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Unmasking the Role of Ubiquitin in Mammalian Systems

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Unmasking the role of ubiquitin in mammalian systems

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Abstract

Ubiquitination is a highly regulated post-translational modification that impinges on a variety of cellular processes. Commonly known for its role in proteolysis, recent studies emphasize the versatility of ubiquitin in non-proteolytic aspects of cell function. Some of these proteolytic-independent roles have been highlighted in recent publications as being critical mediators in stress response pathways including those of DNA repair and NF-κB activation. In these seemingly unrelated pathways it has been shown that a noncanonical linear chain linked through lysine 63(K63) is required in the assembly of multi-subunit complexes which allow eukaryotes to tolerate DNA damage or elicit an immune response. Because the functionality of ubiquitin chains is constrained by length and topology, alteration of these properties greatly alters these cellular pathways. In order to understand the role of these chains in mammalian cells we introduced mutant ubiquitin proteins that interfere with specific properties of ubiquitin chain assembly. These dominant negative mutants revealed interesting biological effects. Here we demonstrate that in human cells, as in yeast, PCNA (proliferating cell nuclear antigen) is polyubiquitinated via K63. Disruption of K63-linked chains impairs the error-free arm of a DNA damage tolerance (DDT) pathway resulting in an increased reliance on error-prone translesion synthesis (TLS), which has significant implications on mutagenesis particularly and in response to common environmental carcinogens such as UV light and BPDE. As a result of our cell culture studies, we have generated transgenic mice expressing the K63R mutant form of ubiquitin to more directly test its roles in cancer development and impaired NF-κB induction in vivo. Recently, several members of error-free DDT including POLη,

SHPRH, hRAD6, hRAD18 have been linked to cancer and the likelihood of chemotherapeutic success in patients. Accordingly, future drug targeting of the error-free DDT pathway in cancer could be of tremendous therapeutic benefit.

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I would like to express a special thanks to my mother and brother for their continued love, unwavering support, and for persevering with me through the worst of times and the best of times.

Finally, I would like to genuinely thank this country of Canada for graciously taking us in after our illegal arrival at Mirabel airport in Montreal shortly after our

defection from communist Czechoslovakia. This country provided hope and a fresh start to a family that lost everything; an altruistic gift for which I am forever indebted and one that transcends any expression of gratitude I could ever offer.

Dedication

A heartfelt dedication to the memory of my father who is missed more with each passing day as I remember how we were, how much your life was and could still have been.

To my grandfather Robert Bartek (Alt) and my late grandmother Eta Bartkova (Berger) whose perseverance, profound strength of character in life and in surviving the concentration camps of Terezin, Auschwitz/Birkenau, and the staged execution of their post-war government in Czechoslovakia by the Stalinist-driven Soviet Union. Their example continues to provide me with the inspiration to persevere and reach goals that would otherwise have seemed unattainable.

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List of Abbreviations

6–4PP pyrimidine-6/4-pyrimidone;
ATM ataxia telangiectasia mutated
ATR ATM and RAD3 related checkpoint kinase
BAP benzo[a]pyrene
BER base excision repair
BPDE benzo[a]pyrene-diol-epoxide
CPD cis-syn cyclobutane pyrimidine dimer
CPT cisplatin
CS Cockaynes Syndrome
DDT DNA damage tolerance
DUB deubiquitinating enzymes
ES embryonic stem
FA Fanconi's anemia
GFP green fluorescent protein
HAT hypoxanthine, aminopterin, and thymidine
HHR6a human homolog RAD6a
HHR6b human homolog RAD6b
HPRT hypoxanthine phosphoribosyltransferase
HR homologous recombination
K63-polyUb lysine 63-linked polyubiquitin
LPS lipopolysaccharide
MM multiple myeloma
MEF mouse embryonic fibroblast
MMR mismatch repair
MMS methyl methane sulfonate
MMC mitomycin C
NHEJ non homologous end joining
NF-κB nuclear factor kappa B
NEM N-ethylmaleimide
NER Nucleotide excision repair
PCNA proliferating cell nuclear antigen
POLη DNA polymerase eta
POLι DNA polymerase iota
POLζ DNA polymerase zeta
PRR post-replication repair
SCE sister chromatid exchange
SDS sodium dodecyl sulfate
SHPRH SNF2, PHD-finger, RING-finger, helicase
TLS translesion synthesis
TT thymine dimers
TTD trichiothiodystrophy
Ub ubiquitin
UBD ubiquitin-binding domain
UBM ubiquitin-binding motif
UBZ ubiquitin-binding zinc-finger

UEV	ubiquitin conjugating enzyme variant
UPP	ubiquitin proteasome pathway
UV	ultra-violet light
VSV	vesicular stomatis virus
WT	wild-type
XP	Xeroderma Pigmentosum
XPV	Xeroderma Pigmentosum variant

CHAPTER 1

Introduction

1.1 Ubiquitin

The activity of many proteins depends on essential chemical labels such as phosphorylation, acetylation, nitrosylation, glycosylation, sumoylation, neddylation, ISGylation, fatylation and ubiquitination [1]. The purpose of my thesis is to unmask the role of ubiquitin in mammalian systems. Ubiquitin serves as a globular 76 amino acid covalent tag that is conjugated to many proteins (**Figure 1**). It is an 8.6 kDa, highly conserved, pleiotropic molecule involved in a variety of biological processes such as cell cycle control, endocytosis, signal transduction and DNA repair. It has essential proteolytic and non-proteolytic functions within the cell that are based on the length, topology, and architecture of the ubiquitin chain formed [2,3]. Substrates can be either mono or polyubiquitinated (**Figure 2**). Monoubiquitination results in various cellular processes such as endocytosis, protein sorting, subnuclear trafficking, gene expression, and possibly processing by the proteasome [4]. In comparison, the formation of polyubiquitin chains results in alternative processes including proteasomal degradation, NF-κB activation and DNA repair [4].

Three enzymatic steps mediate the addition of ubiquitin to a protein substrate **(Figure 3)** [5]. First, the ubiquitin activating enzyme E1 forms a high energy thiolester bond between an internal cysteine residue and the C terminal glycine of ubiquitin via the cleavage of ATP. Ubiquitin is then transferred to an E2, ubiquitin-conjugating enzyme, which forms a similar thiolester bond. E2 together with an E3

