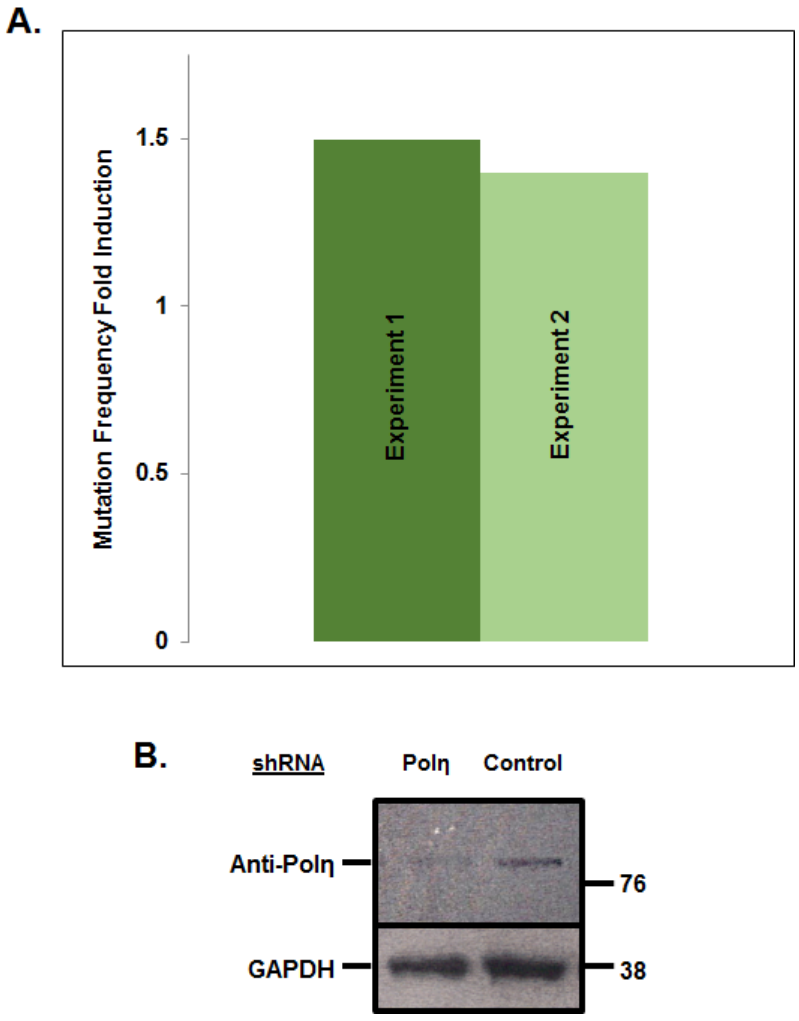
telomere integrity and ultimately, defend the cell from genome instability, senescence, or carcinogenesis. Preventative interventions aimed at telomere preservation following exposure to environmental genotoxicants, could potentially inhibit or delay the onset of diseases and pathologies that are promoted by telomere defects.

APPENDIX: PRELIMINARY STUDY

EVIDENCE THAT POLYMERASE H DELETION INCREASES THE MUTATION FREQUENCY AFTER CR(VI) EXPOSURE – A PRELIMINARY STUDY

O'Brien *et al.* showed that polη was protective against Cr(VI)-induced mutations in *S. cerevisiae*, suggesting that polη was accurately bypassing Cr(VI)-induced DNA adducts (O'Brien et al., 2009). Given our studies indicating polη foci formation after Cr(VI) exposure in human cells (Fig. 12), we asked whether polη was accurately bypassing Cr(VI)-induced adducts in these cells. To address this, we employed the *supF* shuttle vector (SV) mutagenesis assay (described in section 3.5.3, Fig. 18). Our study originally planned to compare mutation frequencies of untreated and Cr(VI)-treated SVs replicated in polη proficient and deficient cells. However, recovery of the untreated SV was not successful. Therefore, we only report mutagenesis data for the Cr(VI) treated SV. First, we exposed the SV to 100 μM Cr(VI) and the dominant reducer of Cr(VI), ascorbate (Standeven and Wetterhahn, 1992), *in vitro* for 30 minutes. Cr(VI)-treated and untreated control SVs were then transfected into human cells. U2OS cells stably expressing either a control shRNA against GFP (Control) or an shRNA targeted against polη (shPolη) (Fig. 19B) were used to replicate the Cr(VI)-treated SV. Cells were

allowed to replicate the SV for 48 h before isolating the SV and transfecting them into the *E. coli* reporter MBM7070 cells for the identification of colonies harboring inactivating supF SV mutations. We observed a 1.5 fold induction in mutation frequency in shPolη cells compared to controls for the Cr(VI) treated SVs (Fig. 19A). Our data provides evidence that, similar to yeast, polη is able to accurately synthesize past Cr(VI)-induced adducts.



