DNA Damage Tolerance in Mammalian Cells

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology, University of Saskatchewan, Saskatoon, Canada.

Prepared by Parker Lyng Andersen,
Summer 2009

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Head of the Department of Microbiology and Immunology
107 Wiggins Road
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5E5

Acknowledgements

For this thesis I am in great debt to many people.

Dr. Wei Xiao has created a fantastic research environment with respect to the research being performed and the collection of individual researchers under his supervision. Dr. Xiao has always been very inspirational to me as he can always build excitment about the research I am doing. Dr. Xiao is a great mentor and a great friend.

My committee members including Dr. V. Misra, Dr. S. Carlsen, Dr. P. Howard and particularly Dr. B. Juurlink have been great inspirations always with kind words and helpful advice, and I thank each of them greatly.

I am grateful for Dr. B. Wouters for agreeing to serve as my external examiner for this thesis.

Dr. B. Ziola has donated a tremendous amount effort and time in the supervision of the antibody production portion of this research work, and without his expertise this research would not have been possible. Additional thanks goes to Dr. F. Xu for assistance in creating the Rev3 antibody.

I am also grateful for every member of the Department of Microbiology and Immunology, and the many other labs I've been associated over the years for continuously helping me and encouraging me without fail, including Hania (a wonderful friend), Michelle (for all the ordering and proofreading this thesis), Landon (all the science arguments), Lindsay (both of them), Rui, Shaun, Mike, Ke, Audesh, Mike, Katherine, Syd, Susan, Lynn, Yuping, Yu Fu, Leslie, James, Treena, Todd, Stacey, Danielle, Dr. Loop, Jill, Henry, Matt, Tristan, Doug gray, Jan, Monique, Emily, Fang, two Sarahs, Noor, Nida, Sabina, Pat, Dilip, Bin, Layne, Harold, Katie and Vern, Bonnie, Arlene, Carmen, Mary, Dawn, Shirley, Corrine, Cynthia, Ron, Ric, Sherry, David, Val, Jayne, Andrew, Brynn, Christine, Adil, Patrick and many others I'm sure I've forgotten to mention.

I am also grateful to the College of Medicine, to the Dept. of Graduate Studies and Research, and to CIHR for funding my position.

I am also very thankful for my family, particularly mom and dad for never-ending continuous support and encouragement. And thanks even to all my rotten little nieces and nephews.

List of Abbreviations

(6-4)PP	Pyrimidine-pyrimidine (6-4) photoproduct
8-oxoG	*8*-oxo-7,*8*-dihydroguanine
A	Adenine
a.a.	Amino acid
Ab	Antibody
AP	Abasic site
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BER	Base excision repair
BRCT	BRCA1 C-terminal
BrdU	Bromodeoxyuridine
C	Cytosine
cDNA	Complementary DNA
Co-IP	Co-immunoprecipitation
CPD	Cyclobutane pyrimidine dimer
CPT	Camptothecin
Cys	Cystine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleotide triphosphate
DDT	DNA damage tolerance
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	Double strand break
DUB	Deubiquitinating enzyme
G	Guanine
γ-H2AX	Phosphorylated histone 2AX
ICC	Immunocytochemistry
Lys	Lysine
$^{\bullet}$OH	Hydroxyl radical
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween-20
PCNA	Proliferating cell nuclear antigen
Pol	Polymerase
PRR	Post-replication repair
recA*	Activated RecA
RPA	Replication protein A
RFC	Replication factor C
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SUMO	Small ubiquitin-like modifier
T	Thymidine
TLS	Translesion synthesis
Ub	Ubiquitin
UBZ	Ubiquitin binding zinc finger
UBM	Ub-binding motif
UV	Ultraviolet
XP	Xeroderma pigmentosum
XPV	XP variant

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Nomenclature

Bacterial genes are written in lower case letters in italics (i.e., *recA)*

Bacterial proteins are written with a capital first letter (i.e., RecA)

Yeast genes are written with capital letters in italics (i.e., *MMS2)*

Defective yeast genes are written in lower case letters in italics (i.e., *mms2*)

Yeast proteins are written with a capital first letter (i.e., Mms2)

Human genes are written in capital letters in italics (i.e., *MMS2)*

Mouse genes are written with a first capital letter in italics (i.e., *Mms2)*

Mammalian proteins are written with a capital first letter (i.e., Mms2)

The target of interference RNA is written as the gene name in italics with the first letter capitalized and preceded by the letter "i" (i.e., *iMms2*).

siRNA refers to interference RNA derived from a DNA coding sequence producing a short-hairpin RNA (i.e., *siUbc13*).

RNAi refers to interference RNA derived from synthetic dsRNA (i.e., *Ubc13i*).

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ABSTRACT

DNA is susceptible to both exogenous and endogenous damaging agents. Damage is constantly reversed by a wide range of DNA repair pathways. Lesions which escape such repair may cause nucleotide mis-pairing and stalled replication, resulting in mutagenesis and cell death, respectively if left unresolved. Stalled replication is particularly dangerous because replication fork collapse can lead to double-strand breaks (DSBs) and chromosome rearrangement, a hallmark of cancer. DNA damage tolerance (DDT) is defined as a mechanism that allows DNA synthesis to occur in the presence of replication-blocking lesions.

DDT, also known as post-replication repair (PRR) in yeast, has been well characterized in the lower eukaryotic model *Saccharomyces cerevisiae* to consist of error-free and error-prone (mutagenic) pathways. Mono-ubiquitination of proliferating cell nuclear antigen (PCNA) by the Rad6-Rad18 complex promotes mutagenesis by recruiting low fidelity translesion synthesis (TLS) polymerases, while continual Lys63-linked poly-ubiquitination of PCNA by the Mms2-Ubc13-Rad5 complex promotes error-free lesion bypass. Since most of the genes involved in DNA metabolism are conserved within eukaryotes, from yeast to human, I tested the hypothesis that mammalian cells also possess two-pathway DDT in response to DNA damage. Namely, the error-free pathway is dependent on the Ubc13-Mms2 complex, while the error-prone pathway utilizes the TLS polymerases, such as Rev3.

By utilizing cultured mammalain cells and producing antibodies against human Ubc13, Mms2 and Rev3, I was able to show that all three proteins associate with PCNA in S-phase cells, and that this association is enhanced following DNA damage. Ubc13-

Mms2 association with PCNA was enhanced in response to DSBs. Furthermore, suppression of Ubc13 or Mms2 using interfering RNA technology resulted in increased spontaneous DSBs. In response to UV exposure, Rev3 co-localized with PCNA and two other TLS polymerases, Rev1 and Pol-η, at the damage site. UV-induced Rev3 nuclear focus formation was dependent on Rev1 but independent of Pol-η. Surprisingly, over-expression of Pol-η was sufficient to induce spontaneous Rev3 nuclear foci. It was further demonstrated that Rev1 and Pol-η were independently recruited to the damage site and did not require Rev3. These observations support and extend the polymerase switch model which regulates the activity of the replicative and TLS polymerases. Finally, simultaneous suppression of Rev3 along with Ubc13 or Mms2 resulted in a synergistic sensitivity to UV, whereas simultaneous suppression of Ubc13 and Pol-η resulted in an additive effect. These results are consistent with those in yeast cells, implying a comparable mammalian two-pathway DDT model.

Additional interesting observations were made. Firstly, Ubc13 interacts with Uev1A, a close homolog of Mms2, which is involved in the NF-κB signaling pathway independent of DNA damage. Secondly, Rev3 appears to be excluded from the nucleus in a fraction of low passage normal non-S-phase cells, whereas in tumor derived cell lines, Rev3 is consistently enriched in the nucleus independent of cell cycle stage. Finally, Rev3 is elevated during mitosis and associates with condensed chromosomes, suggesting a possible novel role in mitosis. Consistent with this notion, chronic ablation of Rev3 resulted in cell death with inappropriate chromosome segregations. The above preliminary observations require further investigation.

CHAPTER ONE

INTRODUCTION

1.1. DNA Maintenance

Typical human cells contain approximately three billion nucleotide base pairs in their genomic DNA in a highly ordered and conserved arrangement, double this directly following DNA duplication. Proficient maintenance and duplication of the genome is required to ensure near permanent storage of all genetic and often epigenetic information, which is ultimately to be faithfully passed on to all progeny through countless generations. Proper maintenance of the genome requires: 1) the stability of nucleotide coding sequences and associated sequences such that correct products (functional peptide-coding and non-coding RNA products) are produced in the correct tissue when appropriate, 2) the minimization of chromosome rearrangements to avoid translocations, duplications, expansion/contractions and insertions of large sections of DNA, 3) the maintenance of the structural portions of DNA (such as telomeres and centromeres) to ensure correct genome segregation in dividing cells, and 4) preservation of epigenetic factors such as nucleotide methylations which coordinate gene expression. All these are required for cell survival and for the avoidance of mutagenic events, which have the potential to lead to cell pathology and cancer progression.

The progression of cancer is largely due to the continued acquisition of mutations in the genome derived from nucleotide alterations. The type of cancer formed is a consequence of the particular genes that have been mutated, how they are mutated

and the tissue in which these mutated genes occur. A single mutation is likely incapable of becoming cancerous. Based on the age of onset of unilateral and bilateral retinoblastoma, a "two hit" mutation hypothesis (loss of both alleles) was originally assumed to be responsible which led to the hypothesis that mutations inactivating tumor suppressor genes are sufficient for tumor growth (Knudson, 1971). However further analysis has indicated that although the two hit model in retinoblastoma may initiate uncontrolled growth, many additional genomic alterations occur (Corson and Gallie, 2007). Analysis of 188 human lung adenocarcinomas for mutations in 623 specific genes thought to be involved in cancer have revealed that many but not all of these genes are mutated in a predictable fashion. Twenty-six of these genes were mutated at a high frequency in most of the samples whereas the remaining appeared to be mutated sporadically from sample to sample (Ding et al., 2008). This has been suggested to reflect that for the progression of this type of cancer, specific mutations must occur in many of these twenty-six genes, with little requirement from the genes which are not continuously mutated across the samples. Although not considered essential for tumor progression, these additional genes exhibiting low and random mutation rates may be important in the tumor diversity with respect to clinical treatment responses.

Mutations most often arise when damage to the DNA results in a permanent alteration in the heritable base sequence such as a transition/transversion event or nucleotide deletion/insertion. Alternatively, mutations may arise from chromosome rearrangements resulting in inappropriate protein products or unsuitable regulation of gene products. Additional factors that can promote cancer include epigenetic alterations to the DNA, such as nucleotide methylation, which affects gene expression

without an alteration in the nucleotide sequence itself. Some cancer causing agents, such as asbestos, are mutagens without directly altering the DNA. Asbestos is taken up by the cell but not metabolized, resulting in a prolonged inflammation reaction and possibly directly inhibiting the normal operation of the mitotic spindle, and possibly resulting in tumor progression (MacCorkle et al., 2006; Shukla et al., 2003).

Although the progression of cancer is largely dependent upon the continued acquisition of mutations, not all mutations are harmful to the cell. Many mutations may occur in introns of peptide coding genes with no effect on the gene product. Also, due to the wobble position of the genetic code leading to alternative nucleotides ultimately coding for the identical amino acid, or silent mutations being introduced resulting in the utilization of an amino acid similar to the native amino acid, mutations may be inconsequential to the gene product. In fact, under certain circumstances, nucleotide sequence alterations are important for the organism. For instance, germ line crossover events between sister chromatids are important for induction of genetic diversity in offspring. Additionally, in somatic cells of the immune system, point mutations and gene rearrangements play critical roles in antibody diversification and maturation, a process known as somatic hypermutation.

Chemical functional groups within the nucleotide bases and along the ribose-phosphate backbone of DNA impart a natural reactivity of DNA with the physical and chemical items in its environment, which in mammalian cells is an oxygen-rich aqueous environment. This makes DNA an inherently unstable molecule. Human DNA damage is estimated to occur in the order of 10^4-10^6 lesions per cell per day (Ames and Gold, 1991; Lindahl, 1993). However, DNA must and does remain a stable molecule

through countless generations, which ensures survival of the cell and of its descendants. Overall, the stability of the genome can be considered as combined activities of: 1) replication fidelity, 2) the type and extent of the damage the DNA is exposed to, and 3) the course of action in response to damage.

DNA damage can occur due to endogenous factors such as normal cellular products (e.g., endogenously produced reactive oxygen species) and exogenous factors such as chemical mutagens (which may directly bind DNA, intercalate between successive base pairs or obstruct the proteins involved in DNA metabolism) or physical relations (such as cosmic radiation). How a particular cell responds to DNA damage depends upon the type and amount of DNA damage, and sometimes on the location of the damage.

A DNA damage response can be initiated by various mechanisms. The majority of DNA damage is identified by specific enzymes which recognize incorrect base pairing, distortions in the DNA superstructure, single strand gaps in the DNA or free DNA ends. Mild DNA damage will be repaired without consequence to the cell. However, if the damage is significant, cell cycle checkpoints may be initiated to halt the cell cycle in order to allow repair before proceeding through the cell cycle. Alternatively, in response to more pronounced DNA damage, either a programmed cell death pathway or a senescence pathway may be initiated, likely in an attempt to impede cancer formation.

If DNA damage is not recognized or the machinery is not available for repair, DNA damage may persist and DNA synthesis may proceed in the presence of damage in a process called DNA damage tolerance (DDT). The polymerase scanning hypothesis

relies on RNA and DNA polymerases to initiate a response (Heddle and Bielas, 2005). Due to their high fidelity, these polymerases typically cannot pass sites of damage and either stall replication fork progression or are released from the DNA lesion site. During transcription across a DNA lesion, RNA polymerase-II (RNA Pol-II) may initiate transcription-coupled repair which results in preferential repair of genes that are actively transcribed irrespective of the cell cycle. "Global genome scanning" by the replicative polymerases may result in replication fork collapse in response to DNA damage. If a single replication fork collapses and DNA duplication is not completed there is a danger of chromosome instability during the next mitotic event. Cells have therefore devised ingenious methods to tolerate damage at a stalled replication fork to ensure complete genome duplication. Damage is thought to be tolerated by various DDT mechanisms in order to allow complete genome duplication to proceed with the likely intent of repair at a more convenient time. However, during DDT, a DNA lesion is thought to have the potential to be converted into a permanent mutation and therefore promote tumor progression.

1.2. The high fidelity replication system

The basic structure of DNA is made up of two long non-covalently bound polymers consisting of covalently bound deoxyribonucleotides. Each nucleotide consists of a single organic base (the purines adenine [A] and guanine [G], and the pyrimidines thymine [T] and cytosine [C]) linked to the C^1 position of 2-deoxyribose and a phosphate group linked to the C^5 position of the ribose. The nucleotides are covalently linked in tandem by phosphodiester bonds between the 3'-OH group of the

deoxyribose and the phosphate group of the preceding nucleotide (derived from a deoxynucleotide triphosphate [dNTP]). This gives polarity to the polynucleotide, such that each polymer has a 3'-OH group at one end and a 5'-phosphate group at the opposite end. Original X-ray crystallographic studies were used to derive the structure of DNA (Watson and Crick, 1953). The basic structure of DNA consists of two polynucleotide chains forming a double helix with opposite polarity (one 5'→3' aligned with a polymer of the opposite 3'→5' orientation). The two sugar-phosphate backbones lie at the external portion of the double helix with the bases lying within the structure. The bases are situated opposite one another, aligned by hydrogen bonding between specific complementary pairs (Watson-Crick base pairing) such that A aligns only with T and G aligns only with C in a step-like manner stabilized by pi-bond interactions between sequential bases of the same polynucleotide chain. This orientation gives DNA a highly ordered structure that has remained conserved in all living organisms, with the nucleotide base sequence as the most distinguishable characteristic between species.

Duplication of DNA proceeds in a semi-conservative manner initiated by the DNA being separated into two single strands followed by reciprocal daughter strand duplication onto each parental strand (the template strands) by the sequential incorporation of single dNTPs by the replicative polymerases (Pols) to complement the template nucleotides based on Watson-Crick base pairing. Each complementary (daughter) strand remains non-covalently associated with the template until the following phase of DNA duplication. Each daughter strand will contain the identical nucleotide sequence of the strand which it replaced. All DNA polymerases synthesize

DNA in a 5'→3' direction (with respect to the sugar-phosphate backbone) from a preexisting primer (RNA or DNA) already hydrogen bound to the template strand in a two-metal-ion dependent nucleotide transfer mechanism (Brautigam and Steitz, 1998).

The majority of DNA duplication is carried out by the replicative polymerases Pol-δ (lagging strand) and Pol-ε (leading strand) (Kunkel and Burgers, 2008; McElhinny et al., 2008). In either case, polymerization occurs only in the 5'→3' direction, therefore the leading strand can continue uninterrupted for thousands of bases, whereas the lagging strand from the same replication fork must be intermittently re-initiated using RNA primers (by the enzyme primase) and the formation of short stretches of DNA (termed Okazaki fragments) which must be later ligated by the enzyme ligase. The RNA primers used to initiate Okazaki fragments are produced by primase (10-20 million times per cell cycle) and later removed by the 3'→5' proofreading activity of Pol-δ. DNA synthesis is therefore much more processive on the leading strand than the lagging strand.

By X-ray crystallography, the bulk of genomic DNA is thought to be occur as a double helix in the B-form configuration (Watson and Crick, 1953). That is to say it consists of a right-handed double helix which completes a single turn every 10.5 base pairs, however this highly ordered structure is likely to be distorted by the presence of DNA interacting proteins, such as histones. The highly efficient and highly accurate duplication of DNA is only possible in the absence of DNA damage (or absence of helical distortions) due to the high stringency of the active site of the replicative polymerases with the pre-existing B-DNA comprised of the template strand and the priming strand which nucleotides will be attached to. Additional physiological forms of

genomic DNA likely include A-DNA and Z-DNA. A-DNA is composed of a slightly relaxed helix often composed of a DNA-RNA hybrid, and may be physiologically relevant during DNA priming of the lagging strand utilized to initiate Okazaki fragment formation during normal duplication. Z-DNA is a tightly wound left-handed helix which has been postulated to be stabilized by methylation in regions with a high density of C and G, termed CpG islands, with a general result of inhibiting transcription, but its unwinding by helicases likely allows DNA synthesis by replicative polymerases. However, some mutational hot spots are associated with CpG islands suggesting the possibility of defective DNA maintenance in these areas (Tsai et al., 2008). Significantly, DNA may also form additional structures such as hairpin loops or crucifix structures in regions of inverted repeats or A-T rich sequences which may lead to polymerase stalling and the formation of fragile sites with increased chromosome translocations (Durkin and Glover, 2007). Any distortion in the template-primer structure from the normal B-DNA will not be tolerated by the highly stringent polymerases and therefore DNA synthesis will cease in the presence of DNA damage or at common or reare fragile sites induced by helix distortions.

A key component of the duplication machinery is a processivity factor which in eukaryotes is termed proliferating cell nuclear antigen (PCNA). PCNA occurs as a homotrimer and is loaded around DNA in a donut fashion by replication factor C (RFC), a heteropentameric complex with DNA-dependent ATPase activity (Yao et al., 2003). Upon loading of PCNA, RFC is released and replaced by the replicative polymerase Pol-δ (Yao et al., 2003). Without PCNA, the Pol-δ processivity drops more

than 100-fold because of a decreased affinity for DNA and a propensity for improper nucleotide incorporation (Lee and Hurwitz, 1990; Lee et al., 1991).

The high fidelity of DNA synthesis is the most important means of DNA preservation (Friedberg, 2006). This is primarily achieved by three mechanisms: 1) correct base insertion, 2) prevention of mismatch extension, and 3) exonuclease proofreading. Prevention of mismatch insertion largely depends upon the correct binding of the incoming dNTP. The polymerases with highest fidelity appear to preferentially bind the correct dNTP, and this step is thought to be the most essential step for correct nucleotide incorporation (Beard et al., 2002).

Due to the molecular spacing of the CG and AT base pairs being very consistent, the double helix has a very conserved architecture regardless of the base sequence. This is crucial when a polymerase bound to the backbone and primer accepts an incoming nucleotide, so that the polymerase will constrain the incoming nucleotide with the template and promote nucleophile attack between the 3'OH of the existing primer and the primary phosphate of the incoming nucleotide. This only occurs if correct Watson-Crick base pairing is preserved. The crystal structures of several DNA polymerases in association with DNA have been deduced and have revealed that a portion of the active site is formed by the existing primer base paired with the template strand (Jager and Pata, 1999). Therefore, if there is a mismatch in the primer-backbone structure resulting in a distortion in the double helix, polymerase activity is significantly inhibited, and the incoming dNTP is not incorporated. Polymerization is therefore dependent on correct base pairing presented on the existing template-primer prior to further elongation of the primer (Goodman et al., 1993).

Additionally, the high fidelity of replication polymerases is enhanced by their 3'→5' proofreading exonuclease activity, which acts as a safeguard in case an inappropriate base is inserted into the nascent strand. A replicative polymerase cannot extend from a mismatched pair between the primer and substrate; therefore it stalls. Upon excision of the non-Watson-Crick base-pair by the exonuclease function of the polymerase polymerization can proceed.

The combination of the above three activities results in high fidelity of the replicative polymerases, amazingly incorporating only a single nucleotide mismatch in every 10^7 bases introduced into the DNA (Friedberg, 2006).

DNA replication occurs in replication factories within the perichromatin regions (between the condensed chromatin and interchromatin space) scattered about the nucleus (Jaunin et al., 2000). These factories are relatively immobile structures, each containing multiple replication forks of up to 5-6 double strands of DNA simultaneously (or more in late S-phase). Utilizing electron micrographs in conjunction with pulse labeling techniques, replication factories were observed to be immobilized and anchored to the nuclear matrix, probably by lamin-B, with replication occurring as the DNA passes from the replication factories into the condensed chromatin (Hozak et al., 1993; Jaunin et al., 2000). Using fluorescence microscopy, the factories were not observed to move or combine with one another, but rather form by disassociation and re-association with the majority of the movement appearing to be DNA passing through the stationary factories, not replication factories traveling along the DNA (Leonhardt et al., 2000). The replication factories have a lifespan of approximately one hour (Jaunin et al., 2000) and by immunoflourescence means using synchronized cells, factories are

described as punctuate throughout the nucleus in early S-phase, become localized near the nuclear periphery in mid S-phase and then appear as large structures often associated with nucleoli in late S-phase (O'Keefe et al., 1992). The replication factories are thought to coordinate sequential enzymes, particularly on the lagging strand which continuously switches between polymerase and primase and the associated DNA synthesis machinery (Frouin et al., 2003).

1.3. Variations of DNA damage

DNA damage can be induced by either endogenously (spontaneous) or exogenously (environmental) derived factors. Continual chemical reactions occur in every cell with many undesirable side products such as the production of reactive oxygen species (ROS) which are highly reactive with the many functional groups on the DNA. Environmental factors may directly damage DNA, for example by causing a photoreaction on nucleotides in response to ultraviolet (UV) irradiation, by chemically reacting with the DNA, or initializing the formation of reactive compounds near the DNA, such as ROS production in response to ionizing radiation. Additionally, some environmental factors are often processed by the cell to produce products that inadvertently interact with DNA. Furthermore, some agents may not interact with DNA but may affect its metabolism such as oxidation of free nucleotides or intercalation between adjacent base pairs. However, lesions to the ribose units or to the phosphodiester linkages may also have a profound effect on the genome resulting in incomplete DNA synthesis, chromosome rearrangements or chromosome non-disjunction during mitosis may occur.

1.3.1. Endogenous *vs.* exogenous damage

Wild type cells living in a favorable environment have the astounding ability to maintain their genome through countless generations despite persistent endogenous and spontaneous damage. Because mammalian DNA is essentially housed in an aqueous environment surrounded and dependent on an oxygen-rich atmosphere, it is very susceptible to hydrolysis and oxidation during normal cellular metabolism. However, when exposed to environmental factors, the DNA damage becomes more pronounced and the induced mutation rate may increase significantly. Environmental exposure has therefore become a major area of research into tumorigenesis. The importance of adapting to the environment by recognizing and processing exogenously derived factors is essential for maintenance of the genome. Environmental factors include radiations and chemical compounds that may directly or indirectly damage the genome.

1.3.2. Deamination

The amino groups of bases are subject to spontaneous deamination. A, C, G and 5-methylcytosine (a variant of cytosine) can undergo deamination reactions to form hypoxanthine, uracil, xanthine and thymine respectively. Each of these will have inappropriate base-pairing characteristics upon duplication and therefore have the potential to alter the DNA sequence. The most notable and most biologically active reaction is the deamination of cytosine to uracil by a hydroxyl ion reacting with the 4-methyl group of cytosine. This, if left unrepared, will produce GC→AT transitions (Duncan and Miller, 1980; Radany et al., 2000). Cytosine deamination occurs slowly at

physiological levels but may increase 100-fold in the presence of ssDNA possibly because the bases become open to the surrounding aqueous solution (Lindahl and Nyberg, 1974). Polymerase stalling, which has the potential to produce sections of ssDNA due to disassociation of the DNA unwinding helicase from the replication machinery may therefore exacerbate spontaneous damage (Byun et al., 2005; Impellizzeri et al., 1991).

Deamination of adenine to hypoxanthine is also potentially mutagenic if it is not repaired as it can cause an AT→GC transition during the next round of DNA synthesis (Lindahl, 1979). Deamination of guanine to xanthine likely cannot form stable hydrogen bonds with any of the bases and therefore is thought to stall DNA synthesis (Greer and Zamenhof, 1962).

1.3.3. Generation of abasic sites

Under physiological conditions, hydroxyl attack on the N-glycosidic bond of DNA, while leaving the phosphodiester backbone intact, can release each of the individual bases leaving abasic (AP) sites (Lindahl and Barnes, 2000), which have been estimated to occur thousands of times spontaneously in human cells every day (Nakamura et al., 1998). Additionally, DNA glycosylases function in removing a single base to initiate the base excision repair pathway resulting in a temporary AP site which must be further restored (Sakumi and Sekiguchi, 1990). AP sites have a very short half-life unless the downstream steps in their repair are jeopardized (Guillet and Boiteux, 2003). If left unrepared, during DNA synthesis AP sites have the potential of leading to mutation by either the incorporation of an incorrect base or due to the induction of a

frame shift mutation by slippage between the template and nascent strand. Additionally, AP sites have the possibility of being unstable, particularly if they occur within ssDNA or close to another AP site on the opposite DNA strand, and therefore have the potential to be converted into DNA strand breaks (Male et al., 1982).

1.3.4. Oxidative damage to DNA

In all aerobic organisms normal metabolism produces reactive oxygen species (ROS). These are often used in biological processes such as pathogen clearance, macromolecule breakdown, and cell signaling. The presence of anti-oxidants and mechanisms to maintain a balanced reduction/oxidation potential is used within the cell so that ROS will not be excessive. ROS are considered a potent and continuous source of endogenous damage, which affect all the major macromolecules including proteins, lipids and nucleic acids. Therefore, ROS have the potential to be very damaging to the cell and have been suggested to play a role in various disease states, particularly degeneration and aging.

Oxidative damage can arise from endogenous and exogenous sources. The most common endogenously produced ROS is likely the hydroxyl radical ($^{\bullet}OH$) produced by the electron transport chain in the mitochondria (Breimer, 1988). However, because the free $^{\bullet}OH$ molecule has a very short diffusion range due to its high reactivity, it likely is not a source of genomic DNA damage (Pryor, 1986). ROS have been proposed however to damage mitochondrial DNA. An additional reaction, the Fenton reaction, produces a $^{\bullet}OH$ from a freely diffusible H_2O_2 molecule in the presence of a metal ion. One metal ion with this capability is thought to be iron (Fe^{2+}) associated with the

negatively charged phosphate groups of the DNA backbone (Dizdaroglu et al., 1991; Izatt et al., 1971). This reaction has the potential to produce a hydroxyl free radical directly adjacent to the DNA and is likely the major source of endogenously derived ROS damaging DNA. The most common source of exogenously derived oxidative damage is ionizing radiation reacting with water to form $^{\bullet}OH$. A single high energy photon derived from radio decay of cobalt^{60} is estimated to produce as many as 36,000 free radicals in an aqueaous solution (Breimer, 1988).

Oxidation damages DNA in a multitude of ways which include strand breaks, production of AP sites (von Sonntag, 1987) and direct base alterations (Bjelland and Seeberg, 2003). One of the most notable biologically relevant examples of oxidative DNA damage is the formation of 8-oxoguanine (8-oxoG). This results in protonation of the N^7-atom of guanine, which if flipped into the *syn* position, allows base pairing with adenine resulting in the potential to be processed into a GC→TA transversion during the next round of DNA synthesis (Kasai and Nishimura, 1984; Shibutani et al., 1991).

1.3.5. Radiation damage to DNA

Radiation damage can be initiated from X-rays, γ-rays and UV exposure. Primary lesions formed from these are due to the production of free radicals (as described above). However, additional damage can occur from the high energy ionizing radiations (X-rays, γ-rays) by a direct interaction with DNA. Direct interactions typically can involve the abstraction of a hydrogen atom from the ribose of the DNA backbone which will be rapidly converted into a single strand break (Breen and Murphy, 1995). Double strand breaks (DSBs) may arise either from dual single event

lesions occurring within close proximity, or indirectly following the production of multiple free radicals from a single photon.

Low energy radiation in the form of UV light is readily absorbed by both aromatic amino acids and aromatic bases of DNA (Kielbassa et al., 1997). Most notable is the formation of covalent bonds between adjacent bases during reduction of their aromatic structures. The most common photoproduct on DNA is the cyclobutane pyrimidine dimer (CPD) and the pyrimidine-pyrimidine (6-4) photoproduct [(6-4)PP] formed between adjacent thymidine bases on the same DNA strand. UV-induced photoproducts can also occur between DNA strands. Typically, when formed in the common *cis-syn* conformation in B-DNA, the TT dimer can still partially interact as Watson-Crick base pairing with the opposite AA dinucleotide with minimal distortion of the double helix (Taylor et al., 1990). However, in ssDNA (such as highly transcribed genes) or Z-DNA/B-DNA regions, a *trans-syn* dimer may be formed, or a dimer may be produced from non-adjacent pyrimidines resulting in a more distorted double helix (Taylor et al., 1990), which may have profound effects on DNA synthesis as it is not expected to fit into the active site of the DNA polymerase as easily as CPDs. (6-4)PPs are formed about a third as frequently as CPDs (Mitchell and Nairn, 1989).

1.3.6. Chemical alteration of DNA

Exogenous or environmental chemical compounds can directly or indirectly damage DNA. Directly, they can bind covalently to the nitrogen or oxygen atoms of the bases. Indirectly, they can be processed from a non-reactive compound into a reactive nucleophile that will then bind the DNA. Alternatively, exogenous factors may

affect the enzymes or substrates that are required for proper DNA metabolism without affecting the DNA directly, which may lead to replication breakdown.

The outcome to chemical modification of the DNA depends largely on the cellular response to the type of adduct formed. Direct modification can occur with alkylating agents such as methyl methanesulfonate, which primarily results in a mono-adduct on the N^7 position of guanine and can be further converted to potentially lethal AP sites by a glycosylase (Shulman, 1993). Methyl methane sulfonate can also methylate the N^3 position of adenine, which results in a replication-blocking lesion (Beranek, 1990). Modification by *N*-methyl-*N*'nitro-*N'*-nitrosoguanidine results in an O^6-methylguanine which has the potential to induce mutations because of its base pairing with thymine during DNA synthesis (Friedberg, 2006). Bi-functional compounds, such as the chemotherapeutic agent cisplatin, are extremely potent as DNA damaging agents because they are able to simultaneously bind two bases of DNA as either intra-strand or inter-strand cross links, or alternatively crosslink to produce a DNA-protein hybrid (Friedberg, 2006). Not only will DNA damage be difficult to repair and DNA synthesis likely impossible without its removal, but chromosome segregation during mitosis will be jeopardized resulting in gross chromosome instability and/or improper cytokinesis.

Various non-polar, hydrophobic compounds are processed by the cell or even another tissue by the cytochrome P450 enzymes that attempt to detoxify hydrophobic xenobiotic compounds by chemically altering them into hydrophilic and polar moleculaes so that they can be excreted from the cell and body. Unfortunately, this conversion may produce a compound capable of binding and distorting the DNA

double helix. One example of this is the conversion of the constituents of incinerated hydrocarbons, benzo[α]pyrene into benzo[α]pyrene dihydrodiolepoxide (BPDE), which is a very potent carcinogen (Phillips, 1983). BPDE binds directly to the N^2-position of guanine with the bulk of the compound lying in the minor groove of the DNA resulting in little distortion of the DNA double helix (Cosman et al., 1992; Graslund and Jernstrom, 1989). Additionally nitrosamines, derived from charcoal barbeques and cigarette smoke are also hydroxylated by the cytochrome P450 system and have the potential to bind DNA and become carcinogenic (Hecht, 1999).

DNA damage can also occur due to chemical modification of the enzymes and processes required for normal DNA synthesis. Topoisomerases are enzymes required for temporary cleavage and religation of the DNA backbone, in either a single-strand or double-strand manner, in order to relax torsional stress as DNA is unwound during duplication or transcription, and to reduce catenation following duplication. Camptothecin (CPT) is a topoisomerase I inhibitor which inhibits the religation of a single strand break in the DNA relxing torsional stress, resulting in a single strand break being processed into DSB as DNA duplication procedes. As with the chemotherapeutic DNA damage inducer cisplatin, CPT has the ability to preferentially kill proliferating cells as opposed to cells removed from the cell cycle (Andersen et al., 2003). Additionally, inactivating the enzyme ribonucleotide reductase by hydroxyurea may result in deoxyribonucleotide depletion with the consequence of stalled synthesis and replication fork collapse. Ultimately, this will produce single strand gaps and possibly double strand breaks in the DNA due to incomplete synthesis (Friedberg, 2006).

1.4. The cellular response to DNA damage

In response to DNA damage, mammalian cells have evolved a series of coordinated responses. Minor DNA damage occurs endlessly within the cell and is continuously repaired without alteration of the cell cycle. If the damage is great enough DNA checkpoint activity will retard progress through the cell cycle, particularly at the G1/S, early-S and G2/M points of the cell cycle. These checkpoints are designed to allow cell cycle progression only after the damage has been corrected. Alternatively, excessive damage may lead to apoptosis as a protection mechanism against tumorigenesis (Bernstein et al., 2002). Accumulated damage over many years is believed to also result in senescence, which may also be a cellular mechanism to forestall cancer progression. The DNA damage response includes: 1) recognition of the damage, 2) propagation of the damage signal by effecter molecules and finally 3) functional processes for removal from the cell cycle and/or repair of the DNA.

1.4.1. Recognition of DNA damage

The primary level of organization of the genome is the double helix with nucleotides pointing inward away from most constituents of the cell with the only accessible portions along the major and minor grooves between the phosphoribose backbones. Presumably, this orientation shields the nitrogenous nucleotides from covalent modifications by reactive molecules. The helix is also associated with numerous protein complexes, most notably histones, which are packaged into the larger nucleosome structures. On a larger scale, the DNA containing regions of the nucleus in

higher eukaryotes is roughly segregated into euchromatin containing the majority of the actively expressed DNA, and heterochromatin, a more tightly condensed and generally non-expressed portion of the DNA. The packaging of the DNA and the orientation of the nucleotides likely reduces the potential damage to the nucleotides from chemical agents due to steric constraints (Friedberg, 2006). Unfortunately, these constraints also have to be overcome so that DNA damage, when and where it occurs, can be recognized and processed accordingly (Wuebbles and Jones, 2004). Cells have evolved various mechanisms to initially locate and analyze damage sites in order to initiate the appropriate DNA damage response.

In some cases DNA damage recognition occurs by enzyme scanning in a linear fashion along the DNA. A few specialized enzymes have the ability to inspect the DNA for particular types of DNA damage and test sequentially by "base flipping" each applicable base pair (i.e. CG bases) into a reactive pocket in which an unwanted base is recognized and removed, as occurs by the bacterial uracil DNA glycosylase (Fuxreiter et al., 2002). The human enzymes apurinic/apyrimidinic endonuclease Apex1 (Carey and Strauss, 1999) and human 8-oxoG glycosylase hOOG1 also will bind intact DNA, and in a processive fashion, scan DNA in a linear manner to detect and excise oxidized bases (Banerjee et al., 2005). To investigate every base pair is likely an energetically unfavorable process due to the shear size of the genome, but has rather been postulated to first scan DNA which has been relaxed from the typical B-form due to reduced base stacking (Yang, 2008).

Polymerase scanning, by either DNA or RNA polymerases, has been suggested as one mechanism to ascertain DNA damage (Heddle and Bielas, 2005). This proposal

is based on the combined observations that 1) transcribed genes containing lesions such as UV-induced CPDs can be more efficiently repaired than non-transcribed genes (Hanawalt and Spivak, 2008), and 2) some premutagenic lesions are not repaired efficiently in non-transcribed genes in quiescent cells (at least in mice) until S-phase is achieved (Bielas and Heddle, 2000). This is likely not applicable to ssDNA and DSBs which are most readily recognized by specific DNA structure-specific binding proteins (detailed below).

Damage recognition may be initiated by binding of nuclear proteins to specific structural damage in the DNA, such as single-strand DNA binding proteins and proteins with affinity for DNA ends. ssDNA and DNA ends are very susceptible to nucleases and are therefore thought to be recognized and confined quickly by these binding proteins as a protection mechanism (Friedberg, 2006). Three important complexes involved in a DNA damage response directed toward these lesions include replication factor A (RPA), which binds ssDNA, Rad51 and the Ku70/Ku80 heterodimer, which differentially bind DSBs.

Specialized DNA binding proteins likely interact with ssDNA and dsDNA within seconds of their creation (Mari et al., 2006). The RPA complex is a heterotrimer with an affinity for ssDNA. ssDNA wraps around the RPA structure as an early event in the response to a ssDNA derived from a lesion or during DNA synthesis. Long stretches of ssDNA bind additional RPA such that little ssDNA is left exposed, likely to protect the ssDNA from nucleases or further damage (Zou et al., 2006). Because the DNA damage response is not necessarily initiated during normal synthesis, its binding alone to DNA is likely not the initial instigator of a DNA damage response. RPA

occurs in high abundance in the nucleus, and probably interacts with ssDNA within seconds of its creation. Rad51 and the Ku70/Ku80 complex both bind DSBs to protect DNA ends from exonuclease activity. Rad51 primarily promotes homologous annealing of breaks with small overhangs, whereas Ku70/Ku80 is required for non-homologous end joining to repair blunt ended DSBs. These two repair mechanisms will be discussed further below. The binding of these proteins may not be the initiator of the DNA damage response, but binding to ssDNA and DSBs are early and essential steps in DNA damage response.

Damaged sites may also be recognized by the heterotrimeric Rad9-Rad1-Hus1 (the 9-1-1 complex, Rad17-Mec3-Ddc1 in budding yeast) complex. The 9-1-1 complex is similar in structure to PCNA, is loaded onto DNA as a trimeric clamp with the aid of the Rad17-RFC (Rad24-RFC in budding yeast) complex and is thought to be able to travel along the DNA in a processive fashion (Bermudez et al., 2003). This Rad17-RFC-9-1-1 complex may be a direct sensor of DNA damage as it has been demonstrated to be required for downstream checkpoint activation (Fu et al., 2008; Melo et al., 2001; Wu et al., 2005; Zou and Elledge, 2003).

1.4.2. DNA damaged-induced checkpoint activation in mammalian cells

If DNA damage is significant a DNA damage response may arise with two generalized outcomes. The first is to activate cell cycle checkpoints to regulate progression through the cell cycle. The second is to regulate repair of the DNA by recruiting repair enzymes to the sites of DNA damage.

With a significant amount of ssDNA, such as formed during replication stress induced by hydroxyurea depletion of dNTPs, checkpoint activation is initiated by the hyperphosphorylation of single strand binding proteins, most notably RPA. Phosphorylation occurs by the complexes Ataxia telangiectasia mutated (ATM, Tel1 in yeast), ATM and Rad3-related (ATR, Mec1 in yeast), and DNA-dependent protein kinase (DNA-PK). Although each may have principal functions, the above three protein kinases appear to have distinct but nevertheless overlapping substrate specificities, possibly because various types of damage are simultaneously produced by any given insult. ATM primarily phosphorylates in response to blunt ended DSBs. ATR is responsible for the most part for lesions which would likely induce replication stress or ssDNA, but also has activity towards UV damage and DSBs. DNA-PK is largely responsible for the phosphorylation at DSBs with overhanging ssDNA. (Abraham, 2001; Adams et al., 2006; Jazayeri et al., 2006). The cell cycle is regulated at G_1/S and G_2/M by DNA damage checkpoints, to limit DNA synthesis and chromosome segregation, respectively, which is thought to be achieved by eventual phosphorylation and activation of the effecter proteins Chk1, Chk2 (Sancar et al., 2004) and p53 (Khanna et al., 1998). However, this appears to be a simplified view of the DNA damage response as ATM and ATR combined have the potential to directly or indirectly phosphorylate more than 700 proteins in response to DNA damage (Matsuoka et al., 2007).

Mammalian checkpoint activation has been most characterized for the ATR response to ssDNA (Zou et al., 2006). Activation of ATR is dependent on the cofactor ATR-interacting protein for its localization to RPA-ssDNA structures (Ball and Cortez,

2005; Ball et al., 2005; Zou and Elledge, 2003) where it aids in the hyperphosphorylation of RPA following various types of damage induction (Binz et al., 2003; Liu and Weaver, 1993; Nuss et al., 2005). The 9-1-1 complex interacting with RPA-ssDNA at the site of DNA damage (Wu et al., 2005) is essential for ATR activity due to its ability to recruit the ATR-activating protein, TopBP1, to ssDNA (Delacroix et al., 2007; Lee et al., 2007). ATR binds and phosphorylates claspin (Chini and Chen, 2006), which in turn reciprocally trans-phosphorylates and activates Chk1 (Kumagai and Dunphy, 2003). Alternatively, claspin may bind Chk1 during S-phase at the replication fork in an RPA/ATR/Rad17 independent manner, suggesting activation of the checkpoint in an alternative control mechanism, or an ability to react quickly to replication stress (Lee et al., 2003). Ultimately ATR promotes phosphorylation of the checkpoint protein Chk1, promoting G_1/S transit restriction and inhibiting replication fork advancement, (Chen and Sanchez, 2004).

The ATM response to DNA damage has been most characterized in response to DNA DSBs by inducing Chk2 at the G_2/M checkpoint. An early step in the recognition of DSBs, such as those induced by ionizing radiation, involves the loading of the proteins Ku70 and Ku80, and the loading and activation of the Mre11-Rad50-Nbs1 complex. The Ku proteins likely protect the free ends against nuclease destruction. The bound Mre11-Rad50-Nbs1 is thought to act as the damage sensor which activates ATM (Bennett and Harper, 2008). Activation of ATM then initiates a cascade of phosphorylation events (Matsuoka et al., 2007). Most notably, ATM phosphorylates the histone H2AX (producing γH2AX), the protein called mediator of damage checkpoint protein-1 and p53. In turn γH2AX and phosphorylated mediator of damage

checkpoint protein-1 recruit additional enzymes including the ubiquitin (Ub) conjugating complex RNF8-Ubc13. RNF8-Ubc13 ubiquitinates γ-H2AX and histone H2A (Mailand et al., 2007). This results in the recruitment of p53BP and the BRCA1-BARD1 E3 ubiquitin ligase complex that initiates checkpoint arrest at G_2/M (Kolas et al., 2007; Mailand et al., 2007).

A second function of the DNA damage response, which is also initiated by the ATM and ATR phosphorylation activities in response to ionizing radiation-induced DSBs, is to localize repair enzymes to sites of DNA damage. As discussed above the downstream effect of ATM is to ubiquitinate H2A and H2AX. Generally, ubiquitination results in protein degradation, however, ubiquitination by Ubc13 is often in a conformation not normally recognizable by the proteosome (as will be discussed below). In fact, using fluorescent microscopy, this cascade effect to ubiquitinate many histone units near the site of damage results in structures that can be observed as stable nuclear foci (Mailand et al., 2007). Additional components are also recruited, such as the RAP80-Abraxis complex, which becomes phosphorylated and ubiquitinated, suggesting these super-complexes are also stabilized structures in the DNA damage response. The removal of these foci is also an enzymatic process and may be influenced by the deubiquitinating (DUB) enzymes USP3 (Nicassio et al., 2007) and BRC36 (Chen et al., 2006), which have been demonstrated to regulate different aspects of ionizing radiation-induced foci.

Using a yeast model system, a single DSB event can be observed as a fluorescent nuclear focus (Lisby et al., 2003). Also in mammalian cells, a single DSB induced by cleavage at an introduced *Sce*I restriction enzyme site, can be observed as

large nuclear foci in the fluorescent microscope (Rodrigue et al., 2006), suggesting the DNA damage response is a cascade effect to promote the formation of regions concentrating repair enzymes. The purpose of this may to be co-localize multiple substrates (lesions) at a single or few foci in the cell with the repair machinery, as would be expected to be required to bring broken ends in proximity to one another (Lisby et al., 2003).

1.4.3. Induction of apoptosis or senescence

Cells of the higher eukaryotes have the amazing capacity to recognize DNA damage and to non-selfishly remove themselves from the cell cycle as an option to becoming tumorigenic. This is achieved by either entering senescence or a programmed cell death pathway termed apoptosis.

Senescence is a permanent removal from the cell cycle such that the cell will be completely incapable of further division, as opposed to the quiescent state which will allow the cell back into the cell cycle. Senescence can be the result of: 1) a limited division potential controlled by telomere length, 2) a non-responsive state to external growth factors or 3) the steady accumulation of DNA damage (Collado et al., 2007). Senescent cells have a G_1 DNA content with no observable increase in DNA repair activity (Friedberg, 2006). DNA damage induced by the topoisomerase inhibitor CPT results in a senescent phenotype by several cancer cell lines and normal fibroblasts, but only in the presence of p53 (te Poele et al., 2002). p53 induces the expression of the cell cycle inhibitor p21, and defective p53 or defective p21 reduces formation of the senescent phenotype. This strongly suggests that the DNA damage checkpoint is one

mechanism to induce the activity of p53 to permanently repress cell division. This has led to the suggestion that some cancer cells have gained the ability to avoid senescence by alteration of this pathway.

Apoptosis in response to DNA damaging agents has been noted for various types of damage, including ionizing radiation, cisplatin, and CPT. A common theme in the DNA damage response is the activation of p53 by ATM, ATR and DNA-PKs, and if consistent induction of p53 occurs it is thought to lead to apoptosis (Sionov and Haupt, 1999). The half life of p53 is normally very short in cells, less than 30 minutes, but it becomes stabilized following DNA damage by inhibition of its interaction with Mdm2. Besides activating p53, ATM inactivates Mdm2, which is normally used to induce degradation of p53 in a ubiquitination dependent manner (Khosravi et al., 1999; Maya et al., 2001). Therefore p53 stabilization and its phosphorylation may remain constant until the damage is repaired and ATM phosphorylation subsides. One model of the induction of apoptosis is therefore a sustained DNA damage response leading to persistence of phosphorylated p53 (Cann and Hicks, 2007). In support of this, several cell lines defective in DNA repair exhibit an increased propensity for p53-dependent apoptosis at reduced levels of damage (Dumaz et al., 1997; McKay et al., 1998). However, the mechanism leading to the decision between apoptosis and senescence is not well characterized.