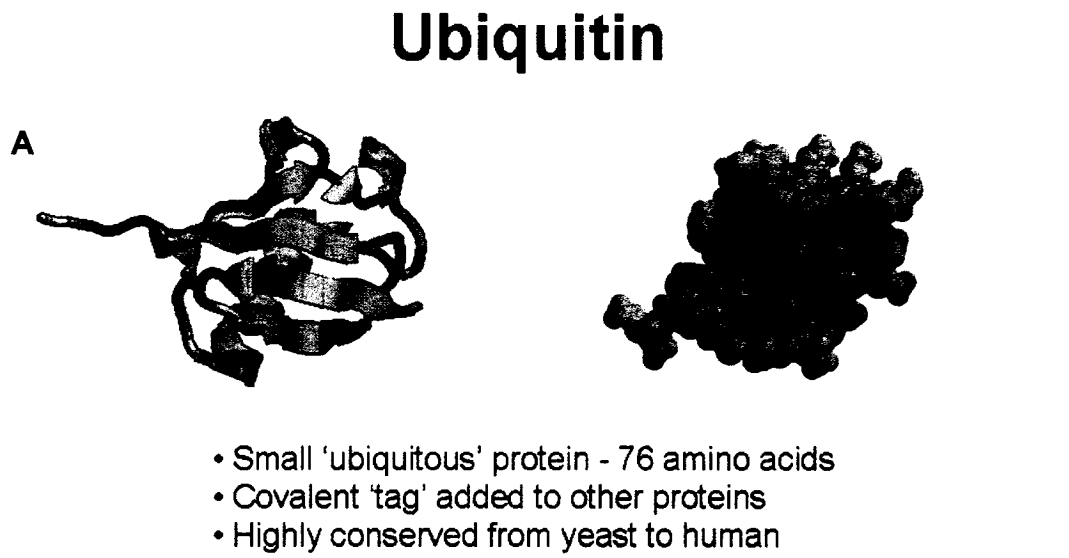

Figure 1. Illustration of ubiquitin. A) 3D model of ubiquitin **B)** Amino acid sequence of ubiquitin in yeast (blue) as compared to human (gray). The sequence is highly conserved between the two species except at the highlighted (orange) amino acids.

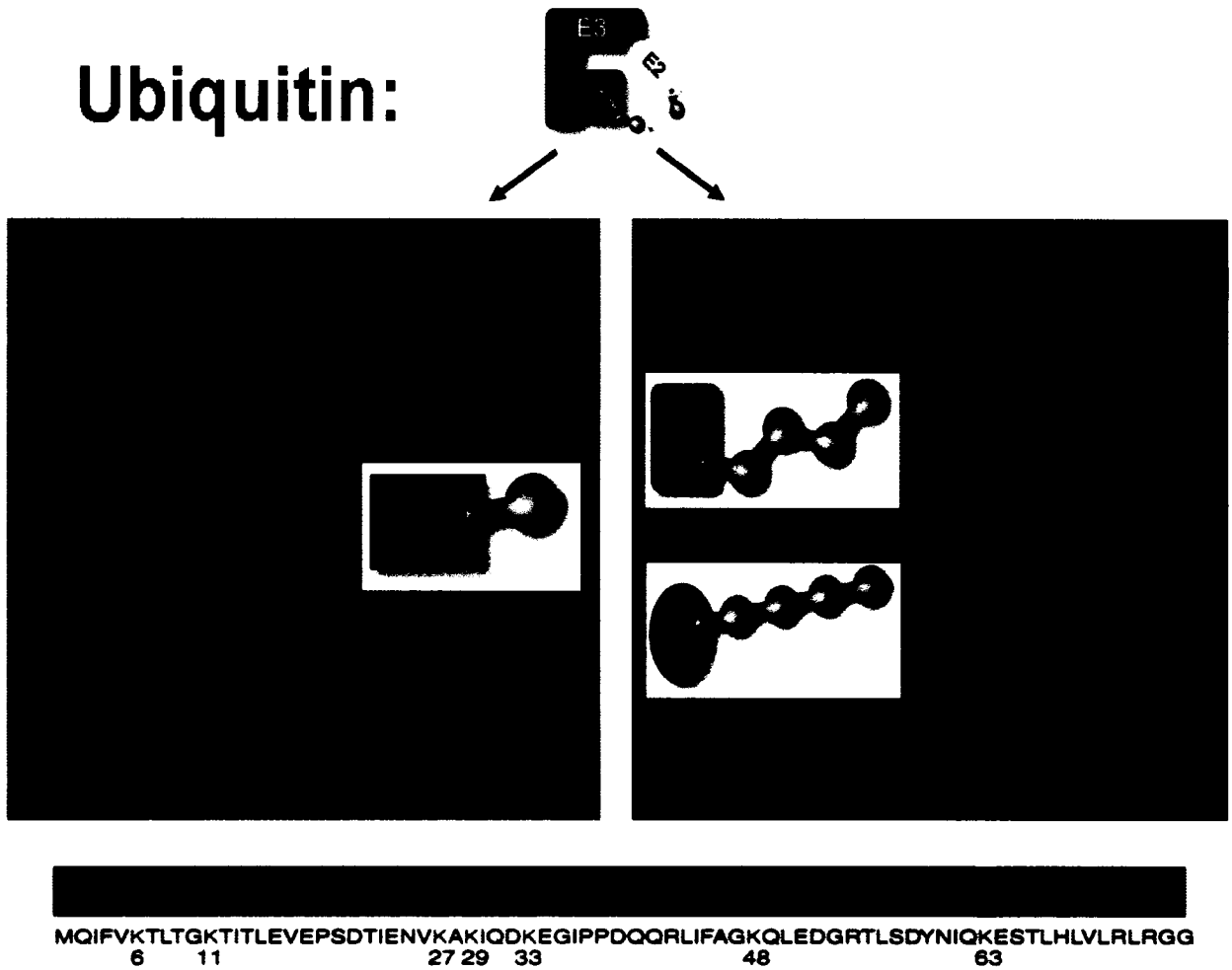

Figure 2. Divergent roles of mono and polyubiquitinated substrates. Monoubiquitination confers a different function upon substrates as compared to polyubiquitination. For example monoubiquitination is involved in processes such as protein sorting and while polyubiquitination can be involved in processes such as signal transduction and proteolysis. The amino acid sequence highlights the 7 lysine residues (red) that can be used to form polyubiquitin chains

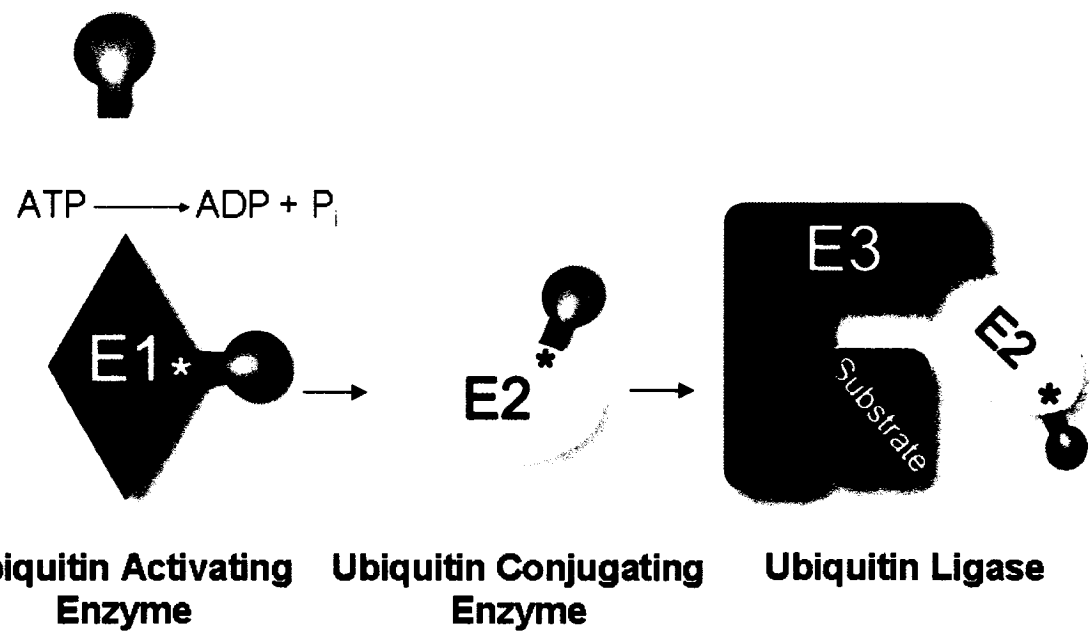

Figure 3. Enzymatic machinery that promotes ubiquitination of target substrates. First an E1 enzyme creates a high energy thiolester bond between an internal cysteine and the C terminus of ubiquitin. Second, an E2 enzyme receives the activated ubiquitin monomer and forms a similiar high energy thiolester bond. Finally an E3 catalyzes the attachment of activated ubiquitin to a protein substrate.