(A) Mutagenesis was examined by propagating pSP189 plasmids in U2OS polη KD and control cells. The number of *supF* mutants and the yield of replicated progeny were scored in the *E. coli* MBM7070 strain. Data indicate the fold induction between Cr(VI)-treated vectors replicated in control cells compared to shPolη cells from two independent experiments. (B) Western blot shows polη protein levels in U2OS cells stably expressing controls or polη shRNAs.

Figure 19. Polymerase η depletion increases the supF mutant frequency of Cr(VI) exposure vectors.

A.1 MATERIALS AND METHODS

A.1.1 Cell Culture

Human U2OS osteosarcoma cells were obtained from ATCC (Manassas, VA). U2OS cells proficient and deficient for polη were generated by stably expressing short hairpin RNA (shRNA) against GFP (Control) and shRNA against polη (shPolη). We obtained five lentiviruses from the University of Pittsburgh Cancer Institute Lentiviral Core that express an shRNA against polη (MISSION® shRNA, Sigma) and a control lentivirus (shRNA against GFP). Stable clones were obtained by lentiviral transduction and selection by culturing in the presence of puromycin (500 ng/ml). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (50 units/ml), and streptomycin (50 units/ml) in humidified chambers with 5% CO_2 and 20% O_2 at 37^0C.

A.1.2 SupF Mutational Analysis of Telomeric and Control Shuttle Vectors

The shuttle vector (SV) plasmids, pSP189, harboring *supF,* a suppressor tyrosly tRNA mutagenic reporter gene, was constructed to contain six telomeric repeats as previously reported (Damerla, 2012). The SV was incubated in the presence of 100 μM Cr(VI) and 1 mM ascorbate for 30 minutes at 37ºC. Cr-exposed SV and untreated controls were

transfected into U2OS polη knock down (KD) cells and controls cells by mixing 2 µg SV with 2 x 10^6 cells in 100 µl of nucleofector kit V solutions and electroporating with the Amaxa Nucleofection system (Lonza). Cells were allowed to replicate in the presence of the SV for 48 h in supplemented DMEM media. SVs were then isolated using PureLink Quick Plasmid Miniprep kit (Invirtogen), and digested with *DpnI* enzyme to separate out unreplicated vectors. Next, purified SV were transfected into *Escherichia coli* MBM7070 strain bacteria that contains the *lacZ* amber mutant stop codon and incubated for 45 minutes at 37ºC. Cultures were plated on selective media containing 50 µg/ml chloramphenicol (chlor), 0.12 mg/ml X-gal and 0.3 mg/ml IPTG permitting white/blue screening (Wang et al., 2006). *SupF* mutant frequency was determined by dividing the number of mutant white colonies by the total number of chlor-resistant colonies.

A.1.3 Western Blotting

To confirm polη depletion by the shRNA against polη, cell lysates were immunoblotted. Cells were collected by scraping, and were washed with cold PBS. Cells were resuspended by whole cell lysis buffer (50 mM Tris [pH 7.4], 20 mM NaCl, 1 mM $MgCl_2$, 0.1% sodium dodecyl sulfate, 50 U/ml benzonase and protease inhibitor cocktail (1 µg/ml chymostatin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.4 µl/ml AEBSF). Lysates were incubated on ice for 30 min and cell debris was centrifuged at 15,000g for 20 min at 4ºC. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to an immunoblot membrane. Proteins were probed using anti-polη (Sigma Prestige Antibodies HPA006721, 1:2000) or anti-GAPDH

(Santa Cruz, 1:1000). Probes were visualized using horseradish peroxidase-conjudated secondary antibodies (1:5000) and enhanced chemiluminescent plus (Amersham Biosciences, NJ).