1.4.4. DNA repair mechanisms

Spontaneous and induced DNA damage rates are estimated to be between tens of thousands to hundreds of thousands of lesions per cell per day. However, organisms

carry on their daily life with most cells intact, in a non-cancerous and non-senescent, often proliferating state. This genomic and cellular preservation is the work of several distinct but overlapping types of DNA repair mechanisms. Generally, the repair mechanisms include: 1) direct damage reversal, 2) mismatch repair, 3) excision repair, and 4) end joining. An additional mechanism responsible for maintenance of the genome is DDT. It is assumed that DDT allows duplication of the genome to proceed during times of damage (or during times of inefficient repair) to allow unrestrained S-phase progression with the intention of repairing the damage at a later time.

1.4.4.1. Direct reversal of DNA damage

Direct reversal of DNA damage can only occur in a very limited fashion such as by photo reactivation or methyl transfer. These reactions do not require a template to replace damaged nucleotides, but instead specifically act directly on the affected base.

Although not occurring in mammalian cells, photo reactivation is notable because historically it initiated the field of DNA repair. Although it was demonstrated earlier that radiation was able to induce hereditary changes (Muller, 1927) the field of DNA repair was not established until nearly 20 years later. Kelner demonstrated that bacterial cells could recover from UV radiation and furthermore that recovery of the fungus *Streptomyces griseus* from UV was dependent on available light conditions (Kelner, 1949). Independently, Dulbecco described the survival of irradiated phage particles but only in the presence of bacteria exposed to light. This was discovered even before the structure of DNA determined (Watson and Crick, 1953). Eventually this repair mechanism was demonstrated to be an enzymatic reaction by a photolyase

which is able to reverse a UV-induced CPD in a light dependent reaction (Cleaver, 2003; Sancar, 2003).

Direct methyl transfer can occur from a base with alkylation damage to a recipient protein. One such recipient protein in mammalian cells is O^6-methylguanine (O^6-MeG) methyl transferase encoded by *MGMT* that removes a methyl group most efficiently from, but not limited to, O^6-MeG. This is a direct transfer, such that each MGMT protein can be used only a single time (Hazra et al., 1997). Methyltransferase activity in bacteria has been described as an "adaptive response to DNA damage". In this scenario, a sub-lethal exposure to a methylating agent such as N-methyl-N'-nitro-N-nitrosoguanidine results in an enhanced survival and reduced mutagenesis following further exposure to the same agent (Jeggo et al., 1977; Samson and Cairns, 1977; Volkert, 1988).

The *alkB* gene product in *E. coli* was first characterized to be responsible for direct reversal of alkylation damage in ssDNA (Friedberg, 2006). Several human homologs have been noted in human cells and somewhat characterized and they are at least partially interchangeable between human and bacterial cells (Chen et al., 1994; Wei et al., 1996). Although at least eight homologs are proposed in human cells, only a few have been properly characterized. The human AlkB homolog ABH2 is likely responsible for repair of alkylation damage to dsDNA (Ringvoll et al., 2006) and ABH3 likely targets alkylation damage of ssDNA and RNA (Friedberg, 2006).

1.4.4.2. Excision repair of DNA damage

Excision repair is essentially the removal of a portion of one strand of the double helix which contains a damage site. This occurs typically in three steps: 1) recognition of the damage, 2) excision of the damaged base, nucleotide or polynucleotide, and 3) repair synthesis based on the complementary strand. Excision repair mechanisms therefore are template-dependent and can be subdivided into mismatch repair, base excision repair and nucleotide excision repair.

1.4.4.2.1. Mismatch repair

Normal DNA replication has a very high fidelity, but inappropriate base pairs sometimes occur by the incorporation of an incorrect or damaged base or due to base misalignment leaving short single strand loops. Mismatch repair enzymes are the first line of defense to remove these damage sites, probably during or shortly after DNA synthesis. Additionally the mismatch repair enzymes are partially responsible for recognition of additional types of DNA damage such as alkylation, cross-linking and UV photoproducts. Mismatch repair involves the recognition of damage, likely initiated by deformities in the helix, followed by excision of various lengths of the damaged strand, from less than ten to thousands of bases. In higher eukaryotes, this results in the coating of ssDNA with RPA followed by the DNA re-synthesis in a PCNA dependent manner (Hsieh and Yamane, 2008) which is often refered to as unscheduled DNA synthesis.

Because the incorrect base must be cut out from the newly synthesized strand, and not the correct one from the parental strand, mismatch repair enzymes must be able

to distinguish between the two. In *E. coli* and some (but not all) other bacteria, the parental strand is identified by a methylated adenine in a GATC sequence. Although the basic excision mechanism is conserved in eukaryotes, the mammalian recognition system differentiating between the two stands is not known. Dysfunctional mismatch repair results in an approximately 1000-fold increase in the spontaneous mutation rate (Friedberg, 2006) and in human cells mismatch repair defects can result in hereditary nonpolyposis colorectal cancers due to an increased mutation rate and microsatellite instability (Bellacosa, 2001).

1.4.4.2.2. Nucleotide excision repair

Nucleotide excision repair was originally characterized in bacteria as a multi-step process responsible for the removal of bulky DNA lesions that significantly distort DNA. Mammalian nucleotide excision repair was first demonstrated as unscheduled DNA synthesis as tritiated thymidine incorporation into the DNA of cells outside of S-phase in the response to UV exposure (Djordjevic and Tolmach, 1967; Rasmussen and Painter, 1964). Analysis of patients suffering from the hereditary disease xeroderma pigmentosum (XP) exhibited lower levels of unscheduled DNA synthesis in response to UV light (Cleaver, 1989) and the mutations found in this disorder were demonstrated to be largely responsible for nucleotide excision repair (Friedberg, 2006). In humans, at least nine genes are involved in nucleotide excision repair, defects in seven of which lead to sensitivity to UV light and result in XP, which led to the identification of the genes XPA through XPG. Two additional genes, discovered in Cockayne syndrome patients with complementation groups CSA and CSB also show sensitivity to UV light.

The majority of damage recognition by nucleotide excision repair is performed by the XPC-Rad23B complex, while some lesions can be recognized by DDB1 and XPE. The XPF-ERCC1 complex produces a nick in the ribophosphate backbone of the DNA 5' to the damage and XPG a 3' nick, which allows the removal of a stretch of the damaged DNA about 25-30 nucleotides long. The DNA is then re-synthesized by either Pol-δ or Pol-ε in a replication protein A (RPA) and PCNA dependent manner. DNA ligase seals the final nick to complete the repair process.

A variation of nucleotide excision repair is transcription coupled repair which utilizes the majority of the nucleotide excision repair enzymes to preferentially repair genes which are actively being transcribed as opposed to non-transcribed DNA (Tornaletti, 2005). Transcription-coupled repair utilizes nearly the same enzymes as nucleotide excision repair except the XPC-Rad23B nucleotide excision repair sensor. During transcription-coupled repair, RNA polymerase-II acts as the damage sensor as it cannot synthesize RNA beyond UV-induced CPDs (Venema et al., 1991; Venema et al., 1990). A stalled polymerase can either be removed or be reversed from the damaged site. Key to the outcome is the association of CSA and CSB with the stalled polymerase (Fousteri and Mullenders, 2008). The CSA-DDB1 complex forms a ubiquitin-ligase which is likely responsible for further processing of the existing mRNA to assist in resumption of transcription (Fousteri and Mullenders, 2008; Groisman et al., 2003). CSB likely helps to anchor the polymerase in place while the remaining repair enzymes are recruited (Fousteri et al., 2006). Following removal of the damaged section of DNA by nucleotide excision repair, DNA in the resulting gap is re-synthesized in an RPA, PCNA and ligase-dependent manner (Friedberg, 2006).

1.4.4.2.3. Base excision repair

Unlike nucleotide excision repair, base excision repair (BER) involves the removal of DNA damage which produces little or no distortion in the structure of the double helix. DNA glycosylases, which initiate the reaction, are quite specific for the damage they recognize. hOOG1 for example, will traverse the DNA in a processive manner until it recognizes specifically 8-oxoG (Banerjee et al., 2005). The base is excised at the N-glycosidic bond between the base and sugar by the glycosylase, resulting in an AP site. An AP-endonuclease then nicks the DNA adjacent to the AP site to initiate repair synthesis and strand ligation (Friedberg, 2006).

1.4.4.2.4. End joining of DSBs

DSBs lead to chromosome mis-segregation during mitosis which is a common feature in tumorigenesis. They often form in response to ionizing radiation or when the replication apparatus collapses near a single strand gap in the DNA. DSBs are repaired by either non-homologous end joining, which brings broken ends together for re-ligation or homologous recombination which uses a sister chromatid as a substrate to synthesize a patch. Non-homologous end joining has the potential to result in a loss of DNA and is therefore considered to be mutagenic. In contrast, homologous recombination is largely error free as the intact sister chromatid will serve as a template for the missing sequences. These two procedures occur in all eukaryotes; however, yeast cells preferentially utilize homologous recombination whereas mammalian somatic cells predominately utilize non-homologous end joining. In contrast, germ line

mammalian cells utilize homologous recombination to enhance genetic variation referred to as crossover events during mitosis.

Non-homologous end joining operates directly on the free DSB ends whether they are complimentary or not. Free DNA ends are susceptible to nuclease degradation and this method is therefore error prone as some genetic material may be lost (Pfeiffer, 1998). Ku70 and Ku80 bind preferentially to DSBs, and with the further addition of ATM and the Mre11-Rad50-Nbs1 complex, initiate the non-homologous end joining cascade pathway. Alignment is regulated by the Ku proteins and ligation is then mediated by DNA-PK (Weterings and Chen, 2008), which, in a non-phosphorylated state represses further processing of the free ends. When aligned as a synaptic complex, the DNA-PKs on opposite free ends trans-autophosphrylate one another and activity of the processing machinery is allowed access. If the ends have overhangs which are not compatible, DNA is either synthesized by a polymerase or removed by the protein Artremis. Finally the ends are ligated with the ligase IV/XRCC4 complex (Weterings and Chen, 2008).

Homologous recombination relies on the presence of a sister chromatid as a source of homologous, undamaged DNA for the repair of DSBs. Homologous recombination is therefore most likely to occur in late S or G_2 phases of the cell cycle while the sister chromatids are still associated by cohesions introduced during DNA synthesis (Byun et al., 2005).

Upon recognition and binding of the DSB by the Mre11-Rad50-Nbs1 complex (Mre11-Rad50-Xrs2 complex in yeast) one or both sides of the break will undergo 5' resection by Exo1 (Fiorentini et al., 1997) [as well as Sae2 in yeast (Clerici et al., 2005)

or CtIP in mammals (Sartori et al., 2007)] to produce 3' ssDNA which is quickly bound with RPA. In yeast, RPA is replaced with Rad51 by the protein Rad52 (Sugiyama and Kowalczykowski, 2002). The mammalian BRCA2 likely has the same function as yeast Rad52 (San Filippo et al., 2006; Yang et al., 2005) and the Rad51-DNA filament invades the undamaged dsDNA of the sister chromatid in search of a homology site (Sung, 1994) which disassociates the double strand so that DNA synthesis can continue along the complementary, undamaged strand. Re-ligation to its original strand results in a Holliday junction that must be resolved by either BLM-Top3 or Mus81-Eme1.

Homologous recombination may also be utilized at the site of replication fork stalling when coordination of the leading and lagging strands is disrupted at single strand breaks or during duplication of fragile sites where secondary DNA structures have formed which inhibit fork advancement (Freudenreich, 2007). Fork stalling can be prevented by fork regression without Rad51 into a chicken foot structure, which will allow re-induction of synthesis upon its unfolding and thus allow lesion bypass (tolerance). Alternatively, template switching by a homologous recombination-like mechanism in a Rad51 dependent manner may allow strand invasion to occur to utilize an intact DNA strand for duplication, thus bypassing the damage. In yeast, genetic studies have demonstrated the Shu1-Psy3-Shu2-Csm2 complex to possibly be involved in this process as they are epistatic to *RAD52* deletion but do not respond to DSBs (Shor et al., 2005). In some instances, inappropriate homologous recombination may occur at a blocked replication fork. In budding yeast, the protein Srs2 exhibits 3'→5' ATP-dependent helicase activity (Rong and Klein, 1993), is recruited to the stalled replication fork when PCNA is modified with a small ubiquitin-like modifier, SUMO,

and functions to displace Rad51 which in turn represses homologous recombination (Byun et al., 2005; Krejci et al., 2003; Veaute et al., 2003). The Srs2 helicase may also have additional activity in unwinding hairpin loops in DNA and therefore possibly preventing replication fork stalling and/or preventing nucleotide expansions/contractions at nucleotide repeat and fragile sites (Dhar and Lahue, 2008). In the absence of Srs2 damage accumulates in a homologous recombination-dependent manner at nucleotide repeat sites (Kerrest et al., 2009). Also in yeast the 3'→5' helicase Mph1 appears to prevent homologous recombination by eliminating Rad51 from D-loops and therefore homologous recombination activity in response to DSBs (Prakash et al., 2005; Prakash et al., 2009). Furthermore, Mph1 appears to promote gross chromosomal rearrangements while inhibiting homologous recombination activity due to the stabilization of RPA at DSBs in place of Rad51 (Banerjee et al., 2008). Human functional homologs of Srs2 and Mph1 have been identified as RTEL1 (Barber et al., 2008) and FancM (Gari et al., 2008), respectively.

1.5. DNA damage tolerance

Cells have developed the astonishing capability to both duplicate their DNA with very high fidelity and also to react to damaged DNA with very specific procedures to coordinate the cell cycle with repair of the DNA. This however may not be sufficient to protect the genome in response to stress. Cells have also surprisingly developed mechanisms to complete genomic replication in the presence of otherwise replication-blocking lesions. This was initially termed DNA post-replication repair (PRR) in bacteria and yeast, but is commonly referred to as DNA damage tolerance

(DDT) in mammalian cells. The main purpose of these DDT pathways is to ensure cell survival during times of genomic stress or when repair mechanisms are not functional, so that the damage can be corrected at a later time without failure of cell duplication. A single stalled replication fork if left without restart has the probability of producing ssDNA and/or a DSB, which can easily lead to partial chromosome loss during the subsequent mitosis. It is also thought that the enhanced mutagenesis during this process may be beneficial for the organisms to respond to environmental stresses, at least in single cell organisms.

1.5.1. Post-replication repair in bacteria

Experiments on the kinetics of DNA synthesis in bacteria following UV-induced damage led to the discovery of PRR. An alkaline sedimentation assay was developed so that the relative size of ssDNA could be analyzed. This experiment led to the demonstration that following UV exposure, DNA synthesis could continue but a significant proportion was fragmented (Rupp and Howard-Flanders, 1968). A complementing experiment utilizing electron microscopy revealed that the reduction in ssDNA size was due to single strand gaps in the nascent DNA strand (Cordeiro-Stone et al., 1999). Importantly, these electron micrographs suggested that replication restart can occur downstream of the blocked replication fork and that the undamaged strand may be duplicated past the lesion site in the opposite strand (Cordeiro-Stone et al., 1999). In both of the above experimental procedures when the damaged cells were allowed additional time to survive, the ssDNA gaps vanished into what appeared to be full length dsDNA. Additional studies suggested that the number of gaps formed

roughly correlated with the UV dose and the expected number of CPDs, confirming that each CPD results in a single gap (Rupp et al., 1971). Elegantly, because the experiment was carried out in nucleotide excision repair-deficient cells, it was proposed that the damage may not have been repaired even though synthesis was complete. It was revealed that although the nascent fragments re-annealed, the original UV-induced pyrimidine dimers, which were responsible for the generation of the single strand gaps, often persisted in the genome (Bridges and Munson, 1968a; Bridges and Munson, 1968b; Ganesan, 1974). The authors interpreted these data and proposed the existence of PRR as a mechanism to allow DNA replication without damage correction. Apparently it is more important to the cell to duplicate its genome in its entirety rather than to allow replication fork collapse.

Supporting experiments led to the further characterization of PRR in bacterial cells and related the activity directly to the bacterial SOS response. First of all, single strand gaps are not repaired in *recA* mutants, the bacterial homolog of eukaryotic RAD51 (Smith and Meun, 1970). Furthermore, the RecFOR complex is required for gap filling (Horii and Clark, 1973; Rothman et al., 1975). We now know that the RecFOR complex is responsible for replacing RecA with single strand binding protein associated with ssDNA (Morimatsu and Kowalczykowski, 2003).

The *E. coli* SOS response is regulated by the RecA protein bound to ssDNA and promotes two parallel pathways to induce specific genes and to activate the gene products (Salles and Defais, 1984). Activated RecA (RecA*) induces the self cleavage of LexA, which in turn allows the transcription of various genes involved in DNA repair and cell survival (Little, 1984). The SOS response induces, among other genes,

dinB and *umuDC* coding for the Y-family polymerases DNA Pol-IV and Pol-V, respectively. These two polymerase genes are not essential for cell survival, but their inactivation has an anti-mutator effect due to their low fidelity translesion synthesis (TLS) activity (Fernandez De Henestrosa et al., 2000; Wagner and Nohmi, 2000). RecA* also stimulates the cleavage of the regulatory subunit UmuD to form a fully active Pol-V (UmuD'$_2$-UmuC) (Reuven et al., 1999; Tang et al., 1998). The RecA-ssDNA filaments are required for both homologous recombination and TLS (Pham et al., 2002). The binding of RecA to ssDNA has been suggested to promote DDT by inducing fork regression and forming a chicken-foot like structure, or to act as a primer for TLS allowing DDT and replication restart downstream of the lesion site (Robu et al., 2001), as illustrated in Figure 1-1. The activities of polymerases IV and V are highly dependent on the β-clamp, the functional equivalent of mammalian and yeast PCNA (Tang et al., 2000; Wagner et al., 2000). Pol-V is thought to be the major mutagenic polymerase in *E. coli*, as it is capable of bypassing many types of damage with a mutator phenotype (Fuchs et al., 2004). Because of their notorious activities, Pol-IV and Pol-V are tightly controlled in the SOS response at both the transcriptional and translational levels (Friedberg, 2006). The SOS response is thought to promote DDT as a backup mechanism to ensure cell survival during times of stress when repair mechanisms are saturated, or in response to a changing environment (Friedberg, 2006).

1.5.2. The ubiquitin conjugation system

The RecA protein of the bacterial SOS response plays a central role in DDT in bacteria cells. Rad51 is the eukaryote equivalent of bacterial RecA, produces a protein-

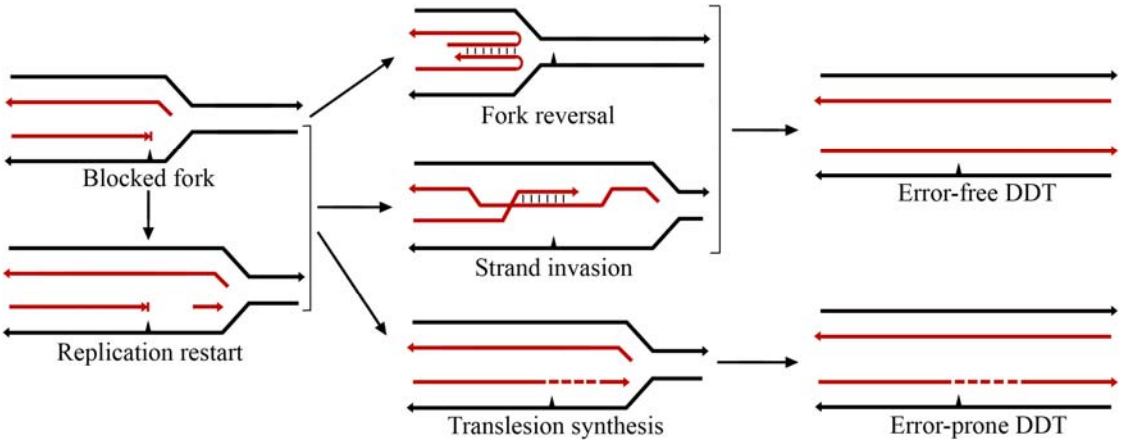

Figure 1-1. Possible DDT mechanisms at a blocked replication fork

During normal DNA synthesis DDT is assumed to occur at sites of DNA damage (represented by a black triangle) blocking replication forks, where continued extension of the non-damaged strand and/or replication fork restart may occur downstream from the lesion. If synthesis continues on the undamaged strand, the newly synthesized strand may serve as a template for error-free lesion bypass. Two possible mechanisms have been proposed, namely fork reversal and strand invasion by the nascent strand or sister chromatid. If the synthesis of the undamaged strand proceeds significantly past the lesion, strand invasion would likely be preferred over fork reversal. Alternatively, error-prone DDT utilizes TLS polymerases to synthesize DNA across from damaged DNA without obligatory high fidelity duplication from the original base sequence which may or may not be mutagenic. Note that in each case the original lesion remains.

DNA filament at sites of ssDNA which is required for homologous recombination; however, unlike bacteria, they do not appear to be required for DDT. Instead, the ubiquitination activities of the Rad6-Rad18 and Rad5-Ubc13-Mms2 complexes appear to be required for PRR/DDT in yeast. Because ubiquitin (Ub) conjugation activity is essential for PRR in yeast, as will be described shortly, a description of the Ub-conjugation pathway is necessary here.

One of the major protein degradation pathways in eukaryotic cells is the ubiquitin-proteosome system. Briefly, proteins targeted for destruction for various reasons (general maintenance, cell cycle regulation, response to stress or mis-folded proteins) are covalently attached to several Ub subunits linked in a linear fashion. *In vitro*, attachment of four or more Ub subunits in a chain increases the target protein affinity for the 26S proteosome. These tagged proteins are then recognized as targets for degradation by the proteosome. The Ub chains are cleaved to monomers for reuse and the target protein is degraded by the 26S proteosome into short peptides which are further degraded by proteases into amino acids for recycling (Hershko and Ciechanover, 1998; Hochstrasser, 1996). The 26S proteosome is composed of at least 65 individual proteins (4.1 MDa total) arranged in three subunits consisting of the 20S core unit in the form of two open barrels with 19S regulatory subunits capping each end (Baumeister et al., 1998). Multiple Ub interacting motifs on the regulatory 19S subunits are responsible for association between the target protein and proteosome and coordinately responsible for recognition of Ub chains rather than monomers. The catalytic 20S core is responsible for degradation of proteins into peptides of less than 10 amino acids in length, which are released into the cytosol to be further cleaved by

cytosolic proteases for amino acid recycling. One variant is the 11S subunit, which is utilized by antigen presenting pathway to process protein for major histocompatibility complex class I-binding peptides for self-non-self recognition (Rock et al., 1994; Wang and Maldonado, 2006). Similar self-compartmentalizing structures resembling the eukaryotic proteosome may also occur in archaea (Ehlers et al., 1997) and bacteria (Lupas et al., 1994).

Ubiquitin is a 76-amino acid, 8.5 kDa protein found in all eukaryotic cells and is highly conserved with only three amino acid differences between humans and budding yeast. Human Ub can substitute for yeast without any observable abnormalities in the yeast. The Ub subunits become covalently attached to the ε-amino group of a lysine residue on a target peptide in a three step manner utilizing the E1, E2 and E3 enzymes. Free Ub is first activated by E1 in an ATP dependent manner. The C-terminal glycine is first ligated to the active site cysteine residue of E1 by a thioester linkage. The Ub is then transferred to the active site cysteine residue of E2. The E3 ubiquitin ligase is responsible for interacting with both E2-Ub and for recognition of the target to facilitate transfer of Ub to the desired protein. All E2s contain a highly conserved core domain of approximately 150 amino acids with possible N- or C-terminal extensions. E3s occur in three forms depending on the existence of a HECT domain (homologous to the E6-AP carboxyl terminus), a RING (really interesting new gene), or a U-box domain. A RING domain will assist in the transfer of Ub directly from the E2 to target, whereas the HECT or U-box E3s form a covalent bond with Ub prior to transfer. Budding yeast contains 13 E2s and several dozen E3s, whereas mammals contain 30-40 E2s and possibly several hundred E3s.

Each Ub molecule contains seven surface lysine residues that have the potential to accept another Ub, and the next Ub can accept yet another Ub, resulting in a short polymer of Ub subunits (poly-Ub). For proteosomal degradation *in vivo* it is widely accepted that the most important recognition substrate by the proteosome is a Ub chain of four subunits with each Ub linked by its C-terminus to the lysine at position 48 (K48) of the previous Ub. However other linkages can also be produced including branching Ub complexes, at least *in vitro* (Kim et al., 2007), and linkages formed at lysine 63 (K63) position appear to be biologically relevant. Initially tetra-Ub of K48 ($K48_4$) chains were demonstrated to have the highest affinity for the 26S proteosome, but *in vitro*, K48 or K63 labeling of either dihydrofolate reductase, luciferase or troponin-I have been described as suitable substrates for proteosomal degradation (Hofmann and Pickart, 2001). Furthermore, linear Ub_5, derived from the yeast *UBI4* gene (which normally undergoes processing to mono-Ub subunits) produces a peptide resembling that of K63 chains, and may be a competitive inhibitor of $K48_4$ binding to the proteosome (Thrower et al., 2000). Although the above analyses suggest that K63-linked chains have the potential to induce protein degradation, several lines of *in vivo* evidence argue against this possibility. In poarticular, several K63-linked proteins remain stable *in vivo*, such as the ribosomal L28 subunit (Spence et al., 2000), and K63-linked Ub chains are often essential for endocytosis and targeting to the lysosome independently of functional activity of the proteosome. Additionally, yeast containing a mutation of Ub (K63R) are viable with no discernable defects in protein degradation, indicating cellular roles of K63 chains other than degradation (Spence et al., 1995). It is now generally accepted that non-canonical poly-Ub chains such as the K63-linked

poly-Ub chain or single Ub addition to proteins (mono-Ub) are not involved in proteosomal degradation *in vivo*, but rather are involved in regulating target protein activity (Broomfield et al., 1998; Chen and Sun, 2009).

An early example of the specific *in vivo* production of K63-linked chains in a non-degradation manner is the activity of the E2 Ubc13 operating in conjunction with the E2-like protein Mms2. Yeast and human Mms2 (or human Uev1) are Ub E2-variants (Uevs), which are defined as having high amino acid homology to E2s, but lacking an active site cysteine residue (Sancho et al., 1998; Xiao et al., 1998b). The Uevs interact specifically with Ubc13 (Pastushok et al., 2007) and possess a unique activity to bind Ub non-covalently (McKenna et al., 2001). In combination with the crystal structures of the Ubc13-Mms2 complexes (Moraes et al., 2001; VanDemark et al., 2001), it was revealed that Ubc13 and Mms2 are spatially positioned such that only K63 of the Mms2-bound Ub is exposed to the C-terminus of the preexisting Ub covalently linked to Ubc13, whereas its K48 residue is embedded in the Mms2-Ub interface (McKenna et al., 2003; Moraes et al., 2001; VanDemark et al., 2001). As will be discussed in the next section, the Ubc13-Mms2 complex is critical for error-free PRR (Broomfield et al., 1998; Hofmann and Pickart, 1999). Mutating Ub in budding yeast such that K63-Ub chain formation cannot be produced (K63R) also effectively repressed error-free PRR (Hofmann and Pickart, 1999).

The discrepancy between *in vitro* observations and *in vivo* data may be explained by the discovery of proteins binding specificly to K63-linked Ub chains. For example, p62 recognizes Ub-K63 bound Traf-6, and affects downstream NF-κB signaling and osteoclast function (Layfield and Shaw, 2007). The specificity and

importance of Ub-K63 linkages also becomes apparent when one considers the K63-specific de-ubiquitination enzymes such as CYLD, which specifically removes K63 linkages from NEMO to regulate NF-κB activity, or USP-1, which removes mono-Ub from PCNA (Huang et al., 2006).

Non-degrading Ub may contribute to the production of cytosolic Ub inclusions, which is a common phenomenon and serves as a hallmark of neurodegeneration (Sherman and Goldberg, 2001). This accumulation of Ub molecules may be due to either a defect in the proteosomal degradation of the target proteins or unregulated production of the alternative lysine linkages. Hence, the ubiquitination system is much more complex than simply targeting proteins to the proteosome for degradation.

Eukaryotic cells also contain several Ub-like molecules that adopt a Ub-like fold with conserved isopeptide bond formation and target modification (Hochstrasser, 2000; Schwartz and Hochstrasser, 2003). One important Ub-like modification is the covalent addition of the small ubiquitin-like modifier (SUMO), which also requires the activities of an E2-E3 complex and modifies the activity of the targeted protein (Bayer et al., 1998; Sheng and Liao, 2002).

1.5.3. PRR in yeast

The majority of research on how cells tolerate DNA damage has been performed in the budding yeast *Saccharomyces cerevisiae*. This yeast species is an attractive eukaryotic model organism because it can be maintained in either a haploid or a diploid stage, and because of the availability of powerful tools for genetic analysis. In addition, its genome can be readily manipulated through homologous recombination to

delete or modify specific genes. In the field of DNA repair and mutagenesis, pioneering studies have identified three main radiation repair pathways named after key genes in each pathway. The *RAD52* epistatic group is responsible for homologous recombination; the *RAD3* epistatic group is responsible for nucleotide excision repair; and the *RAD6* epistatic group is responsible for PRR (Friedberg, 1988; Friedberg, 2006; Prakash et al., 1993). The yeast PRR pathway is highly relevant to this thesis dissertation.

1.5.3.1. The *RAD6* epistasis group

Initially an alkaline sedimentation assay was employed to demonstrate the existence of PRR activity in yeast and genetic analyses indicated that in the absence of nucleotide excision repair (*rad1*), UV-induced ssDNA gaps cannot be filled in *rad6* or *rad18* mutants (Prakash, 1981). *RAD6* was the founding member of the PRR and mutagenesis pathway as one of three major radiation repair pathways. However, historically, *RAD* genes that do not belong to either of the well-defined *RAD3* (nucleotide excision repair) or *RAD52* (homologous recombination repair) groups were assigned to the *RAD6* pathway (Friedberg, 1988; Haynes and Kunz, 1981). The *rad6* mutant exhibits a mutator and slow growth phenotype, is defective in UV-induced mutagenesis, and is extremely sensitive to killing by UV and a variety of DNA damaging agents (Friedberg, 1988; Prakash et al., 1993). The *rad6* diploid is also defective in sporulation (Montelone et al., 1981). *RAD6* was found to encode an E2 enzyme (Ubc2) (Jentsch et al., 1987) and its Ub conjugation activity is absolutely required for all of its functions (Sung et al., 1991). The C-terminal polyacidic tail of

Rad6 is required for its ability to polyubiquitinate histone H2B *in vitro* (Sung et al., 1988) and *in vivo* (Robzyk et al., 2000). However, deletion of the entire Rad6 C-terminal tail has little effect on its role in DNA repair and UV-induced mutagenesis, and affects only sporulation (Morrison et al., 1988). The N-terminal 15 amino-acid sequence is nearly identical among all Rad6 homologs (Koken et al., 1991a; Koken et al., 1991b; Reynolds et al., 1990; Reynolds et al., 1985); and deletion of the first 9 amino acids from Rad6 ($rad6_{\Delta 1\text{-}9}$) abolishes sporulation, reduces cell survival after UV treatment, but surprisingly increases spontaneous and UV-induced mutagenesis (Watkins et al., 1993). Furthermore, the N-terminus of Rad6 is also required for N-end rule protein degradation (Dohmen et al., 1991; Sung et al., 1991; Watkins et al., 1993). And while the full-length Rad6 interacts with the E3 protein Ubr1, the $Rad6_{\Delta 1\text{-}9}$ protein is unable to form a complex with Ubr1 (Watkins et al., 1993). Rad6 is known to form a stable complex with Rad18 (Bailly et al., 1994) and this complex displays Ub conjugation (from Rad6), ssDNA-binding and ATPase (from Rad18) activities (Bailly et al., 1997). However, Rad18 had not been defined as an E3 until the RING finger motif was discovered (Lovering et al., 1993; Saurin et al., 1996) and found in Rad18, and the physical interaction of Rad18 with the substrate Pol30 (PCNA) was demonstrated (Hoege et al., 2002). Like *rad6*, the *rad18* mutant is extremely sensitive to killing by UV and a variety of DNA damaging agents, and displays a mutator phenotype (Jones et al., 1988); however, unlike *rad6*, *rad18* displays a signature spontaneous GC→TA transversion increase (Kunz et al., 1991) and does not display slow growth or sporulation defects (Prakash et al., 1993). Hence, Rad6 appears to be a

multi-functional E2 operaating with various E3 proteins, but its DDT activity is exclusively achieved through interaction with Rad18 (Broomfield et al., 2001).

Further genetic analysis has demonstrated that the *RAD6* pathway can be divided into two parallel pathways, one being error-prone and another error-free. The error-prone or mutagenesis pathway was first discovered through genetic screens of *rev* mutants incapable of reverting the *arg4-17* and *lys1-1* alleles in response to UV irradiation (Lemontt, 1971a; Lemontt, 1971b). *REV1* was cloned and found to encode a 112-kDa protein (Larimer et al., 1989) with deoxycytidyl transferase activity (Nelson et al., 1996a), whereas *REV3* and *REV7* encode two subunits of a non-essential DNA polymerase, Pol-ζ, capable of bypassing thymine dimers more efficiently than Pol-α (Nelson et al., 1996b), The *rev* mutants exhibit moderate sensitivity to a variety of DNA damaging agents but with strongly compromised mutability (Lawrence, 2004). Thus, the yeast mutagenesis pathway relies on nonessential DNA polymerases to bypass DNA replication blocks, by TLS, at the cost of increased mutagenesis.

The *rad6* and *rad18* mutations are epistatic to *rev* mutations; however, it is apparent that TLS is not the only pathway operated by *RAD6-RAD18*, since the *rad6* and *rad18* mutants are much more sensitive to DNA damaging agents than the *rev* mutants (Prakash et al., 1993). An error-free branch within the *RAD6* pathway had been proposed but not convincingly demonstrated until the identification and functional characterization of *MMS2* (Broomfield et al., 1998). The *mms2* mutant is moderately sensitive to a broad range of DNA damaging agents and epistasis analysis places *MMS2* within the *RAD6* pathway. However, unlike *rev3*, the *mms2* mutant displays a massively increased spontaneous mutation rate and this increase is dependent on *REV*

functions. Furthermore, the *mms2* and *rev3* mutations are synergistic with respect to DNA damage sensitivity and the sensitivity of the double mutant is comparable to that of the *rad18* single mutant (Broomfield et al., 1998; Xiao et al., 1999). Based on these analyses, a model was proposed in which the *RAD6* pathway is composed of two independent sub pathways: one is mediated by TLS and requires *REV1, REV3* and *REV7*, whereas the other is mediated by error-free PRR and requires *MMS2* (Broomfield et al., 1998). *MMS2* encodes a protein homologous to Ubc but lacking the active Cys residue (Broomfield et al., 1998). It turns out that Mms2 forms a stable complex with a true Ubc, Ubc13, and the Mms2-Ubc13 complex specifically catalyzes K63-linked Ub chain formation (Hofmann and Pickart, 1999). Indeed, the *ubc13* mutant displays phenotypes indistinguishable from those of the *mms2* mutant (Brusky et al., 2000). The cognate E3 for Mms2-Ubc13 turns out to be Rad5, another RING-finger protein that interacts with both Ubc13 and Rad18 (Ulrich and Jentsch, 2000). *RAD5* encodes a protein with DNA helicase and zinc-binding domains (Johnson et al., 1992) and DNA-dependent ATPase activity (Johnson et al., 1994). Hence, at least two E2-E3 complexes, namely Rad6-Rad18 and Mms2-Ubc13-Rad5, are required for PRR in yeast. In addition, *RAD5* has been reported to promote instability of simple repetitive sequences (Johnson et al., 1992) and to inhibit non-homologous end-joining of DSBs (Ahne et al., 1997). Indeed, Rad5 is involved in double-strand break repair independent of its ubiquitination activity (Chen et al., 2005).

1.5.3.2. Covalent modifications of PCNA and PRR

PCNA (encoded by *POL30* in budding yeast) forms a homotrimer which circles the DNA and operates as a scaffold, often termed a processivity factor, to assemble a multitude of proteins required for DNA unwinding and synthesis, cell cycle progression and chromatin structure maintenance (Moldovan et al., 2007). The involvement of this DNA-polymerase sliding clamp in PRR was first suggested by the isolation and characterization of the *pol30-46* allele (Torres-Ramos et al., 1996). *pol30-46* is epistatic to *rad6* and *rad18*, but synergistic with *rev3*. The *pol30-46* mutant is normal in UV-induced mutagenesis and DNA synthesis but displays significantly reduced PRR activity as judged by the alkaline sedimentation assay (Torres-Ramos et al., 1996).

PCNA can be either ubiquitinated or sumoylated in budding yeast (Hoege et al., 2002). In response to DNA damage, PCNA is modified by a single Ub on the Lys164 residue and this process is dependent upon the Rad6-Rad18 complex (Hoege et al., 2002). Ub modification appears to be limited to the PCNA that has been loaded onto DNA by replication factor C (Garg and Burgers, 2005), suggesting that PCNA is mono-ubiquitinated only at stalled replication forks. In wild type cells, polyubiquitinated PCNA was also observed upon DNA damage, and this modification is also at the Lys164 residue, linked through the K63-linked Ub chain and requiring expression of *MMS2*, *UBC13* and *RAD5* (Hoege et al., 2002). Hence, it is conceivable that the two ubiquitination complexes Rad6-Rad18 and Mms2-Ubc13-Rad5 sequentially ubiquitinate PCNA. Interestingly, the identical residue can also be targeted for sumoylation. The fraction of sumoylated PCNA increases during S phase as well as during extensive DNA damage, and this process requires yet another E2-E3 complex

Ubc9-Siz1 (Hoege et al., 2002; Stelter and Ulrich, 2003). It is noted that PCNA can also be sumoylated at the Lys127 residue (Hoege et al., 2002), and this specific modification does not appear to affect DDT activity, but is required for the establishment of sister chromatid cohesion during S phase (Moldovan et al., 2006).

The discovery of PCNA covalent modifications imposes several functional implications. Firstly, it predicts that the *pol30-164R* mutation is epistatic to all PRR pathway mutations. Indeed, Pol30-164R cannot be ubiquitinated and the *pol30-164R* mutation suppresses the severe sensitivity of *rad6* and *rad18* mutations (Hoege et al., 2002). Secondly, it predicts that monoubiquitinated PCNA promotes TLS, which was subsequently demonstrated (Stelter and Ulrich, 2003). Thirdly, the above model suggests that polyubiquitinated PCNA promotes error-free PRR. To date, this prediction has not been explored. Finally, it indicates that the Pol30-K164 sumoylation plays a role in the regulation of PRR. Interestingly, the *pol30-164R* mutant is less sensitive to DNA damage than *rad6*, *rad18* or the *mms2 rev3* double mutant, suggesting that the Pol30-K164 sumoylation sensitizes cells to DNA damage. This model is further strengthened by analyzing the effects of the *siz1* mutation that specifically affects sumoylation but not ubiquitination (*UBC9* is an essential gene), and is reminiscent of the *srs*2 (*s*uppression of *r*ad *s*ix) mutation that was initially isolated by its ability to suppress the severe damage sensitivity of *rad6* mutants (Lawrence and Christensen, 1979). Srs2 possesses a 3'→5' DNA helicase activity (Rong and Klein, 1993; Rong et al., 1991) that is crucial for recombination (Rong et al., 1991) and suppression of PRR defects (Broomfield and Xiao, 2002; Ulrich, 2001). Genetic data indicate that Srs2 negatively regulates recombination (Aboussekhra et al., 1989;

Aguilera and Klein, 1988) possibly by reversal of intermediate recombination structures (Chanet et al., 1996; Kaytor et al., 1995; Milne et al., 1995; Schild, 1995). Indeed, the DNA strand exchange mediated by Rad51 is inhibited by Srs2 through disruption of the Rad51–ssDNA filaments (Krejci et al., 2003; Veaute et al., 2003), and it turns out that sumoylated PCNA has increased affinity for Srs2 (Papouli et al., 2005; Pfander et al., 2005) and represses the Rad52-dependent recombination pathway (Haracska et al., 2004). These observations collectively support a hypothesis that Srs2 serves as a molecular switch between homologous recombination and PRR (Barbour and Xiao, 2003) and further confirm that the sensor for this switch is the state of PCNA modification. The current model of yeast PRR through covalent modifications of PCNA is depicted in Figure 1-2.

1.5.4. Low fidelity translesion synthesis polymerases and error-prone lesion bypass

Typically, DNA polymerases contain six relatively well conserved domains and their presence is sufficient to assign non-characterized genes as putative DNA polymerases (Hubscher et al., 2002). These regions result in a general structure resembling a human right hand with regions often referent to as the palm, the fingers and the thumb. Based on phylogenetic analysis, polymerases are classified into six major families. Families A, B and C are represented by *E. coli* polymerases I, II and III (α-catalytic subunit) respectively. Eukaryotic replicative polymerases belong to the B family. The Archaea contain D family polymerases. Family X is based of eukaryotic Pol-β which is important in excision repair. Y-family polymerases include the

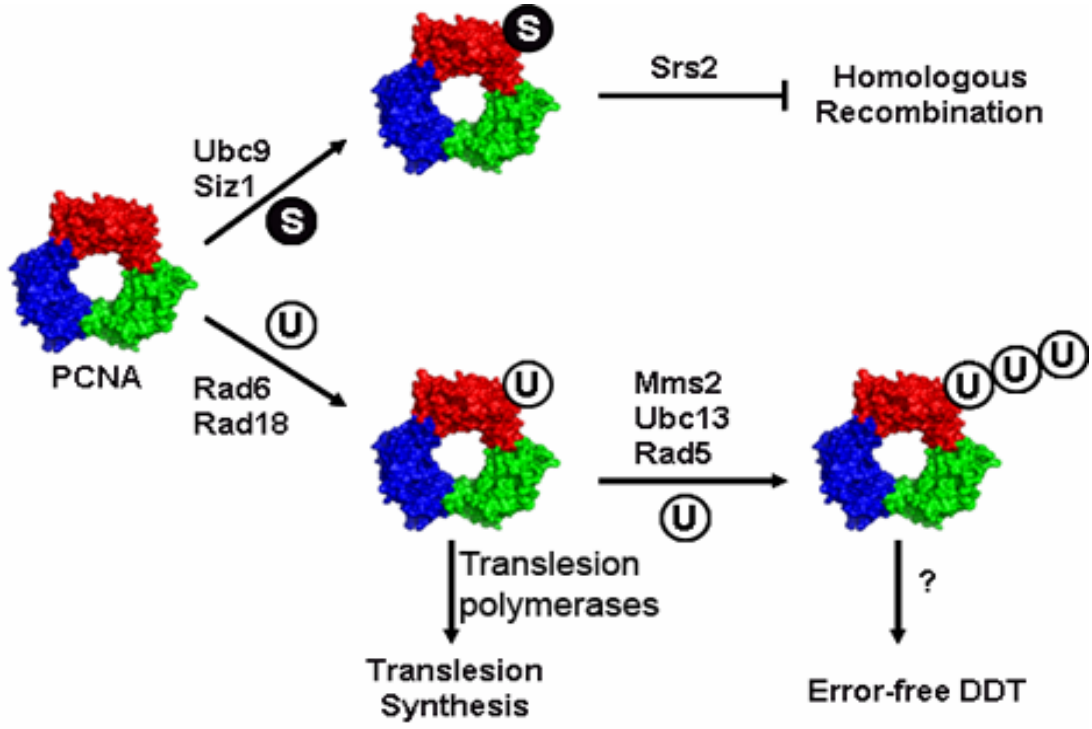

Figure 1-2. Modifications of PCNA regulating PRR in yeast

In yeast, the PCNA homotrimer can be modified by either SUMO (S) or Ub (U) on the Lys164 residue to regulate PRR. A SUMO modification is inhibitory to homologous recombination through recruitment of the Srs2 helicase and disruption of Rad51-ssDNA filaments. Mono-Ub by Rad6-Rad18 will promote TLS by recruiting TLS polymerases, whereas further poly-Ub of K63-linked chains by Rad5-Ubc13-Mms2 promotes error-free PRR. Note that all three subunits of PCNA may be modified simultaneously, but only a single subunit modification is demonstrated here for simplicity. This model is assumed to be conserved in higher eukaryotes.

mutagenic polymerases exhibiting low fidelity on undamaged DNA and the ability to synthesize DNA past lesions which the other polymerasae families cannot in general surpass. (Brautigam and Steitz, 1998; Burgers et al., 2001; Cann and Ishino, 1999; Joyce and Steitz, 1994; Ohmori et al., 2001). Additional DNA polymerases include the reverse transcriptase polymerases found in retroviruses and eukaryotes (as telomerases) which utilize an RNA strand as a template to synthesize DNA. Error-prone TLS can occur by the regular replicative polymerases or specialized, error-prone polymerases. Replicative polymerases include Pol-I, Pol-II and Pol-III in prokaryotes, Pol-1(α), Pol-2(ε), and Pol-3(δ) in yeast and Pol-α, Pol-ε and Pol-δ in higher eukaryotes. Errors can arise by simple incorrect base-pairing and/or lack of proofreading. Frameshift mutations often occur in regions of repeated nucleotide sequences likely from slippage of the template strand or misalignment of the incoming nucleotide with the template. In addition, certain nucleotide repeats can readily form secondary structures that become recombination hotspots and fragile sites in the DNA, among which triplet repeats can also provide sources of extensive amino acid expansion in the coding region (Friedberg et al., 2006). Furthermore, non-replicative polymerases may be required for extension from nucleotide insertion by a low fidelity polymerase, which allows further downstream progression by a replicative polymerase.

1.5.4.1. Y-family DNA polymerases

Essentially all TLS polymerases except one (i.e., Rev3) are Y-family polymerases which lack a 3'→5' proofreading exonuclease activity and contain relatively non-restrictive active sites compared with the replicative polymerases (Yang

and Woodgate, 2007). Surprisingly, although members of this family of proteins have been studied for many years and implicated in mutagenesis or TLS, it was only at the end of the last century that they were reported as a novel class of DNA polymerases and attracted tremendous attention with respect to mutagenesis (Friedberg and Gerlach, 1999; Lehmann et al., 2007; Wang, 2001; Woodgate, 1999; Yang and Woodgate, 2007).

E. coli contains two Y-family polymerases, Pol-IV and Pol-V; both are DNA damage inducible and belong to the SOS regulon. Pol-IV has an extremely low affinity for naked primer-template substrates and relies heavily on the β-clamp (a bacterial functional homolog of PCNA) to load onto DNA (Tang et al., 2000; Wagner et al., 2000). *In vitro* studies indicate that Pol-IV and its archeal homolog Dpo4 are relatively faithful polymerases at the incorporation step and the low fidelity primarily results from poor discrimination between correct and incorrect incoming nucleotides at the binding stage and the capacity to elongate mismatched primer-template, which results in -1 frame shift mutations (Fiala and Suo, 2004; Kobayashi et al., 2002). Hence, Pol-IV promotes mutagenesis through three distinct mechanisms: replication error, TLS and incorporation of base analogs.