ubiquitin ligase mediates the attachment of ubiquitin to the substrate via a covalent bond between the C terminal glycine (G76) and a lysine residue on the substrate. Ubiquitin can also be covalently attached to itself, forming a polymeric chain on specified substrates. Sequential conjugation of monomeric ubiquitin occurs through the covalent linkage of G76 to one of the 7 internal lysine residues of the preceding monomer, forming a polyubiquitin chain. This chain elongation process is driven by the same E1/E2/E3 mechanism. Ultimately, the length and topology of the ubiquitin chain will determine its function.

Ubiquitin has 7 internal lysine residues – K6, K11, K27, K29, K33, K48, and K63, all of which can form polyubiquitin chains [6]. Assembly of ubiquitin polymers can occur through the following lysine residues – K6, K11, K27, K29, K33, K48, and K63 with K48 being the predominant form followed by K11 and K63 linkages, which occur with an equivalent prevalence (**Figure 4**) [3,6-9]. While there is no known function ascribed to polyubiquitin chains linked through K11, K27, K29, and K33 they still remain potential targets for proteasome mediated degradation [10]. Most abundant in the cell are chains linked through lysine 48 (K48) which take on a zig-zag type conformation that results in the degradation of the substrate through the 26S proteasome **(Figure 5 & 6)**. In the case of K48 linkages a threshold length of four sequentially attached ubiquitin monomers is required for recognition by the 26S proteasome [11]. Conversely, K6 linked chains inhibit the targeting of substrates to the proteasome serving a role in DNA repair [12]. From yeast studies, it is also known that alternate linkages, such as those through lysine 63 (K63), form linear chains that may serve as scaffolding for other proteins. These chains assume a non-

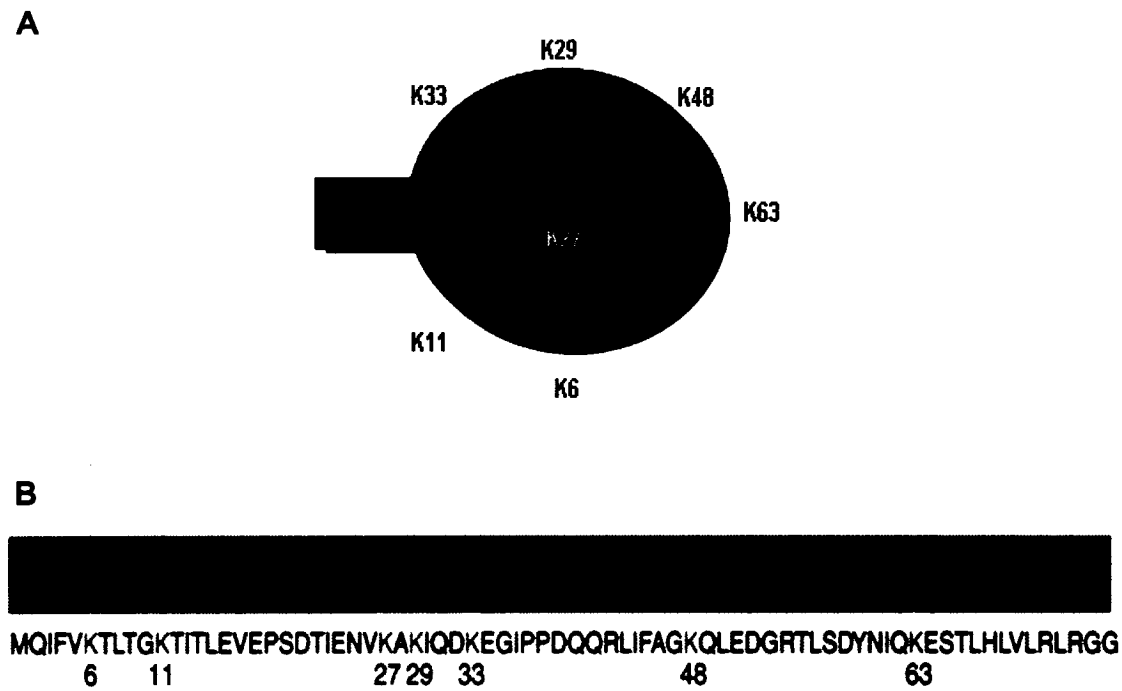

Figure 4. Lysine residues of ubiqutitin. **(A)** 2D illustration of ubiquitin with the hypothesized location of the lysine residues. **(B)** Amino Acid sequence with the highlighted lysine residues (red). All 7 lysine residues are able to participate in ubiquitin chain elongation.