A.2 POLYMERASE H SUPPRESSES CR(VI)-INDUCED MUTAGENESIS. A DISCUSSION

XPV, the autosomal recessive genetic disorder, causes an increased frequency of skin cancer from sunlight exposure by 1000-fold (Cleaver, 2000; van Steeg and Kraemer, 1999). Polη inserts adenines opposite thymine-thymine (T-T) dimers rather efficiently (McCulloch et al., 2004). It is generally accepted that when polη is lacking, other TLS polymerases substitute for this role in spite of their decreased fidelity of DNA synthesis (Cleaver, 2000; Johnson et al., 2000; Zhu and Zhang, 2003). However, polη also exhibits low fidelity of DNA synthesis on undamaged DNA or while bypassing other types of DNA adducts. For instance, polη showed increased mutagenicity on templates containing benzo[a]pyrene adducts, and templates harboring cisplatin cross-linked di-guanine adducts (Masutani et al., 2000; Zhang et al., 2002).

We found the generation of replication blocking lesions by Cr(VI) also caused an increase in the SV supF mutant frequency in cells lacking polη, compared to Wt cells (Fig. 19). This suggests that polη's role in bypass of Cr(VI)-induced lesions is accomplished with a good degree of accuracy, thereby suppressing potential genomic instability and carcinogenicity. In a previous study in yeast, the polymerase ζ homolog

rev3, and polη homolog *rad30*, are both reportedly involved in the response to Cr(VI) exposure (O'Brien et al., 2009). In the absence of *rad30*, the incidence of mutagenesis increased by 2.7-fold. In the presence of a Wt *rad30* gene and the absence of the *rev3*, gene, the incidence of mutations decreased three-fold (O'Brien et al., 2009). This suggests that the Rev3 polymerase contributed to mutagenesis after Cr(VI) exposure, but the Rad30 polymerase achieved more accurate bypass comparatively. We reported a 1.5 fold increase in mutation frequency between Cr(VI)-treated vectors replicated in cells knocked down for polη expression compared to control cells. While this preliminary result suggests a role for polη following Cr(VI) exposure, the magnitude of the difference in mutation frequency between polη proficient and deficient cells was lower than expected. There are several possible reasons for this. First, from a technical standpoint, shRNA gene silencing does not achieve complete depletion of targeted gene. Residual levels of polη are fully functional and may contribute to accurate bypass of the Cr-DNA adducts, thus, lowering the mutation frequency. Second, while polη knock down cells may be deficient in TLS by polη, these cells are still proficient for other mechanisms that are involved in responding to Cr(VI)-induced lesions; namely NER (Reynolds et al., 2004) and MMR (Peterson-Roth et al., 2005). Perhaps in the absence of polη, the mechanisms are upregulated. Third, polη is not the only TLS polymerase available to respond to replication fork blocking lesions as previously mentioned. While the accuracy of other TLS polymerases in bypass of Cr(VI) is completely unknown, they could potentially contribute to successful and accurate bypass of Cr(VI)-induced lesions.

A notable problem in our study is the lack of mutation frequency data for untreated SVs replicated in the polη proficient and deficient cells. Unfortunately, each experimental replicate performed experienced technical problems with regard to the untreated SV conditions, and we were unable to recover accurate results. One could argue that the 1.5-fold increase in Cr(VI)-treated SV mutation frequency for polη deficient cells, compared to control cells, could also be expected for the untreated SVs, suggesting that the mutagenesis difference reflects polη roles in accurately bypassing endogenous damage and rather than the Cr(VI)-induced damage. However, a very recent study reported similar background mutation frequencies in unexposed XPV and normal human fibroblasts (Herman et al., 2014). Consistent with this, mice lacking functional polη did not exhibit an increased incidence in tumorigenesis when they were not exposed to genotoxicants (Lin et al., 2006). Although it has been shown *in vitro* that bypass by polη on undamaged templates is error-prone (Matsuda et al., 2000), polη's function is believed to be highly regulated against synthesizing DNA at undamaged regions in the genome (King et al., 2005; Pavlov et al., 2001; Waters et al., 2009). Taken together, our results require further and more complete analysis on the mutagenicity of XPV cells after Cr(VI), but provide good evidence that polη bypass reduces the mutagenicity of Cr(VI)-induced lesions in human cells.

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