Pol-V plays a critical role in the most characterized damage-induced mutagenesis pathway. Like Pol-IV, DNA synthesis by Pol-V is strictly distributive, requires additional cofactors such as RecA and the β-clamp, and can efficiently bypass essentially all lesions tested to date (Fuchs et al., 2004). Pol-V accounts for the vast majority of UV-induced mutagenesis in *E. coli* (Fuchs et al., 2004). Due to its notorious substrate plasticity, Pol-V must be placed under strict regulation; indeed Pol-

V activity is controlled at both transcriptional and post-translational levels (Friedberg et al., 2006).

Budding yeast also contains two Y-family polymerases. Rev1, the first characterized eukaryotic Y-family member, is not technically a polymerase but a deoxycytidyl transferase that inserts a dCMP efficiently opposite a template abasic site and is probably responsible for 60-85% of the bypass events at AP sites *in vivo* (Nelson et al., 2000; Nelson et al., 1996a). It can also insert dCMP across template G or A, albeit to a lesser extent (Nelson et al., 1996a). Structural analysis indicates that Rev1 does not facilitate base pairing between the template G and the incoming dCTP. Instead, the G is evicted from the DNA helix and the dCTP pairs with a protein "template" arginine residue (Nair et al., 2005), which ensures base selection in a DNA template-independent manner. The yeast *rev1* mutant displays a complete loss of mutagenesis activity comparable to that of *rev3*, which cannot be explained by its dCMP transferase activity. Indeed, analysis of site-specific mutations confirms that Rev1 enzymatic activity is not essential for TLS; but its BRCA1 C-terminal (BRCT) domain (Guo et al., 2006a; Otsuka et al., 2005) and/or a polymerase-associated domain (Acharya et al., 2005) are required for protein interactions. The C-terminal 100 amino acids of human Rev1 are sufficient to interact with all other TLS polymerases (Guo et al., 2003), implying a scaffold role of Rev1 in TLS. The Rev1 structure and functions appear to be highly conserved between lower and higher eukaryotes. Experimental reduction of *REV1* expression in cultured human cells results in a decrease in UV-induced mutagenesis (Gibbs et al., 2000).

Pol-η in yeast is encoded by *RAD30*, whose inactivation (McDonald et al., 1997) or mutation in the corresponding mammalian *XPV* gene (Johnson et al., 1999b; Masutani et al., 1999b) leads to an increased susceptibility to UV-induced DNA damage. Pol-η is able to correctly incorporate AA opposite *cis-syn* thymine-thymine dimers (Johnson et al., 1999c) with incorporation kinetics comparable to that opposite of undamaged template (Johnson et al., 2000c). This insertion fidelity is thought to be achieved through an induced-fit mechanism similar to replicative polymerases (Washington et al., 2001). However, for other types of lesions including those induced by UV, such as TT (6-4)PPs, Pol-η has reduced affinity, poor incorporation rates or low fidelity (Vaisman et al., 2004). Hence, Pol-η appears to be highly specialized and at least in some circumstances an "error-free" Y-family polymerase when bypassing thymine dimers.

Mammals contain two additional Y-family polymerases. Pol-ι is the only known DNA polymerase to date that violates the Watson-Crick base-pairing rule (Tissier et al., 2000). It relies on Hoogsteen base pairing as opposed to typical Watson-Crick base pairing and thus operates with very low fidelity (Nair et al., 2004). This mechanism may facilitate read-through of replication-blocking minor groove purine adducts (Wolfle et al., 2005). *In vivo*, uracil derived from cytosine deamination may be the target of Pol-ι as it inserts a G opposite a template U (Vaisman and Woodgate, 2001).

Pol-κ is thought to be involved in the elongation step following mismatched bases or following damaged bases (Haracska et al., 2002a; Ohmori et al., 2004; Wolfle et al., 2003), and reads through bulky adducts such as modifications by BPDE (Ogi et

al., 2002; Shen et al., 2002). When nucleotides containing dG-N^2-BPDE, the most potent carcinogenic compound produced by industrial and cigarette smoke, are used as template, Pol-κ can bypass the adduct with much higher efficiency than Pol-η or Pol-ι by correctly inserting C opposite the bulky lesion (Ogi et al., 2002). However, when undamaged DNA or DNA containing some common lesions are used as templates, Pol-κ exhibits extraordinarily low fidelity (Ohashi et al., 2000; Zhang et al., 2000a; Zhang et al., 2000b).

In summary, although each of its members has distinct base-pair specificity and specialized functions, Y-family polymerases are highly conserved. These generally allow relatively less stringent DNA duplication and thus result in an elevated mutagenic potential. The low fidelity of these polymerases suggests that their activities must be restricted to highly selective conditions in order to limit mutational events. A schematic diagram of the human Y-family polymerases is depicted in Figure 1-3.

1.5.4.2. Regulated access of Y-family polymerases to the damage site

Because of the high probability of Y-family TLS polymerases being mutagenic, it is expected that these polymerases are tightly regulated, probably at several levels as are the error-prone polymerases in *E. coli*. One example of transcriptional regulation of higher eukaryotic error-prone TLS polymerases is the regulation of human and mouse *POLK* promoters, which contain xenobiotic responsive elements that can be induced by polycyclic aromatic hydrocarbons, among which benzo[*a*]pyrene is the most characterized (Ogi et al., 2001). Hence, *POLK* is induced in response to specific DNA damage that can be bypassed by Pol-κ. A second regulatory mechanism is damage-

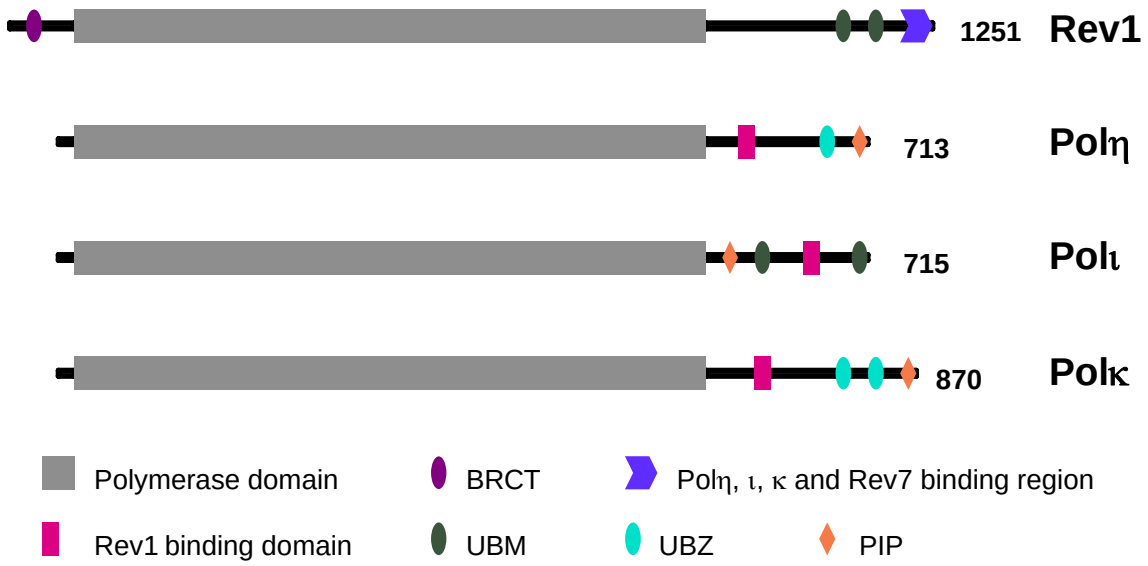

Figure 1-3. A schematic representation of Y-family polymerases

The Y-family polymerases have been partially characterized with respect to their functional domains. Numbers indicate the amino acid (a.a.) content of each protein. Each display variations in their polymerase domains which results in preferential activity on different types of DNA damage. The additional domains listed, the PCNA interacting peptide (PIP), Ub-binding zinc finger domain (UBZ), Ub-binding motif domain (UBM) and the BRCA1 C-terminal domain (BRCT), are thought to perform regulatory roles by influencing interactions with other proteins, most notably Ub-modified proteins such as mono-Ub-PCNA.

induced accumulation at the replication foci stalled at DNA damage. Pol-η forms such foci in response to UV irradiation, and mutations with a functional polymerase motif but lacking the C-terminal domain fail to localize to the nucleus due to loss of a NLS; the NLS operates independently of UV exposure. Additionally, a second sequence in the C-terminal distinct from the NLS is required to form nuclear foci in response to UV exposure (Kannouche et al., 2001). Pol-ι physically interacts and co-localizes with Pol-η to the damage induced nuclear foci (Kannouche et al., 2002), suggesting that Pol-ι plays a role in bypassing UV-induced lesions. In contrast, BPDE treatment specifically induces Pol-κ foci formation but not Pol-η foci (Bi et al., 2005), indicating lesion-specific recruitment of the cognate Y-family polymerase. The mechanism of this lesion-specific recruitment is currently unknown. Budding yeast Pol-η is a short lived protein with a half life of approximately 20 minutes but becomes stabilized following exposure of the cells to UV light (Skoneczna et al., 2007). The TLS polymerases may therefore be regulated by the synthesis, degradation and localization.

One interesting observation is that the Y-family polymerases generally contain both PCNA interacting peptide (PIP) and Ub-binding domains, including Ub-binding motifs (UBMs) or Ub-binding zinc fingers (UBZs) (Figure 1-3). Pol-η specifically interacts with monoubiquitinated but not unmodified PCNA (Kannouche et al., 2004) and the Ub-binding domains are essential for the accumulation of Pol-ι and Pol-η in replication foci. Similarly, the damage-induced foci formation and UV resistance of Rev1 also require UBMs (Guo et al., 2006b). Unlike other Y-family polymerases, Rev1 does not contain a PIP motif; instead, a recent study suggests that Rev1 utilizes its BRCT domain to interact with PCNA (Guo et al., 2006a). Supporting this notion is the

observation that the damage-induced foci formation of Pol-η (Kannouche et al., 2004; Watanabe et al., 2004) and Pol-κ (Bi et al., 2006) is dependent on functional Rad18, presumably because Rad18 is required for the generation of mono-ubiquitinated PCNA. The ultimate support perhaps comes from an *in vitro* study (Garg and Burgers, 2005), in which PCNA was found to be ubiquitinated only when appropriately loaded onto DNA. Mono-ubiquitinated PCNA shows functional interactions similar to those of unmodified PCNA *in vitro* with replication factors such as Fen1, Lig1, RFC, Pol-δ and Pol-ε, but in addition this modification is also able to activate Pol-η and Rev1 (Garg and Burgers, 2005).

Although the above studies provide a paradigm for the restriction and recruitment of TLS polymerases to the damage site, the overall model is challenged by other reports. One study shows that unmodified PCNA is sufficient to stimulate DNA synthesis by Pol-κ, primarily by reducing the *Km* to enhance correct nucleotide incorporation (Haracska et al., 2002b). The direct challenge came from the *in vitro* reconstitution of the DNA synthesis reaction, in which PCNA monoubiquitinated on all three monomers does not enhance affinity for any polymerases examined, nor does it enhance TLS activity by Y-family polymerases (Haracska et al., 2006). Furthermore a recent report showed that mutations in the UBZ motif of yeast Pol-η did not impair its *in vivo* or *in vitro* TLS functions (Acharya et al., 2007a). The authors suggested an alternative model in which PCNA monoubiquitination may disrupt its interactions with a protein(s) that normally are present to inhibit interactions with the TLS polymerases. This may be supported by a recent report (Hishida et al., 2006) that Mgs1, a protein with homology to *E. coli* RuvB and eukaryotic clamp loader protein RFC, as well as

DNA-dependent ATPase activity and DNA-annealing activities (Hishida et al., 2001; Hishida et al., 2002), associates with PCNA and appears to repress the *RAD6* pathway in the absence of exogenous damage. In *E. coli* the RuvA-RuvB complex may promote replication fork reversal and therefore offset TLS activity (Le Masson et al., 2008). Additionally, interaction of monoubiquinated PCNA with p21 may be inhibitory to TLS (Avkin et al., 2006; Livneh, 2006). Other concerns with the above paradigm include the stability of monoubiquitinated PCNA, particularly in mammalian cells, that extends past the expected time required to bypass the damage (Kannouche et al., 2004), which would allow persistent TLS with unnecessarily increased mutation rates. In addition, hydroxyurea treatment, which depletes the nucleotide pool and induces replication fork stalling, also results in PCNA monoubiquitination in the absence of true DNA damage (but the likely production of ssDNA) (Kannouche et al., 2002; Kannouche et al., 2004). The stalled replication fork after this treatment is unlikely to benefit from TLS, raising doubt that mono-Ub is at the heart of polymerase switching.

1.5.4.3. Polymerase-ζ

DNA polymerase-ζ is unique in that it is the only known B-family DNA polymerase and the only non-Y-family polymerase that participates in TLS in eukaryotic cells. Budding yeast Pol-ζ is composed of two subunits (Nelson et al., 1996b): yRev3 (1504 amino acids) is the catalytic subunit of Pol-ζ, whereas Rev7 is considered a regulatory subunit of Pol-ζ. Rev7 has at least two regulatory roles. Firstly, it is required for the *in vitro* polymerase activity of Pol-ζ (Nelson et al., 1996b), although how Rev7 contributes to such an activity is currently unclear. Secondly, Rev7

physically interacts with the polymerase-associated domain of Rev1 (Acharya et al., 2005), although the same research team also reported that yeast Rev3 can directly interact with the C-terminus of Rev1 and that this interaction appears to be essential for Rev3 function (Acharya et al., 2006). In budding yeast Pol-ζ is not essential, but upon its inactivation cells become more susceptible to genotoxic agents (Lawrence et al., 1984; Morrison et al., 1989; Pavlov et al., 2001) and strikingly display significantly reduced mutagenic capacity in response to DNA damage (Lawrence and Hinkle, 1996; Lawrence and Maher, 2001). Unlike Rev1 or Pol-η, yeast Pol-ζ is required for nearly all UV-induced mutagenesis as well as the majority of spontaneous mutagenic events (Quah et al., 1980).

Human Rev3 (Figure 1-4) has an expected size (3130 amino acids) double that of yeast Rev3 which is mainly due to an additional internal region not shared by yRev3. By sequence analysis, Rev3 is considered a member of the polymerase B-family based homology similarities to Pol-δ in the N-terminal non-catalytic domain (Lin et al., 1999) and on two conserved amino acid sequences, SLYPSI and YGDTDS in the catalytic domain (Hubscher et al., 2002; Morrison et al., 1989). The predicted full length sequence of hRev3 includes a Rev1 binding domain which is partially similar with yRev3, six conserved DNA polymerase motifs identifying it as a DNA polymerase, and a cysteine-rich region, possibly forming two zinc finger motifs (Gan et al., 2008; Morrison et al., 1989). The similarity with the B-family polymerases suggests a high fidelity of insertion events, however a lack of 3'→5' proofreading ability presumably allows Rev3 to be proficient in extention from non-Watson-Crick base pairs

Figure 1-4. Human Rev3 amino acid sequence (continued on next page)

MFSVRIVTADYYMASPLQGLDTCQSPLTQAPVKKVPVVRVFGATPAGQKTCLHLHGIFPYLYVPYDGYGQ
QPESYLSQMAFSIDRALNVALGNPSSTAQHVFKVSLVSGMPFYGYHEKERHFMKIYLYNPTMVKRICELL
QSGAIMNKFYQPHEAHIPYLLQLFIDYNLYGMNLINLAAVKFRKARRKSNTLHATGSCKNHLSGNSLADT
LFRWEQDEIPSSLILEGVEPQSTCELEVDAVAADILNRLDIEAQIGGNPGLQAIWEDEKQRRRNRNETSQ
MSQPESQDHRFVPATESEKKFQKRLQEILKQNDFSVTLSGSVDYSDGSQEFSAELTLHSEVLSPEMLQCT
PANMVEVHKDKESSKGHTRHKVEEALINEEAILNLMENSQTFQPLTQRLSESPVFMDSSPDEALVHLLAG
LESDGYRGERNRMPSPCRSFGNNKYPQNSDDEENEPQIEKEEMELSLVMSQRWDSNIEEHCAKKRSLCRN
THRSSTEDDDSSSGEEMEWSDNSLLLASLSIPQLDGTADENSDNPLNNENSRTHSSVIATSKLSVKPSIF
HKDAATLEPSSSAKITFQCKHTSALSSHVLNKEDLIEDLSQTNKNTEKGLDNSVTSFTNESTYSMKYPGS
LSSTVHSENSHKENSKKEILPVSSCESSIFDYEEDIPSVTRQVPSRKYTNIRKIEKDSPFIHMHRHPNEN
TLGKNSFNFSDLNHSKNKVSSEGNEKGNSTALSSLFPSSFTENCELLSCSGENRTMVHSLNSTADESGLN
KLKIRYEEFQEHKTEKPSLSQQAAHYMFFPSVVLSNCLTRPQKLSPVTYKLQPGNKPSRLKLNKRKLAGH
QETSTKSSETGSTKDNFIQNNPCNSNPEKDNALASDLTKTTRGAFENKTPTDGFIDCHFGDGTLETEQSF
GLYGNKYTLRAKRKVNYETEDSESSFVTHNSKISLPHPMEIGESLDGTLKSRKRRKMSKKLPPVIIKYII
INRFRGRKNMLVKLGKIDSKEKQVILTEEKMELYKKLAPLKDFWPKVPDSPATKYPIYPLTPKKSHRRKS
KHKSAKKKTGKQQRTNNENIKRTLSFRKKRSHAILSPPSPSYNAETEDCDLNYSDVMSKLGFLSERSTSP
INSSPPRCWSPTDPRAEEIMAAAEKEAMLFKGPNVYKKTVNSRIGKTSRARAQIKKSKAKLANPSIVTKK
RNKRNQTNKLVDDGKKKPRAKQKTNEKGTSRKHITLKDEKIKSQSGAEVKFVLKHQNVSEFASSSGGSQL
LFKQKDMPLMGSAVDHPLSASLPTGINAQQKLSGCFSSFLESKKSVDLQTFPSSRDDLHPSVVCNSIGPG
VSKINVQRPHNQSAMFTLKESTLIQKNIFDLSNHLSQVAQNTQISSGMSSKIEDNANNIQRNYLSSIGKL
SEYRNSLESKLDQAYTPNFLHCKDSQQQIVCIAEQSKHSETCSPGNTASEESQMPNNCFVTSLRSPIKQI
AWEQKQRGFILDMSNFKPERVKPRSLSEAISQTKALSQCKNRNVSTPSAFGEGQSGLAVLKELLQKRQQK
AQNANTTQDPLSNKHQPNKNISGSLEHNKANKRTRSVTSPRKPRTPRSTKQKEKIPKLLKVDSLNLQNSS
QLDNSVSDDSPIFFSDPGFESCYSLEDSLSPEHNYNFDINTIGQTGFCSFYSGSQFVPADQNLPQKFLSD
AVQDLFPGQAIEKNEFLSHDNQKCDEDKHHTTDSASWIRSGTLSPEIFEKSTIDSNENRRHNQWKNSFHP
LTTRSNSIMDSFCVQQAEDCLSEKSRLNRSSVSKEVFLSLPQPNNSDWIQGHTRKEMGQSLDSANTSFTA
ILSSPDGELVDVACEDLELYVSRNNDMLTPTPDSSPRSTSSPSQSKNGSFTPRTANILKPLMSPPSREEI
MATLLDHDLSETIYQEPFCSNPSDVPEKPREIGGRLLMVETRLANDLAEFEGDFSLEGLRLWKTAFSAMT
QNPRPGSPLRSGQGVVNKGSSNSPKMVEDKKIVIMPCKCAPSRQLVQVWLQAKEEYERSKKLPKTKPTGV
VKSAENFSSSVNPDDKPVVPPKMDVSPCILPTTAHTKEDVDNSQIALQAPTTGCSQTASESQMLPPVASA
SDPEKDEDDDDNYYISYSSPDSPVIPPWQQPISPDSKALNGDDRPSSPVEELPSLAFENFLKPIKDGIQK
SPCSEPQEPLVISPINTRARTGKCESLCFHSTPIIQRKLLERLPEAPGLSPLSTEPKTQKLSNKKGSNTD
TLRRVLLTQAKNQFAAVNTPQKETSQIDGPSLNNTYGFKVSIQNLQEAKALHEIQNLTLISVELHARTRR
DLEPDPEFDPICALFYCISSDTPLPDTEKTELTGVIVIDKDKTVFSQDIRYQTPLLIRSGITGLEVTYAA
DEKALFHEIANIIKRYDPDILLGYEIQMHSWGYLLQRATFRVLSDWFDNKTDLYRWKMVDHYVSRVRGNL
QMLEQLDLIGKTSEMARLFGIQFLHVLTRGSQYRVESMMLRIAKPMAALSIDLCRMISRVPDDKIENRFA

AERDEYGSYTMSEINIVGRITLNLWRIMRNEVALTNYTFENVSFHVLHQRFPLFNYIPVTPSVQQRSQMR
APQCVPLIMEPESRFYSNSVLVLDFQSLYPSIVIAYNYCFSTCLGHVENLGKYDEFKFGCTSLRVPPDLL
YQVRHDITVSPNGVAFVKPSVRKGVLPRMLEEILKTRFMVKQSMKAYKQDRALSRMLDARQLGLKLIANV
TFGYTSANFSGRMPCIEVGDSIVHKARETLERAIKLVNDTKKWGARVVYGDTDSMFVLLKGATKEQSFKI
GQEIAEAVTATNPKPVKLKFEKVYLPCVLQTKKRYVGYMYETLDQKDPVFDAKGIETVRRDSCPAVSKIL
ERSLKLLFETRDISLIKQYVQRQCMKLLEGKASIQDFIFAKEYRGSFSYKPGACVPALELTRKMLTYDRR
SEPQVGERVPYVIIYGTPGVPLIQLVRRPVEVLQDPTLRLNATYYITKQILPPLARIFSLIGIDVFSWYH
ELPRIHKATSSSRSEPEGRKGTISQYFTTLHCPVCDDLTQHGICSKCRSQPQHVAVILNQEIRELERQQE
QLVKICKNCTGCFDRHIPCVSLNCPVLFKLSRVNRELSKAPYLQQLLDQF

Figure 1-4. Human Rev3 amino acid sequence (continued from previous page)

The hRev3 amino acid sequence is derived from Gibbs *et al*. (1998) (GenBank accession No. AF058701.1). Amino acid regions with high homology to yeast Rev3 are underlined and the Rev7 binding region is highlighted in blue. Within the C-terminal region, which has homology to yeast Rev3, are six sequences characterizing Rev3 as a DNA polymerase (not indicated). Similarities with Pol-δ occur in the N-terminal domain (not indicated) The boxed sequences in the C-terminal identify Rev3 as a B-family polymerase.

(Lawrence and Maher, 2001; Ohmori et al., 2001). A schematic comparision between yeast and human Rev3 is depicted in Figure 1-5. The *hREV3* transcript is inducible upon some types of DNA damage, possibly in a p53-dependent manner due to a p53-binding site upstream of the promoter (Krieg et al., 2006; Yu et al., 2004). Human Rev3 is presumed to function similarly to yeast Rev3. Reduced levels of Rev3 protein in cultured cells results in a compromised UV-induced mutation rate without apparent loss of viability (Diaz et al., 2003; Gibbs et al., 2000; Li et al., 2002b). However, viability was reduced following treatment with the cross-linking agent cisplatin (Wu et al., 2004). Although *Rev3* knockout results in mouse embryonic lethality (Esposito et al., 2000; Van Sloun et al., 2002; Wittschieben et al., 2000), concurrent knockout of *Rev3* and *p53* yields cells exhibiting enhanced spontaneous chromosomal translocations as well as enhanced sensitivity to UV exposure and cross-linking agents (Wittschieben et al., 2006; Zander and Bemark, 2004).

The *in vitro* activities of Pol-ζ have only been studied with the yeast protein to date and in higher eukaryotes research has been limited to knocking down *Rev3* expression. Purified yeast Pol- ζ has the ability to bypass UV-induced thymine-thymine dimers much more efficiently than Pol-α (Nelson et al., 1996b). It can also correctly incorporate dAMP opposite an ROS-inducedthymine glycol lesion in an error-free manner (Johnson et al., 2003) and pol-ζcan synthesize past BDPE-induced lesions in an error-prone manner (Li et al., 2002b). Minimal mutation induction by Rev3 may depend upon its interaction with Rev1, as demonstrated in chicken DT40 cells: when

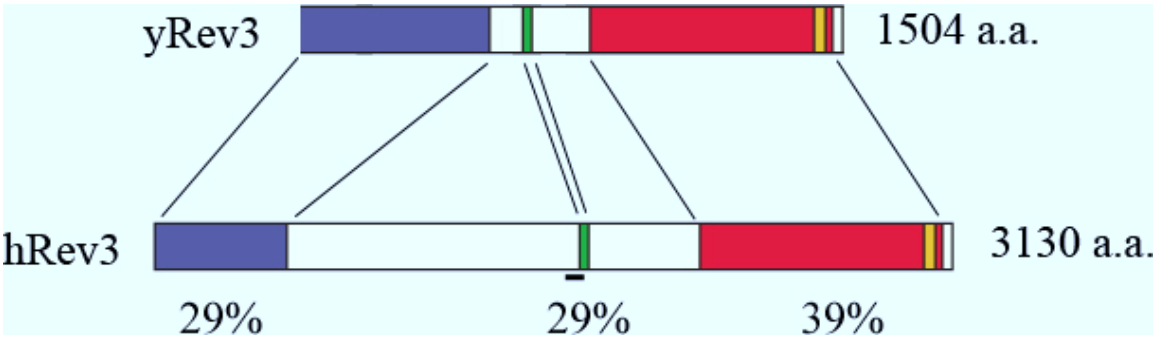

Figure 1-5. Schematic comparison of yeast and human Rev3

Human Rev3 shares significant amino acid identity to budding yeast in three areas including regions in the N-terminal (colored blue, amino acids 1-333), an internal region (colored green, amino acids 1888-1943) and the C-terminal (colored red, amino acids 2276-3125) with percent identity as indicated. The Rev7 binding region (Black bar, amino acids 1847-1892) partially overlaps with the internal similar region (green). Within the C-terminal domain lie six domains characterizing Rev3 as a DNA polymerase, including two domains which put it into the B family of polymerases (not shown). Also present is a putative Zinc finger (yellow).

Rev1 is deleted frame shift mutations are induced but only in the presence of Rev3 (Szuts et al., 2008). The trend appears to be that each of the TLS polymerases has a preferred function operating on a specific lesion, and mutagenesis occurs when activity is imposed on a sub-optimal substrate. Although *in vitro* assays have demonstrated that yeast Pol-ζ itself is capable of bypassing some selected lesions, in a polymerase switch model it is thought to be primarily responsible for the extension step of TLS. The two-step model of TLS polymerase activity also appears to be a general trend, as outlined in the next section.

1.5.4.4. The polymerase switch model

The amount of endogenous damage that DNA experiences (Ames and Gold, 1991) and the observation that spontaneous mutations occur in a Pol-ζ dependent manner (Zhong et al., 2006) suggest that TLS polymerases are present and active with or without exogenous genotoxic agents. Presumably TLS polymerases must be tightly regulated to minimize their activity in favor of the high fidelity replicative polymerases in order to minimize mutagenesis. The mechanism that controls the exchange between these two classes of polymerases is largely unknown but likely involves at least three distinct steps in eukaryotes. First is the loss of function of the replicative polymerase and gain of function of a TLS polymerase to insert one or a few nucleotides across from a lesion site. Second is the use of a second TLS polymerase to extend from the non-Watson-Crick base pair at the lesion site, which the replicative polymerases cannot accomplish. Third is the timely reversal from a TLS polymerase to a replicative

polymerase to ensure restoration of high fidelity duplication. This proposed process is collectively referred to as the polymerase switch model (Lehmann et al., 2007).

In *E. coli* two mechanisms have been proposed to regulate the switch from the replicative polymerase Pol-III to the TLS polymerases Pol-IV and Pol-V. The first model suggests that the Pol-III holoenzyme completely dissociates from the β-clamp at a stalled replication site followed by the access of Pol-V that proceeds with synthesis for less than 100 bases before disassociating, which is followed by replacement with Pol-III (Fujii and Fuchs, 2004). The second model, termed the tool belt model, utilizes the homodimer structure of the β-clamp such that Pol-III and Pol-IV each bind one of the subunits simultaneously, which allows utilization of both polymerases as required without the need to dissociate and re-associate at the lesion site (Indiani et al., 2005). This model has also been described in *Sulfolobus solfataricus*, an archeabacteria whose PCNA is a heterotrimer (PCNA1, PCNA2 and PCNA3) and each subunit binds preferentially to either primase, DNA polymerase or Fen1, supporting the tool belt model at least during lagging stand DNA synthesis (Dionne et al., 2003). The observation in the same organism that the uracil DNA glycosylase, UDG1 preferentially binds to PCNA3 in a yeast two hybrid study may indicate the tool belt model occurs also during a DNA damage response (Dionne and Bell, 2005).

The mechanism controlling the polymerase switch from a replicative to a TLS polymerase in eukaryotes is unknown but TLS appears to require monoubiquitination of PCNA at a stalled replication site in a Rad6-Rad18 dependent manner (Hoege et al., 2002; Stelter and Ulrich, 2003). Over-expression of Rad18 induces monoubiquitination of PCNA in the absence of damage (Bi et al., 2006). Although this modification of

PCNA does not appear to modify its interaction with the replicative polymerases (Garg and Burgers, 2005; Zhuang et al., 2008), it is thought that this modification increases the affinity of PCNA for the Y-family polymerase either directly or indirectly, particularly when the PCNA is non-mobile (Zhuang et al., 2008). Pol-η and Pol-κ both contain UBZ domains; Pol-ι contains two UBM domains and each of the three contain a PIP domain (Figure 1-3), which in combination are thought to be important for TLS (Bienko et al., 2005). Depletion of Rad18 not only eliminates BPDE-induced PCNA monoubiquitination, but also inhibits PCNA interaction with Pol-κ (Bi et al., 2006). *In vivo* Pol-η binds chromatin-associated PCNA in a Rad18 dependent manner (Watanabe et al., 2004) and *in vitro* monoubiquinated PCNA increases the polymerase activity of Pol-η (Kannouche et al., 2004). Furthermore, the deubiquitinizing enzyme (DUB) Usp-1, which is responsible for removal of Ub from PCNA, is down-regulated following UV exposure (Huang et al., 2006). Although the UBZ domain of Pol-η has been demonstrated to be essential for recovery in exogenously delivered Pol-η in XPV cells (Bienko et al., 2005), the UBZ domain of Pol-η may not be necessary for a direct interaction with mono-Ub PCNA (Acharya et al., 2007b). The PIP domain of Pol-η is thought to be more important for interacting with PCNA than the UBZ (Acharya et al., 2008); however polymerase activity may be enhanced in the presence of monoubiquinated PCNA (Garg and Burgers, 2005). The PIP domain of Pol-ι has also been shown to be essential for co-localizing with PCNA *in vivo* and for Pol-ι polymerase activity *in vitro* (Vidal et al., 2004). Rev1 contains two UBM domains for binding Ub, an N-terminal BRCT domain, and a C-terminal polymerase-associated domain domain (Figure 1-3). The polymerase-associated domain is responsible for

binding Pol-ι, Pol-κ and Rev7 (Acharya et al., 2005; Friedberg et al., 2005). The BRCT domain of Rev1 is required for direct interaction with PCNA in non-damaged cells, with no increase in affinity following irradiation, but the UBM domain enhances binding to mono-Ub PCNA (Guo et al., 2006a; Guo et al., 2006b). Additionally, Pol-κ, Pol-ι and Pol-η contain Rev1-interacting regions (Ohashi et al., 2009). The Rev1-interacting region in Pol-κ was essential in reconstitution of exogenously supplied Pol-κ in a Pol-κ knockout mouse (Ohashi et al., 2009). In response to DNA damage Rev1 interacts with Pol-η, Pol-ι, Rev7 and PCNA (Friedberg et al., 2005; Kannouche et al., 2002) and Rev7 can compete for binding to Rev1 with Pol-κ (Guo et al., 2003). These observations collectively suggest that Rev1 acts as a scaffolding protein to enhance the interaction of the TLS polymerases with PCNA in a monoubiquitin-modified dependent manner. Interestingly, mouse Rev1 can also become monoubiquitinated, but this function has not been linked to the UBZ or UBM domains of the other polymerases (Guo et al., 2006a).

In response to damage, chromatin bound PCNA likely becomes mono-ubiquitinated on each of the subunits of the homotrimer (Kannouche et al., 2004). At the replication fork Rev1 interacts with the other polymerases and Pol-η and Pol-ι also interact directly with one another (Kannouche et al., 2003). The tool belt model described in *E. coli* therefore becomes an attractive model to the eukaryotic system, although no evidence in support of this eukaryotic model has so far been presented except that many proteins are able to physically interact and localize with PCNA-containing foci concurrently. Although the mono-Ub of PCNA appears to directly induce TLS, an alternative possibility may involve mono-Ub induced release of a

PCNA-interacting molecule, which, in its absence, allows access of TLS polymerases. One candidate molecule is the PCNA-interacting protein p21, the loss of which results in enhanced TLS activity (Avkin et al., 2006; Livneh, 2006).

PCNA modification may not be the only means of promoting TLS; DNA damage checkpoints have been implicated in TLS. For instance, Rad9 of the *S. pombe* 9-1-1 complex, which forms a PCNA-like heterotrimeric clamp, associates with Mms2, and a mutant form of Rad9 incapable of interaction promotes mutagenesis in a TLS-dependent manner (Kai et al., 2007). In budding yeast the phosphorylation of protein kinase Mec1 induces the re-localization of Rev1 and Pol-ζ to sites of DNA double strand breaks independently of mono-Ub PCNA (Hirano and Sugimoto, 2006). Furthermore, the budding yeast 9-1-1 clamp physically interacts with the Rev7 subunit of Pol-ζ and is partially required for spontaneous mutagenesis in a Pol-ζ-dependent manner (Sabbioneda et al., 2005). In this context, it is of great interest that our laboratory recently reported the DNA damage-induced monoubiquitination of the Rad17 subunit of 9-1-1 by Rad6-Rad18 in budding yeast (Fu et al., 2008), reinforcing a possible alternative TLS regulation. We wish to emphasize that the above observations did not directly conflict with the PCNA-TLS model.

In vitro the second step of TLS has been partially characterized as the exchange of activity from a Y-family polymerase inserting nucleotides at the lesion site to the use of a second polymerase to elongate from a DNA mismatch, which otherwise the replicative polymerases cannot initiate. This was first demonstrated by the combined efforts of Pol-ι and Pol-ζ to synthesize past a (6-4)PP or AP site, whereas either alone failed to accomplish this (Johnson et al., 2000b). Pol-ζ requires an interaction with the

polymerase-associated domain of Rev1 for the elongating step (Acharya et al., 2005; Acharya et al., 2006). Interestingly, the mutagenic activity of Rev3 in human cells is dependent on the polymerase with which it associates with. In response to a cisplatin-linked GG, the initial step of TLS can be carried out with either Pol-η or Pol-κ, followed by an elongation step by Pol-ζ. However, the Pol-κ/Pol-ζ pair exhibits a significantly higher mutation rate than the Pol-η/Pol-ζ pair in response to the same lesion (Shachar et al., 2009). In the absence of Rev3 the same lesion was repaired in a non-mutagenic manner. The mechanism controlling which polymerases are utilized is unknown but fundamentally important for the understanding of mutation formation. Pol-κ can also function as an elongating TLS polymerase (Haracska et al., 2002a).

The final stage in TLS is the reversion to the replicative polymerases. Without this step the potential to induce many more mutations would be enhanced. In fact excessive activity (by over expression) of Pol-κ results in reduced replication fork progression, which presumably leads to both nucleotide mutations, chromosome rearrangements and possibly induction of dormant replication initiation sites (Pillaire et al., 2007). However the molecular mechanism of this switch is largely unknown. After UV damage chromatin bound mono-ubiquitinated PCNA may persist for more than 24 hours, well after the damage has been removed, suggesting that the covalent modification of PCNA by Ub removal is not the regulating factor of the TLS to replicative polymerase switch (Niimi et al., 2008). This has led to the hypothesis that the mono-Ub PCNA has simply been retained on the DNA because the TLS polymerase has reached a downstream replication origin, where DNA synthesis has already initiated. Although DNA initiation sites are well separated at an average of

12.5-25 kb apart in human cells, dormant origins have been estimated by computer modeling to occur 1-3 times for every active replication site (Blow and Ge, 2009). Furthermore, reduced replication speed has been reported to induce silent replication origins, likely with the function to complete S-phase in a timely manner (Courbet et al., 2008). This would imply that DNA synthesis becomes intermittent due to sites of replication fork stalling with gap repair at a later stage, as opposed to a fully processive mechanism that would require the recruitment of TLS polymerases repeatedly at each lesion, which would likely slow S-phase considerably.

1.5.5. Error-free DNA damage tolerance

Despite the advances made with PCNA and TLS in past years, little is known about the molecular events leading to error-free DDT following PCNA polyubiquitination. Apparently the error-free bypass mechanism utilizes newly synthesized sister chromatid as a template, and, much like PCNA mono-Ub, poly-Ub of PCNA may provide a signal to initiate the process. Two possible models, namely template switching and replication fork regression, have been proposed (Broomfield et al., 2001). Template switching involves homologous sister chromatid invasion/cohesion, high-fidelity DNA synthesis and the subsequent resolution of a resulting Holliday junction (Figure 1-1). Fork regression (Figure 1-1) is thought to operate much as it does in bacteria, requiring ssDNA binding protein and RecA to produce a characteristic chicken-foot structure (Robu et al., 2001). Experimental evidence to support a chicken-foot structure in eukaryotes came from a recent report that yeast Rad5 has a DNA helicase activity that facilitates replication fork regression

(Blastyak et al., 2007). In contrast, several recent reviews suggest that the DNA damage checkpoint acts to prevent stalled replication fork regression, while error-free DDT is mediated by template switching (Branzei and Foiani, 2007; Cobb and Bjergbaek, 2006; Klein, 2006). Alternatively, the two error-free DDT models may not be as mechanistically different as they appear. Regardless of the mode of reaction, it is abundantly clear that the error-free DDT process is highly conserved in the entire eukaryotic kingdom, from yeast to human. Sequence and functional homologs of all proteins involved in error-free DDT, including Mms2 (Xiao et al., 1998b), Ubc13 (Oh et al., 1994) and Rad5 (Motegi et al., 2006; Unk et al., 2006), have been found in mammals, plants and other higher eukaryotes (Kunz and Xiao, 2007; Pastushok and Xiao, 2004). For a few limited examples, suppression of the above genes resulted in phenotypes reminiscent of the corresponding yeast mutants (Andersen et al., 2005; Ma et al., 1998; Motegi et al., 2006).

1.5.6. DDT, genomic instability and cancer

Studies in the yeast model have clearly demonstrated the significance of DDT in maintaining genomic stability. The two branches within DDT, with one being highly mutagenic and another error-free, are likely kept in a dynamic balance in wild type cells. However, in yeast cells defective in error-free DDT, spontaneous mutation rates can be elevated by 30-fold, which would be viewed as a predisposition to cancer. It was postulated that error-prone TLS may constitute a major source of genomic instability and cancer (Lawrence and Hinkle, 1996), although direct evidence for this is lacking.

Perhaps the best studied example of TLS and tumorigenesis is the discovery of mammalian Pol-η, whose gene was found mutated in all xeroderma pigmentosum variant (XPV) patients examined. Pol-η co-localizes with Rev1 (Tissier et al., 2004), Polι (Kannouche et al., 2002) and monoubiquinated PCNA (Watanabe et al., 2004), suggesting that mutations in these genes may also be associated with cancer. The level of translesion polymerases in normal and matched tumor cell lines has been investigated. Several lung cancer cell lines were found to over express Pol-κ, suggesting a role in promoting genomic instability and cancer (O-Wang et al., 2001). In another study, however, transcript levels of TLS polymerases η, ι, κ and ζ are significantly reduced in various lung, stomach and colorectal cancers (Pan et al., 2005). Clearly, more research is required to establish the role of DDT in tumorigenesis and carcinogenesis.

DDT in mammalian cells is assumed to operate in both error-prone and error-free mechanisms as does in the budding yeast. However, very little published research is available of the error-free pathway, and the research on error-prone pathway is largely restricted to the identification and characterization of Y-family TLS polymerases. Nevertheless, almost all mammalian gene homologous to those involved in the yeast error-free and error-prone PRR have been identified, which allows us to investigate the DNA damage tolerance pathway in cultured mammalian cells.

1.6. Objectives

The primary purpose of this thesis research was to extrapolate what is known about PRR mechanisms in budding yeast to mammalian cells. This was achieved by first investigating both the error-prone and error-free pathways individually and then both pathways simultaneously.

1.6.1. Objective #1: To characterize the human homologs of the yeast Ubc13 Mms2

The mammalian homologs of yeast Ubc13 and Mms2 have been discovered by our laboratory as well as other laboratories. When transformed into corresponding yeast mutant cells the mammalian derived genes functionally replace the respective null mutations with respect to PRR (Ashley et al., 2002; Franko et al., 2001; Xiao et al., 1998b). Furthermore, two human homologs of Rad5, a prerequisite for PRR as judged by the yeast model, have also been identified (Ashley et al., 2002; Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2008; Unk et al., 2006). This has led to the hypothesis that Ubc13-Mms2 is involved in DDT in human cells. To investigate this objective we sought to characterize the Ubc13-Mms2 complex in cultured mammalian cells during normal growth and following DNA damage in order to determine if there is a functional role of the complex in genome maintenance.

1.6.2. Objective #2: To determine if Uev1 and Mms2 are functional equivalents of yeast Mms2

Surprisingly, human (Xiao et al., 1998b) and mouse (Franko et al., 2001)genomes contain two homologs of the yeast *MMS2* gene which we named *MMS2* and *UEV1*. In addition, human *UEV1* potentially codes for two splicing variants Uev1A and Uev1B (Rothofsky and Lin, 1997; Xiao et al., 1999), and their core domain shares 91% identity with hMms2 (Figure 1-6). Each contains a unique N-terminal extension not present in Mms2. Early experiments have found that hMms2 and Uev1A are capable of physically interacting with Ubc13 and are functionally active in the respective yeast null mutations (Franko et al., 2001; Xiao et al., 1998b). The reason for the N-terminal extension is unknown, but Ubc13-Uev has been implicated in NF-κB signaling (Deng et al., 2000), a pathway not present in yeast. Since previous biochemical analyses suggest that the Ubc13-mediated K63-linked Ub chain assembly absolutely requires a Uev as co-factor, we sought to determine if the *in vivo* Ubc13 activity is dependent upon a Uev, and if so, whether it is Mms2, Uev1A or Uev1B, that Ubc13 partners with for different biological activities.

1.6.3. Objective #3: To characterize human Rev3

A putative human homolog of yeast *REV3* has been cloned but not yet well characterized and knockdown studies using anti-sense RNA constructs indicates a TLS activity (Gibbs et al., 1998; Li et al., 2002b). The mutagenic potential of yeast Rev3 and the presence of the other TLS polymerases in human cells strongly suggest that Rev3 may be the primary inducer of mutagenesis in human cells. We therefore sought to characterize human Rev3.

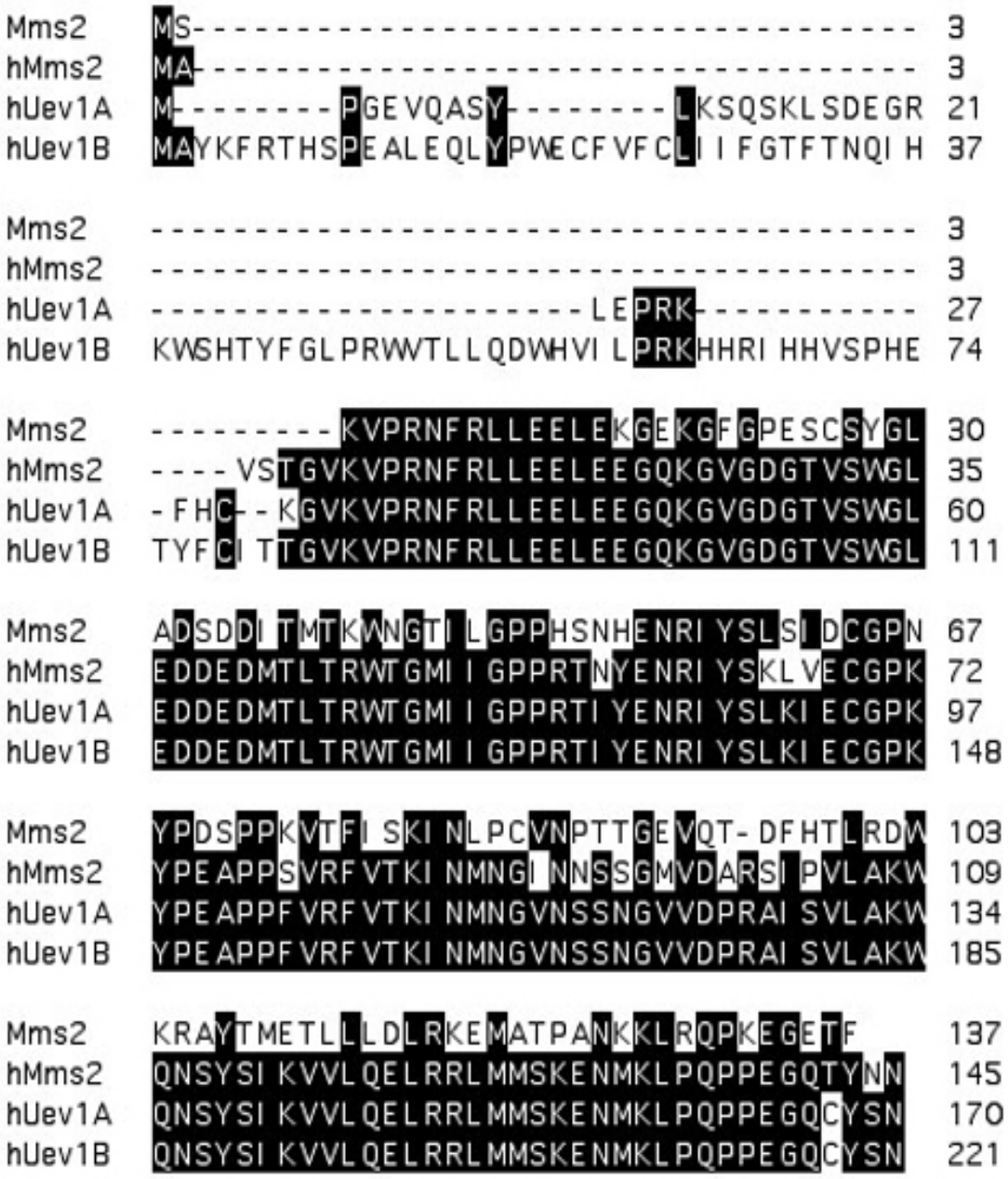

Figure 1-6. Amino acid comparison of human Mms2 and Uevs with yeast Mms2

Amino acid sequences of yeast and human Mms2 proteins aligned with the two human Uev1 isoforms demonstrate a high degree of similarity in the core region, particularly between Uev1 and hMms2. However Uev1A and Uev1B contain different N-terminal extensions that are not found in either yMms2 or hMms2.