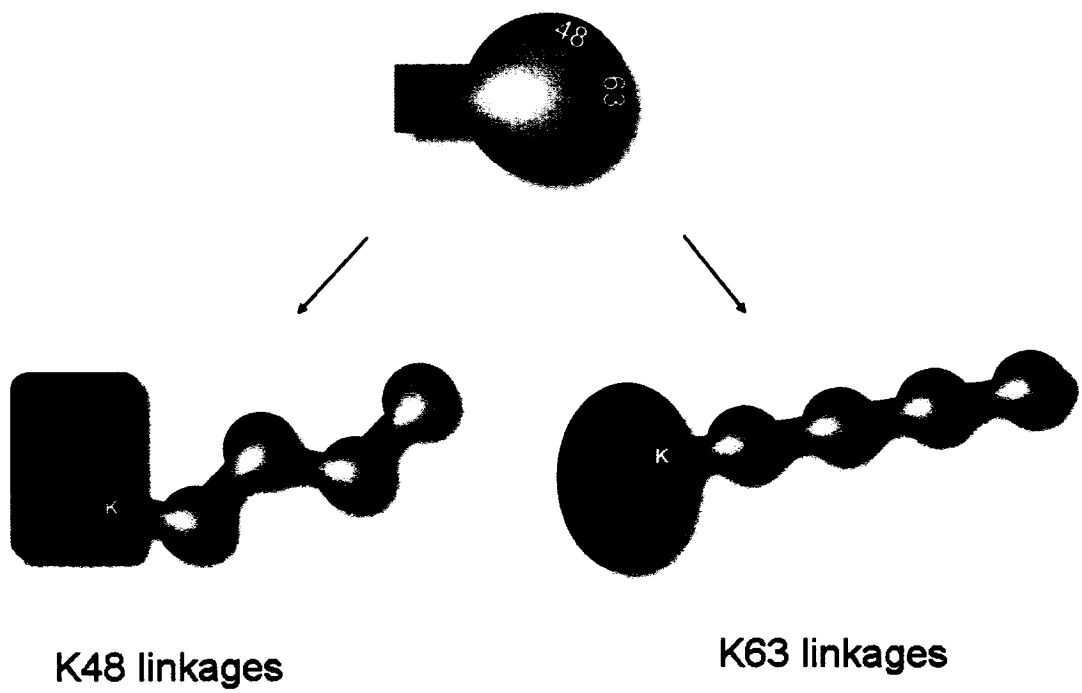

Figure 5. Varying topologies of ubiquitin chains based on the involved lysine linkage. A substrate is first monoubiquitinated via a lysine in the target protein and the C terminal G76 of ubiquitin. Depending on the cellular process, subsequent ubiquitin monomers are either added via a K48-G76 (zig-zag chains) or K63-G76 (linear chains) isopetide bond. Based on the linkage, the ubiquitin polymer acquires different topology and architecture, which consequently confer different functions within the cell.

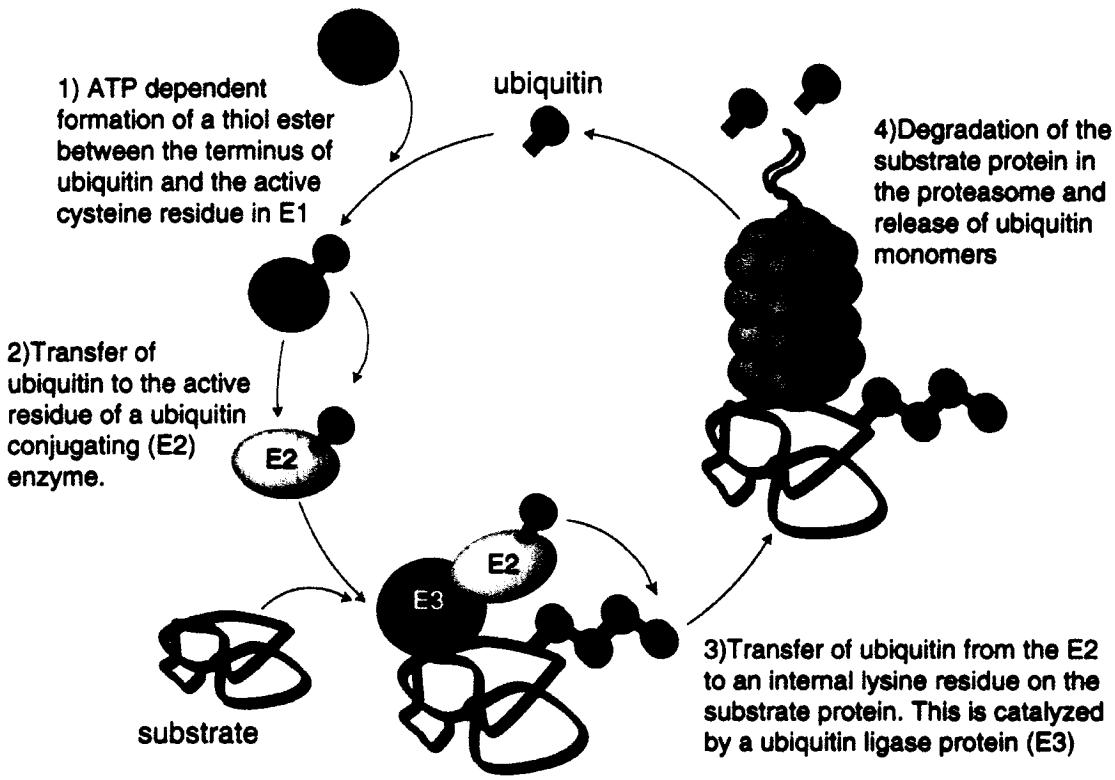

Figure 6. The UPP pathway. This illustration shows the E1, E2 and E3 enzymatic reaction that drives K48-linked polyubiquitination of a target substrate followed by subsequent targeting and degradation by the 26S proteasome.

proteolytic role in several seemingly unrelated processes such as DNA damage tolerance and the inflammatory response via NF-κB activation **(Figure 5 & 7)** [13,14]. In summary, ubiquitin chain assembly plays critical roles in cellular pathways and this role depends greatly on the type of lysine linkage, the length and the architecture that these ubiquitin chains assume.

1.2 DNA repair

Mammalian cells possess a number of distinct DNA repair systems to maintain the fidelity and integrity of the genome in response to endogenous and exogenous agents that produce DNA lesions. These systems include nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), non-homologous end joining (NHEJ), and translesion synthesis (TLS) [15] . Each of these pathways has been well conserved throughout evolution and in the past decade many of the *Esherichia coli (E.coli)*, *Saacharomyces cerevisiae (S. cerevisiae)*, murine, and human genes in these specific pathways have been cloned and their biochemical functions established.

NER is the primary and most versatile mechanism by which bulky helix deforming lesions are removed from the genome, including ultraviolet (UV) induced 6-4 photoproducts (6-4PPs), cis-syn cyclobutane pyrimidine dimers (CPDs), chemical adducts, and certain types of DNA cross-links [16-18] . There are three human autosomal recessive diseases that result in a disruption of NER: xeroderma pigmentosum (XP), Cockaynes syndrome (CS), and Trichothiodystrophy (TTD) [15]. The frequency of XP is $1:10^6$ in North America and Europe, and $1:10^5$ in Northern Africa, and Japan. Patients with the autosomal recessive disease XP have increased

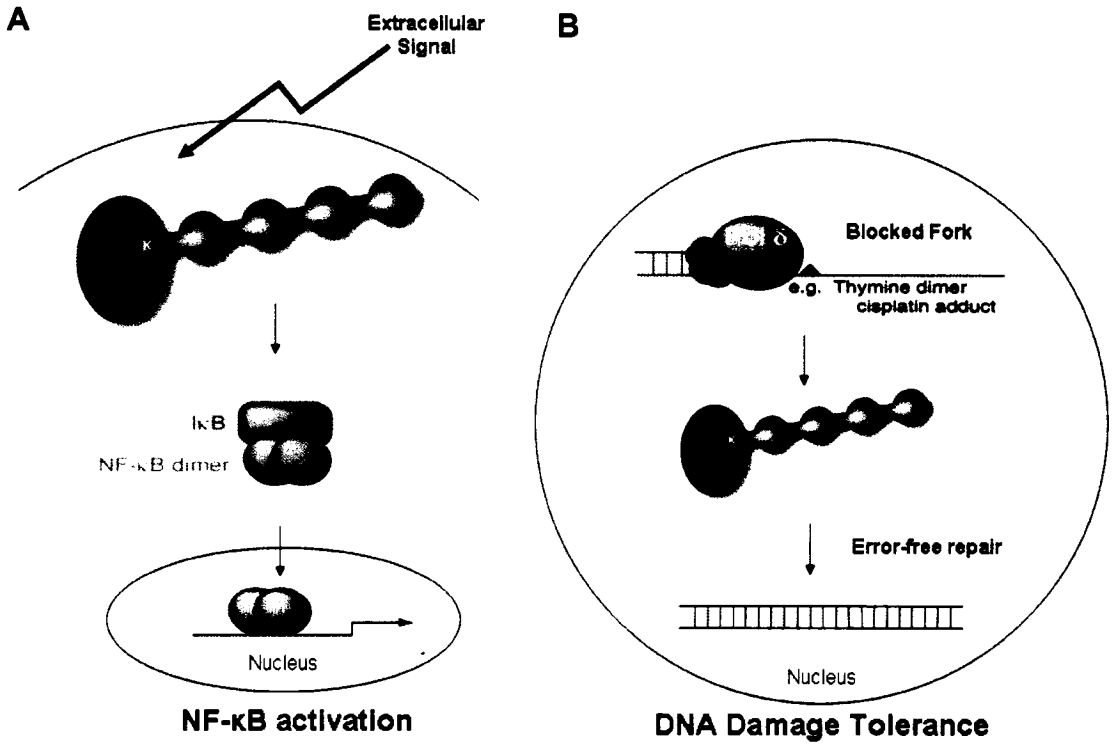

Figure 7. Role of K63 linked polyubiquitin chains in cellular stress response. A) K63-linked ubiquitin chains play critical roles in activating an inflammatory response via NF-kB activation, a transcription factor that activates a variety of cytokines involved in immune response. **B)** K63-linked ubiquitin chains are also critical at blocked replication forks where they mediate error-free repair of damaged DNA.

rates of morbidity and mortality, reducing their overall survival by 30-40 years compared to normal individuals. These patients are clinically associated with extreme sensitivity to UV light, cutaneous and ocular deterioration and highly prone to developing skin cancers prematurely [15]. About 20 % of XP patients also experience progressive neurological deterioration. These patients fall into 8 complementation groups XPA-XPG and the XP variant (XPV). All 8 genes in these complementation groups have been cloned, 7 of which are directly involved in NER (XPA-XPG) [17]. In recent reviews of this pathway, it has become clear that each of the XP proteins plays an active part in the recognition or removal of a DNA patch containing the lesion [17]. Patients with XPV are distinct from other patients with XP because their cells are only slightly more sensitive than normal cells to UV light and they have no overt NER defect. However, like most XP patients, they develop sunlight induced skin cancer at high rates. Hannoaka et al, discovered that the gene defective in XPV patients is DNA polymerase η (POLη) [19,20]. POLη functions as an A rule polymerase inserting adenines across sites of DNA damage. Thus, for UV light the polymerase activity has a high probability of bypassing the damage accurately and efficiently. Since the loss of POLη is not a lethal event, patients lacking POLη may use an alternative more error-prone polymerase resulting in the increased predisposition to skin cancer in the XPV syndrome.

In addition to NER, several other genes have been genetically implicated in repair of lesions that are substrates for NER. These genes do not function in NER but in an alternate pathway termed DNA damage tolerance (DDT), formerly known as post-replication repair (PRR). Unlike NER, these proteins do not directly remove the

damage, or reverse the damage like DNA photolyases, but they tolerate the damage in order to efficiently replicate DNA. Specifically, DDT enables cells to synthesize intact DNA daughter strands, despite the presence of persisting damage in the template strand during S phase. Consequently, these genes impact significantly on the toxicity and mutagenicity of agents such as UV light and cisplatin. Typically, DNA replication stalls when cells are exposed to such agents. This occurs when DNA replication machinery meets a lesion before appropriate repair has taken place on the mother strand. The consequences of failing to repair such damage are typically catastrophic for an organism. For example, they can result in a gap on the daughter strand which can lead to double-strand breaks and to eventual programmed cell death or gross chromosomal instability. In order to restore replication eukaryotes have evolved the DDT pathway to maintain the fidelity and integrity of the genome in response to endogenous (reactive oxygen species) and exogenous (UV, cisplatin) agents that produce DNA lesions. DDT is believed to be governed by two subpathways: (1) an error-free homologous recombination (HR) repair where fork reversal and formation of Holliday junctions allow the use of the undamaged template to bypass the lesion [21-23], and (2) error-prone translesion synthesis (TLS) where polymerase switching (from high fidelity replicative polymerase δ→Y or B family polymerase) permits efficient, but often unfaithful, lesion bypass (**Figure 8**) [24-27].

The importance of DDT is highlighted by extreme sensitivity of *S. cerevisiae* mutants of the RAD6 epistasis group of the DDT pathway. RAD6 and RAD18 play important roles in this pathway and mutations in these genes leads to extreme sensitivity to a variety of DNA damaging agents and defects in induced mutagenesis