1.6.4. Objective #4: To determine if the two parallel DDT sub-pathways are operational in mammalian cells

In budding yeast, null mutations in either the error-free PRR pathway, by deletion of *UBC13* or *MMS2*, or the error-prone PRR pathway, by deletion of *REV1*, *REV3* or *REV7*, results in mild sensitivity to genotoxic agents. However, when the two sub-pathways are eliminated simultaneously the cells become extremely susceptible to killing by a variety of DNA damaging agents (Broomfield et al., 1998; Brusky et al., 2000), suggesting that the two pathways can compensate for one another. For this reason we sought to determine if the synergistic interaction between the two sub-pathways is also true in cultured human cells. This may have important clinical implications, since if the error-prone DDT pathway (Rev3-dependent) can be down-regulated while the cells are still capable of DDT by an error-free bypass mechanism (Ubc13/Uev-dependent), the mutagenic load may be reduced in human cells.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Rational for methodologies used

The model systems used in this research were based on cultured mammalian cell lines because direct observation can be done following manipulations such as transfections or controlled exposure to genotoxic agents. Initially mouse 3T3 cells were utilized as a reasonably normal cell line. When possible normal low passage human cells (GM08402) were utilized as the model cellular system. Several cell types are available with defined genetic abnormalities, such as XPV cells that lack functional Pol-η. However, due to the slow growth and extended population doubling time of the normal cells several established cell lines were utilized, such as HEK-293F cells. These and several other lines widely reported in the literature allow for a direct comparison with published results and reduce the likelihood of cell-line specific phenomenon.

2.2. Optimization of detergent pre-extraction

A significant portion of the results presented in this thesis is dependent upon a modified immunocytochemistry (ICC) technique, which includes a detergent pre-extraction step prior to fixation of the cultures (Figure 2-1). This allows the majority of the soluble cytoplasmic and nuclear constituents to be removed while leaving the presumably relatively stable complexes in place, such as the cytoskeleton, the nucleus and many nuclear complexes. In the literature several detergents are utilized, including

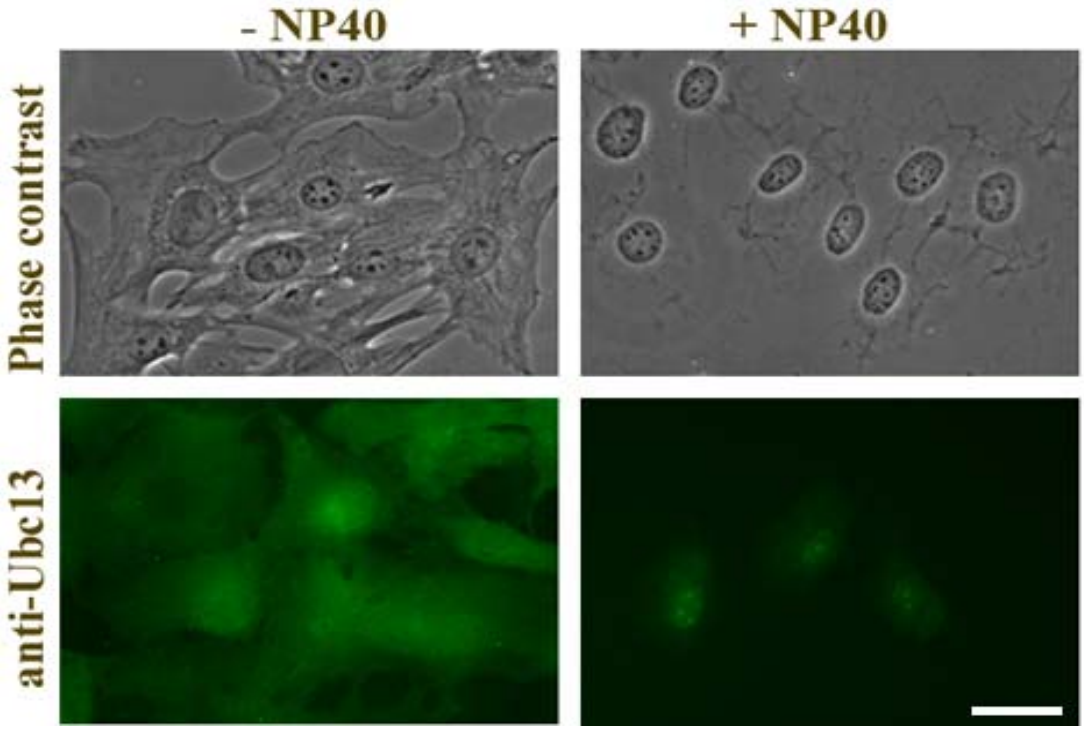

Figure 2-1. Demonstration of detergent pre-extraction to reveal nuclear structure

To eliminate highly soluble components of the cell, cultures are treated with the detergent NP40 before fixation, which allows a significant portion of the contents of the cell to be released as demonstrated in the phase contrast images of mouse 3T3 cells. ICC on the NP40 extracted cells demonstrate a portion of the reactivity is retained following detergent pre-extraction, in this case CPT-induced nuclear foci, which is assumed to be in a more stable complex than the soluble portion. Note that the field of view is different in each image. Bar = 10 μm.

NP40 and Triton X-100, at various concentrations and durations. Here I made use of NP40 at either 0.1% or 0.4% to give a low- or high-stringent pre-extraction, respectively.

Initially the pre-extraction was performed at room temperature and resulted in nuclear foci of Rad51 and Mre11 in the published literature; however utilizing this method many cells were often lost from the cover slip which made subsequent analysis difficult. For this reason the procedure was altered such that the pre-extraction was carried out on ice for a longer duration. Pre-extraction on ice for 40 minutes closely reproduced what is observed from a pre-extraction at room temperature for 3.5 minutes, except that virtually all of the nuclei remain in place for further analysis. An example of optimization procedure is given in Figure 2-2 which demonstrates cultures were optimized with respect to UV exposure (Figure 2-2A), time of analysis following treatment (Figure 2-2B) and pre-extraction time in 0.4% NP40 (Figure 2-2C). In each panel presented, the optimized conditions of the other variables are presented. This procedure was designed to maximally reveal nuclear foci.

Analysis also required the enumeration of surviving cells following various treatments. Cell counts were routinely performed with fixed cells stained with 4',6-diamidino-2-phenylindole (DAPI) to identify the nucleus and assist in counting the cell population. Cells were considered healthy and viable if the nuclei were not fragmented and contained distinct nucleoli.

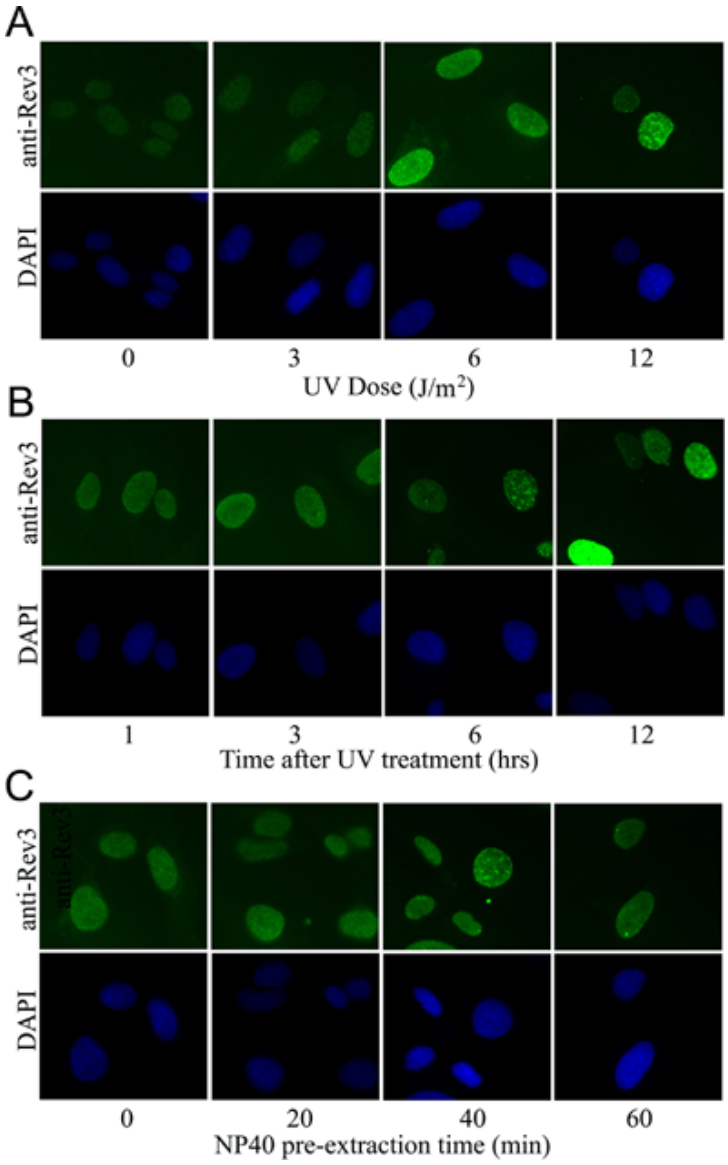

Figure 2.2. Optimization of treatments and detergent extraction

Cultures were optimized by varying the conditions individually while keeping all other conditions constant. In this example The UV exposure **(A)**, Time after UV exposure **(B)** and Time of detergent pre-extraction prior to fixation **(C)** was utilized while keeping the other variables constant at the optimized conditions.

2.3. Mammalian cell cultures

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 4.5 g/L glucose, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% horse serum (Gibco) with sodium bicarbonate reduced to 2.1 g/L in a humidified 5% CO_2 incubator. Mammalian cell lines used in this study are summarized in Table 2-1. HCT116 cells were a gift from Dr. S. Carlsen (University of Saskatchewan). HepG2 cells we a gift from Dr. L. Qualtiere (University of Saskatchewan). Mouse 3T3 cells were a gift from Dr. L Loh (University of Saskatchewan). HEK-293F cells were purchased from InVitrogen (USA). Apparently normal GM08402 cells and the XPV cell line GM03617 were purchased from the Coriell Institute for Medical Research (USA). NF1604 and its Rz20 derivative line expressing ribozyme directed to down-regulate *REV1* mRNA were gifts from Dr. W.G. McGregor (University of Louisville, USA) and characterized elsewhere (Clark et al., 2003). The ribozyme was maintained by culturing in 100 μg/mL geneticin. Microglia were isolated from newborn CD1 mice by aseptically pressing neoplia through 70 μm nitex mesh (BD Biosciences) and allowing the cultures to become confluent before feeding was stopped. Approximately 10 days later the majority of surviving cells remaining are microglia with macrophage-like morphology (Neuhaus and Fedoroff, 1994).

2.4. Immunocytochemistry

For routine immunocytochemistry, cells grown on 11x22 mm cover slips (cleaned with nitric acid) were fixed by adding formaldehyde directly to the culture

Table 2-1. Cells used

Although many cell types were investigated during the course of this thesis work, only those reported in the results are listed here.

Cell name	Species	Description	Source
3T3	Mouse	Fibroblast	Dr. L. Loh, University of Saskatchewan
Microglia	Mouse	Brain macrophage derived from newborn CD1 mice	Dissected in this lab
HepG2	Human	Hepatocellular carcinoma	Dr. L. Qualtiere, University of Saskatchewan
HCT116	Human	Colorectal carcinoma	Dr. S Carlsen, University of Saskatchewan
HEK-293F	Human	Embryonic kidney transformed with adenovirus 5 DNA and harboring the FRT integration site	InVitrogen, USA
NF1604	Human	SV40 transformed fibroblast	Dr. W.G. McGregor, University of Louisville, USA
Rz20	Human	NF1604 derivative expressing ribozyme directed to suppress Rev1	Dr. W.G. McGregor, University of Louisville, USA
GM08402	Human	Normal low passage number fibroblast	Coriell Institute for Medical Research, USA
GM03617	Human	XPV patient derived fibroblast	Coriell Institute for Medical Research, USA

media to a final concentration of 4% for 30 minutes, and then rinsed thoroughly. All rinsing steps were done with 4 changes of PBST [Dulbecco's phosphate buffered saline (PBS) with 0.25g/L Tween-20] over a period of 30 minutes. Cells were then permeabilized with 0.5% Triton X-100 for 5 minutes, blocked in 5% horse serum in PBST for 30 minutes and the primary antibody was applied in blocking solution except where specified. All antibodies are listed in Table 2-2. After washing the secondary antibodies were applied for 20 minutes with 2 μg/mL DAPI in blocking solution. After rinsing cells were mounted and sealed with clear nail polish and observed using an inverted fluorescence microscope (model IX70, Olympus) fitted with the appropriate filters and a LC PlanFL 40x objective (air) or a UPlanFLN 60x/1.25 oil immersion objective. Digital images were taken using the RT Slider "Spot" camera and Image Pro-Plus version 4.1 software (Diagnostic Instruments) and compiled using Adobe Photoshop software. To visualize the incorporation of bromodeoxyuridine (BrdU), cells were treated with 50 μg/mL DNAse-free RNAse-A immediately after the permeabilization step and the DNA then denatured by treating with 2N HCl for 15 minutes at 65°C before the blocking step. To differentiate the mouse monoclonal antibodies 4E11 and 2H11 from mouse anti-BrdU, the anti-BrdU antibody was not applied until after Alexa 488 conjugated secondary antibody was added, washed double the normal amount, and blocked with 2% normal mouse serum for 30 minutes to obstruct un-occupied anti-mouse Fab regions of bound Alexa 488 anti-mouse antibody. To visualize damage induced nuclear foci cultures were permeabilized using NP40 in PBS before fixation. NP40 was applied as either 0.1% or 0.4% at either room temperature or on ice for the duration as specified in the results section.

Table 2-2. Antibodies used

Antibodies were used for Western blotting (WB) or ICC at the indicated dilutions and applied for 60 minutes unless otherwise stated.

	Use (dilution)	**Source** (catalogue number)
Primary antibodies:		
mouse anti-Uev1A/Mms2 (2H11)	WB (1:2000) ICC (1:200)	produced in this lab
mouse anti-Ubc13 (4E11)	WB (1:5000) ICC (1:400)	produced in this lab
mouse (polyclonal) anti-Rev3	WB (1:1000) ICC (1:500)	produced in this lab
mouse anti-Actin (AC-74)	WB (1:5000) ICC (1:200)	Sigma (A5316)
anti-BrdU Alexa 594-conjugated	ICC (1:400)	Molecular Probes (A-21308)
rabbit anti-γH2AX	ICC (1:400)	Bethyl Labs (BL178)
rabbit anti-β-Tubulin	ICC (1:100)	Santa Cruz Biotech (sc-9104)
rabbit anti-hMre11	ICC (1:200)	Oncogene (PC388)
rabbit anti-Myc tag	ICC (1:400)	Upstate (06-549)
rabbit anti-C-Myc	ICC (1:400)	Santa Cruz Biotech (sc-789)
mouse anti-c-Myc (9E10)	WB (1:200) ICC (1:2000)	Calbiochem (OP10L)
rabbit anti-p65	ICC (1:400)	Santa Cruz Biothech (sc-372)
mouse anti-PCNA (PC10)	ICC (1:100)	Abcam (ab9288)
mouse anti-PCNA (PC10)	ICC (1:200)	Calbiochem (NA03)
rabbit anti-PCNA	WB (1: 4000) ICC (1:200)	Santa Cruz Biotech (sc-7907)
rabbit anti-Pol-ι	ICC (1:50, no signal)	Orb Gen (Rb1895)
mouse anti-Pol-η	ICC (1:00, overnight)	Abcam (ab17725)
rabbit anti-Rad51	ICC (1:100)	Santa Cruz Biotech. (sc-8349)
goat anti-Rev1	ICC (1:50, overnight)	Santa Cruz Biotech (sc-13827)
rabbit anti-Ubiquitin	WB (1:4000) ICC (1:400)	Sigma (U-5379)
Secondary antibodies:		
Alexa 546-conjugated goat anti-mouse	ICC (1:2000, 20-30 minutes)	Molecular probes (A11030)
Alexa 546-conjugated donkey anti-mouse	ICC (1:2000, 20-30 minutes)	Molecular Probes (A10036)
Alexa 488-conjugated goat anti-mouse	ICC (1:3000,20-30 minutes)	Molecular Probes (A11001)
Alexa 546-conjugated goat anti rabbit	ICC (1:2000, 20-30 minutes)	Molecular Probes (A11035)
Alexa 488-conjugated donkey anti-rabbit	ICC (1:3000, 20-30 minutes)	Molecular Probes (A21206)
Alexa 488-conjugated donkey anti-goat	ICC (1:3000, 20-30 minutes)	Molecular Probes (A11055)
HRP-conjugated goat anti-mouse	WB (1:10,000, 40 minutes)	Upstate (12-349)
HRP-conjugated goat anti-rabbit	WB (1:10,000, 40 minutes)	Upstate (12-348)

2.5. Interference RNA

Short hairpin (siRNA) constructs were created by cloning double stranded oligonucleotides obtained from Intergrated DNA Technologies (USA) into the *Xba*I and *Bbs*I restriction sites of the plasmid vector mU6pro (a gift from Dr. D. Turner, University of Michigan, USA) which produce short RNA hairpin loops efficiently initiating the RNA interference pathway (Yu et al., 2002).

The target sequences for short hairpin RNA (siRNA) sequences and synthetic RNAi are listed in Table 2-3. The target sequences to suppress Rev3 are indicated in the nucleotide gene sequence of Rev3 in Figure 2-3. The intentional base mismatch in the mutant siRNA (*siUbc13m*) is in bold. Synthetic interference RNA molecules were purchased from Santa Cruz Biotech, including those targeting hRev3 (sc-37790), hUbc13 (sc-43551), and a scrambled interference RNA (sc-37007). The efficacy of target gene disruption was monitored by either Western blot analysis or ICC.

2.6. Constructs to express Myc-tagged proteins

Mms2, Uev1A, Uev1B, Uev1B-N and Uev1Δ30 open reading frames without stop codons were PCR amplified as *Bam*HI-*Xho*I or *Bam*HI-*Sal*I fragments and cloned into pcDNA3.1/Myc-His(+)A (InVitrogen) such that they were under the control of a CMV constitutive promoter and fused in-frame with the Myc-6xHis tag coding sequences to produce the C-terminal fusion proteins. All insert sequences were confirmed by DNA sequencing.

Table 2-3. iRNA target sequences

The interference RNA target sequences used are shown. Note that the inactive (*siUBC13m*) construct has a single nucleotide difference (bold and underlined) from the active construct (*siUBC13*). Each RNAi source was comprised of a mixture to target three sequences.

Target gene	Target sequence
siUBC13 (human/mouse)	AAT CCA GAT GAT CCA TTA GCA
siUBC13m (human/mouse)	AAT CCA GAT GAT CCA **A**TA GCA
siMms2 (mouse)	GC CTT GAA GAT GAT GAA GAC
siUev1A (human/mouse)	CA CTT ACA AGA TGG ACA GGC
siRev3 (human/mouse)	GAG TAC CAC TTA TCC AGC TT
Ubc13i (human)	CAT CTG GAT TGT TGT GAA A GCT TGT GTG TCA TCA GAA A GTA GCC AGT CAT AAA UAC A
Rev3i (human)	CTA TGG TGC ATT CTC TTA A GTC ATC AAT CGG AAA GTT A GGA TGT AAG TCC ATG TAT A

Figure 2-3. Human *REV3* protein coding sequence (continued on next two pages)

```
0001  atgttttcag taaggatagt gactgcagac tactacatgg ccagcccgct gcaggggctg
0061  gatacctgcc aatcccccct cacccaggcc cctgtcaaga aggtgccggt ggtgcgagtc
0121  ttcggagcga ccccggcagg tcagaagaca tgtcttcatc tacatggcat ctttccttac
0181  ctctatgtgc catacgatgg ttatggacag cagccagaaa gctatctttc tcagatggca
0241  ttcagtatcg acagagcact taatgtggct ttaggcaatc catcttccac tgctcagcat
0301  gtgttcaaag tgtcattagt atcaggaatg ccttttatg gttatcatga gaaggaaaga
0361  cactttatga agatctatct ttacaatcct acaatggtga aaaggatatg tgaacttttg
0421  caaagcggag ccataatgaa taaattttac cagcctcatg aagcgcatat tccctacctc
0481  ctacagctct tcattgacta caatctttat ggcatgaatt taataaatct ggctgctgtc
0541  aagttccgaa aagcaagaag gaaaagtaat acattgcatg caactggatc ctgcaagaat
0601  catttatcag gaaattctct tgctgatact ttatttcggt gggaacaaga tgaaatacca
0661  agctctttaa tattggaagg tgttgaacca cagagtacat gtgaattaga agtggatgct
0721  gtagctgctg atatcttaaa tcgtctggac attgaagctc aaattggtgg aaaccctggt
0781  ctacaggcca tatgggaaga tgaaaagcaa cggcgaagaa acagaaatga aacttctcaa
0841  atgagccaac ctgagtcaca agatcacagg tttgtgccag caacagaaag tgaaaaaaaa
0901  tttcagaaga gacttcagga aattctcaaa cagaatgatt tctctgtaac attatcagga
0961  tctgtggact acagcgatgg atcccaggag ttctctgctg agttaacatt gcactctgag
1021  gttctgtctc ctgaaatgct tcagtgtaca ccagccaata tggtagaagt tcacaaagac
1081  aaagagtcaa gcaaaggtca cactagacac aaagtggaag aagctcttat taatgaagaa
1141  gcaattttga accttatgga aaatagtcag acttttcagc ctttgaccca aagactgagt
1201  gagtcacctg ttttcatgga cagtagtcct gatgaggctc tggtacatct tcttgctggt
1261  ttggaaagtg atggatatcg gggggaaaga aataggatgc catcaccatg tcgctccttt
1321  ggaaataata aatatccaca aaatagtgat gatgaagaaa atgaaccaca gattgaaaaa
1381  gaggaaatgg agcttagttt ggtgatgtcc cagagatggg acagcaatat tgaagaacat
1441  tgtgccaaaa agagatcact gtgcagaaat acccacagaa gttcaactga agatgatgac
1501  tcatcttcag gagaagaaat ggaatggagt gataacagtt tgcttctagc cagtctttct
1561  atacctcagt tagatggaac tgcagatgaa aatagtgaca atccattgaa caatgaaaat
1621  tctagaaccc actcttctgt aattgcaaca agcaagcttt cagttaaacc ctccatcttt
1681  cacaaagatg ctgctacatt agaaccctca tcttctgcta agattacctt tcagtgtaaa
1741  cacacaagtg ccctttcttc ccatgttttg aacaaggaag atttaattga agacctttca
1801  cagacaaaca aaaatacaga aaaaggtcta gataactcag tcacttcttt tacaaacgaa
1861  agcacttatt ctatgaaata ccctggatct ttaagcagta ctgttcattc agaaaattct
1921  cataaagaga atagtaagaa agagatcctc ccagtatctt cctgtgaaag tagtattttt
1981  gattatgaag aagatattcc atctgttaca agacaagtac caagtagaaa atatacaaac
2041  attagaaaaa tcgaaaagga ttccccttt atacatatgc accgtcaccc taacgagaat
2101  acattgggca aaaattcttt caacttttct gacttaaatc attcaaaaaa taaagtatcc
2161  tctgaaggaa atgaaaaagg aaacagcaca gctctgagta gtttattccc ttcatcattt
2221  actgaaaatt gtgaattact gtcatgctca ggggagaata gaaCTATGGT GCATTCTCTT
2281  AAtagcactg ctgatgaaag tggactaaat aaacttaaaa ttaggtatga agaatttcaa
2341  gaacataaaa cagaaaagcc aagcctcagc cagcaagcag cacactatat gttttttccc
2401  agtgttgttc tttctaactg tcttactaga ccacagaaac tatctcctgt cacatataaa
2461  ttacaacctg gcaataaacc atcccggtta aaattgaata aaaggaaact tgcaggtcat
2521  caggagactt ctaccaaaag tagtgagact ggatccacaa aagataattt tatacaaaat
2581  aatccttgta atagtaatcc tgagaaggat aatgcattgg ctagtgattt aactaaaacc
2641  actcgtggag cttttgaaaa taaaacaccc acagatggtt ttatagactg tcactttgga
2701  gatgggacgt tagaaactga gcagtccttt ggactatatg gaaataaata cacacttaga
2761  gccaaacgca aggtaaatta tgagactgaa gacagtgagt caagttttgt aactcacaac
2821  tcaaaaatta gtctacctca tcccatggaa attggtgaaa gtttagatgg aactctcaaa
2881  tcccgaaaac gaagaaaaat gtctaaaaag ctgccccctg tcatcataaa gtatattatt
2941  attaatagat ttagagggag aaaaaatatg cttgtgaagc taggaaaaat agactctaaa
3001  gaaaaacaag taatattaac agaagaaaaa atggaactat ataaaaagct tgcacctttg
3061  aaggactttt ggccaaaagt tcccgactcc cctgcaacca aatatcccat ttatccacta
3121  acaccaaaga aaagtcacag aagaaagtca aaacataaat ctgctaagaa aaaaactggt
3181  aaacaacaaa ggacaaataa tgaaaatatt aaaagaactt tgtctttcag gaaaaaacgg
3241  tcacatgcta ttctttctcc tccctcacca tcttacaatg ctgaaaccga agattgtgac
```

3301 ttgaattata gtgatgttat gtctaaacta ggttttcttt ctgagagaag cacaagtccc
3361 ataaattctt ctccacctcg ctgctggtct cccacagatc caagagctga agaaatcatg
3421 gctgctgcag aaaaagaggc aatgcttttt aagggtccta atgtatataa gaagactgtt
2481 aattctcgta taggaaaaac tagtcgcgca agagcacaga ttaagaaatc aaaagcaaag
3541 cttgctaatc cctctatagt tactaagaaa aggaacaaac gaaatcagac aaataaacta
3601 gtagatgatg gaaaaaagaa accaagagca aaacaaaaaa caaatgagaa aggtacatcg
3661 agaaagcata taacacttaa ggatgaaaaa ataaaatctc agtctggtgc tgaggttaag
3721 tttgtactga aacaccagaa tgtgtctgaa tttgcaagta gttctggagg ctctcaacta
3781 ctttttaaac agaaagatat gccactaatg ggctctgctg tagatcatcc cctttctgct
3841 tccctaccca ctggaattaa tgcacaacag aagttatctg gctgcttttc ttctttctta
3901 gaaagcaaga agtctgtaga tttgcagaca ttccccagtt cacgagatga tttgcatcca
3961 tcagttgttt gtaattctat aggacctgga gtctcaaaaa ttaatgttca aaggcctcat
4021 aatcaaagtg ctatgtttac tctaaaggaa tcaacgttaa ttcaaaaaaa tatatttgac
4081 ctttccaatc atttatctca ggtagcacag aatacacaga tatcttctgg tatgtcctca
4141 aagatagaag ataatgcaaa taatatacaa agaaactatt tGTCATCAAT CGGAAAGTTA
4201 agtgaatatc gcaattccct agaatcaaag ctggaccaag catatacccc taatttttg
4261 cattgcaaag acagtcagca gcagattgta tgcatagcgg aacagtcaaa gcacagtgaa
4321 acttgttctc cgggaaatac agcttcagag gaaagccaaa tgcctaataa ttgctttgta
4381 acttccttga gaagtccaat caaacaaata gcatgggagc aaaagcaaag gggctttatt
4441 ttagatatgt caaattttaa acctgaaaga gtaaaaccga ggtcattatc agaagcaatt
4501 tcacaaacca aagcactttc tcagtgtaaa aatcgaaatg tgtcaacacc ttcagcattt
4561 ggtgaaggac agtctggact ggcagttcta aaagaattgt tacaaaaaag acagcagaaa
4621 gcacaaaatg caaatactac acaagaccca ttatccaata aacatcaacc aaataaaaat
4681 atttctggtt cccttgagca taacaaagca aataaacgga cacgatcggt aacgtcccca
4741 agaaaacctc gaactcccag aagtacaaaa caaaaagaaa aaatccccaa acttctcaaa
4801 gtagactctt taaatttaca aaactctagc cagttggata actctgtatc agatgatagt
4861 cccatctttt tttcagatcc aggctttgaa agttgttact cacttgaaga tagtttatct
4921 cctgaacata attataattt tgatattaac acaataggtc agactggatt ttgtagcttt
4981 tattctggaa gtcagtttgt cccagctgat cagaatttgc ctcagaagtt cctaagtgat
5041 gctgttcagg atctttttcc aggacaagct atagaaaaaa atgagttttt aagtcatgac
5101 aaccagaaat gtgatgaaga caagcatcat accacagact cagcctcatg gattagatct
5161 ggtactttaa gtcctgaaat ttttgagaag tcaaccatag atagcaatga gaatcgtcgc
5221 cacaaccagt ggaaaaatag ctttcatcct ctaacaactc ggtctaactc aataatggat
5281 tctttctgtg ttcagcaggc agaagactgt ctaagtgaaa aatctagatt gaataggagt
5341 tcagtaagca aagaagtgtt tcttagcctc ccacagccaa acaattcaga ctggattcaa
5401 ggtcacacca gaaaagaaat gggacagtct cttgactcag ccaatacctc ttttactgca
5461 atactctcct cccctgatgg tgaacttgta gacgtggcct gtgaagattt agaactgtat
5521 gtttcaagaa acaatgatat gttgacacca actcctgata gttcaccaag atctactagc
5581 tctccttcac aatctaaaaa tggcagcttc acccctcgaa ctgctaacat tctgaaacca
5641 cttatgtccc ccccaagtag ggaagaaatt atggcaactt tgttggatca tgacctgtct
5701 gagactattt accaggaacc attttgcagt aatccttctg atgtaccaga aaagcccagg
5761 gagattggtg gacggctcct catggtagaa actcgacttg caaatgatct ggctgagttt
5821 gagggagact tttccttgga aggacttcgt ctttggaaaa cagcattctc agcaatgact
5881 cagaatccaa ggccagggtc acccccttcgc agtggccaag gagttgtcaa taaagggtca
5941 agtaatagcc ctaagatggt tgaagataaa aaaattgtga ttatgccttg caaatgtgcc
6001 ccaagtcgac aactggttca agtgtggctt caagccaaag aagaatacga acgttccaag
6061 aaactgccta aaaccaagcc aactggagtt gtaaaatctg ctgagaactt tagctcttca
6121 gttaacccag atgacaaacc tgtagtgcct ccaaaaatGG ATGTAAGTCC ATGTATActc
6181 cccactacag cacataccaa ggaggatgtt gataattctc agattgcttt acaagcacca
6241 accacgggat gtagtcaaac tgcaagtgaa agtcagatgc tgccaccagt tgcctctgca
6301 agtgatcccg aaaaagatga agatgatgat gataactatt acattagtta tagctcccct
6361 gattctccag taattccccc ttggcaacaa ccaatatccc cagattccaa agcattaaat
6421 ggagatgata gaccctcatc accagtagag gagctgcctt cattggcttt tgagaacttc
6481 ttaaagccaa taaaagatgg tatacaaaaa agcccctgca gtgagcctca agagcctcta
6541 gtgatatctc caattaatac tagggcaaga actgggaaat gtgaatcact ttgctttcat
6601 agtacaccaa tcatacagag aaaacttctg gaaaggcttc ctgaagcacc tggccttagc
6661 ccattatcaa cagaaccaaa aacacagaag ttgagtaata agaaaggaag taatactgac

6721 actcttagaa gagtactgtt aacacaagca aagaatcaat ttgcagcagt aaatacccca
6781 cagaaagaaa cttctcagat tgatggacca tctttaaaca atacttacgg tttcaaagtc
6841 agcatacaaa acttacagga ggcaaaagct ttacatgaga tacaaaatct taccctaatc
6901 agtgtggagt tgcatgctcg aactagacga gacttagaac cggatcctga atttgaccca
6961 atctgtgctc tgttctactg catctcatct gacactccac tgccagatac agaaaaaaca
7021 gaactcacag gtgtaatagt gattgataaa gacaagacag ttttcagtca agatatcaga
7081 tatcagactc cattacttat tagatctgga attacaggac tcgaagtcac ctatgctgct
7141 gatgagaagg cacttttca tgaaattgca aatataataa agaggtatga tcctgatatt
7201 ctgctaggat atgagattca gatgcattcc tggggttacc tcttacaaag ggctgccgct
7261 ttaagtattg acttatgtcg gatgatctct cgggtgccag atgacaaaat tgagaacaga
7321 tttgcagctg aaagagatga gtatggatca tatacaatga gtgagataaa tattgttggc
7381 cgaattacac taaatctttg gagaatcatg agaaatgagg tggctctaac taactacacc
7441 tttgaaaatg tgagctttca tgttcttcat cagcgttttc ccctctttac ctttcgagtc
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7561 gttagccgtg tccgtggaaa tctccaaatg ttagaacagc tggacctgat tgggaaaacc
7621 agtgagatgg ctagactttt tggcattcag tttttacatg tactgacaag gggttcacag
7681 taccgtgtgg aatcaatgat gttgcgtatt gctaaaccaa tgaactatat tcctgtgaca
7741 cctagtgttc agcaaagatc ccagatgaga gccccacagt gtgttcctct aattatggag
7801 cctgaatccc gcttctatag caactctgtt ctcgttttgg atttccaatc actttatcct
7861 tctattgtga ttgcatataa ctactgcttt tccacctgcc ttggccatgt ggagaacttg
7921 ggaaagtatg atgagttcaa atttggctgt acctctctga gagtacctcc agatttactt
7981 taccaagtta ggcatgatat cacagtgtcc cccaatggag tagcttttgt caagccttca
8041 gtaagaaaag gtgtactacc aagaatgctt gaagaaattt tgaagactag atttatggtg
8101 aagcagtcaa tgaaggctta caagcaagac agagccctgt cacgaatgct tgatgcgcgt
8161 cagttgggac ttaagctgat agcaaatgtc acatttggct atacatctgc taatttttct
8221 gggagaatgc catgcattga ggttggcgat agtattgttc acaaagccag agagaccttg
8281 gaacgagcta ttaaactggt gaatgatacc aagaaatggg gggctagggt tgtatatggc
8341 gatactgaca gtatgtttgt gctactgaaa ggagccacta aggagcagtc ttttaagatt
8401 ggtcaggaaa ttgccgaagc tgtaactgct accaatccta aaccagtgaa attgaagttt
8461 gaaaaggtat atttgccctg tgttttacaa acaaaaaaga ggtatgtggg ttacatgtat
8521 gaaacactgg atcagaagga cccagtattt gatgcaaaag gaatagaaac agtcagaaga
8581 gattcctgcc ctgctgtttc taagatactt gagcgttctc taaagctgct atttgaaacg
8641 agagatataa gtctaattaa acagtatgtt cagcgacaat gtatgaagct tctggaagga
8701 aaggccagca tacaagactt tatctttgcc aaggaataca gaggaagttt ttcttataaa
7761 ccaggagctt gtgtgccagc ccttgaactt acaaggaaaa tgctgactta tgaccggcgc
8821 tctgagcctc aggttgggga gcgagtgcca tacgtcatca tttatgggac ccccg**GAGTA**
8881 **CCACTTATCC** **AGCTT**gtaag gcgcccagtg gaagtcctgc aggacccaac tctgagactg
8941 aatgctactt actatattac caagcaaatc cttccaccct tggcaagaat cttctcactt
9001 attggtattg atgtcttcag ctggtatcat gaattaccaa ggatccataa agctaccagc
9061 tcctcgcgaa gtgaacctga agggcggaaa ggcactattt cacaatattt tactacctta
9101 cactgtcctg tgtgtgatga cctaactcag catggcatct gtagtaaatg tcggagccaa
9161 cctcagcatg ttgcagtcat cctcaaccaa gaaatccggg agttggaacg tcaacaggag
9221 caacttgtaa agatatgcaa gaactgtaca ggttgctttg atcgacacat cccatgtgtt
9281 tctctgaact gcccagtact tttcaaactc tcccgagtaa atagagaatt gtccaaggca
9321 ccatatctcc ggcagttatt agaccagttt taa

Figure 2-3. Human *REV3* protein coding sequence (contimued from previous two pages)

The *hREV3* coding sequence is from Gibbs *et al*. (1998) (GenBank accession No. AF058701.1). The short hairpin interference RNA target sequence is capitalized and highlighted in blue. The three synthetic RNAi target sequences are capitalized and highlighted in red. The region cloned for recombinant protein production to which antibody was produced is underlined.

2.7. Western blot analysis

Cell cultures were lysed in 1% sodium dodecyl sulfate in the presence of a protein inhibitor cocktail for mammalian cells (Sigma). Total protein concentration was determined using the Bradford method (BioRad). Cell extracts underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis, were transferred to Polyvinylidene fluoride membrane, blocked in 5% skim milk in PBST for one hour and then incubated with primary antibody overnight in the blocking solution. After washing (4 times over 30 minutes with PBST), horse radish peroxidase conjugated secondary antibody (anti-mouse or anti-rabbit) was applied in blocking solution for 40 minutes. After washing with PBST, the membrane was submerged in Western Lightning Chemiluminescent Plus reagent (PerkinElmer Life Sciences) for 1 minute and exposed to Kodak BioMax MS scientific imaging film for various times before developing with an automatic processor. In cross-linking experiments, dithiobis succinimidyl propionate (Pierce Biotechnology) was applied at a final concentration of 1 mM on ice for 60 minutes before cells were harvested. Glycine was added to a final concentration of 20 mM to stop the reaction and samples were separated by electrophoreses in the presence or absence of 10 mM dithiothreotol.

2.8. Antibody production

Recombinant human Ubc13 and Mms2 proteins were obtained by expressing the ORF in *E. coli* cells as described (McKenna et al., 2001). The GST-Ubc13 and GST-Mms2 fusion proteins were subject to protease cleavage; Ubc13 and Mms2 were

further purified (Andersen et al., 2005) and emulsified in Freund's incomplete adjuvant. The C-terminal 0.9 kb coding region of *hREV3* (Xiao et al., 1998a) was cloned into the *Eco*R1-*Xho*I sites of pET30a (Novagen) to form pET-hREV3C, which was transformed into *E. coli* strain BL21(DE3)-RIPL (Strategene) to produce a His_6-hRev3C fusion protein. After Isopropyl β-D-1-thiogalactopyranoside induction, the fusion protein was found to be for the most part insoluble. Crude cell extract was centrifuged repeatedly to remove soluble proteins and the resulting pellet was resuspended, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose band corresponding to His-immunoreactivity was collected, crushed in liquid nitrogen and used as an immunogen in CD1 mice. Serum was collected as a source of mouse polyclonal antiserum 30-40 days after initial immunization. For monoclonal antibody production, spleen cells from the immunized mice were fused using polyethylene glycol to Fo cells and the resulting hybrids screened for a secreted monoclonal antibody with reactivity to the protein of interest using standard enzyme-linked immunosorbant assay techniques in 96 well plates. Approximately one million of the derived hybridoma cells were injected into the peritoneal cavity of mice pre-injected with Freund's incomplete adjuvant for ascites fluid production as a monoclonal antibody source. The Rev3 antibody preparation procedure was assisted by visiting Professor F. Xu (Ningxia Medical College, China).

2.9. Growth analysis

Initiated from transfected or control cultures, cells were trypsinized and seeded at 60,000 cells per 35 mm dish pre-coated with poly-lysine. After 24 hours of

incubation to allow for attachment, all sister cultures were treated identically and fixed after treatments as indicated. Culture plates were withdrawn every 24 hours and fixed by the direct addition of 37% formaldehyde to the media to a final concentration of 3.7% and plates were rinsed with PBST 30 minutes later. To aid in counting the cells, plates were exposed to 2 μg/mL DAPI and fluorescent images taken with a low power objective. Plates were observed under phase contrast and cells with non-fragmented nuclei containing distinct nucleoli were considered healthy. All statistical data were complied and analyzed using Microsoft Excel and Graphpad QuickCalcs Software (GraphPad Software, Inc.).

Alternatively, to determine survival of slow growing cells (XPV and GM08402) a survival assay was organized. Three days following RNAi transfection cells were passaged and the following day were exposed to UV light. Two days later the number of apparently viable cells was determined by phase contrast microscopy and DAPI staining. Cells with intact nuclei and nucleoli were deemed viable.

2.9. Cell transfection

Log phase cells at approximately 90% confluence were transfected using either Lipofectamine 2000 (InVitrogen) or Genejuice Transfection Reagent (Novagen). DNA prepared from a Quantum Preparation kit (BioRad) was further cleaned by phenol-chloroform extraction and ethanol precipitation. For each 35 mm dish approximately 5 μg of plasmid DNA was suspended in 125 μL serum-free media. Lipofectamine was diluted in a second 125 μL serum-free media and after 5 minutes the two were combined drop-wise. Fifteen minutes later the solution was applied to the culture in

complete growth media and the cultures were left undisturbed overnight. Alternatively using Genejuice, 3 μL Genejuice solution was vortexed with 100 μL serum-free media and let sit for 5 minutes at room temperature before the addition of 1 μg of DNA followed by gentle mixing. Fifteen minutes later the solution was added directly to the culture dish and left undisturbed overnight.

RNAi transfection was performed using the transfection reagent supplied by the manufacturer (Santa Cruz). In each case 6 μL of this reagent was diluted in 100 μL serum-free media in one tube and 6 μL RNAi duplex diluted in a second tube of 100 μL serum-free media. After 5 minutes the duplex DNA was added directly to the diluted transfection reagent with gentle mixing and 30 minutes later the solution was added to cells in serum-free media. Media containing serum was re-introduced the next morning.

To produce isogenic cell lines the Flp-In system was utilized (InVitrogen). HEK-293F cells contain a single integrated FRT site that can be recognized by the yeast-derived Flp recombinase. The short hairpin constructs were cloned into the pcDNA5/FRT expression plasmid and then co-transfected using Genejuice transfection reagent with the plasmid pOG44 which expresses the Flp recombinase and which mediates the recombination between the vector FRT and the pre-integrated genomic FRT site in HEK-293F cells. After transfection, stable clones were selected by their resistance to 400 μg/ml hygromycin B and simultaneous loss of Zeocin resistance.

CHAPTER THREE

RESULTS

As discussed earlier the working model of DNA damage tolerance (DDT) is largely based on the budding yeast system. Although various DNA repair mechanisms exist cells still require DDT to allow for DNA synthesis to proceed when damage cannot be repaired readily. The high fidelity DNA replication machinery cannot duplicate damaged DNA efficiently and therefore a damage tolerance mechanism may play a critical role for cell survival during and shortly after DNA synthesis.

Budding yeast utilize error-free and error-prone pathways to temporarily circumvent the damage to allow the cell cycle to proceed with the likely objective of repair at a later time. The error-free mechanism utilizes an intact daughter strand of newly synthesized DNA as the template to produce a DNA strand complimentary to the damaged strand. Although the mechanism is poorly characterized, it is dependent upon the activities of the Rad5-Ubc13-Mms2 complex to poly-Ub PCNA with largely unknown downstream activities. The error-prone pathway utilizes the Rad6-Rad18 complex to mono-Ub PCNA, which promotes TLS past the site of damage. The mono-Ub addition to PCNA likely functions to enhance localization of TLS polymerases to sites of DNA damage. The TLS polymerases exhibit low fidelity and often incorporate mutations in the genome. In fact, without TLS polymerases like Rev1, Rev3 or Rev7, induced mutations become largely eliminated. Therefore, it is also speculated that the

DDT mechanism may play an important role in balancing individual conservation and species evolution.

Based on the facts that 1) DNA is essentially chemically identical from bacteria to humans; 2) most cells have to respond to same sources of DNA damage; 3) DNA synthesis and repair enzymes appear to be conserved from bacteria to humans; and 4) the PRR/DDT mechanisms are present (although rather different) in *E. coli* and budding yeast, **I hypothesize that human cells possess a DDT mechanism comparable to that of budding yeast.** The central objective of this thesis work is to test this hypothesis. The results of this thesis have been subdivided into three sections to investigate the putative error-free and error-prone pathways of DDT in mammalian cells. First I wanted to establish if Ubc13-Mms2/Uev1 of the putative error-free pathway is required for the maintenance of genomic stability during normal DNA synthesis and following DNA damage. Secondly I investigated human Rev3 of the putative error-prone DDT pathway in response to DNA damage. Finally, by attempting to repress these two pathways simultaneously I sought to critically examine whether the two pathways act cooperatively to protect the genome from DNA damage. Combined data supports the hypothesis that these two pathways do exist in mammalian cells; however some differences between yeast and human DDT were also observed as will be discussed. If these two pathways can be adequately understood, perhaps they may be regulated in the future such that the error-free pathway is preferentially exploited over the error-prone pathway, which possibly may lead to a reduced mutation load in mammalian cells.