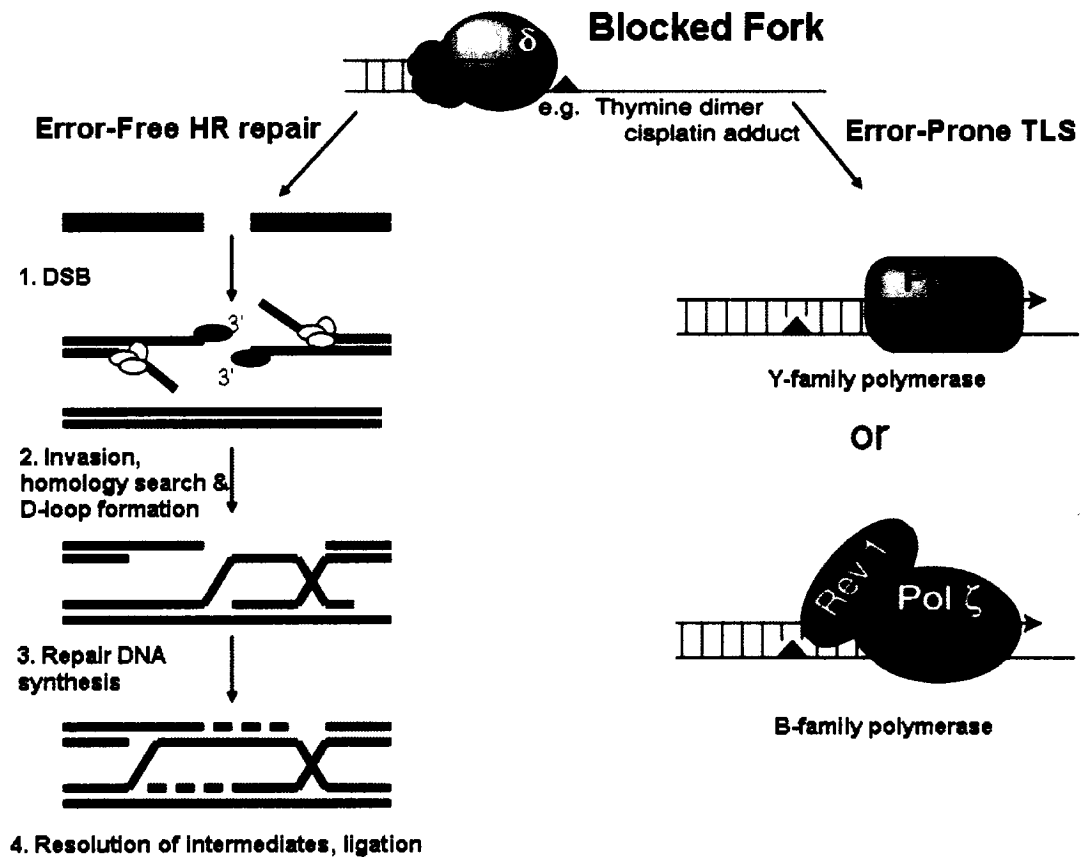

Figure 8. The two arms of the DNA damage tolerance pathway. Lesions produced by UV light or cisplatin lead to replication blocks. Synthesis across these lesions requires either the recruitment of lesion bypass polymerases or homologous recombination proteins for error prone or error free repair respectively. Double strand breaks initiate HR repair as a result of catastrophic replication blocakge. Strand invasion, homology search, D loop formation, followed by synthesis and resolution of recombination intermediates. Alternatively, DNA lesions can be bypassed by more liberal polymerases such as POLη or POLζ from the Y and B family of polymerases respectively. This process is mostly error-prone as these polymerases have poor processivity, and low fidelity as a result of the absence of 3'-5' proofreading exonuclease.

[28,29]. In addition, RAD6 mutants have defects in sporulation and a slow-growth phenotype [28]. Biochemical studies in yeast have demonstrated a stable interaction between RAD6 and RAD18 [30]. Many of the genes downstream of RAD6/RAD18 have been identified and have been placed in either the error-free or error-prone arm of DDT based on the ability of these genes to affect sensitivity or mutagenesis in response to genotoxic agents, such as UV light [31]. Genes included in the error-free pathway consist of RAD5, MMS2, and UBC13 while error-prone pathway consists of RAD30, REV1, REV3 and REV7 [32]. Genes that are common to both branches of DDT include RAD18, RAD6, PCNA and ubiquitin [32].

1.3 Ubiquitin and DNA repair

1.3.1 Fanconi anemia pathway requires ubiquitination

There is increasing evidence that the role of the ubiquitin pathway in DNA repair, in some contexts at least, is unrelated to protein degradation in the proteasome. For example, the monoubiquitination of the gene product FANCD2 on lysine 561 is an important component that complements a DNA repair defect in patients with Fanconi anemia (FA) [33]. FA is an autosomal recessive disease which results in genomic instability and a high predisposition to leukemia and squamous cell carcinomas [34]. Similar to NER, FA has at least 8 complementation groups (FANCA-C, FANCE-G, FANCL and M). They do not function in the direct removal of the damage, however they seem to form a multi-subunit E3 ubiquitin ligase that ubiquitinates FANCD2 [33]. Monoubiquitinated FANCD2 is targeted to the nucleus where it interacts with other DNA repair proteins, namely BRCA1, BRCA2, PCNA, RAD51 and others, to co-ordinate the removal of DNA-crosslink damage inflicted by

agents such as UV, cisplatin or mitomycin C (MMC) by either an HR or TLS mediated response [35-37].

1.3.2 NER requires ubiquitination

Another example of a non-proteolytic role of ubiquitin in DNA repair comes from the ubiquitination of the XPC protein in the NER pathway. The gene product XPC is part of a protein complex with RAD23 that complements the NER defect, in XPC cells [38]. Known as the primary DNA damage sensor in NER, the multi-subunit ligase consisting of ROC1, CUL4A, DDB1 and DDB2 binds to the DNA lesion and proceeds to auto-polyubiquitinate DDB2 and CUL4a, followed by the recruitment and polyubiquitination of the XPC/RAD23 heterodimer [39]. Despite being ubiquitinated by the same multi-subunit ligase, strikingly the ubiquitinated proteins of DDB2, CUL4a and XPC suffer different fates. DDB2 and CUL4a are targeted to the proteasome for degradation, while polyubiquitinated XPC is bound to the DNA lesion and required for optimal NER activity [39]. Although the topology of these XPC bound ubiquitin chains has yet to be determined, it is interesting to speculate that these chains are either linked through K6 or K63 as they can both serve non-proteolytic roles. Recently, sumoylation has also been speculated to play a role in XPC stability [40]. Alternatively, XPC could be ubiquitinated similarly to DDB2 or CUL4a, but protected from degradation by its heterodimeric partner RAD23, as has been suggested by several recent studies [41,42]. In fact, RAD23 contains an ubiquitin-like sequence at its amino terminus, a domain that is conserved between yeast and human [43]. Recently, it was shown that the interaction of the RAD23 ubiquitin-like domain and the proteasome is essential for NER [44]. Moreover, the

requirement of this interaction was independent of the proteolytic functions of the proteasome, thus it was suggested that the proteasome may be playing a chaperone-like function to increase the rate or fidelity of NER [45]. Overall, these combined observations indicate a unique role for ubiquitination in NER.

1.3.3 DDT requires ubiquitination

The final example of a role for ubiquitin in DNA repair comes from the RAD6 governed DDT pathway. The RAD6 protein itself is an E2 ubiquitin conjugating enzyme. Mutational inactivation of the active site cysteine 88 clearly indicates that the conjugation activity is essential for its function in variety of biological processes [46-48]. RAD6 is able to mono and polyubiquitinate histones 2A and 2B *in vitro* and *in vivo* [47]. Furthermore, in yeast, RAD6 mediates the N-end rule protein degradation in a complex with Ubr1 protein [46]. However, the function of RAD6/UBR1 complex is likely distinct from the role of RAD6 in DNA repair. In the scenario of DNA damage, RAD6 is bound and recruited by a different partner, the single strand DNA binding protein RAD18, which regulates DDT via ubiquitination [30,49].

1.4 DNA damage tolerance in yeast

During normal replication, the molecular sliding tool-belt PCNA serves as a processivity factor for replicative polymerases δ and ε [50]. In yeast, PCNA is typically sumoylated by UBC9 on K127 and K164 during S phase, a process that serves to recruit SRS2 and suppresses unwanted recombination [51]. Upon encountering DNA damage, RAD6 and RAD18 catalyze the monoubiquitination of PCNA on the same K164 [52]. This reaction activates the error-prone arm of DDT,

which helps recruit bypass polymerases such as POLη or POLι, a member of the Y family of lesion bypass polymerases [53,54]. Recruitment is facilitated by the recently discovered ubiquitin binding motif (UBM) and ubiquitin binding zinc finger (UBZ) in TLS polymerases (POLη and POLι) that leads to physical interaction with monoubiquitinated PCNA at sites of stalled replication forks [55-57]. Presumably, after efficient bypass with these low fidelity polymerases, ubiquitin is cleaved by deubiquitinating enzymes (DUBs) and the temporarily displaced POLδ or ε resumes replication. However the exact mechanism of replicative resumption remains unclear (**Figure 9**).

There is substantial evidence from yeast that monoubiquitinated PCNA can be extended by noncanonical polyubiquitin chains linked through K63 [52]. In yeast, a K63R mutation results in an ubiquitin monomer that can function in a majority of pathways where chains are assembled through K48 but which are incapable of forming chains through K63 [7,14]. The K63R mutant did not have a general defect in ubiquitination as it grew at wild type rates, and it mediated protein turnover [14]. Intriguingly, the K63R mutation resulted in a mutant that was hypersensitive to UV irradiation and MMS which was indistinguishable from a loss of RAD6, RAD18, MMS2 or UBC13 [14,52]. This was the first reported role for ubiquitin chains in DNA repair and suggested the possibility that novel chains linked through K63 may serve as a recruiting signal that acts as a scaffold for a multi-subunit complex that bypasses DNA lesions.

In yeast, PCNA polyubiquitination is catalyzed by the RAD5/UBC13/MMS2 complex consisting of the E3 ring finger ubiquitin ligase RAD5 and the unique E2

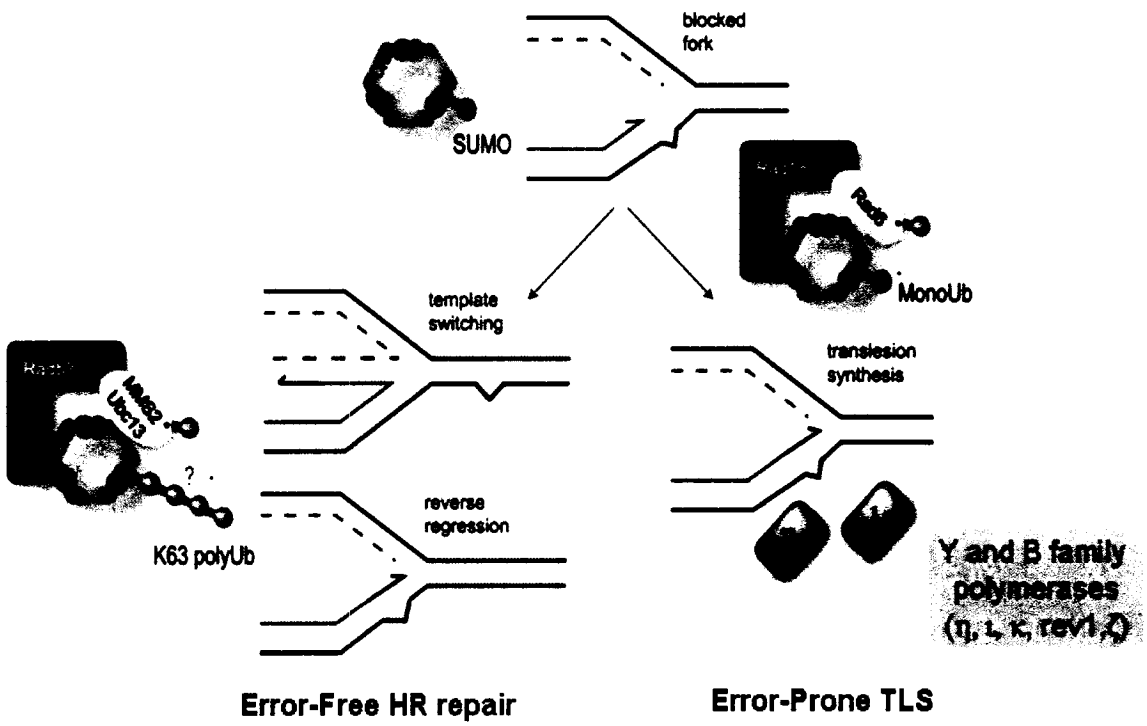

Figure 9. DNA damage tolerance in yeast. In undamaged cells PCNA sumoylation allows normal S-phase progression by preventing unwanted recombination. Upon encountering a DNA lesion PCNA is ubiquitinated and acts a molecular switch that governs the error-prone or error free resolution of DNA damage during replication.

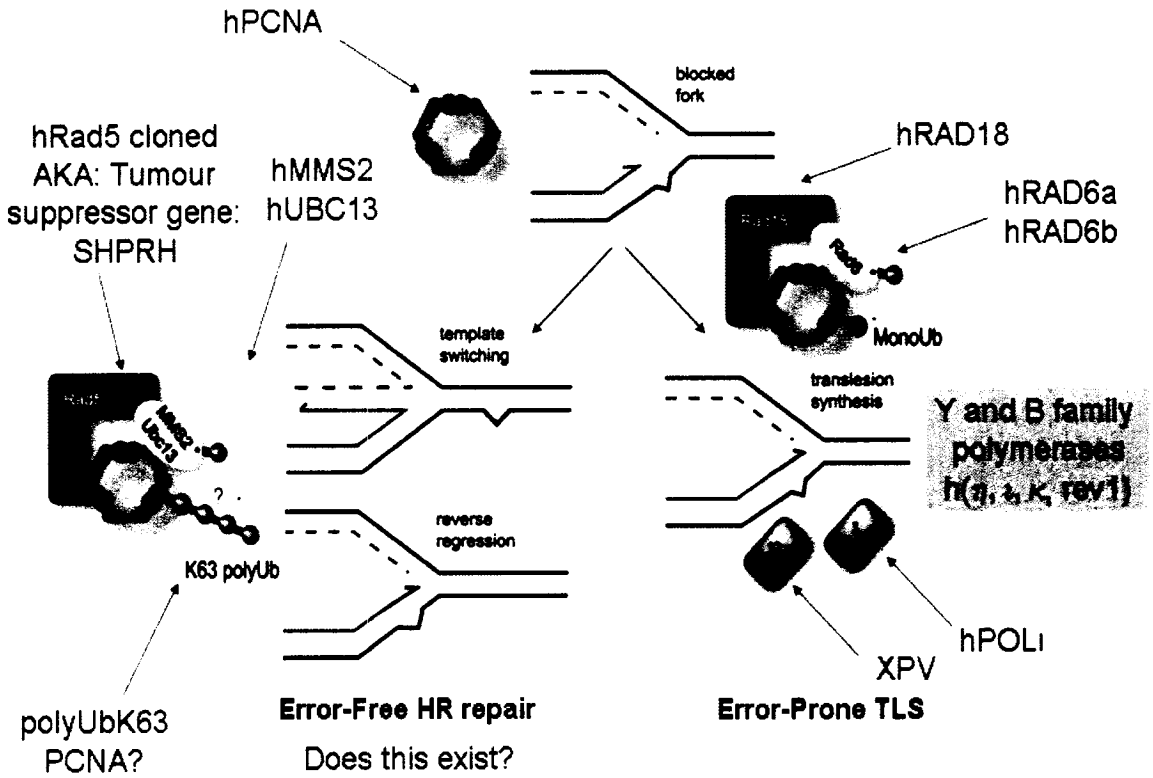

Figure 10. DNA damage tolerance in humans. Many of the genes involved in DDT are conserved from yeast to humans and at the start of this project the TLS arm of DDT was shown to be functional in human cells. We sought to resolve whether the error-free arm of DDT was functional.

conjugating heterodimer of UBC13/MMS2 [52,58]. MMS2 is a ubiquitin conjugating enzyme variant (UEV) that lacks a catalytic cysteine and thus requires UBC13, which contains the appropriate cysteine to carry out the ubiquitin conjugation [59-61]. While biochemical studies demonstrate that both partners are required for ubiquitination, structural studies reveal that both enzymes are oriented in such a fashion that only chains linked through K63 can be formed [62]. Interestingly, UBC13 and MMS2 are largely cytosolic and are recruited to the nucleus following DNA damage where they interact with RAD5 and are subsequently brought into close proximity with the RAD18/RAD6 complex [63]. Interaction of RAD5/UBC13/MMS2 with RAD6/RAD18 then promotes the assembly K63-linked ubiquitin chains at K164 of PCNA (**Figure 9**) [52]. K63-linked polyubiquitinated PCNA is essential for the error-free arm of DDT as evidenced by increased sensitivity and increased mutagenesis in yeast mutants of MMS2 and UBC13 to a variety of DNA damaging agents. The presence of UBC13/MMS2 in the cytoplasm is likely related to the use of this complex in other biological processes, most notably the cell cycle [64] and signal transduction [13].