3.1. Ubc13-Mms2 and Ubc13-Uev1A display two distinct biological functions in mammalian cells

3.1.1. Characterization of anti-Ubc13 and anti-Mms2 antibodies and interference RNAs

It has been well characterized that the budding yeast Ubc13-Mms2 complex modifies PCNA by addition of a K63 poly-Ub chain on the Lys164 residue to promote error-free DDT. To investigate the possible mammalian error-free DDT we first sought to characterize human Ubc13 and the three human homologs of Mms2, namely hMms2, Uev1A and Uev1B. To do this we raised monoclonal antibodies against recombinant hUbc13 and hMms2. Among several hybridomas characterized in each group, we chose to use clone 4E11 as the anti-Ubc13 monoclonal antibody and clone 2H11 as the anti-Mms2 monoclonal antibody sources. As revealed below, 2H11 also recognizes Uev1 efficiently and hence is regarded as an anti-Mms2/Uev1 monoclonal antibody. ICC using 4E11-derived ascites fluid on cultured mammalian cells (Figure 3-1A) revealed Ubc13 immunoreactivity throughout the cytoplasm and nucleus of most cells. Occasionally, nuclei exhibited reduced or enhanced Ubc13 immunoreactivity. ICC utilizing 2H11-derived ascities fluid showed a very similar immunoreactivity pattern throughout the cytoplasm and nuclei with occasional nuclei exhibiting enhanced or reduced immunoreactivity (Figure 3-1B). A Western immunoblot (Figure 3-1C) revealed that the 4E11 antibody recognized a single band in whole cell lysates derived from 3T3 cells that corresponds to the migration of purified Ubc13. This band could be reduced from whole cell lysates short hairpin interference RNA treatment to suppress Ubc13 (*iUbc13*) while an inactive variant (*iUbc13m*) could not. Furthermore, siRNAs

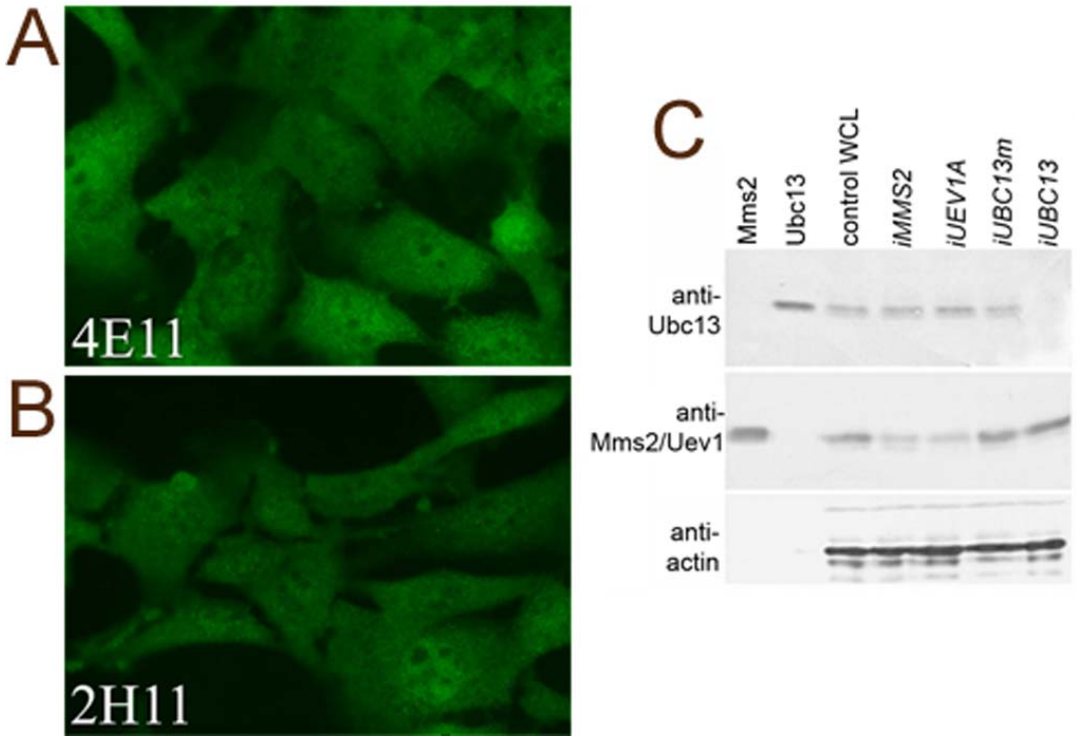

Figure 3-1. Characterization of anti-Ubc13 (4E11) and anti-Mms2/Uev1 (2H11) monoclonal antibodys

ICC on 3T3 cells (without detergent pre-extraction) indicates both Ubc13 **(A)** and Uev1A **(B)** are distributed throughout the nucleus and cytoplasm. Virtually indistinguishable results have been observed with several human cell lines. Western blotting **(C)** demonstrates Ubc13 and Mms2/Uev1A immunoreactivity in 3T3 cells with each corresponding to a single major band. iRNA application indicates both Uev1A and Mms2 are recognized as a single band migrating identically on a 10% acrylamide/bisacrylamide gel which can be partially reduced with iRNA directed against Mms2 or Uev1A. *siUbc13m* is an inactive iRNA control which does not repress Ubc13 or Mms2/Uev1A. The three Westerns blots are sister blots probed with the indicated antibody, anti-actin was used as an internal loading control. Bar = 10 μm.

directed toward Uev1A or Mms2 were incapable of reducing Ubc13 immunoreactivity (Figure 3-1C). These observations collectively confirm the specificity of both 4E11 and s*iUBC13*. Similarly, a parallel study with the 2H11 antibody also resulted in a single band in the whole cell lysate co-migrating with purified Mms2 (Figure 3-1C). However, utilizing the siRNA technology to suppress either Mms2 or Uev1A reduced the immunoreactivity of the same size band, which could not be affected by *iUbc13* or *iUbc13m*. Because Mms2 and Uev1A are very similar in size as well as amino acid sequence, we believe the two proteins co-migrate. The 2H11 antibody therefore recognizes both Mms2 and Uev1A.

3.1.2. Subcellular localization of Mms2, Uev1A and Uev1B

The pan-substrate nature of the 2H11 monoclonal antibody makes it impossible to distinguish between Mms2 and Uev1 by ICC. To differentiate between Mms2 and the two Uev1s, we cloned *hMMS2*, *UEV1A* and *UEV1B* in a mammalian expression vector so that each of the above gene products is fused at the C-terminus to Myc and 6xHis epitopes. Figure 3-2A illustrates the expected fusion gene products of these constructs after transfection into mammalian cells. The core regions of each are very similar; however, Uev1A has a 35 amino acid N-terminal extension and Uev1B has a 76 amino acid N-terminal extension (Figure 1-5). The two extended sequences are completely different and derived from alternative splicing (Rothofsky and Lin, 1997; Xiao et al., 1998b). Transient transfection of these constructs into mouse 3T3 cells resulted in sufficient expression to allow investigation into their localization. ICC directed against the Myc-tag revealed that Mms2-Myc and Uev1A-Myc occur

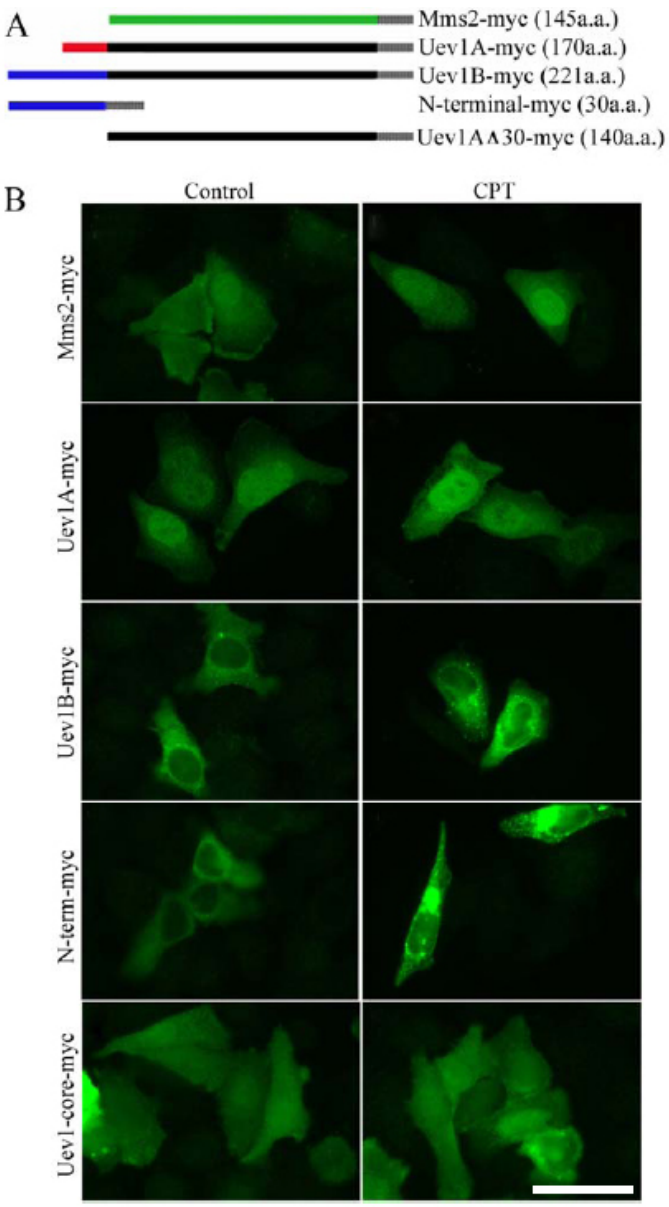

Figure 3-2. Expression of Mms2 and Uev1 Myc-tagged constructs

(A) A schematic diagram demonstrates the constructs used. **(B)** ICC on 3T3 cells without detergent pre-extraction directed against the Myc antigen reveals that Mms2-Myc and Uev1A-Myc are distributed throughout the cytoplasm and nucleus, while Uev1B-Myc appears to be largely excluded from the nucleus due to its N-terminal unique region. DNA damage treatment by the addition of 5μM CPT for 6 hours does not appear to significantly alter the subcellular cocalization of any fusion proteins. Bar = 10 μm.

throughout the cytoplasm and nucleus of transfected cells with occasional enhanced or reduced immunoreactivity in the nucleus (Figure 3-2B), much as the 2H11 ICC pattern of immunoreactivty (Figure 3-1). In contrast, Uev1B-Myc appeared to be predominately cytosolic in all transfected cells (Figure 3-2B).

Since the core domains between Uev1A and Uev1B are identical, we suspected that the unique N-terminus of Uev1B is responsible for its exclusion from the nucleus. To test this hypothesis, we created two fusion constructs in the same Myc/His-tagged vector, with one containing the 145 amino-acid Uev1 core domain and the second containing the 76 amino acid Uev1B N-terminus (Figure 3-2A). Parallel transient transfection experiments with 3T3 cells revealed that the 76 amino acid Uev1B sequence alone is sufficient to restrict the fusion protein from entering the nucleus, whereas the localization pattern of the Uev1 core domain fusion is indistinguishable from that of Uev1A or Mms2 (Figure 3-2B).

Since the purpose of this study is to examine the involvement of Ubc13-Uev in DDT, I also examined Mms2-myc and Uev1A-myc localization after DNA damage treatment. Under the experimental conditions, I was unable to observe obvious differences or re-localization of any fusion protein to the nucleus in response to UV or CPT exposure (Figure 3-2B). The above observations collectively suggest that Mms2 and Uev1A are potentially capable of participating in DDT, while Uev1B is unlikely to be a candidate.

Parallel studies on Ubc13, Mms2 and Uev1 in this laboratory have also revealed important aspects of these proteins including physical interaction studies between these proteins and functional assays in yeast cells (Andersen et al., 2005). In these

experiments bacterial extracts expressing either GST-Mms2 or GST-Uev1A, but not GST-Uev1B, were capable of co-purifying with Ubc13. Additionally, in a yeast two-hybrid assay Ubc13 was shown to interact with Mms2 and Uev1A, but not with Uev1B. In an *in vivo* assay, *hMMS2* and *UEV1A* were able to functionally complement a yeast *mms2* null mutant in response to killing by methyl methane sulfonate, whereas once again the expression of *UEV1B* could not. Interestingly, upon deletion of the N-terminal 80 amino acid coding region of *UEV1B*, it was then able to complement the *mms2* null mutation in yeast (Xiao et al., 1998b). Since Uev1B does not appear to interact with Ubc13, it is excluded from the nucleus, and given the previous observation that Uev1B transcript and cDNA were not found in a mouse cDNA library (Franko et al., 2001), we decided to remove Uev1B from subsequent studies. However, subsequently Uev1B mRNA has been described in rat tissues (Watanabe et al., 2007).

3.1.3. Ubc13 and Mms2-Myc in S-phase

Initial observations suggested that Ubc13 is distributed throughout the cytoplasm and nucleus of cultured cells. To investigate these further, cells were subjected to mild detergent pre-extraction before fixation and ICC was performed. Ubc13 was found to be retained in nuclei corresponding with PCNA positive nuclei (Figure 3-3A). Since in undamaged cells PCNA positive immunoreactivity has been routinely used as a marker for S-phase cells, retention of Ubc13 in these nuclei suggests that Ubc13 may have a function during DNA synthesis. Although 2H11 immunoreactivity was also demonstrated to be retained in PCNA positive cells (Figure 3-3B), it does not distinguish between Uev1A and Mms2 as described above. 3T3 cells

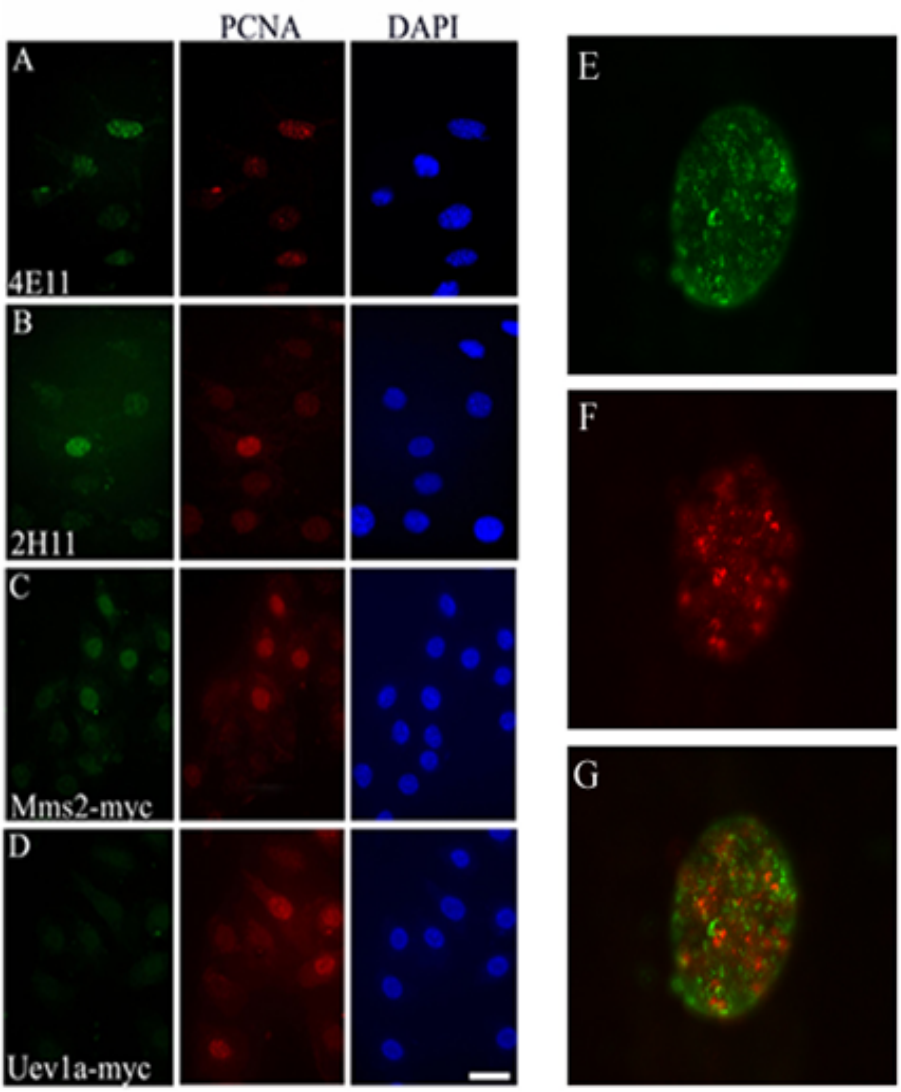

Figure 3-3. Ubc13 and Mms2 are retained in S-phase nuclei

ICC following mild NP40 pre-extraction (0.1%, 20 minutes on ice) demonstrates that Ubc13 **(A, 4E11)** and Mms2/Uev1 **(B, 2H11)** positive nuclei are also positive for PCNA in mouse 3T3 cells, suggesting that Ubc13 acts with Mms2 and/or Uev1A and plays a role in S-phase nuclei in untreated cells. To differentiate the involvement between Mms2 and Uev1A, cells were first transfected with Myc/His-tagged constructs and then ICC against the Myc tag was performed, which demonstrated that Mms2-Myc is retained in PCNA positive nuclei **(C)** whereas Uev1A is not **(D)**. Under higher magnification, it was observed that although Ubc13 **(E)** is retained in PCNA positive nuclei **(F)**, very few Ubc13 foci merge with PCNA **(G)**. Bar = 10 μm.

were transfected with the Myc-tagged constructs and analyzed by anti-Myc ICC. Again utilizing a similar mild detergent pre-extraction procedure, Mms2-Myc was found in PCNA positive nuclei (Figure 3-3C) whereas Uev1A-Myc was not (Figure 3-3D). These results suggest that Ubc13 and Mms2 play a role during S-phase and provide the first clue that Mms2 and Uev1A may not function identically in mammalian cells as they do when expressed in yeast cells.

Upon closer inspection of S-phase nuclei, it was discovered that although Ubc13 is retained in PCNA positive cells, the two do not strictly co-localize with one another (Figure 3-3E-G). This was unexpected as Ubc13 was thought to be responsible for the ubiquitination of PCNA, at least following DNA damage. To investigate this further a co-immunoprecipitation (Co-IP) assay was performed in an attempt to demonstrate a possible physical interaction. A HEK-293F whole cell lysate was incubated with anti-Ubc13 antibody (4E11) and PCNA was identified by Western blotting as a Ubc13-interacting protein since PCNA was not detected in negative controls lacking either the antibody or the cell lysate (Figure 3-4). These results suggest that Ubc13 and PCNA co-exist in a subset of replication complexes in S-phase undamaged cells but are NP40-sensitive. Alternatively, mouse (3T3) cells may react rather different from human (HEK-293F) cells under our experimental conditions.

3.1.4. Ablation of Ubc13 or Mms2 causes increased spontaneous double strand breaks

Because Ubc13 and Mms2 appear to be retained in S-phase nuclei, we wish to determine if there is a physiological function of these two proteins during S-phase. Our

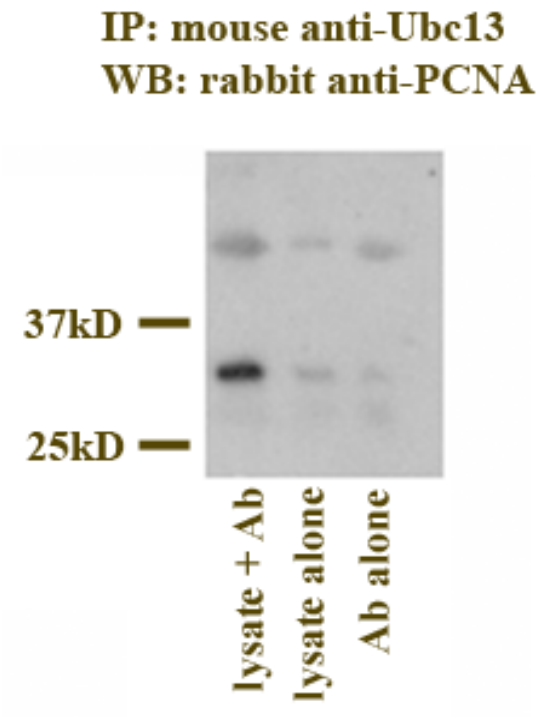

Figure 3-4. Physical interaction between Ubc13 and PCNA

A co-IP was performed using whole cell lysates from HEK-293F cells. The lysate was immunoprecipitated with anti-Ubc13 (4E11) and probed with rabbit anti-PCNA. The resulting immunoreactivity indicates enrichment of a band of approximately 30kDa, which is diminished in lanes without lysate or without the precipitating antibody, indicating that Ubc13 has the potential to complex with PCNA.

rationale was that if the Ubc13-Mms2 complex acts to maintain genomic stability, suppression of either protein would result in an increased spontaneous DNA damage. 3T3 cells were treated with each iRNA construct to suppress the intended target protein and then assayed for DNA damage. We chose to monitor cells with spontaneous Rad51 positive nuclear foci as it may detect both ssDNA and DSBs. Following siRNA-mediated down-regulation of Ubc13 or Mms2 cultures contained many cells with strong Rad51-positive nuclei suggesting the presence of DNA damage (Figure 3-5). In contrast, this increase in Rad51 immunoreactivity was not observed in cultures treated for siRNA-mediated reduction in Uev1A or utilizing the control s*iUbc13m* construct (Figure 3-5). To determine whether the observed DNA damage as detected by Rad51 immunoreactivity is ssDNA or DSBs, we performed ICC using a γ-H2AX specific antibody that detects early events of DSBs (Li and Heyer, 2008). Suppression of Ubc13 resulted in an increased number of cells with γ-H2AX-positive nuclear foci (Figure 3-6), suggesting that Ubc13 and Mms2 prevent DSB formation. Quantitative analysis was performed by scoring approximately 1000 individual cells under each treatment for percentage of strong Rad51 or γ-H2AX immunoreactivity signal in cells with significantly reduced 4E11 or 2H11 immunoreactivity. This result (Figure 3-7) shows that suppression of Ubc13 or Mms2 causes 4-fold increase in Rad51-positive cells and suppression of Ubc13 causes more than 3-fold increase in γ-H2AX positive cells. Hence, I was able to conclude that compromised expression of Ubc13 or Mms2, but not Uev1, leads to spontaneous genomic instability in the form of DSBs.

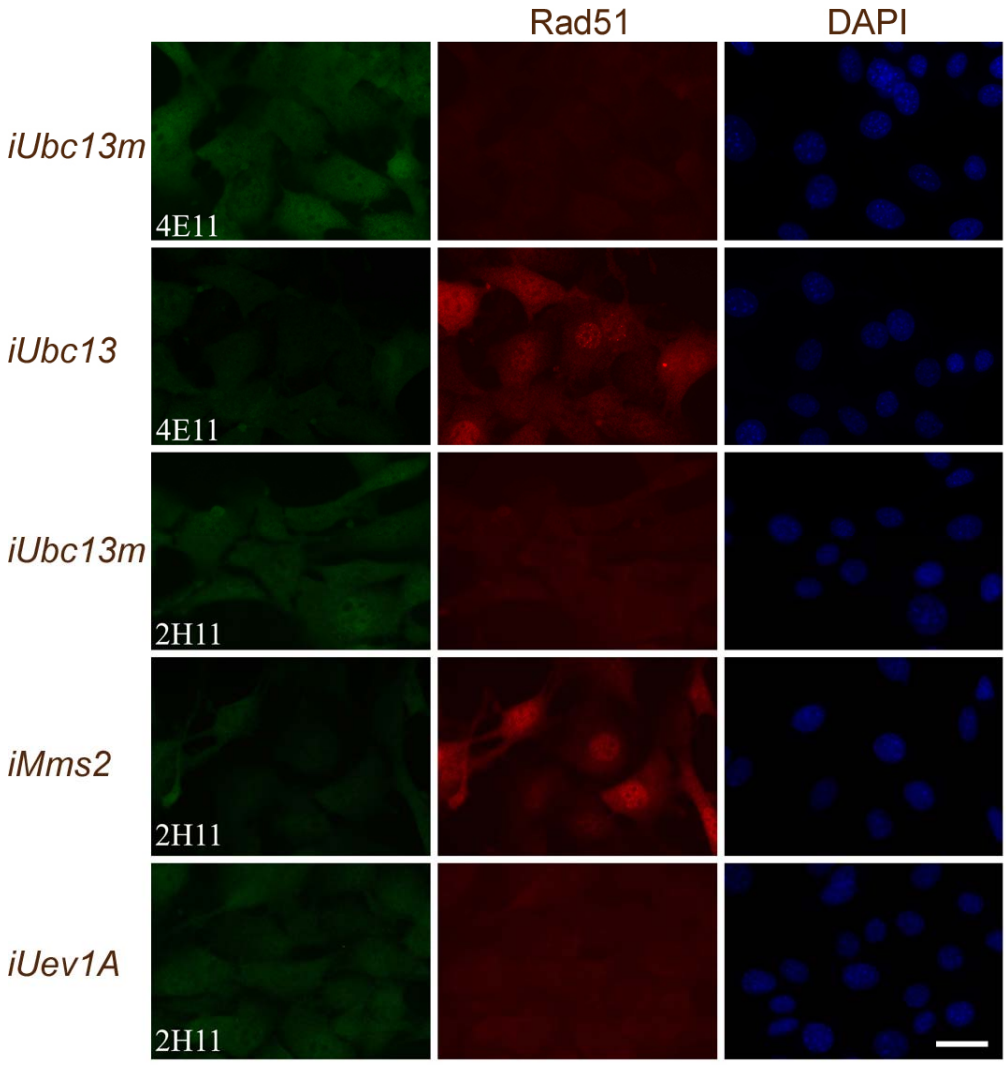

Figure 3-5. Ubc13 and Mms2 are required to maintain genomic stability

Log-phase 3T3 cells were examined for Rad51 immunoreactivity (center column) without detergent pre-extraction following repression of Ubc13, Mms2 or Uev1A using siRNA (si*Ubc13*, si*Mms2* and si*Uev1A*, respectively). Following Ubc13 (second panel) or Mms2 (fourth panel) reduction a significant increase in the number of Rad51-positive nuclei were observed, indicating the presence of a significant amount of DNA damage. Application of a mutant siRNA (si*Ubc13m*) incapable of suppressing 4E11 (first panel) or 2H11 (third panel) immunoreactivity did not result in an increase in Rad51 staining. Additionally, application of siRNA targeting Uev1A (si*Uev1A*, fifth panel) did not affect Rad51 immunoreactivity, suggesting that the Ubc13-Mms2 complex only is involved in genome maintenance. Bar = 10 μm.

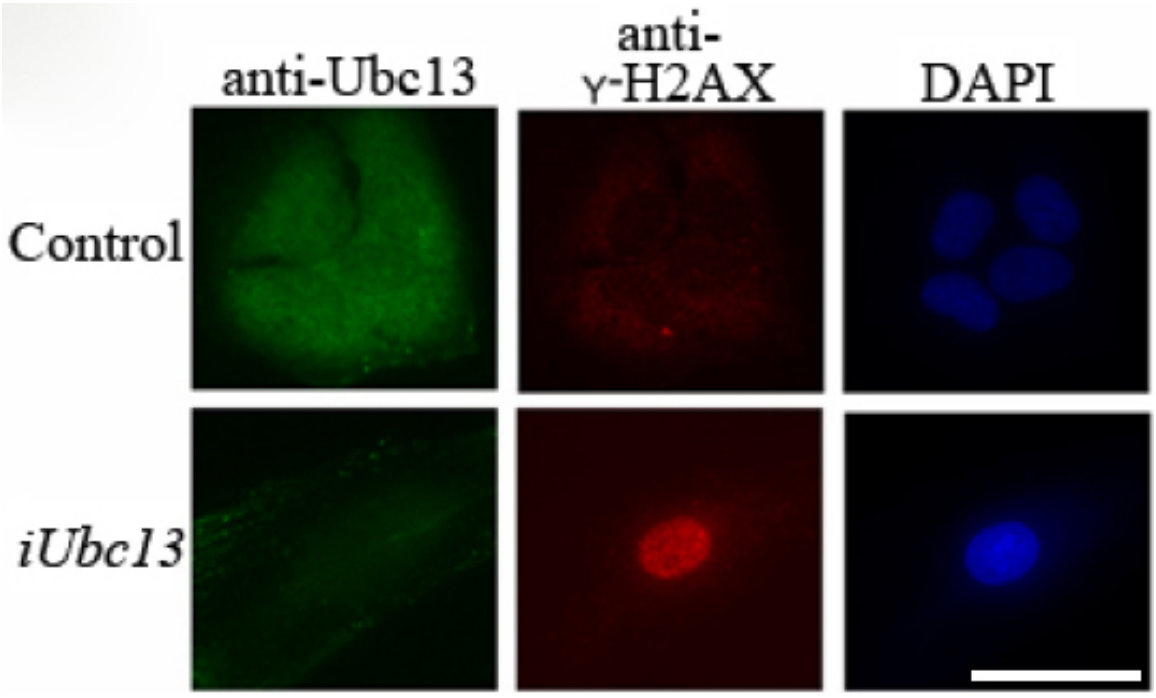

Figure 3-6. Repression of Ubc13 results in spontaneous γ-H2AX positive nuclei

Two days after interference RNA treatment to suppress Ubc13 expression, mouse 3T3 cells exhibited γ-H2AX positive nuclei (without detergent pre-extraction), which were rare in control cultures. This indicates a spontaneous increase in double-strand breaks in Ubc13-compromised cells in the absence of external damage. Bar = 10 μm.

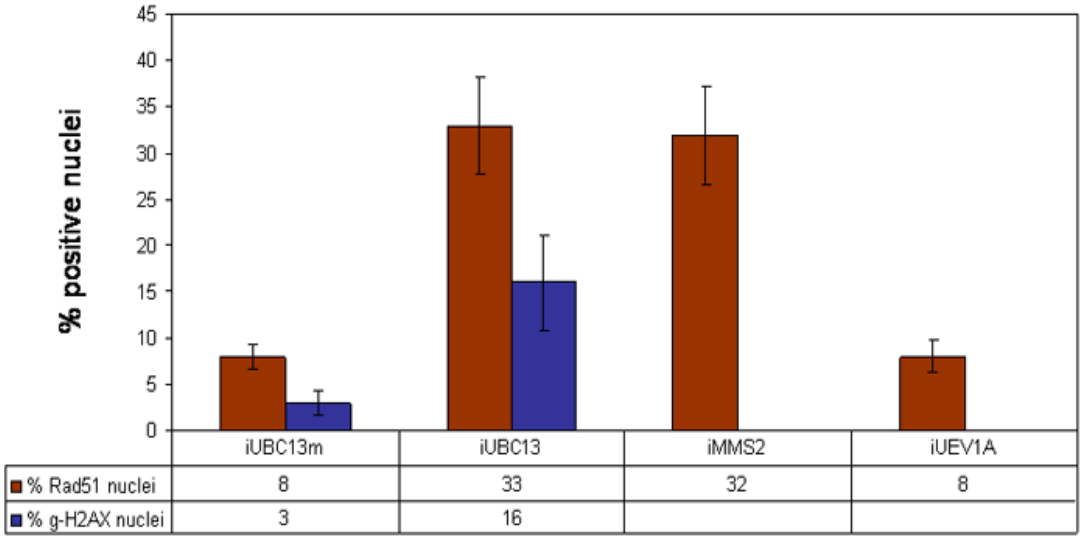

Figure 3-7. Quantitative analysis of spontaneous DNA damage following Ubc13, Mms2 or Uev1 reduction

Cultures were analyzed using ICC as illustrated in Figures 3-5 and 3-6 by counting nuclei in random fields of view in a predetermined manner (non-overlapping) following reduction of the indicated protein using iRNA and scored as either positive or negative for either Rad51 or γ-H2AX. This graph is representative of one of four individual experiments. Each bar represents approximately 1000 cells and error bars represent standard deviation.

3.1.5. DNA damage induced nuclear focus formation containing Ubc13 and Mms2

In the budding yeast Ubc13-Mms2 is involved in DDT of both spontaneous and induced DNA damage. To determine if Ubc13 and the Uevs play a role in response to increased DNA damage, several cell lines were exposed to various DNA damaging agents and analyzed using ICC with and without detergent pre-extraction. Exposure of 3T3, HepG2, HCT116 and L929 cells to methyl methane sulfonate, UV irradiation, hydroxyurea or bleomycin had no noticeable effect on Ubc13 immunoreactivity. However, exposure of 3T3 cells to the topoisomerase-I inhibitor CPT had a noticeable effect. The function of the topoisomerase-I is to regulate supercoiling of DNA by first binding the DNA, cleaving either one or both backbones without being removed and allowing the strands to rotate around one another such that supercoiling can be relaxed, and then catalyzing their re-ligation to restore genome integrity. CPT binds topoisomerase-I and prevents re-ligation of the DNA ends following cleavage, therefore inducing a single strand break in the DNA sugar-phosphate backbone, which can be further converted into a DSB at a replication fork (Ryan et al., 1991; Tsao et al., 1993). Exposure of cells to CPT therefore results in specific and predictable DNA lesions.

Exposure of 3T3 cells to 5 μM CPT resulted in the accumulation of nuclear Ubc13 immunoreactivity that was resistant to mild (0.1%, 20 minutes, on ice) NP40 pre-extraction in a time dependent manner (Figure 3-8). Nuclear accumulation was observable within four hours of treatment and became very prominent at eight hours. By 12 hours after treatment virtually all cells became Ubc13 positive with no observable effect on cell survival. Continual incubation often resulted in the rounding

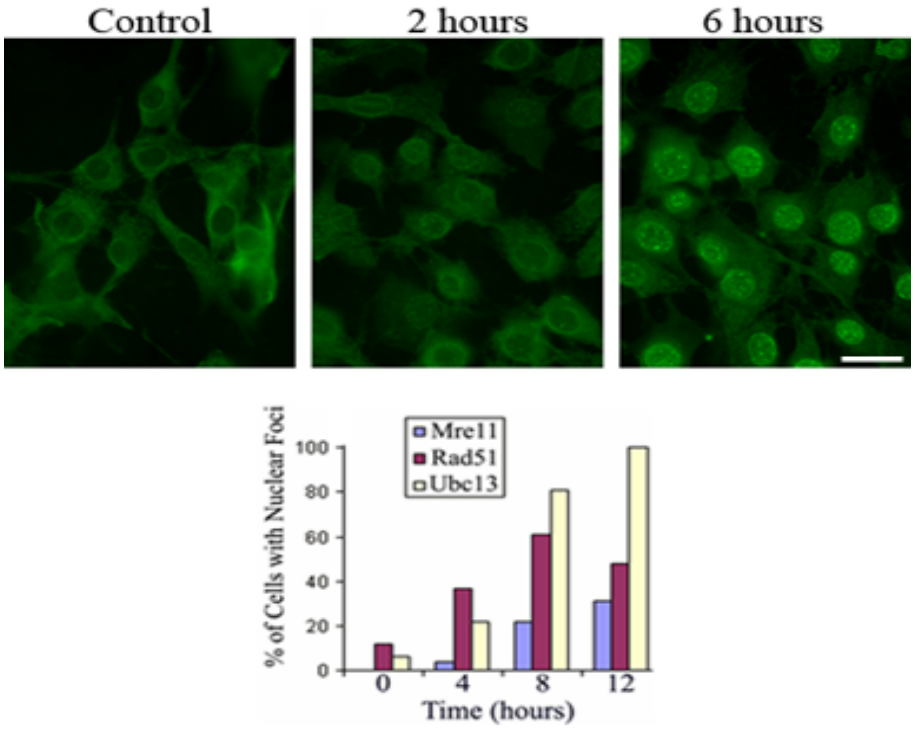

Figure 3-8. CPT-induced nuclear Ubc13 accumulation

3T3 cells were exposed to 5 μM CPT for various times and assayed for Ubc13 immunoreactivity following mild NP40 pre-extraction (0.1%, 20 minutes on ice) before fixation resulting in accumulation of nuclear Ubc13 in a time dependent manner. Within 12 hours virtually 100% of the cells are positive for Ubc13 nuclear foci in cells without a significant reduction in cell survival. Mre11 and Rad51 nuclear accumulation was also assayed as a positive control for DNA damage and also demonstrated a time-dependent accumulation response. The graph represents one representative experiment. Bar = 10 μm.

of cells, detaching from the culture dish and the appearance of surface bleb-like structures reminiscent of apoptotic cell death. Coinciding with this was an increase in the number of nuclei containing significant Mre11 and Rad51 immunoreactivity, which served as an internal control demonstrating the gradual appearance of DNA damage. To investigate the nature of the CPT-induced Ubc13 nuclear foci, cultures were treated with a more stringent NP40 pre-extraction (0.4%, 3.5 minutes at room temperature) before fixation and processed for ICC. CPT-induced NP40-resistant Ubc13, Mre11 and Rad51 nuclear foci remained. Similar CPT-induced nuclear foci were also observed in human HepG2 cells (not shown). Co-localization analysis demonstrated the formation of distinct, non-overlapping foci between Ubc13 and either Mre11 or Rad51 (Figure 3-9). However, if cells were pulsed with bromodeoxyuridine (BrdU) for the identical treatment time of CPT administration and then analyzed for its incorporation using ICC, BrdU was demonstrated to co-localize with Ubc13 (Figure 3-9C). This is in agreement with a previous report that CPT-induced Rad51 foci are distinct from BrdU incorporation (Sakamoto et al., 2001). Additionally, transfection of 3T3 cells with the Mms2-Myc construct two days prior to CPT treatment resulted in anti-myc immunoreactive NP40-resistant nuclear foci that co-localized with Ubc13 immunoreactivity (Figure 3-9D). These observations strongly support the notion that Ubc13 and Mms2 are involved in a DNA damage response in addition to a function during normal DNA replication.

3.1.6. Interdependence of DNA damage-induced Ubc13 and Mms2 nuclear focus formation

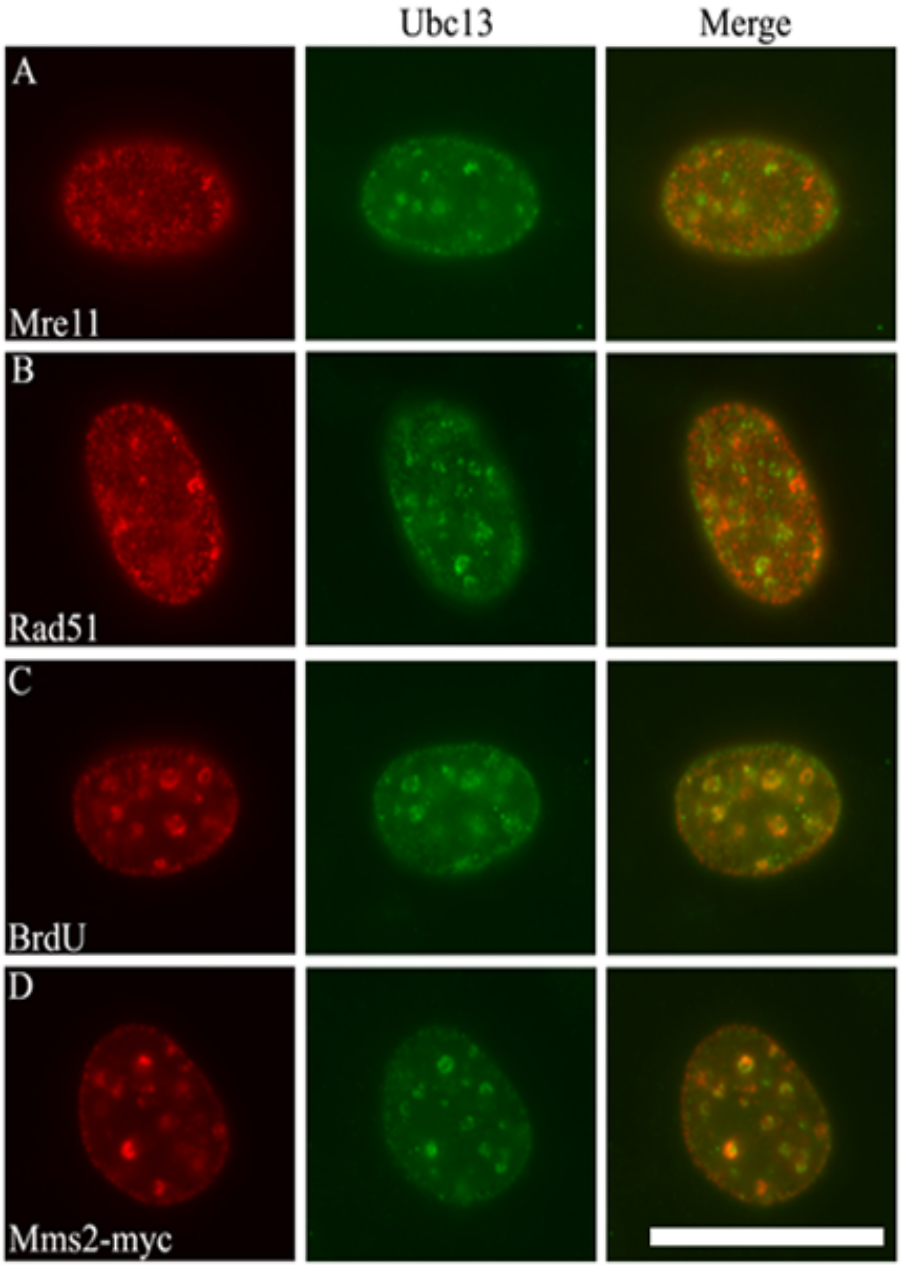

Figure 3-9. CPT induced Ubc13 nuclear focus formation and its co-localizatios

High stringency NP40 pre-extraction (0.4%, 3.5 minutes at room temperature) prior to fixation of 3T3 cells resulted in Ubc13 nuclear focus formation (center column) in comparison with focus formation of Mre11, Rad51, BrdU and Mms2-Myc (left column). Merged nuclear images (right column) reveal that a significant portion of Ubc13 nuclear foci co-localize with BrdU and Mms2-Myc **(panels C, D)**, but not with Mre11 or Rad51 **(panels A, B)**. Bar = 5μm.

The fact that Ubc13 and Mms2, but not Uev1A, co-localize in the same nuclear foci following DNA damage, together with their stable complex formation *in vitro*, strongly suggests that these two proteins form a DNA damage-induced complex *in vivo*. In an attempt to determine a direct interaction between the two proteins, each was analyzed following experimental reduction of the other by iRNA methodology. In this experiment, 2H11 was used and its positive immunoreactivity was regarded as positive detection of Mms2 but not Uev1, since we have previously demonstrated that only Mms2-Myc, but not Uev1A-Myc, form DNA damage-induced nuclear foci under the same experimental conditions. As shown in Figure 3-10, without siRNA treatment or with siRNA treatment against Uev1, both Ubc13 and Mms2 form expected co-localizing nuclear foci, in response to CPT treatment. However, prior ablation of either Ubc13 or Mms2 abolished the formation of either foci. This observation supports the notion that Ubc13 and Mms2 are acting interdependently at the site of DNA synthesis (BrdU incorporation) in response to DNA damage.

3.1.7. DNA damage-induced PCNA modification and interaction with Ubc13/Mms2

To further investigate the interactions of Ubc13 and Mms2 in a DNA damage response, cross linking experiments were conducted in an attempt to detect their interaction with PCNA and hopefully to demonstrate the poly-Ub addition of PCNA in a damage and Ubc13-Mms2 dependent manner. From the literature it is well documented that PCNA from various cell types becomes modified with a single Ub in response to DNA damage. Using several cell lines including 3T3, HepG2 and HEK-

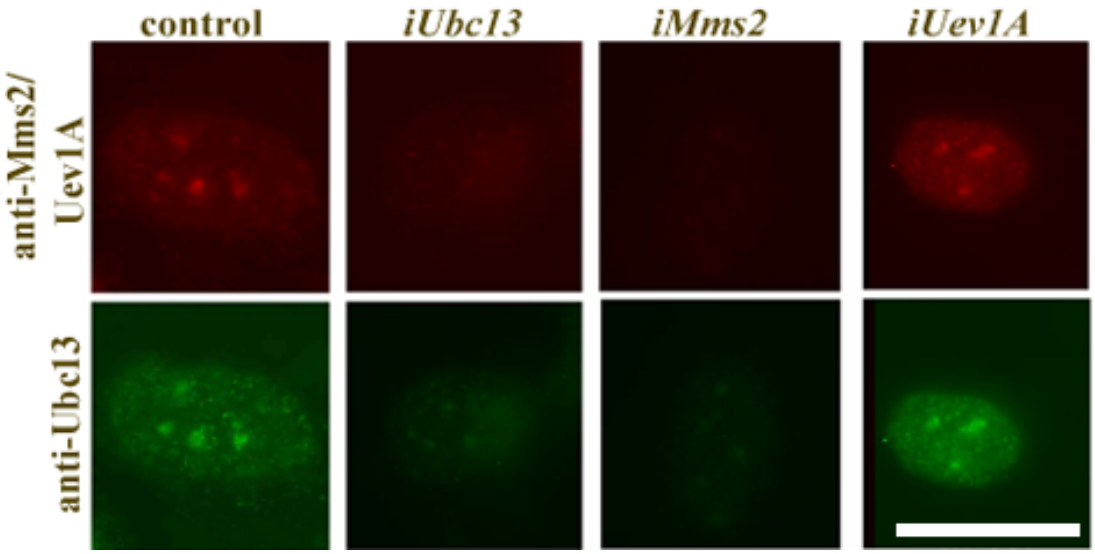

Figure 3-10. Co-dependency of Ubc13 and Mms2 in CPT-induced foci formation

High stringency NP40 pre-extraction (0.4%, 3.5 minutes at room temperature) prior to fixation and ICC on 3T3 cells reveals CPT-induced 4E11 and 2H11 (Mms2/Uev1A) positive focus formation (left column). Suppression of either Ubc13 or Mms2 using iRNA results in loss of both proteins in the nuclear foci (center two columns). However, treatment of cells with iRNA targeting Uev1A does not affect the focus formation. Bar = 5 μm.

293F, in response to various damaging agents such as UV irradiation, methymathane sulfonate, CPT and hydroxyurea, PCNA was most readily seen to be modified in HEK-293F cells in response to UV exposure in a dose dependent manner (Figure 3-11A). Eight hours after UV exposure, cultures were lysed and Western blotting reveals the steady increase of a slow migrating PCNA band with the apparent molecular weight as expected for monoubiquinated PCNA (Figure 3-11A). Although I could not definitively identify this upper band to contain Ub immunoreactivity, the current literature agrees with its identification as mono-Ub PCNA (Brun et al., 2008). In no combination of cell line and treatment was I able to consistently observe the expected laddering appearance indicative of poly-Ub modified PCNA as observed in *in vitro* experiments (Zhang et al., 2008). I also attempted, without success to demonstrate the polyubiquitination of PCNA by immunoprecipitation of the nuclear fraction (data not shown) and with assistance from visiting the laboratory of Dr. D. Gray (Ottawa Hospital Research Institute) in order to learn a detailed method of identifying poly-Ub modified substrates. To assess the possibility of Ubc13-Mms2 operating together with PCNA at the replication fork, cultures were treated with the reversible cross-linking agent dithiobis succinimidyl propionate 8 hours after exposure to a 12 J/m^2 UV dose, which gave the maximal Ub modification response of PCNA without an obvious loss in cell viability. Immunoblotting of the lysates demonstrated the majority of the PCNA, Ubc13 and Mms2 not to be cross-linked; however, at least one high molecular weight band was observed in the presence of dithiobis succinimidyl propionate, which is aligned with PCNA, Ubc13 and 2H11 immunoreactivity (arrow in Figure 3-11B). Furthermore this upper band was eliminated following 10 mM

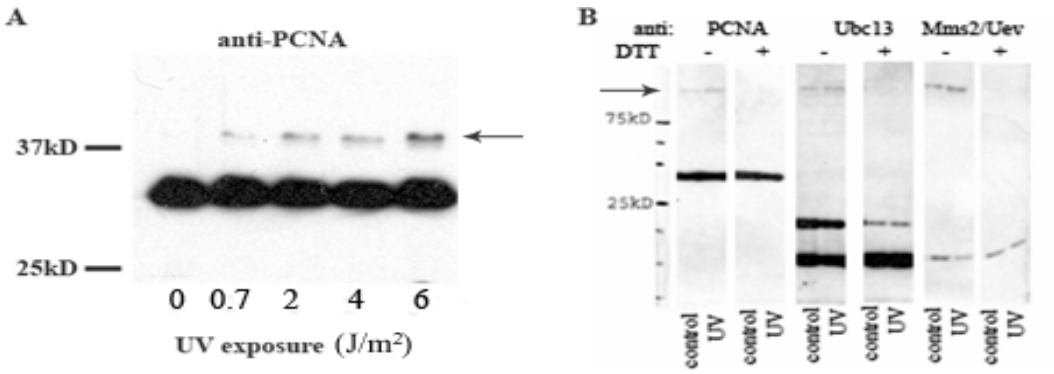

Figure 3-11. PCNA modification and its interaction with Ubc13 and Mms2/Uev1

PCNA from HEK-293 was analyzed by Western blotting 8 hours after UV exposure. **(A)** A modified PCNA band appears in a dose dependent manner. This band is 8 kDa larger than PCNA, indicating the likelihood of being modified by a single Ub. A characteristic ladder pattern of immunoreactivity indicating a poly-Ub modification was not routinely witnessed under any experimental conditions. **(B)** Chemical cross-linking with the reversible cross linking agent dithiobis succinimidyl propionate for 1 hour on ice before lysate collection resulted in a slower migrating band of PCNA (arrow) which appeared to co-migrate with 4E11 and 2H11 immunoreactivity. These slow migrating bands, were not enhanced by UV exposure (12 J/m^2) and were reversible by DTT treatment.

dithiolthreitol treatment as a reducing agent to cleave cross-linking by dithiobis succinimidyl propionate. Unfortunately, I was unable to observe a UV-induced increase in the cross-linked band (Figure 3-11B). Taken together, no conclusive evidence was obtained through this study to support the involvement of Ubc13-Mms2 in PCNA polyubiquitination following DNA damage, despite the fact that ICC analyses have strongly hinted at this possibility.

3.1.8. The N-terminus of Uev1 regulates its pathway-specific activity

From the yeast two hybrid experiments, GST pull down assays and reconstitution of yeast error-free DDT pathway in the presence of human Ubc13 and Uev1A or Mms2, it becomes clear that Ubc13 interacts with both Mms2 and Uev1A (Andersen et al., 2005). However, in mammalian cells Uev1A is obviously not part of the DNA damage response in the same manner as Mms2 (Figures 3-3, 3-5 and 3-7). To determine the defining factor that differentiates Uev1A from Mms2 we created a Uev1-Myc tagged construct without its N-terminal amino acid extension beyond the core region, leaving the core region which contains only a 14 amino acid difference from that of Mms2. This results in 91% amino acid identity between the core region of the newly constructed Uev1AΔ30-Myc and Mms2-myc (Figure 1-5). While 3T3 cells express Uev1AΔ30-Myc in a cellular distribution pattern indistinguishable from that of Mms2-Myc and Uev1A-Myc (Figure 3-2), surprisingly CPT-induced NP40-resistant Uev1AΔ30-Myc nuclear focus formation is similar to that of Mms2-Myc but different from that of Uev1A-Myc (Figure 3-12). Like Mms2-Myc, these CPT-induced Uev1AΔ30-Myc foci co-localize with Ubc13, indicating that the N-terminal unique

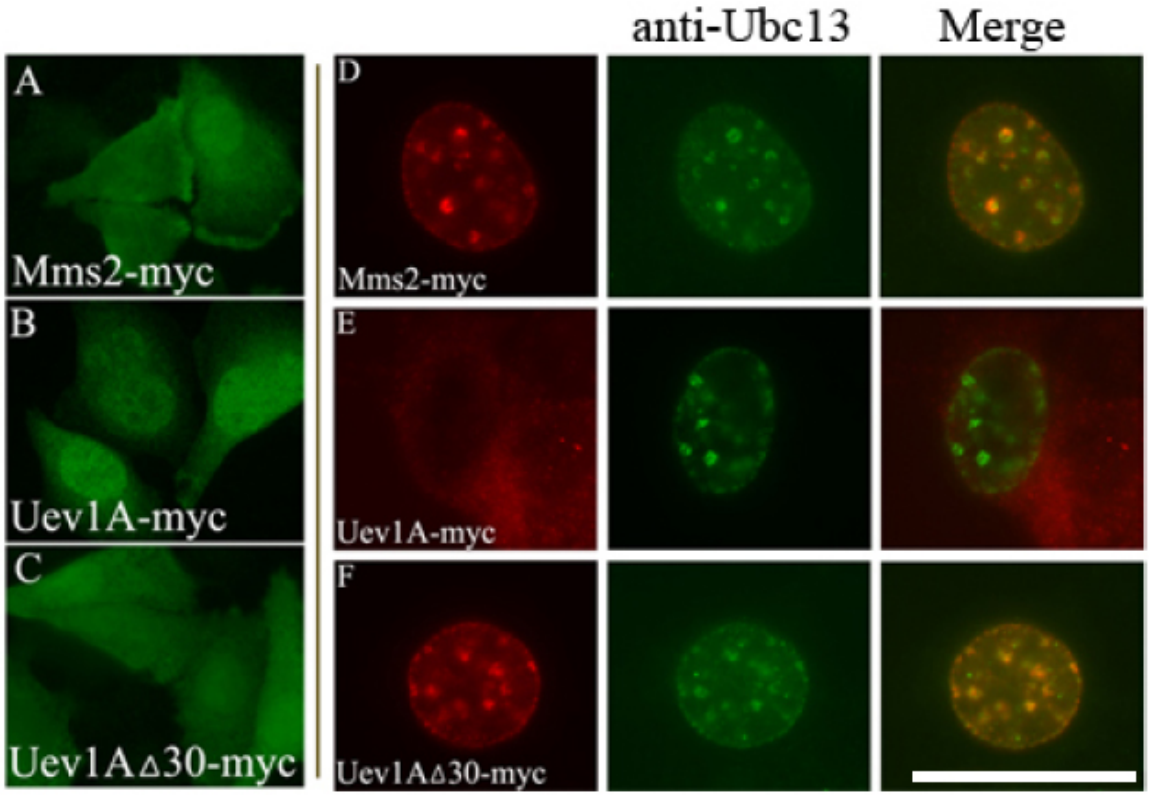

Figure 3-12. The N-terminal domain of Uev1A prevents its access to CPT-induced nuclear foci

Mouse 3T3 cells were transfected with **(A)** Mms2-Myc, **(B)** Uev1A-Myc or **(C)** the N-terminal deletion construct Uev1AΔ30-Myc, and processed for ICC against the Myc-tag monoclonal antibody (clone 9E10) which resembled 2H11 immunoreactivity as previously described. High stringency NP40 pre-extraction (0.4%, 3.5 minutes at room temperature) prior to fixation resulted in CPT-induced (5 μM, 4 hours) Ubc13 focus formation co-localizing with Mms2-Myc **(panel D)** and Uev1AΔ30-Myc **(panel F)**, but not with the full length Uev1A **(E)**. Bar = 5 μm.

region of Uev1A prevents this protein from being involved in the DNA damage response similar to Mms2.

3.1.9. Ubc13 and Uev1A, but not Mms2, are required for NF-κB activation

It was reported that the TNF associated factors 2 and 6 (TRAF2 and TRAF6) (Deng et al., 2000; Shi and Kehrl, 2003) may operate as E3 proteins for Ubc13-mediated NF-κB signaling following activation of TNF receptors, Toll-like receptors and several interleukin receptors (Sun and Chen, 2004). In T-cells and B-cells virus-induced NF-κB activation also requires Ubc13-Uev, possibly via polyubiquitination of NEMO (Zhou et al., 2004). Interestingly, in reported *in vitro* assays, either Uev1A or Mms2 are able to serve as a cofactor of Ubc13 to facilitate K63-linked polyubiquitination of the E3 (Deng et al., 2000) or target (Zhou et al., 2004) protein. However, it is unclear which Uev (or both) serves as an *in vivo* cognate partner of Ubc13 for the NF-κB signaling pathway. To address this issue we utilized a lipopolysaccharide model on cultured mouse microglia to activate NF-κB. Lipopolysaccharide (1 μg/ml) induces the migration of p65, a subunit of NF-κB, to the mouse microglia nucleus within 1.5 hours of treatment (data not shown, but see Figure 3-13). However, when the cells were subject to a prior siRNA treatment to suppress either Ubc13 or Uev1, lipopolysaccharide was no longer able to entice the translocation of p65 into the nucleus (Figure 3-13). In sharp contrast, siRNA targeted to suppress Mms2 expression had no effect on lipopolysaccharide-induced re-localization of p65 to the nucleus (Figure 3-13). Because of the difficulty in identifying each of Uev1 and Mms2 with 2H11, verification of the suppression was done using FLAG-tagged Mms2

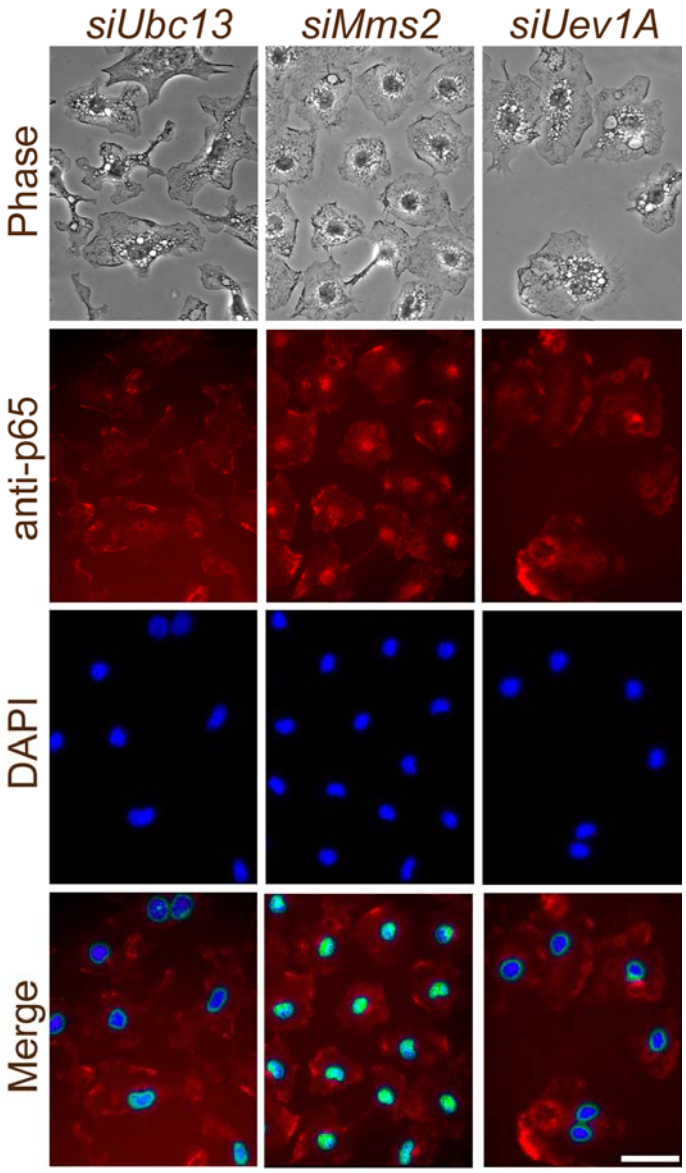

Figure 3-13. The Ubc13-Uev1A complex is required for NF-κB signaling

Cultured mouse (CD-1) microglia were used to assay the activities of Ubc13, Mms2 and Uev1A in the lipopolysaccharide-induced NF-κB pathway. Four days after transfection with iRNA targeting Ubc13, Mms2 or Uev1A, phase contrast, anti-p65 ICC (without detergent pre-extraction and DAPI staining were performed following 1.5 hours treatment with 1 μg/ml lipopolysaccharide. The merged panel illustrates p65 immunoreactivity in the cells treated with s*iMms2*, but not with s*iUbc13* or s*iUev1A*, Note that identical adjustment was made to each merged image to enhance differential co-localization of NF-κB and DAPI. Bar = 10 μm.

or Myc-tagged Uev1A constructs in parallel experiments performed in collaboration with Dr. Honglin Zhou of the Dixit laboratory (Genetech, USA). This collaboration confirmed that suppression of either Ubc13 or Uev1A, but not Mms2, represses the Ub modification of NEMO in the NF-κB pathway in response to TRAF2 or TRAF6 activation (Andersen et al., 2005). Taken together, the above data demonstrate that the Ubc13-Uev1A complex is involved in NF-κB activation independently of Mms2.

3.2. Characterization of mammalian TLS polymerases

3.2.1. Characterization of anti-Rev3 polyclonal serum

Based on cDNA analysis (Gibbs et al., 1998), *hREV3* is expected to encode a 3,130 amino-acid protein with an estimated molecular mass of 353 kDa, which is consistent with its detected transcript size (Xiao et al., 1998a). To investigate endogenous Rev3 protein dynamics we produced a mouse polyclonal antiserum directed against the recombinat C-terminal portion of the protein cloned from a portion of the Rev3 gene (underlined in Figure 2-3). Western blotting analysis of the human colorectal carcinoma HCT116 cells demonstrated a major immunoreactive band that would agree in size with the predicted Rev3 protein (Figure 3-14A). ICC revealed that the polyclonal antiserum is primarily localized to the nucleus of HCT116 cells (Figure 3-14B). In order to further address the specificity of the polyclonal antiserum against Rev3, we transfected HCT116 cells with a mixture of anti-Rev3 interference RNA and found that the immunoreactivity was effectively diminished, while a mixture of non-specific interference RNA did not affect the Rev3 level (Figure 3-14A,B). Hence, we

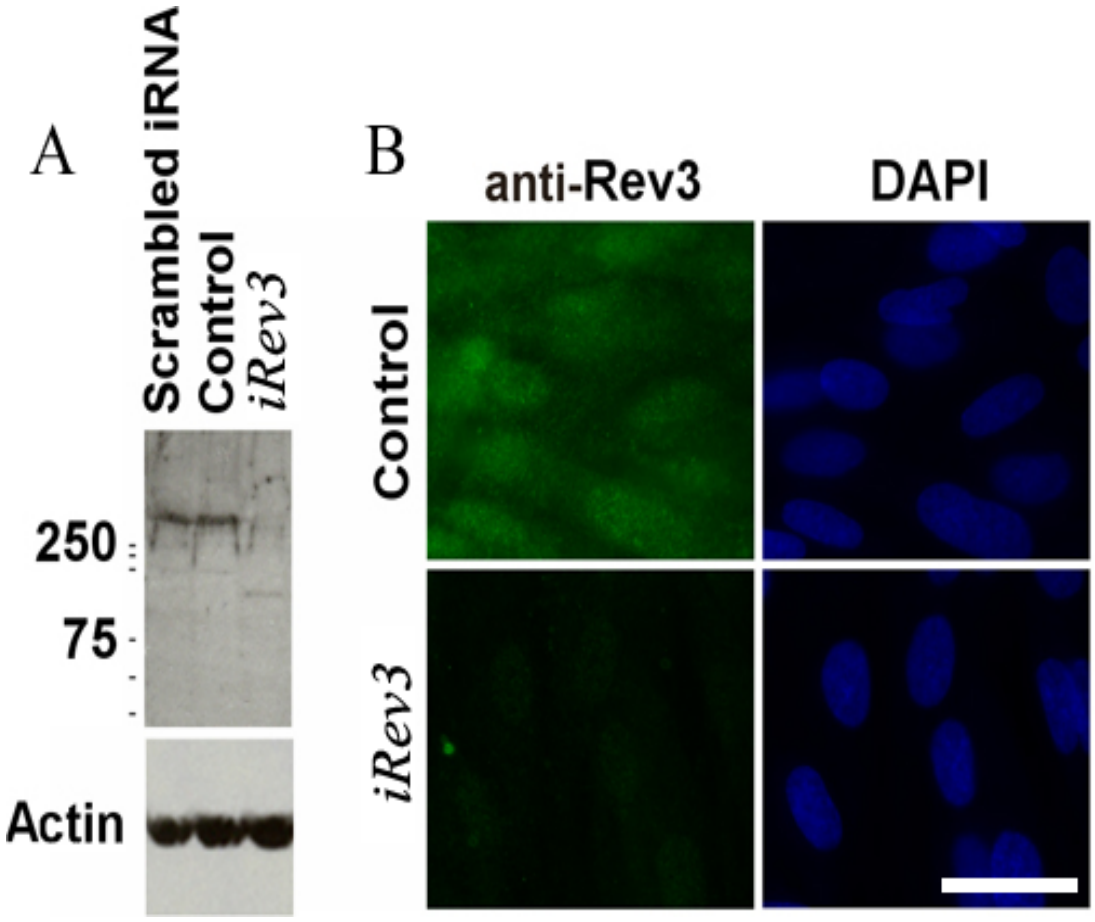

Figure 3-14. Characterization of anti-Rev3 antibody

(A) Western blotting analysis of HCT116 cell lysates separated on a 4% acrylamide/bisacrylamide gel and blotted using polyclonal-Rev3 antibody resulted in a major immunoreactive band with apparent molecular weight in excess of 250 kDa (350 kDa was expected), which could be reduced using Rev3-specific siRNA six days after transfection, but not with control non-specific siRNA. **(B)** ICC on log phase HCT116 cells (without detergent pre-extraction) indicates immunoreactivity throughout the cytoplasm, which could be decreased following siRNA treatment. This strongly supports the assertion that the polyclonal anti-serum to Rev3 is specific for Rev3. bar = 10 μm.

were able with different cell lines with respect to Rev3 localization, to conclude that the polyclonal antiserum prepared for this study contains antibodies specific for Rev3 and that the *iRev3* used in this experiment is capable of suppressing endogenous Rev3 expression. Herein we will refer to the positive detection using this antiserum as identifying Rev3.

3.2.2. Nuclear exclusion of Rev3 in a subpopulation of normal cells

Immunocytochemistry on low passage normal GM08402 cells revealed that Rev3 was partially or completely excluded from the nucleus in a subpopulation of the culture (Figure 3-15). In this case, the cellular Rev3 level was not necessarily low but predominantly distributed in the cytoplasm. Careful inspection indicated that this population accounted for about 20-30% of cycling cells and all of them stained negative for nuclear PCNA, suggesting that they were not in S-phase. On the other hand, not all nuclear PCNA-negative cells displayed Rev3 nuclear exclusion. Hence, we conclude that in a subpopulation of GM08402 cells, possibly representing a particular cell cycle stage, Rev3 is excluded from entering the nucleus. Interestingly, the phenomenon of Rev3 nuclear exclusion was not observed in HCT116 cells (Figure 3-14 and data not shown). In order to ask whether the differential Rev3 distribution between the two cell lines is due to normal *vs*. tumor derived cells, we analyzed Rev3 immunoreactivity in several available cell lines. The low passage non-immortal XP30RO cells (XPV patient-derived) exhibited Rev3 immunoreactivity similar to GM08402, whereas all tumor-derived cell lines examined, including HepG2, SAOS2, U2OS, and the SV-40

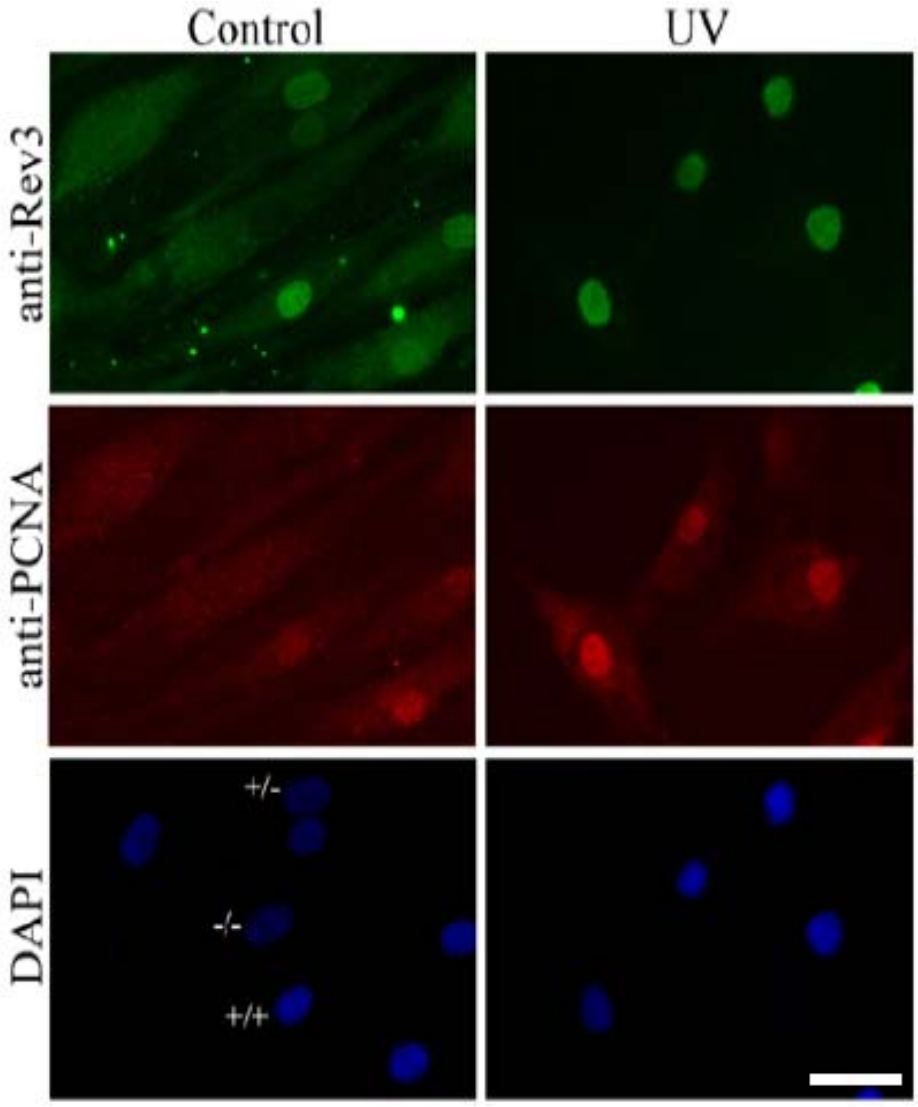

Figure 3-15. Rev3 distribution in normal cells

In the low passage number normal human fibroblast cell line GM08402, Rev3 immunoreactivity (without detergent pre-extraction) dominates in the nucleus of PCNA positive cells, suggesting an activity during S-phase of the cell cycle (left column). Representative cells in the DAPI image are labeled as either -/-, +/- or +/+ to indicate Rev3/PCNA immunoreactivity. In a small population of cells, Rev3 and PCNA was found to be excluded from the nucleus. In tumor-derived lines such as HCT116 (Figure 3-14), the population of cells lacking nuclear Rev3 immunoreactivity was not observed. Eight hours following a UV exposure of 7 J/m^2 Rev3 becomes concentrated in the nucleus (right column). Bar = 10 μm.

immortalized NF1604 cell line exhibited staining similar to that of HCT116 (data not shown), suggesting that the difference may be coordinated with immortalization.

Rev3 is a very large protein and cannot passively diffuse into the nucleus through the nuclear pore. Hence, we propose that an active nuclear transportation system is not active in this subpopulation of cells under our experimental conditions. Since the nuclear transportation of many nuclear proteins is induced by certain signals, we sought to determine whether DNA damage is able to induce Rev3 nuclear localization. Log-phase GM08402 cells were treated with 7 J/m^2 UV and incubated for a further eight hours resulted in the majority of cells (>90%) staining positive for nuclear Rev3 (Figure 3-15). Given the fact that the doubling time for GM08402 under our culture conditions is about 48 hours, we argue that the UV treatment is unlikely to simply synchronize cells to S phase. Hence, Rev3 nuclear localization can be induced by DNA damage treatment by an unknown mechanism.

3.2.3. UV-induced Rev3 nuclear foci and co-localization with PCNA

With the available antibody against Rev3, we sought to characterize the cellular distribution of Rev3 and its response to DNA damaging agents utilizing the low-passage normal human fibroblast cell line GM08402 wherever possible. As explained above, during non-DNA damage treatment conditions Rev3 is generally not distributed evenly between the nucleus and cytoplasm, but is enriched in the nucleus in S-phase (PCNA immunoreactive) cells (Figure 3-15, left column). Eight hours after a sub-lethal dose of UV (7 J/m^2), Rev3 immunoreactive nuclei were observed in PCNA positive

cells, and the majority of them appear to overlap (Figure 3-15, right column), suggesting an association with the chromatin following damage.

In order to determine whether Rev3 co-localizes with PCNA to the nuclear foci following UV treatment, cultured normal human fibroblasts were treated with ice-cold 0.4% NP40 for 40 minutes before fixation, a procedure effectively removing the majority of the soluble protein from the cells and resulting in a NP40-insoluble fraction that represents nuclear foci in proximinity to PCNA immunoreactivity (Figure 3-16), suggesting that these proteins are in the same super-complex. No NP40-resistant Rev3 nuclear focus was observed from cells not exposed to UV, regardless of their cell cycle stages although PCNA positive structures remained (data not shown). Based on the above observation and previous reports (Kannouche et al., 2001), we suspect that the UV-induced NP40-resistant PCNA nuclear foci represent stalled replication forks and further speculate that Rev3 also operates at these forks. Repeated attempts to co-IP Rev3 with PCNA were unsuccessful despite employing the cross-linking agent such as dithiobis succinimidyl propionate (data not shown). This may be due to a low abundance of endogenous Rev3 or the unsuitability of the anti-Rev3 antibody for immunoprecipitation. Furthermore, there is no evidence that Rev3 directly interacts with PCNA. In order to further address whether nuclear foci containing both Rev3 and PCNA represent stalled replication forks, we examined the co-localization of PCNA with Pol-η and Rev1. It has been previously established that following low dose UV treatment, Pol-η accumulates at replication foci stalled at DNA damage (Kannouche et al., 2001); that Rev1 co-localizes with Pol-η to the same replication foci

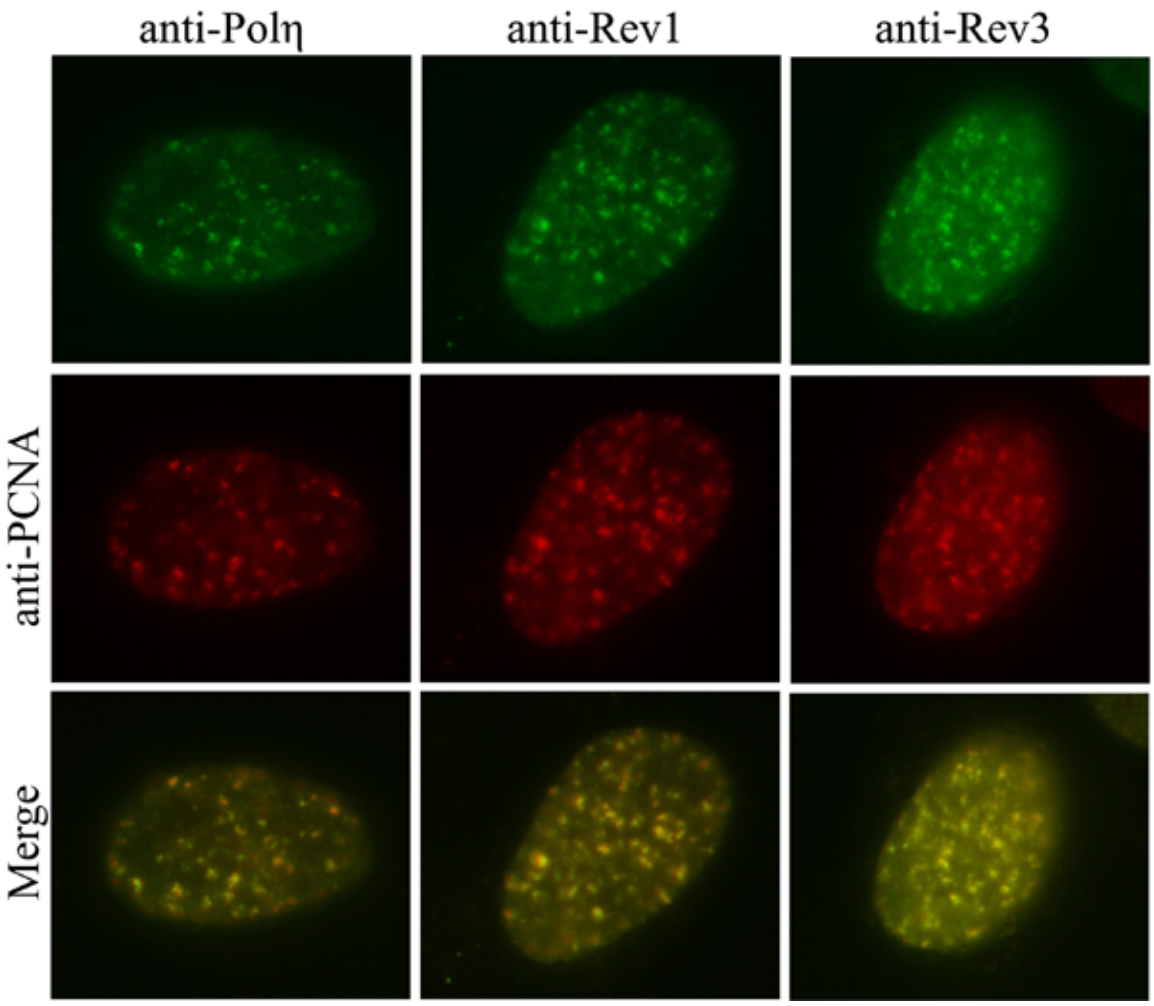

Figure 3-16. UV-induced nuclear co-localization of TLS polymerases and PCNA

ICC with detergent pre-extraction) on normal human fibroblast cells exposed to 7 J/m^2 UV followed by incubation for 6 hours result in nearly the entire population of cells containing NP40-resistant PCNA nuclear foci. These foci co-localize with Pol-η, Rev1 and Rev3, which are indistinguishable from one another except for a fainter staining pattern using either anti-Rev1 or anti-Pol-η, which was enhanced to demonstrate nuclear foci. Bar = 3 μm.

For control images following UV exposure but without NP40 pre-extraction refer to figure 3-15.

(Tissier et al., 2004), and that both co-localize with PCNA. Indeed, under our experimental conditions, both UV-induced Pol-η and Rev1 (Figure 3-16) nuclear foci co-localize with PCNA in a manner similar to that of Rev3 (Figure 3-16, right column). The above observations collectively indicate that upon DNA damage Pol-η, Rev1 and Rev3 all accumulate at replication forks as revealed by NP40-insoluble nuclear foci containing PCNA immunoreactivity.

3.2.4. UV-induced Rev3 nuclear focus formation is dependent on Rev1 but independent of Pol-η

The C-terminal 100 amino acid region of Rev1 has been reported to physically interact with a number of Y-family polymerases as well as Rev7 (Guo et al., 2003), a presumed regulatory subunit of Pol-ζ that binds to Rev3 in an *in vitro* assay (Murakumo et al., 2000). The above observation predicts that Rev3 is co-localized with Rev1; however, such a physical interaction has not been reported *in vivo* and it is unclear whether the interaction is dependent on DNA damage. We found that under untreated conditions, both Rev1 and Rev3 are distributed in the cytoplasm and the nucleus, but their co-localization is not obvious (Figure 3-17, left column). After UV treatment, the Rev3 nuclear foci co-localize with Rev1 foci and these nuclear structures are resistant to NP40 pre-extraction (Figure 3-17, third column), suggesting that these two proteins may coexist in the same complex.

In order to determine whether Rev1 is required for Rev3 localization to the damage site, we utilized the SV40 immortalized human lung cell line NF1604 and its

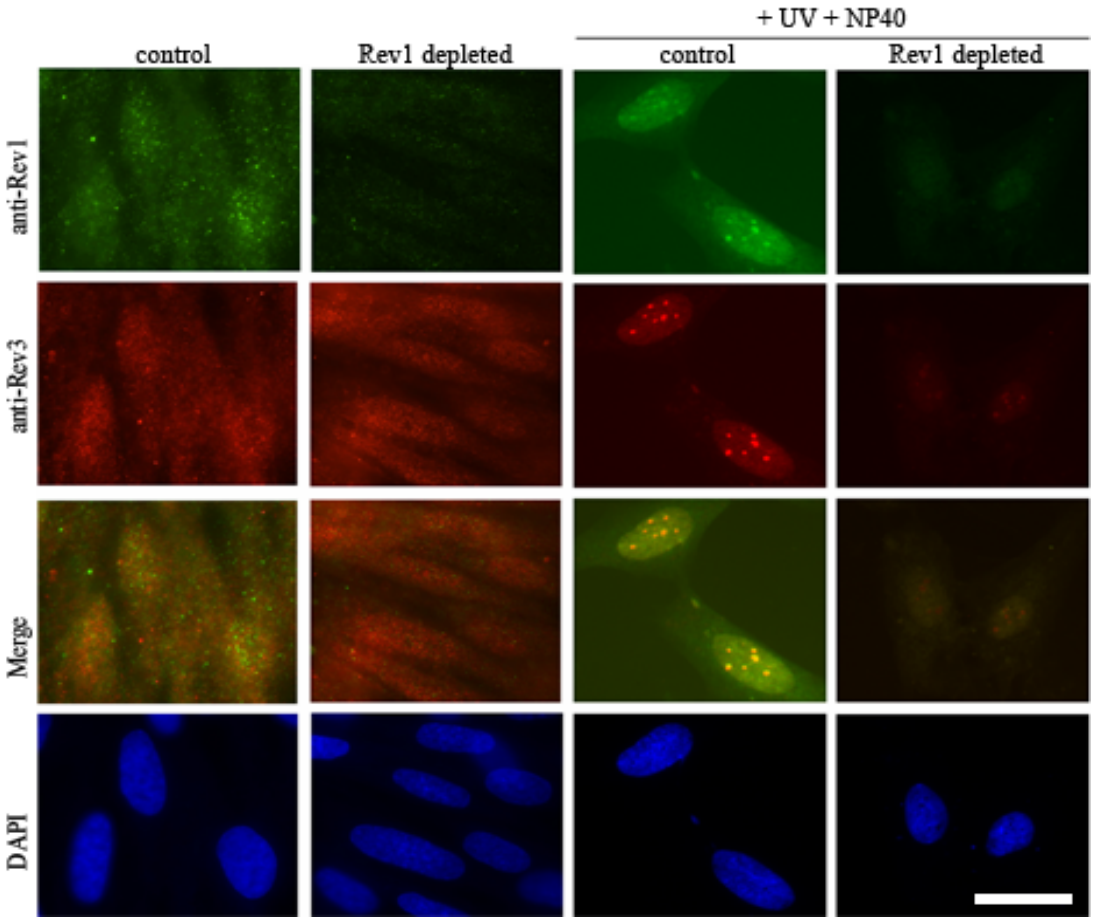

Figure 3-17. Rev1 is required for UV-induced Rev3 focus formation

The immortalized fibroblast cell line NF1604 (Control) and a daughter cell line Rz20 (Rev1 depleted) expressing a ribozyme designed to degrade Rev1 mRNA were used to analyze Rev3 localization by ICC without (left two columns) and with (right two columns) detergent pre-extraction. Although Rev1 immunoreactivity is depressed in the knockdown cells, Rev3 immunoreactivity levels do not appear to be affected (first two columns). Following UV exposure and NP40 pre-extraction before fixation and ICC, UV-induced Rev3 foci did form in the control cells (third column) but did not form in the absence of Rev1 (fourth column). This suggests that damage-induced localization of Rev3 is dependent upon the presence of Rev1. Bar = 10 μm.

derivative, Rz20, stably expressing a *hREV1*-specific ribozyme that results in the suppression of Rev1 mRNA (Clark et al., 2003). Under the condition that the cellular Rev1 mRNA level is reduced by up to 90% (Clark et al., 2003) and the Rev1 protein is barely detectable in Rz20 cells, total cellular Rev3 levels do not appear to be affected (Figure 3-17, second column). However, under these conditions, UV-induced nuclear Rev3 focus formation is severely compromised (Figure 3-17, right column). Hence, Rev1 appears to play a pivotal role in recruiting Pol-ζ to the damage site. Pol-η is recruited to the damage site after UV treatment (Kannouche et al., 2001). It has been reported through *in vitro* translesion DNA synthesis analysis that yeast Pol-ζ may act in concert with a Y-family polymerase in the two step model, allowing for DNA synthesis from an insertion event across the damaged template base(s) by any of the Y-family polymerases (Johnson et al., 2000b). These observations predict that Pol-ζ is co-localized with Pol-η at the damage site. Indeed, we found that as expected, normal human fibroblasts exhibit UV-inducible Pol–η foci that co-localize with Rev3 foci, which are persistent after NP40 extraction (Figure 3-18, third column). To ask whether Pol-η is required for the Rev3 nuclear focus formation, I utilized an XPV cell line derived from a XP variant patient. This mutation contains a 4-base pair deletion in *XPV*, resulting a truncation at amino acid position 42 (Masutani et al., 1999a) and the loss of Pol-η functional domains including the polymerase, Rev1-binding, PIP and UBZ domains. ICC using an anti-Pol-η antibody revealed that endogenous Pol-η is indeed undetectable in this XPV cell line (Figure 3-18, second column); however, UV treatment was still able to induce the NP40-resistant Rev3 focus formation at a level indistinguishable from that of matched normal human fibroblasts (Figure 3-18, right

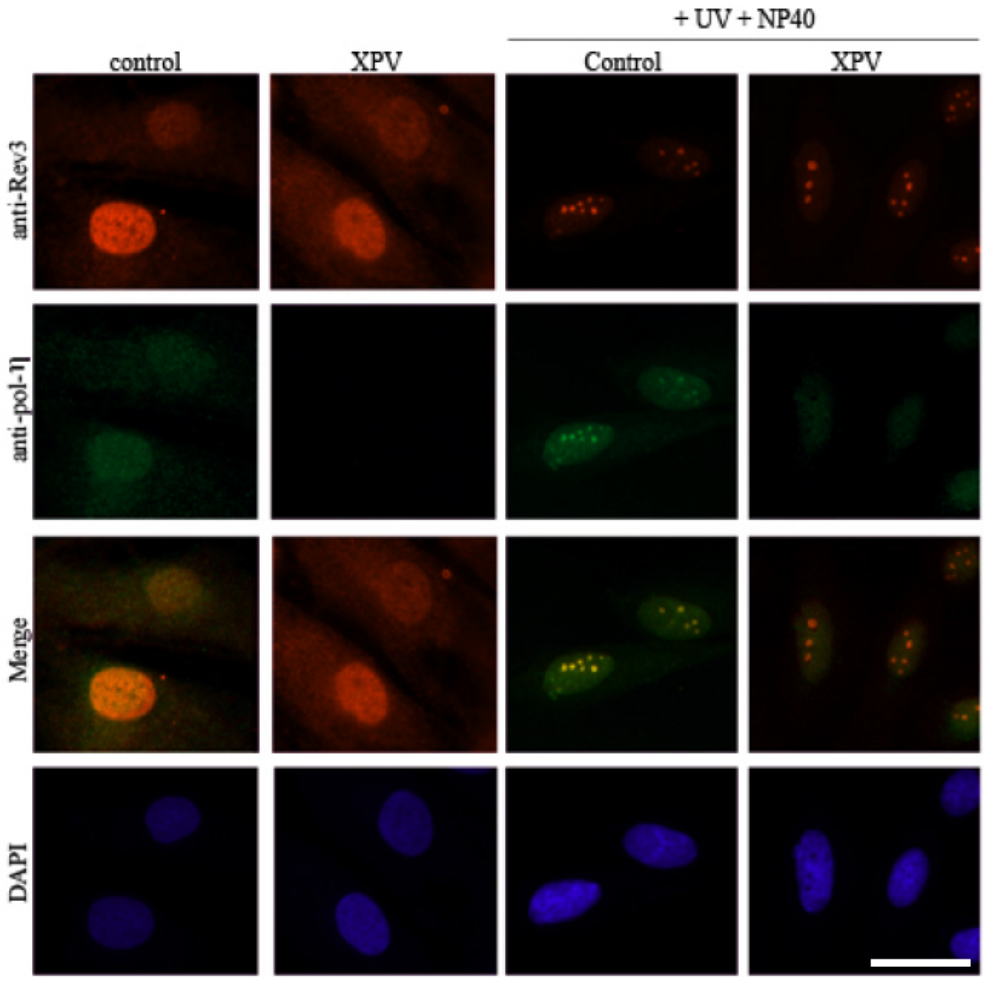

Figure 3-18. Rev3 co-localizes with, but is independent of Pol-η

Low passage fibroblasts derived from an apparently normal person (GM08402, Control) and from an XPV patient, expressing only an N-terminally truncated Pol-η, were used to analyze Rev3 localization using ICC without (left two columns) and with (right two columns) detergent pre-extraction. Although Pol-η protein is absent in XPV cells, Rev3 expression appears to be normal (first two columns). Following UV exposure and NP40 pre-extraction before fixation, UV-induced Rev3 and Pol-η foci did form and co-localized in the control cells (third column). The XPV cells lacking Pol-η still developed UV-induced Rev3 nuclear foci (fourth column). Bar = 10 μm.

column). This result demonstrates that although Rev3 co-localizes with Pol-η following UV treatment, its recruitment to the damage site is independent of Pol-η.

3.2.5. Pol-η and Rev1 are independently recruited to the stalled replication fork

The differential requirement of Rev1 and Pol-η for UV-induced Rev3 nuclear focus formation raised an interesting question of interdependence between Pol-η and Rev1. Surprisingly, despite numerous reports from different laboratories on the nuclear dynamics of Rev1 and Pol-η in response to DNA damage, very little attention has been paid to the *in vivo* interdependence of the two proteins. One report (Tissier et al., 2004) examined the subcellular localization of various YFP-hRev1 derivatives in wild-type and XPV cells and the authors concluded that Rev1 nuclear localization occurs independently of the presence of Pol-η. However, this study relied on experimentally transfected YFP-REV1 cell lines and the authors observed that ectopic over expression of YFP-hRev1 is sufficient to induce nuclear foci in up to 40% of cells in the absence of DNA damage (Tissier et al., 2004), making its physiological relevance questionable. In contrast, spontaneous nuclear foci are not detected for endogenous Rev1 in our experiment (data not shown). In this study, we examined UV-induced co-localization of Pol-η and Rev1 in the nuclear foci and their interdependence. As shown in Figure 3-19 (and Figure 3-16), Pol-η and Rev1 indeed co-localize as nuclear foci. However, suppression of Rev1 does not affect Pol-η nuclear focus formation (Figure 3-19A), nor does inactivation of Pol-η affect Rev1 (Figure 3-19B). These observations allow us to conclude that Pol-η and Rev1 are independently recruited to the stalled

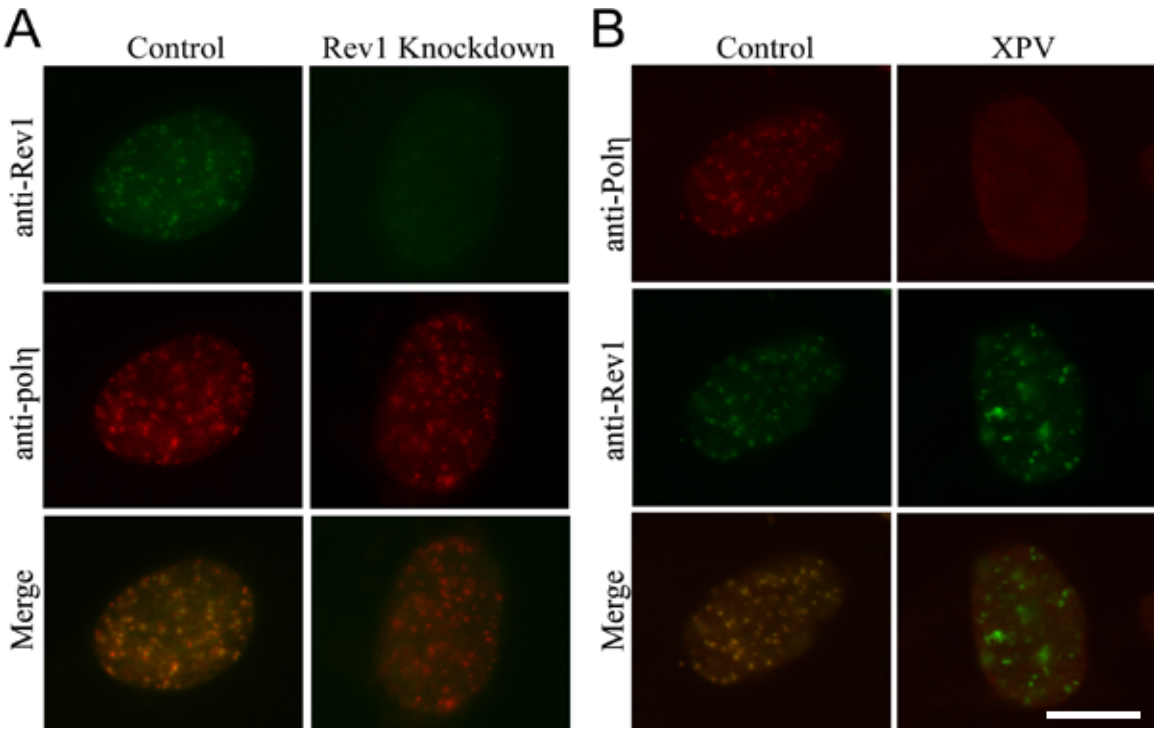

Figure 3-19. UV-induced nuclear foci of Rev1 and Pol-η are co-localized but independent of one another

(A) UV-inducible NP40-resistant nuclear focus formation of Pol-η in the presence (NF1604, left column) or absence (Rz20, right column) of Rev1. Pol-η focus distribution and intensity remains unaltered regardless of the status of Rev1. **(B)** Rev1 nuclear foci are detected after UV treatment and NP40 pre-extraction in both normal human fibroblast cells (control GM08402, left column) and the corresponding XPV cells (GM03617, right column). Note that in both types of cells, UV-induced NP40-resistant nuclear foci of Rev1 and Pol-η co-localize. Bar = 3 μm.

replication forks upon UV-induced DNA damage.

3.2.6. Rev3 is not required for UV-induced nuclear focus formation of Rev1 or Pol-η

After finding that Rev3 is recruited to the stalled replication fork in a Rev1-dependent manner, it would be of great interest to learn whether Rev3 is also required for the recruitment and/or retention of other TLS polymerases, particuarily since Rev3 is postulated in operating in the elongation step of the two-polymerase model of polymerase switching. To this end, we examined the Rev1 and Pol-η nuclear focus formation in NF1604 cells and when the expression of Rev3 is suppressed using interference RNA. We found that suppression of Rev3 does not affect total cellular levels of Rev1 or Pol-η regardless of UV treatment by ICC (data not shown). More importantly, UV-induced NP40-resistant Pol-η (Figure 3-20A) and Rev1 (Figure 3-20B) focus formation, both in terms of approximate number of foci and the focus intensity, is not affected by ablation of Rev3. These observations are consistent with a notion that Rev3 acts downstream of Y-family polymerases in TLS.

The interdependence of UV-induced mammalian TLS polymerase (Pol-η, Rev1 and Rev3/Pol-ζ) focus formation data are summarized in Table 3-1.

3.2.7. Over-expression of GFP-Pol-η induces spontaneous Rev3 nuclear foci

Although we have shown that UV-induced Rev3 nuclear focus formation is dependent on Rev1 but independent of Pol-η, it still remains unclear whether Pol-η can recruit Rev3 to the chromatin in the absence of exogenous DNA damage. To address

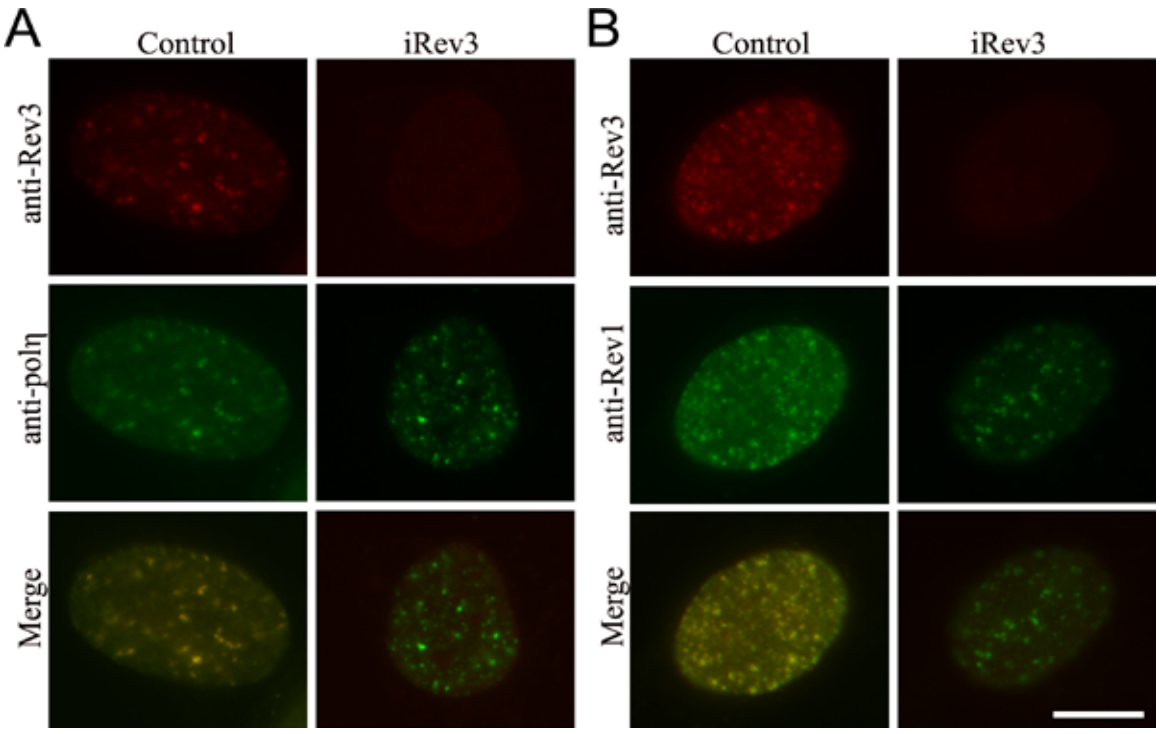

Figure 3-20. UV-induced nuclear focus formation of Pol-η and Rev1 does not depend on Rev3 in normal fibroblasts

ICC on Normal human fibroblasts (GM08402) following detergent pre-extraction was preformed to determine the dependancy of Pol-η and Rev1 localization on Rev3. **(A)** Detection of UV-induced NP40-resistant Pol-η nuclear foci in the presence (left column) and absence (right column) of Rev3. **(B)** Detection of UV-induced NP40-resistant Rev1 nuclear foci in the presence (left column) and absence (right column) of Rev3. Bar = 3 μm.

Table 3-1. Summary of interdependence of UV-induced mammalian TLS polymerase focus formation based on this study.

Experimental data from Figures 3-17, 3-18, 3-19 and 3-20 were compiled to form this table.

		Dependent on		
		Pol-η	Rev1	Rev3
UV-induced foc	Pol-η	--	no	no
	Rev1	no	--	no
	Rev3	no	yes	--

this question, we expressed GFP-Pol-η in HEK-293F cells in an isogenic manner so that all transfected clones are expected to express the same level of GFP-Pol-η. Indeed, the level of GFP-Pol-η expression appears to be homogenous in the cell population, among which appropriately 30% displayed a similar number of spontaneous nuclear foci even without detergent pre-extraction (Figure 3-21). Surprisingly, spontaneous Rev3 nuclear foci were also observed in the same cell population, which co-localize with that of GFP-Pol-η (Figure 3-21A). We suspected that the 30% of cells displaying spontaneous GFP-Pol-η and Rev3 foci were in the S phase. Indeed, these cells turned out to be also PCNA positive and the PCNA nuclear foci co-localize with those of GFP-Pol-η (Figure 3–21B). Taken together, the above observations indicate that in the absence of exogenous DNA damage, excessive GFP-Pol-η is associated to the replication forks and is sufficient to recruit Pol-ζ. Co-migration of Pol-η and Pol-ζ (and possibly Rev1 and other TLS polymerases) with undamaged replication forks may have the potential to enhance mutagenesis due to their low fidelity.

3.2.8. Rev3 in mitotic cells

During the course of Rev3 ICC analysis, we noticed that Rev3 expression is significantly higher in mitotic cells and is localized to condensed chromatin (Figure 3-22A). To confirm this observation, I performed several experiments. First of all, I showed that the strong Rev3 immunoreactivity indeed reflected the Rev3 protein, since synthetic interference RNA specifically against Rev3 suppressed this signal in mitotic cells which are recognizable by a distinct metaphase plate with DAPI staining (Figure

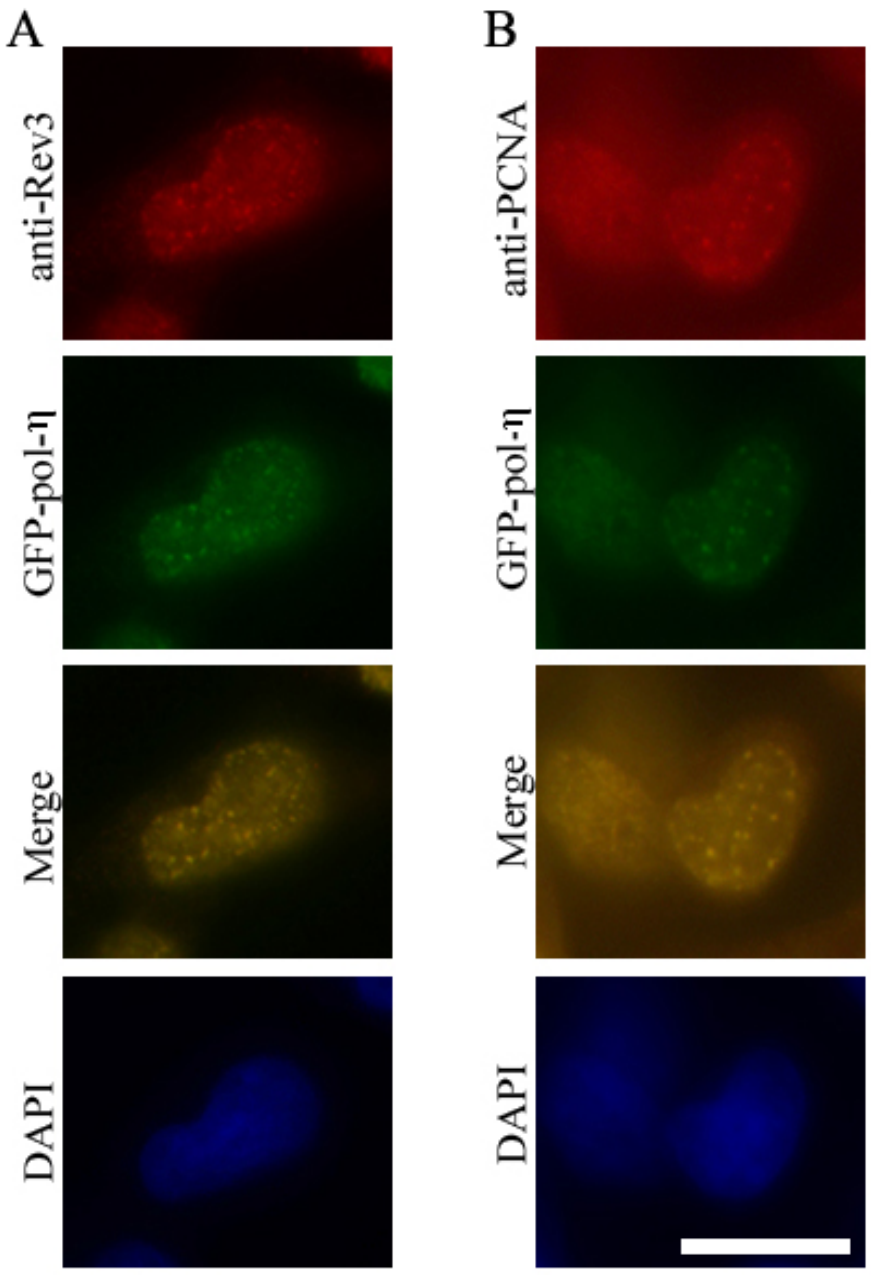

Figure 3-21. Over-expression of Pol-η influences Rev3 localization

HEK-293F cells were stably transfected with GFP-linked Pol-η and processed for ICC without exogenous DNA damage or detergent pre-extraction prior to fixation. GFP-Pol-η nuclear foci spontaneously form as small punctuate nuclear dots that co-localize with (**A**) Rev3 and (**B**) PCNA. Bar = 5 μm.

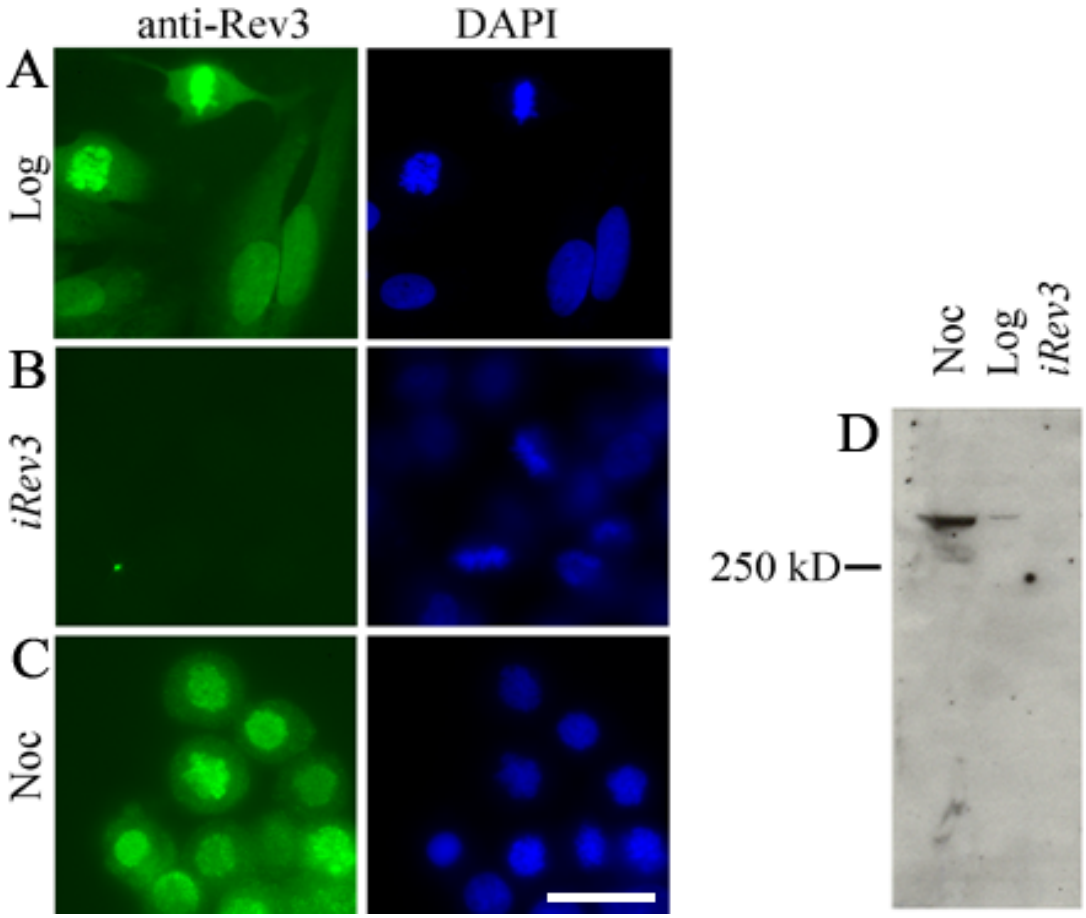

Figure 3-22. Increased levels of Rev3 in mitotic cells and its association with condensed chromatin

(A) Mitotic HCT116 log phase cells revealed strong Rev3 immunoreactivity without detergent pre-extraction (top and left two cells), which is co-localized with DAPI-stained condensed chromatin. **(B)** iRev3 also abolishes Rev3 immunoreactivity in mitotic cells. **(C)** Nocodazole (Noc) treatment (0.5 μg/ml for 16 hours) resulted in 80% of cells arrested in mitosis and all of them displayed the chromosome-associated Rev3 immunoreactivity. **(D)** Western blot analysis reveals that mitosis-enriched HCT116 cells contain a much higher level of Rev3 than the log-phase cells. Sidebars indicate molecular size marker standard used in this experiment. Bar = 10 μm.

3-22B). Secondly, by treating HCT116 cells with 0.5 μg/ml nocodazole for 16 hours, which resulted in up to 80% cells arrested at mitosis as judged by DAPI staining, and these mitotic cells all displayed strong Rev3 immunoreactivity (Figure 3-22C). Finally, in Western blot analysis, cell extract from the above mitosis-enriched HCT116 cells have significantly stronger Rev3 band compared with log-phase cells (Figure 3-22D). It should be noted that unlike the cell line-specific phenomenon of Rev3 nuclear exclusion as described in the previous section, the mitotic Rev3 expression and localization to condensed chromatin in mitotic cells has been observed in all cell lines examined regardless of their immortal or tumorigenic status. The increased level of Rev3 and its association with chromatin in mitotic cells suggest that Rev3 may play a role during mitosis; however, such a mitotic role of Rev3 has not been previously reported.

In order to further address whether the surprising observation of the increased Rev3 level in mitotic cells is related to its reported embryonic lethality, chromosomal instability and cell cycle catastrophe, as asked whether experimental suppression of Rev3 would result in cell death. Stable Rev3 siRNA colonies were established in the HEK-293F cells by site-specific Flp recombinase-mediated transfection and subsequent selection. Within four to six weeks following transfection, cells from Rev3 negative colonies died and the only surviving hygromycin B-resistant colonies regained Rev3 expression, suggesting that these cells lost s*iRev3*-induced Rev3 suppression. In the dying colonies, individual cells exhibited abnormal mitotic patterns with multinucleated

cells, possibly due to improper chromosome segregation (Figure 3-23A). Since cells may die due to reasons other than chromosomal instability, we compared *siRev3* treated cells with cells stably expressing iRNA against Ubc13 (*siUbc13*) in HEK-293F cells. As previously described, Ubc13 appears to be essential. Stable suppression of endogenous Ubc13 in the HEK-293F cells caused death earlier than Rev3 suppressed cells; however, there were no apparent defects associated with cell division as in the *siRev3* clones. (c.f. Figure 3-23A and B). These results are consistent with a notion that Rev3, unlike other Y-family polymerases, plays a critical role in mitotic cells in addition to its role in TLS of DNA damage-induced cells.

3.3. Genetic interactions between mammalian TLS and Ubc13-Mms2 mediated DNA damage response

3.3.1. The parallel-two branch DDT model

One of the characteristic features of budding yeast DDT is the synergistic interaction between mutations in the error-free DDT and TLS pathways. While each single mutant is moderately sensitive to killing by DNA damaging agents, the corresponding *rev3 mms2* (Broomfield et al., 1998) and *rev3 ubc13* (Brusky et al., 2000) double mutants become 10^3-10^4 fold more sensitive than if the effect of two mutations were simply additive; hence, the interaction between error-free DDT and TLS pathways is deemed synergistic (Xiao et al., 1999). This synergistic interaction is explained by the sequential modifications of PCNA (Hoege et al., 2002). Mammalian

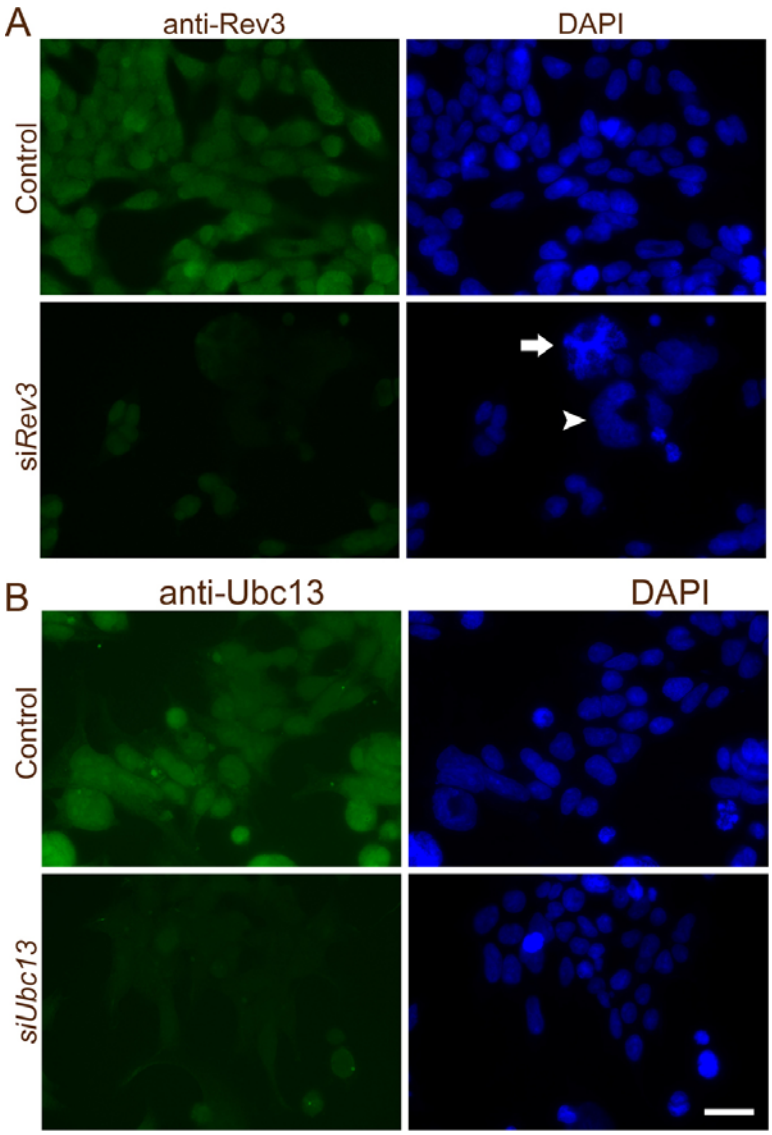

Figure 3-23. Long-term suppression of Rev3 leads to mitotic instability and cell death

(A) HEK-293F cells were stably transfected with a short hairpin construct to permanently repress Rev3. After four weeks Rev3 is apparently suppressed as judged by Rev3 ICC without detergent preextraction and the colonies display multinucleate cells (arrowhead) and abnormal mitotic figures (arrow) characteristic of chromosome non-disjunction. After six weeks of suppression few cells remain viable and those surviving exhibit positive Rev3 immunoreactivity. **(B)** Chronic suppression of Ubc13 in a similar manner also results in cell death within approximately four weeks but without the nuclear abnormalities observed in the iRev3 populations. Bar = 10 μm.

homologs of yeast genes (*RAD5*, *UBC13* and *MMS2*) involved in error-free DDT have been isolated and characterized, including two *MMS2* homologs *MMS2* and *UEV1* (Xiao et al., 1998b), one *UBC13* homolog (Andersen et al., 2005) and two *RAD5* homologs, *HLTF* and *SHPRH* (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2008; Unk et al., 2006). Genes encoding mammalian TLS polymerases, including *XPV/RAD30A/POLH* (Johnson et al., 1999a; Masutani et al., 1999a), *RAD30B/POLI* (Johnson et al., 2000b; McDonald et al., 1999), *DINB1/POLK* (Johnson et al., 2000a; McDonald et al., 1999; Tissier et al., 2000; Zhang et al., 2000a; Zhang et al., 2000b), *REV1* (Gibbs et al., 2000), *REV3* (Gibbs et al., 1998; Xiao et al., 1998a) and *REV7* (Murakumo et al., 2000), have also been isolated. With the experience of characterizing members in each of the above two pathways as presented in this thesis and reagents generated throughout the study, I was then in a position to critically test the hypothesis that the parallel-two branch DDT model also operates in mammalian cells.

3.3.2. Genetic interactions between *Rev3* and *Mms2* or *Ubc13* in mouse cells

During the course of characterizing the distinct functions of Mms2 and Uev1 in cultured mouse 3T3 cells, we have attempted to suppress Ubc13, Mms2 or Uev1 individually by specific RNA interference construct to see if compromised expression of these genes results in an enhanced sensitivity to DNA damaging agents. Transient suppression of each of the above three genes using siRNA did not cause apparent growth retardation within one week following transfection (Figure 3-24A for Ubc13 and Rev3; others not shown). To our surprise, these siRNA treated cells displayed very

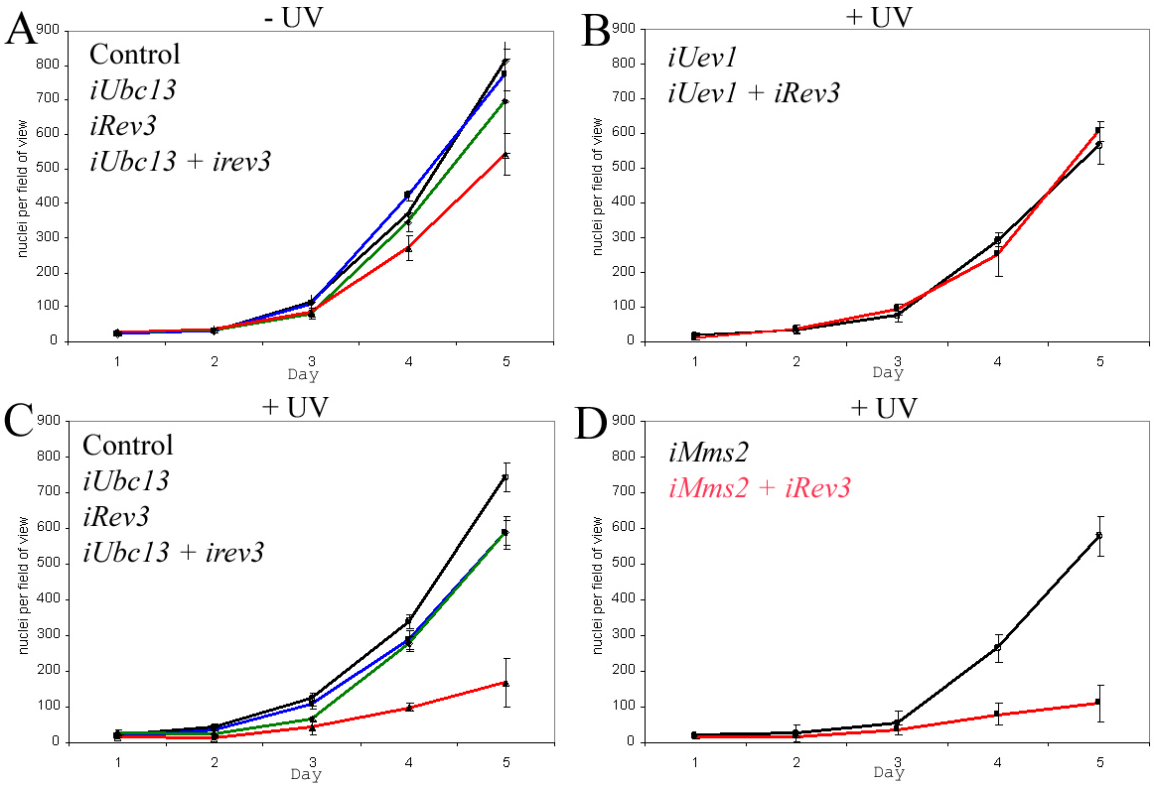

Figure 3-24. Combined effects of Rev3 and Ubc13-Uev suppression by interference RNA in mouse 3T3 cells

Log-phase 3T3 cells were transfected with the siRNA construct(s) as indicated, passaged and analyzed for growth in response to sub-lethal UV irradiation (12J/m^2). Each data point represents an average of ten fields of view and is representative of three individual experiments. Error bars represent standard deviation. **(A)** Growth of non-irradiated cells demonstrating the RNA constructs do not significantly affect cell growth. **(B)** Reduction of Uev1A with and without reduction of Rev3 does not affect the growth response following irradiation. Reduction of either **(C)** Ubc13 and Rev3 or **(D)** Mms2 and Rev3 suppresses the growth response to UV irradiation.

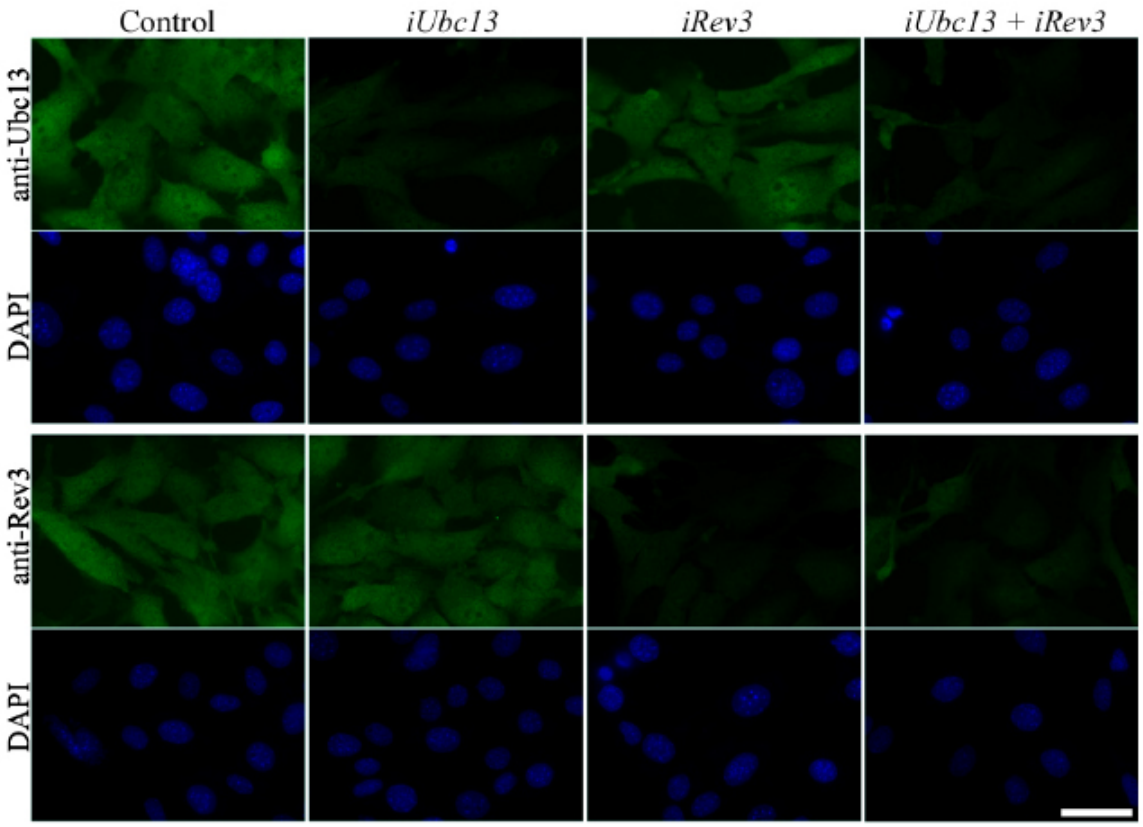

Figure 3-25. Suppression of *REV3* and/or *UBC13* by interference RNA

Cultured HCT116 cells were treated with either *Rev3i*, *Ubc13i* or both as indicated and ICC was performed 3 days after the treatment without detergent pre-extraction. Successful suppression as illustrated in this figure allowed the subsequent assessment of UV-induced growth arrest in matched sister cultures as presented in Figure 3-27.

moderate, if any, sensitivity to 12 J/m^2 UV irradiation (Figure 3-25B-D). In order to enhance the phenotypic effect of suppression of Ubc13-Uev with respect to a DNA damage response, I attempted the simultaneous suppression of the Ubc13-Mms2 complex with Rev3 in mouse 3T3 cells. As shown in Figure 3-24, suppression of Rev3 alone by interference RNA did not result in significant growth retardation within the experimental period (Figure 3-24A) and the enhanced sensitivity to UV was moderate and comparable to that of Ubc13 (Figure 3-24B). Simultaneous suppression of both Rev3 and Ubc13 resulted in a slightly slower growth (Figure 3-24A) but extreme sensitivity to UV irradiation (Figure 3-24B). The combined interference RNA effects are considered to be synergistic. Furthermore, we demonstrated that simultaneous suppression of Rev3 and Mms2 resulted in similar synergistic sensitivity to UV irradiation (Figure 3-24C), whereas the combined suppression of Rev3 and Uev1 resulted in UV sensitivity indistinguishable from that of suppressing Uev1 or Rev3 alone (Figure 3-24D). These observations allow us to draw three important conclusions. Firstly, although Ubc13-Uev1 and Rev3 appear to be essential for cell survival, transient suppression of these gene products, either individually or in combination, does not cause dramatic cell growth defects, although long-term and homogenous suppression (using the Flp integration system) may cause growth arrest. Secondly, individual suppression of any of Ubc13, Uev1, Mms2 or Rev3 did not confer severe UV sensitivity, which is consistent with previous reports that antisense suppression of *REV3* (Gibbs et al., 1998) or *MMS2* (Li et al., 2002a) does not sensitize cells to noticeable UV-induced killing. Finally, in sharp contrast, simultaneous suppression of both Rev3 and Ubc13/Mms2, but not co-suppression of Rev3 and

Uev1A, results in a DNA damage-dependent growth reduction and this effect is apparently synergistic, suggesting that Rev3 and Ubc13-Mms2 form two alternative pathways in mammalian cells to bypass DNA damage-induced replication blocks.

3.3.3. Genetic interactions between *REV3* and *UBC13* in human cells

In order to see whether similar synergistic interaction between Rev3 and Ubc13-Mms2 also exists in cultured human cells, we examined the effects of simultaneous suppression of Rev3 and Ubc13 in HCT116 cells. The interference RNA suppression of each target gene was successful, as judged by ICC against Rev3 and Ubc13 (Figure 3-25). As with mouse 3T3 cells, suppression of either Rev3 or Ubc13 alone in HCT116 had no obvious effects on cell growth or response to UV treatment (Figure 3-26). However, when both Ubc13 and Rev3 were suppressed, the cells became extremely sensitive to UV exposure (Figure 3-26C). This observation confirms that Ubc13 and Rev3 represent two alternative pathways which respond to UV-induced DNA damage.

3.3.4. Genetic interaction between the inactivation of Pol-η and suppression of Ubc13

Pol-η has been demonstrated to play a pivotal role in protecting cells from UV-induced mutagenesis. Indeed all XPV patients were found to contain mutations in the *XPV/POL30A* gene encoding Pol-η and XPV cells display enhanced UV sensitivity and UV-induced mutagenesis (Johnson et al., 1999a; Masutani et al., 1999a). Interestingly, although *RAD30* belongs to the *RAD6* PRR pathway and deletion of the yeast *RAD30* gene also results in a moderately increased sensitivity to UV and UV-induced

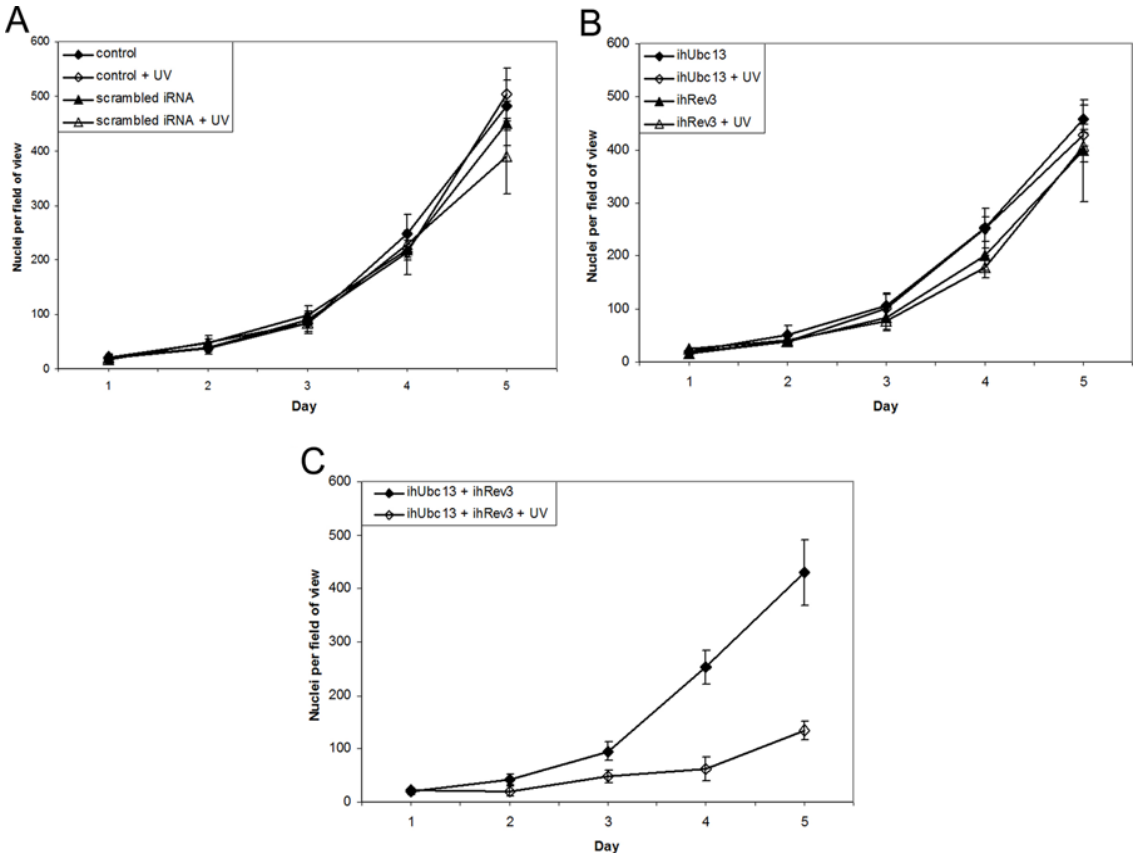

Figure 3-26. Suppression of both Rev3 and Ubc13 exacerbates UV damage in HCT116 cells

HCT116 cells were depleted of either Rev3 or Ubc13 alone, or both as indicated using RNAi and then fixed daily to determine their plating efficiencies and growth in response to a sub-lethal dose of UV irradiation at 13 J/m^2. Each data point represents an average of ten fields of view per treatment and is representative of three individual experiments. Error bars represent standard deviations. **(A)** Growth curve of control (untreated) cells and cells treated with scrambled iRNA demonstrating that neither the iRNA transfection protocol nor the UV dose significantly affects cell growth. **(B)** iRNA directed against either Ubc13 or Rev3 alone had no effect on cell growth regardless of UV irradiation. **(C)** Simultaneous suppression of both Ubc13 and Rev3 resulted in attenuated survival or proliferation in response to UV irradiation. Only experiments that displayed significant reduction in immunoreactivity in sister cultures were assayed.

mutagenesis (McDonald et al., 1997), *rad30* is additive to both *mms2* (Xiao et al., 2000) and *rev3* (McDonald et al., 1997) with respect to killing by UV. Here we examined the genetic interaction between Pol-η and Ubc13 in response to killing by UV irradiation. As shown in Figure 3-27, the low dose UV treatment of wild type cells did not cause observable sensitivity and the interference RNA suppression of Ubc13 did not result in a a statistically significant increase of UV sensitivity. In contrast, XPV cells displayed much stronger UV sensitivity and furthermore, suppression of Ubc13 by synthetic iRNA in the XPV cells resulted in a significant increase in UV sensitivity. This result clearly indicates that Pol-η and Ubc13 act in different survival pathways in response to UV-induced DNA damage.

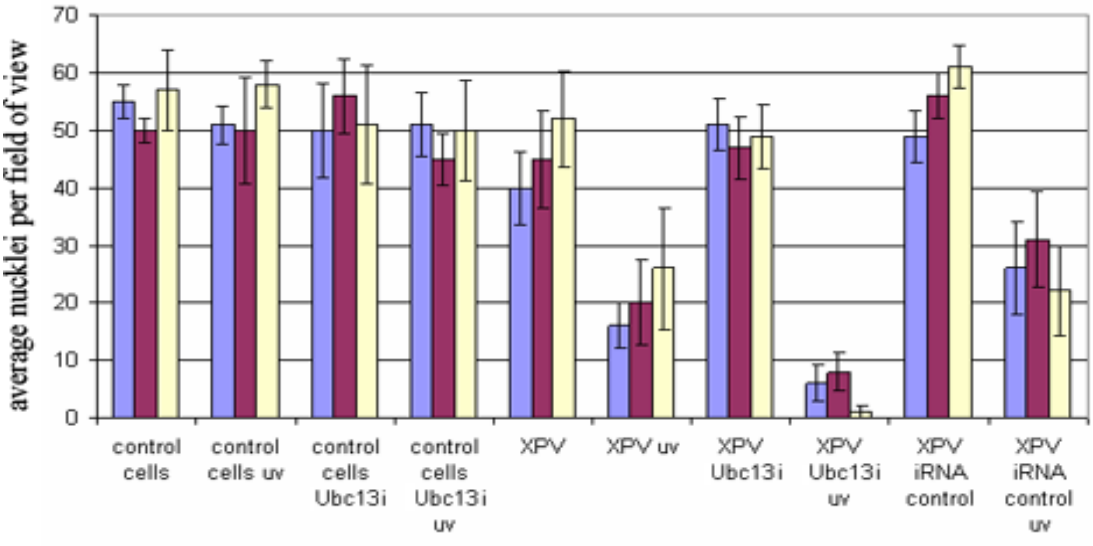

Figure 3-27. Inactivation of Ubc13 and Pol-η results in an enhanced sensitivity to UV irradiation

XPV cells (XP3RO) lacking Pol-η and low passage wild type control cells (GM08402) were treated with interference RNA followed by UV treatment and score for survival. A steady decline in viability from control cells + UV to XPV cells + UV to XPV cells + *Ubc13i* + UV demonstrates an additive effect between Pol-η inactivation and Ubc13 suppression. Three independent experimental results are presented by three bars with different colors, while each bar represents samples from ten fields of view with standard deviations indicated by error bars.

CHAPTER 4

DISCUSSION

4.1. Ubc13-Uev mediated K63 polyubiquitination and cellular stress responses

Cells are facing two rather different types of pressure from either environmental or endogenous influences. Genotoxic stress threatens genome stability, evokes cell cycle arrest and influences DNA repair activity, whereas cellular responses to non-genotoxic stresses influence cell survival and proliferation. Covalent modification of target proteins by Ub and Ub-like proteins is often involved in stress responses. It has been reported recently that Ubc13-Uev and its cognate K63-Ub chain assembly is required for two important stress responses, namely DNA repair and NF-κB activation. It is unclear, however, how Ubc13 is involved in these two seemingly contradictory pathways. Error-free DDT in yeast prevents spontaneous and DNA damage-induced mutagenesis (Broomfield et al., 1998), whereas activation of NF-κB has been described as a primary pro-survival and anti-apoptotic response, and its activity has been linked to various cancers (Dixit and Mak, 2002). Is the error-free PRR pathway conserved in mammals? If it is, how do mammalian cells regulate the two opposite pathways? The discovery of two yeast *MMS2* homologs in human cells, *hMMS2* and *UEV1* (Xiao et al., 1998b) provides a key to solve the paradox; however, the sequence alignment and reports to date do not provide adequate information as to which Uev is involved in which pathway. The situation becomes even more complicated by the observation that

expression of either *hMMS2* or *UEV1* is able to rescue the yeast *mms2* mutant from killing by DNA damage, and that both Mms2 and Uev1 are able to support K63 poly-ubiquitination *in vitro* leading to NF-κB activation (Deng et al., 2000; Zhou et al., 2004). Here, we provide evidence that different Ubc13 activities are modulated by the two Uevs that act as regulatory subunits for K63-mediated target modification. This discovery may reveal a novel regulatory mechanism for the stress response in human cells.

4.1.1. Ubc13-Mms2 and the DNA damage response

Lower eukaryotes such as budding and fission yeasts contain a single Ubc13 and its Uev partner, Mms2, which is essential for error-free PRR (Broomfield et al., 1998; Brown et al., 2002; Brusky et al., 2000). The Ubc13-Mms2 activity in yeast results in poly-ubiquitination of PCNA following its mono-ubiquitination at the Lys164 residue by the Rad6-Rad18 complex (Hoege et al., 2002); PCNA modified by K63-linked poly-Ub chain probably acts to switch the mode of damage tolerance from translesion DNA synthesis and genome instability mediated by mutagenic DNA polymerases (Haracska et al., 2004; Stelter and Ulrich, 2003) into an error-free DDT mode via sister chromatid exchange and/or template switching (Pastushok and Xiao, 2004). We demonstrate in this study that the Ubc13-Mms2 complex in mammalian cells probably inherits the same activity. First, upon DNA damage, Ubc13 and Mms2 form nuclear foci with newly synthesized DNA, suggesting that this complex resides at or near the replication fork. Second, ablation of either Ubc13 or Mms2 results in increased spontaneous DNA strand breaks that induce Rad51 and γH2AX nuclear focus

formation. Third, Mms2 and Ubc13 are retained in the S-phase nucleus and partially co-localize with PCNA. Finally, we also observed that suppression of either Ubc13 or Mms2 results in increased sensitivity to killing by UV irradiation which appears to be synergistic with simultaneous suppression of Rev3. This is comparable to the corresponding yeast mutant phenotypes (Broomfield et al., 1998) and agrees with observations (Li et al., 2002a) that antisense suppression of *hMMS2* results in phenotypes characteristic of error-free PRR defects. In contrast, iRNA suppression of *UEV1* does not share the above phenotypes, nor is Uev1 found in S-phase or damage-induced nuclear foci. Hence, Uev1 is not involved in a DNA damage response.

In summary, we found that *UBC13* and *MMS2* not only protect mammalian cells from genome instability caused by environmental DNA damage, but probably also prevent spontaneous DNA damage or replication fork collapse during S-phase of the cell cycle. When *UBC13* or *MMS2* expression is compromised, cells probably accumulate DSBs that in turn induce Rad51 and γH2AX nuclear focus formation. Our observation that interference RNA suppression of *UBC13* and *MMS2* only induces Rad51 foci but not Mre11 foci, indicates that DSBs accumulated, likely due to lack of K63-Ub chain signaling, and would have to be repaired by homologous recombination instead of non-homologous end joining. This is consistent with a previous observation that suppression of *hMMS2* completely abolishes UV-induced gene conversion (Li et al., 2002a).

4.1.2. Other Ubc13-mediated DNA damage responses

Biochemical and structural analyses of the Ubc13-Uev complex indicate that in the absence of a Uev (Mms2 or Uev1A), Ubc13 likely behaves as a typical E2 Ub-conjugating enzyme capable of conjugating an activated Ub to its active site and interacting with cognate E3s. However, Ubc13 forms an efficient and stable complex with a Uev (Mms2 or Uev1A), which specifically promotes K63-linked poly-Ub chain formation. It is conceivable that Ubc13 alone may confer biological functions independent of a Uev; however, it is most likely that this activity is also independent of the K63-linked Ub chain formation. Conversely, if K63-Ub chain is involved in any given process, one would predict that a Uev cofactor is required.

Recently, several laboratories reported E3 mediated DNA damage responses that require Ubc13 as a cognate E2. Specifically, RNF8, an FHA-containing E3, is required for the recruitment of BRCA1 and 53BP1 to γ-ray induced DSBs and this function of RFN8 requires Ubc13 as cognate E2 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). However, a thorough analysis indicates that this process is independent of Uev (Huen et al., 2008). This appears to be consistent with an earlier report (Zhao et al., 2007), in which chicken DT40 cells deleted for Ubc13 or HeLa cells suppressed for Ubc13 by interference RNA resulted in compromised homologous recombination. However, suppression of Mms2 and/or Uev1 did not lead to the same phenotype. More recently, RNF168, another FHA-containing E3 was identified as a cofactor of RFN8 (Doil et al., 2009; Stewart et al., 2009). The current model indicates that although RFN8 is necessary to trigger DSB-associated ubiquitination of H2A and H2AX, it requires RNF168 to sustain and amplify

the signal, likely by promoting the formation of K63-linked poly-ubiquitination of H2A/H2AX. In this context, it remains unclear whether or which Uev is required for the RNF168 function.

Although several reports have shown that ionizing radiation induced BRCA1 nuclear focus formation required RNF8, RNF168 and Ubc13, one report (Zhao et al., 2007) demonstrated that spontaneous Rad51 foci did not increase in DT40 or HeLa cells lacking Ubc13 activity (Zhao et al., 2007), which is in contrast to our own observations. The authors also showed that inactivation of Ubc13 resulted in a reduced DSB repair and lack of RPA/ssDNA nucleofilament formation, which has been attributed to the lack of resection to generate 3' ssDNA at DSBs following loss of Ubc13. We noticed that the authors also reported a severe growth defect in these Ubc13 deficient cells, which is consistent with our long-term Ubc13 suppressed cells (3-4 weeks) but different from our short-term Ubc13 suppressed cells (within one week). To further confirm that lack of Ubc13 indeed results in an increased DSB, we monitored the γH2AX nuclear focus formation in control and Ubc13 suppressed cells, which is a direct detection of spontaneous DSBs. Lack of Ubc13 resulted in a more than threefold increase in cells with γH2AX nuclear foci, which supports our initial conclusion (Andersen et al., 2005). Interestingly and in support of our argument, it appears that suppression of RNF168 also increases spontaneous γH2AX nuclear focus formation (Figure 1D of Stewart et al., 2009), although this phenomenon was not elaborated by the authors.

4.1.3. Ubc13-Uev1 and NF-κB activation

Prior to this study, it was unclear which Uev is involved in TRAF2- and TRAF6-mediated NF-κB activation, since both Mms2 and Uev1A are able to mediate the activity *in vitro*. Genetic analysis clearly demonstrates that it is Ubc13 and Uev1, but not Mms2, that are required for TRAF2- and TRAF6-mediated NF-κB activation, and that this activity is in a step upstream of IKK but downstream or in concert with TRAF2 and TRAF6 (Andersen et al., 2005). This study further demonstrates that Uev1, but not Mms2, is required for TRAF6-induced NEMO poly-ubiquitination. This conclusion fits well with our previous observation that NEMO serves as the K63 chain target (Zhou et al., 2004) and supports a recently proposed model (Sun and Chen, 2004), in which the MALT1 oligomers bind to TRAF6, induce TRAF6 trimerization and activate the E3 activity of TRAF6 to poly-ubiquitinate NEMO in the presence of Ubc13-Uev.

NF-κB activation has been described as a pro-survival and anti-apoptotic response to bacterial and viral infections and other environmental stresses. To address the physiological relevance of Ubc13-Uev1 in stress response, we showed that lipopolysaccharide-induced NF-κB activation in primary microglia cells requires both Ubc13 and Uev1, but not Mms2. Constitutive activation of NF-κB is linked to cancers such as lymphoma and other human diseases (Dixit and Mak, 2002). Interestingly, human *UEV1* has been independently isolated by its ability to transactivate the *c-fos* promoter (Rothofsky and Lin, 1997), and its transcript level increases when SV40-transformed human embryonic kidney cells undergo immortalization (Ma et al., 1998) and decreases upon differentiation of the human colon carcinoma cell line HT-29-M6

(Sancho et al., 1998). Furthermore, the *UEV1* mRNA level is elevated in all human tumor cell lines examined compared to normal tissues (Xiao et al., 1998b). Furthermore, the gene is located to chromosome 20q13.2, a region where gene amplification is frequently observed in breast cancer (Brinkmann et al., 1996; Kallioniemi et al., 1994; Tanner et al., 1994) and other tumors (El-Rifai et al., 1998), as well as in virus-transformed immortal cells (Savelieva et al., 1997). These observations collectively place *UEV1* as a candidate proto-oncogene. Indeed, our laboratory found that experimental over-expression of *UEV1A* is sufficient to activate NF-κB and inhibit apoptosis (Syed et al., 2006). Conversely, the tumor suppressor gene product CYLD appears to be a K63-specific deubiquitination enzyme responsible for the removal of Ub from NEMO (Brummelkamp et al., 2003; Kovalenko et al., 2003). Hence, Uev1 serves as a regulatory subunit for Ubc13-mediated K63-linked Ub chain assembly and may be an excellent target for cancer therapy.

4.1.4. Mms2 vs. Uev1A: a novel regulatory mechanism?

Protein ubiquitination and its related processes have been unveiled as a versatile mechanism to regulate protein activities in eukaryotes. Our data present a previously undescribed mechanism by which different Uev molecules act as mutually exclusive regulatory subunits of an E2 (Ubc13) to different subcellular locations and/or to modify different target proteins. While Uev1A is required for NEMO poly-ubiquitination in the cytoplasm, mammalian Mms2, if it behaves like its yeast homolog, may be involved in the ubiquitination of PCNA in the nucleus.

Sensing and repairing DNA damage and NF-κB activation are two rather distinct cellular processes and lead to opposite cellular consequences. While the former will arrest cell cycle progress until DNA synthesis is complete or, if the damage is too severe to repair, cause apoptosis, the latter will promote cell survival by inhibiting apoptosis. How do cells sense these different stresses and respond correctly? Our findings suggest that the two highly conserved but functionally distinct Uevs may play a central role in this decision-making process. Three alternative, but not mutually exclusive mechanisms may be envisioned to achieve such regulation. First, Uev1A and Mms2 may compete for binding to limited Ubc13 in the cell. It is interesting to notice that structural analyses (McKenna et al., 2001; Moraes et al., 2001; VanDemark et al., 2001) have shown that the Ubc13-Uev heterodimer formation is in a 1:1 ratio, that the binding affinity of Ubc13 for Mms2 and Uev1A is comparable (McKenna et al., 2003), and that Mms2 and Uev1A are distributed in both cytoplasm and the nucleus. Hence, Uev1A can readily compete with Mms2 to prevent it from forming a complex with Ubc13 in the nucleus. The fact that Uev1A is found in the nucleus with no defined role described thus far suggests that perhaps Uev1A competes with Mms2 in the nucleus for binding to Ubc13 and acts as an antagonistic factor. This possibility is particularly attractive, since it would also explain its oncogenic property, as inhibition of error-free PRR in yeast cells results in a massive increase in spontaneous mutagenesis (Broomfield et al., 1998), which would lead to genome instability and tumorigenesis in mammals. Second, cellular Ubc13 may not be limited; however, it is not activated until binding to a cognate Uev. This hypothesis predicts that Mms2 and Uev1A are differentially activated depending on the source of stress (e.g., genotoxic vs. non-

genotoxic), and that the activated Ubc13-Uev complex determines pathway specificity by either associating with a specific E3, or other cellular components. The third possible mechanism is that Mms2 and Uev1 selectively activate target proteins through di- and poly-ubiquitination, respectively, as demonstrated in this study. We have demonstrated that NEMO is poly-ubiquitinated by Ubc13-Uev1A *in vivo*. In contrast, despite repeated reports on mono-ubiquitinated PCNA (Kannouche et al., 2004; Watanabe et al., 2004), it is not yet known whether PCNA is further di- or poly-ubiquitinated *in vivo* or *in vitro*. Nevertheless, we are able to show that the N-terminal extension of Uev1A is probably the determinant of functional specificity, and that the core domain of Uev appears to play a default function in DNA repair.

In summary, we demonstrate that Ubc13-mediated ubiquitination can coordinate cellular responses to both DNA damage as well as non-genotoxic stresses; its target selection and mode of response (e.g., DNA repair or cell proliferation) is determined not only by E3 proteins, but also by a Uev as its regulatory subunit. Hence, we propose that the Uevs serve as an essential modulator of E2 ubiquitination activity. A working model based on the above analyses is depicted in Figure 4-1. It should be noted that the above possibilities are not mutually exclusive. For example, Uev1A can recruit Ubc13 to a process that directly promotes tumorigenesis and meanwhile prevent Ubc13-Mms2-mediated error-free DDT.

Conventional selectivity of ubiquitin addition is thought to be under the direct influence of E3 enzymes, which target specific substrates. Here we describe a novel mechanism that regulates the type and length of ubiquitin chains and potentially the target proteins. Given recent reports that K63-Ub chains are involved in diverse

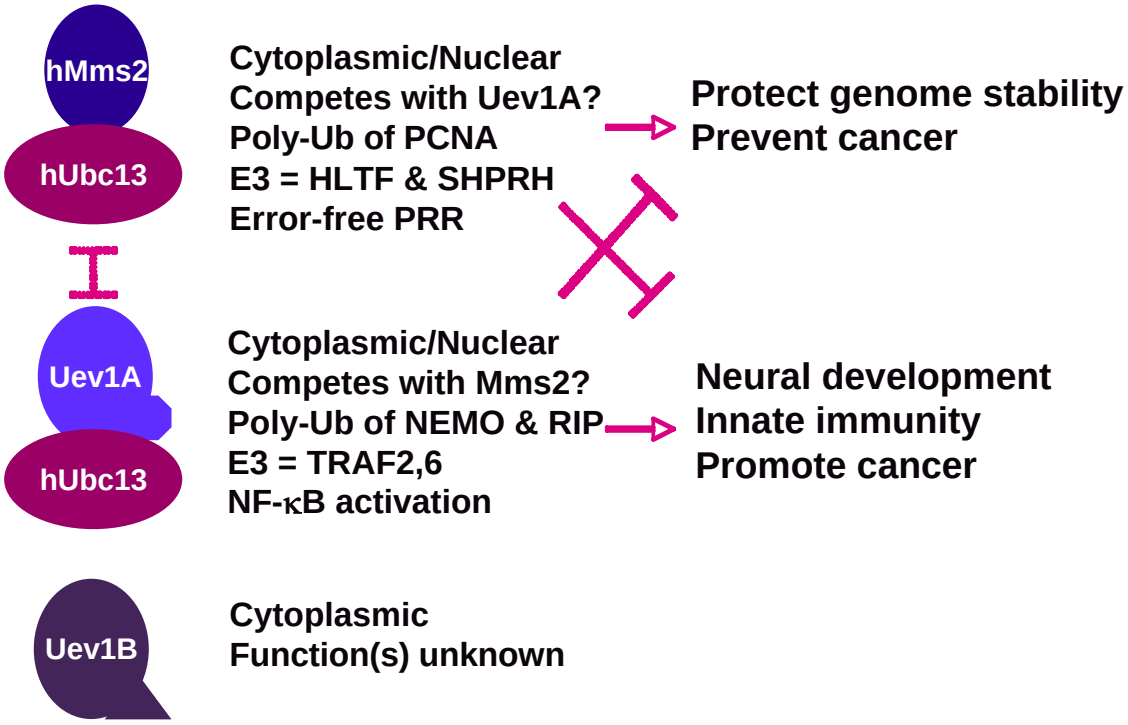

Figure 4-1. A working model of Ubc13-Uev functions in mammalian cells

This model is based on data and discussion presented in this thesis as well as some previous reports. Additional Ubc13-Uev functions accumulated lately in the literature are not included in this model.

functions such as DNA repair (Broomfield et al., 1998), stress response and immunity (Deng et al., 2000; Wang et al., 2001; Zhou et al., 2004), neurodegeneration (Doss-Pepe et al., 2005; Lim et al., 2005), ribosomal activity (Spence et al., 2000), endocytosis (Galan and Haguenauer-Tsapis, 1997), that some of them do require Ubc13-Uev (Bothos et al., 2003; Doss-Pepe et al., 2005), and that additional Uev proteins have been identified with known or unknown activities (Wong et al., 2003), our findings shed light on the diversity and complexity of the ubiquitination pathways.

4.2. Translesion DNA synthesis in response to UV irradiation

4.2.1. Nuclear focus formation of endogenous TLS polymerases

DNA damage-induced nuclear focus formation has been employed as an important tool to characterize a number of proteins involved in DNA damage response. Lehmann and his colleagues used this method to elegantly demonstrate that following treatment of cells with UV, Pol-η accumulates at replication foci stalled at the damage site (Kannouche et al., 2001). Subsequently, it was shown that Rev1 co-localizes with Pol-η at stalled replication forks (Tissier et al., 2004). One caveat for both of the above studies is that the authors used cells ectopically expressing the genes of interest to facilitate functional domain analysis and, to our knowledge, DNA damage-induced nuclear focus formation of endogenous Pol-η or Rev1 has not been reported. Furthermore, it is unclear whether UV-induced Pol-η nuclear focus formation is dependent on Rev1. Here we report the generation of Rev3-specific antibody and characterization of three TLS polymerases, Pol-η, Rev1 and Rev3, in their native form. We found that UV-induced detergent-resistant (hence chromatin-containing) nuclear

foci of all three TLS polymerases co-localize with each other as well as with PCNA, which provides strong evidence that the TLS factory is assembled at the stalled replication fork. A few discrepancies are noted between this and previous reports (Kannouche et al., 2001; Tissier et al., 2004). Firstly, unlike previous reports, we did not observe detergent-resistant nuclear foci in the absence of UV treatment, which is probably due to the low level of endogenous TLS polymerases detected in this study in contrast to the transfected cell lines in the published reports. Secondly, there are fewer but apparently larger detergent-resistant nuclear foci in our study compared to the previous reports. Although the difference is most likely attributed to variations in the detergent pre-extraction procedures, we also feel that the transfected cell lines used in other studies may be primarily responsible for the discrepancy, since under our experimental conditions the GFP-Pol-η transfectants also display nuclear focus images similar to those reported.

4.2.2. Dynamic recruitment of TLS polymerase to UV-induced damage sites

The current protein interaction data could be compatible with several possibilities on how the TLS polymerases are assembled. To determine the actual order of such an assembly, we utilized different methods to reduce or eliminate one TLS polymerase and examined the UV-induced nuclear focus formation of the two remaining polymerases. As summarized in Table 3-1, we found that Pol-η and Rev1 are independently recruited to the stalled replication fork, that Rev3 recruitment requires Rev1 but not Pol-η, and that suppression of Rev3 does not affect the assembly of either Pol-η or Rev1. This order of assembly could be consistent with observations that both

the PCNA interaction domain and the Ub-binding domain of Pol-η (Bienko et al., 2005; Kannouche et al., 2004) and Rev1 (Guo et al., 2006a; Guo et al., 2006b) are required for the nuclear focus formation and *in vivo* functions, as well as for their *in vitro* TLS activities (Garg and Burgers, 2005; Haracska et al., 2001), but cannot explain the significance of the direct interaction of Rev1 with Pol-η (Guo et al., 2003). Our result does not favor a notion that Pol-η interacting with Rev1 stabilizes the complex at the stalled replication fork, since depleting either component does not appear to affect the number or intensity of the other within nuclear foci. It is highly possible though that Rev1 may assist Pol-η for translesion synthesis, or alternatively that once both Pol-η and Rev1 are independently recruited to the stalled fork by ubiquitinated PCNA, their physical interaction provides a functional bridge for polymerase switching (Figure 4-2). Indeed, we found that Rev3 is recruited by Rev1 to the stalled fork independently of Pol-η. This observation invokes two competitive scenarios, as illustrated in Figure 4-2. Firstly, it supports the notion that Rev1 serves as a trading place for the polymerase switch (Friedberg et al., 2005), in this case between Pol-η and Pol-ζ, for the translesion insertion and extension of the two step model, respectively. Alternatively, if the lesion is not a UV-induced thymine dimer, other TLS polymerases may be preferentially used over Pol-η at the insertion step. For example, it has been speculated that Pol-κ is specialized for lesions induced by chemicals such as benzo[*a*]pyrene (Ogi et al., 2002), which induces Pol-κ nuclear foci instead of Pol-η foci (Bi et al., 2005). Secondly, when the preferred TLS polymerase is not available or the lesion cannot be preferentially recognized by a specialized TLS polymerase, Rev1 may insert Cs

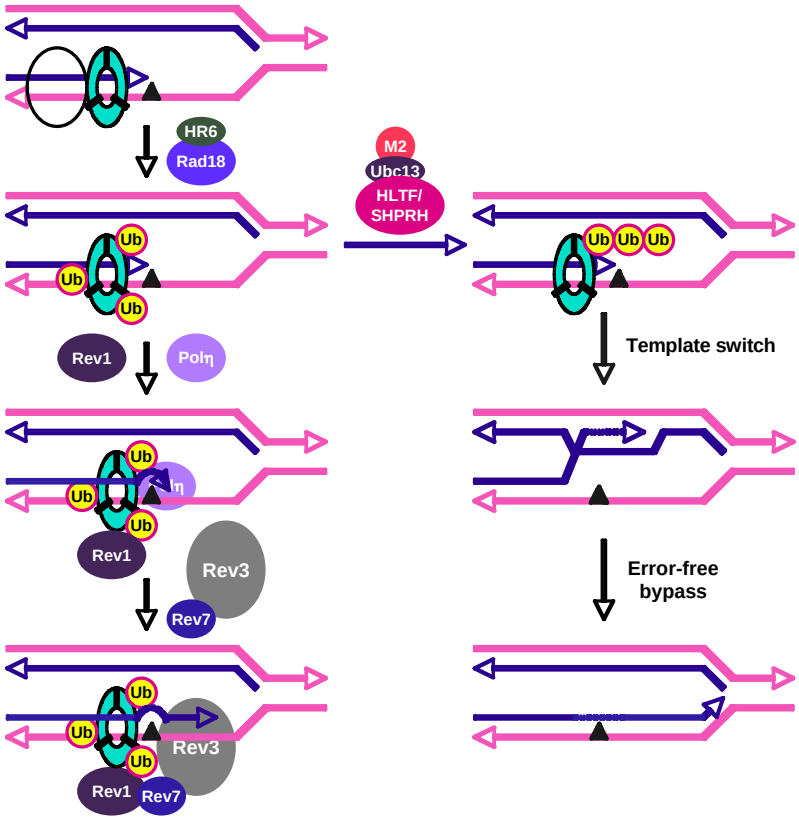

Figure 4-2. A proposed model of DDT

Upon UV irradiation, the replication machinery is stalled at the damaged template and invokes PCNA mono-ubiquitination by the HR6A/HR6B-Rad18 complex. Rev1 and Pol-η are recruited to the damage site and Pol-η is able to insert AA across from the thymine dimer with or without assistance from Rev1. Meanwhile, Rev1 is able to recruit Rev3 to the damage site probably through its interaction with the Rev7 subunit, and the Rev1-Pol-η interaction brings Rev3 into proximity to replace Pol-η for primer extension. It is noted that for a different type of DNA damage, another preferred TLS polymerase may replace Pol-η for a similar reaction. This model also predicts that in the absence of Pol-η, Rev1 (or another TLS polymerase) may serve to perform translesion insertion, followed by Rev3 extension. On the other hand, mono-ubiquitinated PCNA can be further modified by Mms2-Ubc13-HLTF/SHPRH to form Lys63-linked poly-Ub chain(s), which appears to promote error-free lesion bypass through template switching. The detailed molecular events of this process are unclear.

opposite the damaged template, followed by Pol-ζ extension, which is expected to be highly mutagenic. The latter scenario may account for the observed XPV phenotypes with enhanced mutagenesis and predisposition to cancer. Indeed, Rev1 and Pol-ζ are responsible for most spontaneous and damage-induced mutagenesis in budding yeast (Lawrence, 2004), and *in vitro* studies indicate that yeast PCNA (Garg and Burgers, 2005) or Rev1 (Acharya et al., 2006) is able to enhance TLS by Pol-ζ. Additionally, experimental suppression of mammalian Rev3 results in a decrease in mutagenesis induced by either benzo[α]pyrene (Li et al., 2002b) or UV (Diaz et al., 2003), suggesting that Rev3 is responsible for bypassing a broad range of lesions. The above argument is further strengthened by a recent report (Shachar et al., 2009) of two-polymerase mechanisms dealing with different lesions in mammalian cells, in which Pol-ζ (Rev3) appears to be required for bypass of several lesions examined except a TT cyclobutane dimer, regardless of wether the bypass is error-prone or error-free.

Currently it is unclear how Rev3 is recruited to the stalled replication fork by Rev1. A likely candidate is Rev7, since it binds to the C-terminal domain of Rev1 (Guo et al., 2003) and also forms a stable complex with Rev3 (Murakumo et al., 2000). Nonetheless, we notice that in addition to the reported Rev1-Rev7 interaction (Acharya et al., 2005), the yeast Rev3 can also directly interact with Rev1 and this interaction appears to be essential for Rev1 function (Acharya et al., 2006). Unfortunately, due to the extremely large size of *hREV3*, its functional domains remain to be further characterized.

4.2.3. Rev3 recruitment to the replication forks in the absence of DNA damage

TLS polymerases are inherently mutagenic so they are likely regulated very stringently to minimize their activity unless they are required. However, over-expression of GFP-tagged Pol-η and Rev1 have been shown to spontaneously form punctate nuclear foci in the absence of DNA damage treatment with unknown consequences (Tissier et al., 2004). DNA damage-induced focus patterns (e.g., more and smaller foci) using these GFP-Pol-η over-expressing cells are also different from that reported in this thesis. We were able to reproduce the reported focus formation pattern by using the GFP-Pol-η construct. Since the Flp-mediated integration system was used, we were able to achieve homogenous expression in transfected cells. This expression resulted in nearly all S-phase cells with spontaneous GFP-Pol-η nuclear foci co-localizing with PCNA, suggesting that GFP-Pol-η co-migrate with most if not all replication forks. One possibility is that GFP-Pol-η slows down the replication fork migration and causes the assembly of damage response factors, such as Rev3, to form foci. Another possibility is that GFP-Pol-η directly recruits other factors to form large protein-DNA complexes. To our surprise, regardless of the mechanisms of GFP-Pol-η and PCNA focus formation, Rev3 is recruited to the same sites. It would be of great interest of know how Rev3 is recruited to the sites since under UV treatment, Pol-η is not required for the Rev3 focus formation. Obviously a candidate would be Rev1, which interacts with both Pol-η and Rev3.

4.3. Reconstitution of the mammalian DDT model

4.3.1. Two alternative DDT pathways in mammals

As discussed before, the parallel-two branch model of PRR/DDT has been well established in yeast and the molecular mechanisms are relatively understood. In genetic terms, the error-prone PRR pathway is represented by *REV1, 3* and *7*, whereas the error-free pathway is represented by *RAD5*, *UBC13* and *MMS2*. Interestingly, RAD30 encoding yeast Pol-h (the equivalent of mammalian Pol-η) does not appear to belong to either of the above pathways (Xiao et al., 2000). Given the central role played by *REV3* in TLS and *UBC13/MMS2* in error-free PRR in yeast, we hypothesized that mammalian DDT also consists of two parallel pathways. In this study, we have demonstrated that mammalian *REV3* plays a central role in TLS. We have also documented that the mammalian Ubc13/Mms2 complex likely plays a role in an error-free mode of DDT like its yeast counterpart (Andersen et al., 2005; Li et al., 2002a). Here we demonstrate, using two different interference RNA systems and two different cell lines, that while ablation of either Ubc13 or Rev3 has little effect on cell growth following a sub-lethal dose of UV irradiation, simultaneous suppression of both genes results in a synergistic interaction with respect to UV sensitivity, which is reminiscent of the synergistic interaction observed in yeast (Broomfield et al., 1998; Brusky et al., 2000) and testifies that the molecular mechanisms of DDT are highly conserved within eukaryotes, from yeast to human. Interestingly, we found that suppression of Ubc13 in a Pol-η null mutant (XPV) cell line resulted in only moderate increase of UV sensitivity compared to XPV cells, which is also consistent with the additive effect observed in yeast when both *MMS2* and *RAD30* are deleted (Xiao et al., 2000).

4.3.2. Sequential modifications of PCNA in mammalian cells

It has been well accepted that in budding yeast, PRR/DDT is achieved by sequential modifications of PCNA. While mono-Ub facilitates TLS, poly-Ub promotes error-free bypass. The former is thought to be mediated by an increased affinity of ubiquitinated PCNA for Y-family TLS polymerases (Bienko et al., 2005), whereas the molecular events leading to error-free bypass of replication-blocking lesions by poly-ubiquitinated PCNA are largely unknown. In addition, yeast PCNA can be sumolated at the same Lys164 residue, which is thought to recruit the Srs2 helicase (Papouli et al., 2005; Pfander et al., 2005) which prevents Rad51-ssDNA filament formation (Krejci et al., 2003; Veaute et al., 2003) and inappropriate recombination.

The mechanisms of PCNA mono-ubiquitination and its recruitment of Y-family polymerases have been the subject of extensive research in mammalian cells (Andersen et al., 2008). In contrast, inducers and factors required for PCNA poly-ubiquitination have not been well characterized and current literature, in our judgment, is inconsistent. First of all, reports on the detection of poly-ubiquitinated PCNA frequently show a smear on Western blots instead of the expected ladders representing PCNA poly-Ub. Secondly, the cognate E3 responsible for PCNA poly-ubiquitination remains unclear. Two laboratories independently identified SHPRH (Motegi et al., 2006; Unk et al., 2006) and HLTF (Motegi et al., 2008; Unk et al., 2006) as cognate E3s for Ubc13 and both appear to be absolutely required for PCNA polyubiquitination; however, how these two E3s coordinate in the same process is unclear. Furthermore, another RING and FHA-containing E3, RNF8, was also reported to be required for damage-induced

PCNA polyubiquitination (Zhang et al., 2008). Last but not least, it has been reported that inactivation of Mms2 does not affect PCNA poly-ubiquitination in mammalian cells (Brun et al., 2008), which is inconsistent with the structural model and data presented in this thesis work. Applying reported experimental conditions, we readily detected UV-induced and dose-dependent PCNA mono-ubiquitination. However, numerous attempts to detect PCNA poly-ubiquitination have failed, which has prevented us from asking what are required for PCNA poly-ubiquitination in mammalian cells.

4.3.3. Lesion-specific nuclear focus formation

Throughout this study, we have utilized a variety of cell lines to examine Ubc13/Mms2 and Rev3 focus formation in response to a wide range of DNA damage. Among DNA damaging agents employed (UV, methyl methane sulfonate, mitomysin C, CPT and cisplatin), only CPT treatment yields detergent-resistant Ubc13 and Mms2 nuclear foci, whereas UV treatment instead of CPT generates ideal TLS polymerase positive nuclear foci. In contrast, without cell damage different cell lines do not display dramatic differences in the immunoreactivity of Ubc13 or Rev3. CPT and UV cause two very different types of DNA damage, which suggests that Ubc13-Mms2 and Rev3 may be involved in DDT of different types of DNA damage. However, we cannot rule out the possibility that the observed difference simply reflects some unknown technical issues. This technical difficulty prevented us from asking whether Ubc13/Mms2 and Rev3 co-localize to the same nuclear foci following the same DNA damage treatment. Nevertheless, in the genetic studies reported here, suppression of Ubc13/Mms2 and

Rev3 resulted in a synergistic interaction with respect to UV-induced cell survival, indicating that both pathways indeed are involved for alternative bypass mechanisms for the same type of DNA damage.

4.4. Possible additional functions revealed by Rev3 immunoreactivity

The availability of the first known Rev3-specific antibody allowed us to discover some unexpected patterns of Rev3 distribution during cell cycle, which are extremely exciting and require further investigation.

4.4.1. Nuclear exclusion of Rev3 in primary cells

The observation that Rev3 immunoreactivity is excluded from the nucleus in a fraction of two primary cell lines but not in any of the tumor-derived or immortal cell lines indicates a potential important regulatory mechanism that governs Rev3 accessibility to DNA. To date, we have shown that these cells are not in S phase or in mitosis, as judged by PCNA and DAPI staining, therefore they are most likely in G_1 or G_2, although we cannot rule out the possibility that these cells are in a G_0 state. Both primary cell lines grow slowly with a doubling time of nearly 48 hours. Hence, it is important to determine at which cell cycle stage (or a fraction of cell cycle stage) that Rev3 is excluded from the nucleus.

Rev3 is a very large protein and therefore cannot enter the nucleus by simple diffusion, it must be actively transported into the nucleus. We thus propose that in primary cells, this transporter system is inactivated in a specific cell cycle stage to prevent Rev3 access to the nucleus whereas in the immortal and tumor cells, this

negative regulation appears to be lifted. Alternatively, it remains possible that an additional exporter system may pump Rev3 out of the nucleus in the primary cells following mitosis, which becomes inactive in immortal and tumor cells. This would suggest that in "normal" cells, Rev3 is actively transported to the nucleus during S phase or in response to DNA damage, when it is needed for TLS. The implication of this regulation is far-reaching as in yeast the Rev3 (Pol-ζ) activity accounts for two-thirds of spontaneous and over 95% of DNA damage-induced mutations (Lawrence, 2004). Significantly, it is well known that genomic instability is a hallmark of tumor and immortal cells. Genomic instability was initially characterized primarily at the chromosome level and reflected by chromosome insertion, deletion, translocation and abnormal karyotype; however, Dr. Loeb and his colleagues recently developed a highly sensitive method called "random mutation capture" to resolve random point mutations (Bielas and Loeb, 2005). They were able to demonstrate that human cancers display an increase in random point mutations of at least two orders of magnitude compared to the matched normal tissues (Bielas et al., 2006) and argued that malignant cells exhibit a mutator phenotype resulting in the generation of random mutations throughout the genome (Loeb et al., 2008). Our observation of possible deregulation of cell-cycle dependent Rev3 nuclear distribution during malignancy may provide an underlying mechanism of such random point mutations. Obviously, this is only a preliminary observation and more work is required to establish the correlation between Rev3 distribution and tumorigenesis.

4.4.2. Unexpected function of Rev3 during mitosis

Our Rev3 ICC also revealed unexpected immunoreactivity in mitotic cells regardless of their immortal or tumorigenic status. Mitotic cells express a much higher level of Rev3 than cells in other cell cycle stage and Rev3 is clearly associated with the condensed chromosomes. This phenomenon has not been reported previously. It is interesting in the context that *Rev3* knockout mice are embryonic lethal whereas *Rev1* (Delbos et al., 2005) and other TLS polymerase mutant mice (Jansen et al., 2006; McDonald et al., 2003; Ogi et al., 2002) are not, suggesting that it is not the TLS activity of Rev3 that causes the lethality (Esposito et al., 2000; Van Sloun et al., 2002; Wittschieben et al., 2000). Furthermore, *Rev3* knockout mouse embryonic fibroblast cells are also unviable (Van Sloun et al., 2002) unless the p53 gene is inactivated (Wittschieben et al., 2006; Zander and Bemark, 2004), indicating that it is the genomic instability in the *Rev3* null mouse cells that triggers the p53-mediated damage checkpoint. Indeed Rev3 null mouse MEF cells display enhanced chromosome rearrangement (Van Sloun et al., 2002; Wittschieben et al., 2006), a phenotype that cannot be readily explained by the lack of TLS activity. In addition, chicken DT40 *REV3*$^{-/-}$ cells are viable and also display a significant increase in spontaneous sister chromatid exchange and chromosome breaks (Sonoda et al., 2003), which has been interpreted as Rev3 playing multiple roles in DNA metabolism. Our observation offers an unexplored possibility that Rev3 may play a critical role during mitosis in the maintenance of genomic stability. Our preliminary data show that constitutive suppression of Rev3 results in chromosome instability most likely at the stage of chromosome segregation, suggesting that the association of Rev3 with condensed

chromosomes is critical in the completion of telophase. Since a single unresolved chromosome bridging opposite genomes at telophase is sufficient to inhibit nuclear division and cytokinesis (Huang et al., 2008), lack of proper chromosome segregation would be detrimental to the cell and result in polyploidy. The exact cause of death by depleting Rev3 needs to be carefully characterized. More importantly, it is of great interest to investigate whether the polymerase activity of Rev3 or the unique domains of hRev3 not shared by yRev3 are required for this mitotic function. It will also be interesting to learn whether this Rev3 mitotic function requires its accessory partner Rev7.

4.5. Advantage of utilizing the Flp-mediated integration and expression system

One of the most difficult technical tasks in working with mammalian cells is the creation of transfected cells. Three major challenges are obvious compared with working with microorganisms such as *E. coli* and budding yeast. First of all, the transfection efficiency is often low. Transient transfection efficiency is typically below 10% and is heavily dependent on the host cells. Low passage and primary cell lines, for example, are difficult to transfect. Secondly, it is a challenging task to establish stably transfected cell lines. Often one has to select numerous drug-resistant colonies to obtain a few true transfectants, and the success rate is unpredictable. Finally, among the stable transfectants, the level of gene expression is often variable, which results in heterogeneous cells lines. Although sometime this has been regarded as an advantage since one can select cell lines with the desired level of expression, the nature of

heterogeneity and stability has been a concern. In this study, we attempted to utilize a HEK-293F cell line containing a single *FRT* target site in the host genome that allows site-specific integration of the plasmid-based constructs to integrate at this site mediated by co-transfection with a plasmid pOG44 expressing the Flp recombinase. A pioneer study using a plasmid expressing GFP indicates that indeed all transient and stable transfectants express the target gene relatively uniformly. In addition, the transfection efficiency, particularly the rate of obtaining stable transfectants, appears to be higher than the conventional approaches due to specific recognition of the FRT site and insertion by the recombinase. The homogenous expression of target genes as well utilizing iRNA has allowed us to obtain several valuable sets of data that otherwise would have been unattainable. Currently, we were limited to use a single HEK-293F cell line. However, several other common cell lines are also available commercially and it is possible to create our own cell lines containing an integrated *FRT* site in the genome.

4.6. Summary

Major conclusions obtained through this study are summarized as follows:

- Human and mouse cells contain a single *UBC13* gene and two yeast *MMS2* homologs, *MMS2* and *UEV1*.
- Deduced Mms2 (150 a.a.) and Uev1 (170 a.a.) sequences share >90% identity in their core domain of 145 amino acids.
- In human, *UEV1* encodes at least two splicing variants, Uev1A and Uev1B with identical core domains but different N-terminal sequences.

- We successfully raised polyclonal and monoclonal antibodies against Ubc13 and hMms2; the latter recognizes both Mms2 and Uev1.

- Mms2 and Uev1A are distributed in both nucleus and cytoplasm, while Uev1B is excluded from the nucleus due to its unique N-terminal sequence.

- Ubc13 and Mms2, but not Uev1A, are associated with a subset of chromatin-bound PCNA in S-phase cells.

- In response to CPT treatment, Ubc13 and Mms2, but not Uev1A, are found in detergent-resistant nuclear foci and co-localize with PCNA and newly synthesized DNA, but not with Rad51 or Mre11 foci.

- Deletion of the unique N-terminal sequence from Uev1A results in its localization to the CPT-induced nuclear foci in a manner indistinguishable from that of Mms2.

- Ubc13 and Mms2 are dependent on each other for damage-induced nuclear focus formation.

- Suppression of Ubc13 or Mms2, but not Uev1A, results in increased spontaneous nuclear focus formation of Rad51 and γH2AX, indicating that inactivation of Ubc13 or Mms2 results in increased double strand breaks, probably at the site of stalled replication forks.

- Suppression of Ubc13 or Uev1A, but not Mms2, results in the loss of lipopolysaccharide-induced NF-κB nuclear localization, indicating that Ubc13-Uev1A is required for the NF-κB signaling pathway.

- Mms2 and Uev1A both form stable complexes with Ubc13 but confer distinct cellular functions.

- We successfully raised polyclonal antibodies against human Rev3 and validated its specificity against endogenous Rev3 in cultured human cells.

- In cultured immortal and tumor-derived cells, Rev3 is found to be enriched in the nucleus throughout the cell cycle.

- In cultured low passage, non-imortal cells, Rev3 is found to be enriched in S phase and most other cell cycle stages; however, a small percentage (about 20%) of cells display Rev3 nuclear exclusion. The precise cell cycle stage of this cell population is currently unknown, but may have significant implications with regard to tumorigenesis.

- In all cultured cells, the Rev3 level is found to be significantly higher in mitosis than in other cell cycle stages and Rev3 is exclusively associated with condensed chromosomes, suggesting that it plays a critical role in mitosis.

- Constitutive suppression of Rev3 by interference RNA results in cell death within 4-6 weeks, with chromosome abnormalities likely due to abnormal chromosome segregation, consistent with a putative role for Rev3 in mitosis.

- After sub-lethal UV treatment, all cells display Rev3 nuclear localization and association with PCNA in S-phase cells.

- Under the above UV treatment conditions, endogenous Rev1 and Pol-η also co-localize with PCNA in S-phase cells; hence, all three TLS polymerases examined in this investigation co-localize in UV-irradiated S-phase cells.

- After UV irradiation, Rev1 and Pol-η are independently recruited to the damage site while neither requires Rev3. In contrast, Rev3 nuclear focus formation is dependent on Rev1 but independent of Pol-η.

- The above results support and extend the current polymerase switch model.

- Over-expression of GFP-Pol-η is sufficient to induce spontaneous nuclear focus formation in S-phase cells; it also induces and co-localizes with discrete PCNA foci. Furthermore, over-expression of GFP-Pol-η is sufficient to induce spontaneous Rev3 nuclear foci that co-localize with Pol-η and PCNA.

- Individual suppression of Rev3, Ubc13, Mms2 or Uev1 by interference RNA does not result in apparent growth retardation within one week of suppression.

- Simultaneous suppression of Rev3 along with Ubc13/Mms2, but not with Uev1A, results in a significant increase in sensitivity to UV-induced killing, supporting the existence of a two-branch DDT model in mammalian cells.

- XPV cells display significant UV sensitivity; suppression of Ubc13 in XPV cells further enhances this sensitivity. However, unlike the synergistic interaction between Rev3 and Ubc13/Mms2, the effect of Pol-η and Ubc13 inactivation appears to be additive with respect to UV irradiation, consistent with that observations in yeast cells.

4.7. Future directions

This thesis has initiated investigation into DDT in mammalian cells and the results collectively suggest that the Ubc13-Mms2 complex and Rev3 are operational in a DNA damage response, but admittedly these findings are preliminary and call for further investigation to achieve the originally stated overall objectives. Most notably, additional research is required to characterize functional activities of the Ubc13-Mms2 complex and Rev3 in DDT. Furthermore, through this study, several interesting

observations were made that deserve attention and further investigation, which may lead to significant discoveries if experimentally pursued.

4.7.1. Is the Ubc13-Mms2 complex involved in error-free DDT?

Although the Ubc13-Mms2 complex has been shown to respond to endogenous and exogenous DNA damage, a true activity in error-free DDT has not been observed other than the altered growth in response to UV when Rev3 or Pol-η is inactivated. Functional assays are required to demonstrate DDT directly, and particularly to characterize DDT as error-prone or error-free. In budding yeast, DDT/PRR is defined as the ability to convert ssDNA gaps into double-strand large molecules in the presence of damage but absence of repair, and experimentally demonstrated by an alkaline sucrose gradient assay (Yamada and Takezawa, 2006). This type of activity has been demonstrated for mammalian Rad18 (Yoshimura et al., 2006). It is anticipated that inactivation of Ubc13 or Mms2 alone in cultured mammalian cells will be insufficient to cause a significant shift in the molecular size of ssDNA in this assay system; however, if the two-branch DDT model is correct, simultaneous inactivation of Ubc13 or Mms2 along with Rev3 is expected to result in a significant increase in ssDNA of reduced molecular size. In mammalian cells, two alternative techniques may be developed to investigate DDT. One is an alkaline comet assay (Singh et al., 1988) and another is pulse field gel electrophoresis (Carle et al., 1986) under alkaline conditions. Under alkaline conditions DNA is denatured to ssDNA, and hence the number of ssDNA breaks will be directly reflected by altered migration patterns in both assays. Genomic DNA would need to be monitored from cells with or without suppression of

Ubc13-Mms2 and/or Rev3 at various intervals following DNA damage (e.g., UV) treatment. The DDT activity would be observed as the ability to regain genomic DNA migration patterns resembling undamaged control DNA. Although the comet assay is relatively uncomplicated, pulse field gel electrophoresis may be more challenging technically.

The above DDT assay does not distinguish if the DDT activity is error-prone or error-free. A mutation assay has been tailored to measure Mms2 functions in cultured chicken cells (Simpson and Sale, 2005), in which Mms2 was found to be incapable of UV-induced gene conversion. A plasmid-based mutagenesis assay was reported very recently (Shachar et al., 2009), in which a plasmid with a specific lesion is introduced into the cell which is later recovered, re-introduced into bacteria for amplication, and then sequenced to determine exactly how the repair has been made (i.e. with or without a mutation across or near the lesion site). This method has been utilized to assess the relative contributions of various TLS polymerases to the lesion bypass, and can be adapted to assess relative contributions of error-free (Ubc13-Mms2) and error-prone (Rev3) bypass. The experimental protocol appears to be a complicated and significantly time consuming procedure, but has the potential to demonstrate exactly how a given DNA lesion is bypassed in mammalian cells. The advantage over exogenously applied damage is that the lesion introduced can be very specific. However, the disadvantage of this approach is that the lesion bypass occurs in a non-chromosomally dependent manner.

4.7.2. How is Rev3 involved in TLS?

Although it been clearly demonstrated whether Ubc13-Mms2 is involved in error-free DDT like its yeast counterpart, it is rather convincing that Rev3 is involved in TLS, or the error-prone branch of DDT, like yeast Rev3, based on previous (Lin et al., 2006) and recent (Shachar et al., 2009) mutagenesis assays as well as data presented in this thesis. The detailed molecular mechanisms by which Rev3 and other TLS polymerases exert their activitie remain to be elucidated.

A recent review article (Gan et al., 2008) summarizes questions remain in the study of mammalian Pol-ζ: "Studies of the higher eukaryotic REV enzymes have been hampered by the inability to express the very large mammalian Rev3 homologs or to detect the protein in cells. What is the function of the large non-conserved region of Rev3L? Do levels of Rev3L protein, which are presumably kept low through alternative splicing and translation controls, increase in response to DNA damage? While sequence analysis suggests that it is highly likely that REV3L is a functional DNA polymerase, the ability to incorporate and/or extend nucleotides opposite different types of damaged template is unexplored. Potential stimulation by REV7, and even whether full-length REV3L interacts with mammalian REV7 and REV1 remain important unanswered questions."

With the production and characterization of a polyclonal anti-Rev3 antibody and the established ability to suppress endogenous Rev3 by different interference RNA techniques in this study, I feel that we will be able to directly and indirectly answer several of the above critical questions.

This study has provided strong evidence supporting the polymerase switch model in the context of UV-induced DNA damage. In order to address whether the two-polymerase TLS model also applies to other types of lesions, the genetic relationship between Pol-κ, Rev1 and Rev3 in the bypass of BPDE-induced DNA damage should be tested. It has been reported that BPDE induces Pol-κ, but not Pol-η nuclear focus formation in a Rad18-dependent manner (Bi et al., 2006). However, Pol-κ itself has been suggested as an extender of TLS (Lone et al., 2007). Experimental strategies as described in this study will be able to tell whether Rev1 and Rev3 are involved in BPDE-induced lesion bypass.

This study relies heavily on damage-induced co-localization as evidence of protein complex assembly for the lesion bypass, which cannot distinguish between direct and indirect interactions. The current model is based on interactions between the C-terminus of Rev1 and other Y-family polymerases as well as Rev7 (Andersen et al., 2008), and *in vitro* evidence of Rev7 interaction with both Rev1 and Rev3 (Murakumo et al., 2001). However, a direct and functionally relevant interaction between yeast Rev1 and Rev3 has been reported (Acharya et al., 2006; Murakumo et al., 2001), and our GFP-Pol-η over-expression data suggest a possible Pol-η and Pol-ζ direct interaction. The above interactions can be systematically examined by cloning *hREV3* into yeast two-hybrid vectors (Young, 1998) and as a glutathione S-transferase (GST) fusion protein for the *in viv*o and *in vitro* assays, respectively. However, the ultimate goal is to perform the *in vivo* interaction assay in living mammalian cells. In this case, Rev3 can be fused to GFP (or YFP, RFP for co-localization studies) or an epitope (e.g.,

Myc, Flag or HA) tag in a mammalian cloning vector and tested by co-IP with other proteins containing alternativet tags.

The possible interaction domains present within the human Rev3 protein have not been characterized and preliminary motif alignments have not been able to identify significant prospects except for partial sequence homologs with budding yeast Rev3. Of particular importance are the likely binding domains present in Rev3 that allow its interactions with other proteins, most notably Rev1 and Rev7, and possibly Pol-η and PCNA. For Rev3 protein interaction domains, the deletion and site-specific mutagenesis analyses can be initially performed in the yeast two-hybrid and *in vitro* binding assays and then confirmed in mammalian cells. Other functional domains will also be characterized in a similar manner (see below).

4.7.3. Is Rev3 nuclear localization regulated differently in normal vs. tumor cells?

Initial observations have indicated that in normal cells, Rev3 may be excluded from the nucleus under unknown conditions, possibly during a particular phase of the cell cycle and therefore restricted from TLS. Rev3 is clearly present in the nucleus of all S-phase cells (as judged by PCNA positive nuclei) and preliminary observations indicated that exclusion occurs shortly following cytokinesis (as judged by paired cells) and/or in a quiescent-like state of G_0 (as judged by large flattened cells), but this has not been clarified. Further experiments should be designed to identify this localization pattern with respect to cell cycle stage, possibly by use of non-invasive synchronization

methods, such as the mitotic shake off to enrich cells in mitosis, or by fluorescent cell sorting (labeled with Hoescht 33342) to separate cells followed with ICC.

Initial observations also indicate that the Rev3 nuclear exclusion is only notable in apparently normal, low passage number cells, whereas in tumor derived or immortalized cell lines Rev3 was found throughout the cytoplasm and nucleus. If Rev3 is regulated by its localization as predicted, this may indicate an alteration of its regulation in immortalized cells. In these cells Rev3 may therefore be overactive in the nucleus, which potentially increases the mutation load and has therefore the potential to promote tumorigenesis. Alternatively, the altered localization pattern may be the result of immortalization and is a normal response of the cell. This should be investigated, possibly by directly comparing genetically matched low passage normal cells with immortal cells, or by staining tissue sections from normal and matched tumors with the anti-Rev3 antibody.

The availability of a GFP-Rev3 fusion system may assist the investigation of the dynamic Rev3 nuclear localization. Furthermore, an *in vivo* assay using GFP-Rev3 and its deletion and mutation derivatives can address which domain(s) is required for the localization to (or exclusion from) the nucleus during the cell cycle or in response to DNA damage. The GFP-Rev3 deletion construct can also be assayed for localization to nuclear foci following DNA damage, but a strong NLS may be needed to ensure its entry into the nucleus, as the NLS may occur in a distinct fragment from the domain(s) required for the focus formation.

4.7.4. Role(s) of Rev3 in mitosis

The localization of Rev3 with condensed chromatin during mitosis was a surprising discovery and this is presumed significant because when Rev3 was reduced mitotic abnormalities were observed. Interestingly, the *REV3* mRNA is also up-regulated in the testis (Xiao et al., 1998a), suggesting that Rev3 also coincides with meiotic chromatin and functions during meiosis. However, the exact function of Rev3 during mitosis remains completely unknown, although one can predict that Rev3 plays a pivotal role. Characterization of Rev3 activity during mitosis may prove to be very challenging. One speculation would be that DNA damage occurs continuously throughout mitosis during decatenation of intertwined DNA with the primary utilization of topoisomerases (Luo et al., 2009), and Rev3 may be required to ensure completion of DNA repair during this time. Another possibility is that the unique region in hRev3 not shared with yRev3 may confer a mitotic role independent of TLS. To distinguish these two possibilities, one can suppress endogenous Rev3 by interference RNA and introduce the full-length wild type Rev3, Rev3 mutated in its conserved polymerase motifs or containing the deletion of the hRev3 unique region to see which clone is able to rescue the survival and mitotic defects. Under conditions in which the wild type Rev3 clone restores the Rev3 mitotic function but the vector control cannot, one can critically examine whether the polymerase activity or the unique region of unknown function(s) is required for the Rev3 mitotic activity. To avoid the suppression of the cloned Rev3 by interference RNA, one can choose one of at least two strategies. One is

to use 3' UTR as the target sequence, while another is to mutate the target DNA sequence in the cloned *REV3* gene without altering its amino acid sequence.

In order to investigate how Rev3 exerts its function(s) during mitosis, one can use the full-length or the defined functional domain(s) to perform co-IP in living cells, followed by mass spectrometry to identify its binding partners. Since Rev3 is associated with mitotic chromosomes, an *in vivo* chromatin immunprecipitation assay may be performed to identify candidate proteins. Because Rev3 may be the single most important enzyme involved in spontaneous and damage-induced mutagenesis, the Rev3 protein interaction map would be extremely important as these proteins may serve as possible therapeutic targets to restrict TLS activity without inadvertently inhibiting any additional functions of Rev3, such as its predicted mitotic function.

4.7.5. The sequence critical for Uev1A to be involved in the NF-κB signaling pathway

This thesis was successful in demonstrating that Mms2 operates distinctly from Uev1A, although both interact with Ubc13. The N-terminal deletion of Uev1A resulted in its altered localization to resemble that of Mms2 in response to DNA damage. However the determining factor of Uev1A has not been well characterized. For example, in addition to the unique N-terminal sequence of Uev1A, there is a stretch of amino acid sequence difference (7 out of 14 residues are different) between Mms2 and Uev1A, which may be of biological significance (Pelzer et al., 2009). A critical test would be to fuse the N-terminus of Uev1A with Mms2 and ask whether this chimeric protein behaves like Uev1A or Mms2 for the nuclear focus formation in response to

CPT treatment. Similarly, all the above four constructs can be employed for an NF-κB activity assay as described (Syed et al., 2006). It has been reported that over-expression of Uev1A is sufficient to activate NF-κB (Syed et al., 2006) while over-expression of Mms2 is not (N. Syed, personal communication). If the unique N-terminus determines Uev1A activity, one will predict that Uev1AΔ30 will not be able to activate NF-κB, while the Uev1A-Mms2 fusion will behave like Uev1A with respect to NF-κB activation.

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