1.5 DNA damage tolerance in humans

The absolute requirement of ubiquitination in DDT is well established in yeast but much less is known about its role in humans. However, there is fast evolving evidence that this pathway is functional and important in humans. First, there is a high degree of sequence conservation of the RAD6 pathway genes from yeast to humans, and most homologs in this pathway have been identified (**Figure 10**) [65-69]. Second, disruption of lesion bypass repair has been implicated in the NER

positive xeroderma pigmentosa variant syndrome. Patients with XPV have a mutation in the lesion bypass POLη (member of Rad6 epistasis group in yeast) and display a mild photosensitivity and a greatly increased risk for developing skin cancer [20]. The XPV syndrome has recently been modeled in mice [70]. Third, recent studies of the DDT pathway in human cells indicate the full conservation of the TLS arm of the pathway from yeast to humans [26,71]. These landmark studies by Kannouche et al., and Watanabe et al., demonstrate that hRAD18/hRAD6-dependant PCNA monoubiquitination co-ordinates a switch from highly processive and fidelity-driven POLδ to low fidelity polymerases POLη at sites of DNA damage. Fourth, a report showed that stable expression of a hRad18 (human homolog of RAD18) mutant that was unable to bind HHR6 resulted in increased sensitivity to DNA damaging agents [72]. Fifth, human homologs hMMS2 and hCROC1 are able to functionally complement loss of yeast MMS2 with regard to UV sensitivity and spontaneous mutagenesis [68]. Sixth, decreased expression of hRAD6B is associated with lung cancer [73]. Finally, hRAD18 maps to chromosome 3p24-25 which is frequently deleted in lung, breast, ovary and testis cancers suggesting a potential role of this pathway in preventing carcinogenesis [72].

One could explore the role of polyubiquitination in the error-free arm of the DDT pathway by generating null mutations in UBC13 or MMS2 but as in yeast similar sensitivities should be imparted by expression of dominant negative K63R mutant ubiquitin. The use of such a mutant should provide us with the opportunity to establish a role for K63-linked chains in human cells and answer some of the following questions: (1) is the error-free arm of DDT conserved from yeast to

humans? (2) If so, are K63-linked chains a key component of DDT in human cells as they are in yeast? (3) Is PCNA the key substrate involved?

1.6 General Hypothesis

By disrupting polyubiquitin chain assembly we will affect specific cellular pathways. The introduction of dominant negative ubiquitin mutants can be used to disrupt this chain assembly and be used as a tool to study ubiquitin in vivo and unmask its role in mammalian systems.

Several studies, most of which have been performed in yeast, support this hypothesis. In yeast, the overexpression of a K48R ubiquitin mutant in wild type cells acts in a dominant negative manner and results in a general disruption of protein turnover [74]. On the other hand, the expression of a K63R ubiquitin mutant results in the impairment of post-replication DNA repair processes [14]. Notably, *S. cerevisiae* can be exploited in a manner such that one can express the ubiquitin mutants as the sole source of ubiquitin. In such a case, the expression of a K48R mutant leads to cell death, while the expression of the K63R mutant leads to a different phenotype demonstrated by sensitivity to DNA damaging agents, such as UV light and methyl methanesulfonate (MMS) [14,74]. Although we are unable to express ubiquitin mutants as the sole source of ubiquitin in mammalian cells, our contribution lies in developing analogous mammalian systems that exploit the use of dominant negative mutant forms of ubiquitin, which serve as chain terminators, resulting in a disruption in ubiquitin chain formation (**Figure 11**).

We have created such a system by using site-directed mutagenesis to generate a series of lysine to arginine ubiquitin mutants in the pUbGFP vector (**Figure 12**)

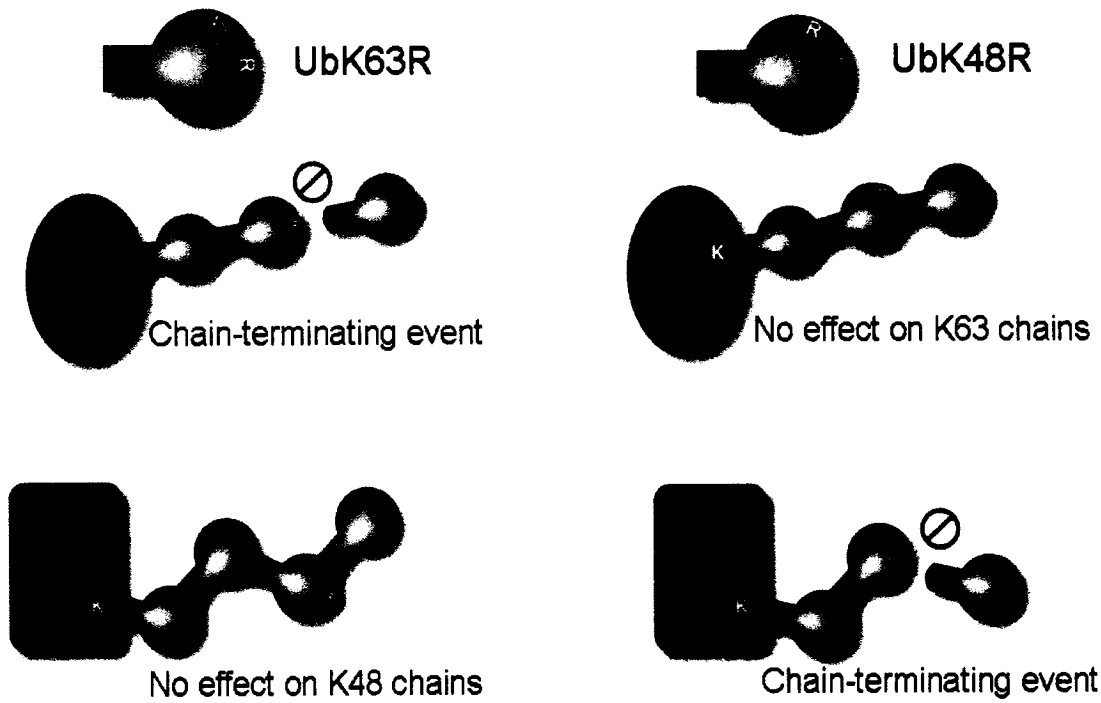

Figure 11. Selective disruption of ubiquitin chains. Ubiquitin that is mutated at specific lysine resides creates a dominant negative ubiquitin that serves as a chain terminator. For example UbK63R will terminate K63 linkages while having no effect on chains linked through K48. Alternatively an UbK48R mutant will disrupt K48 linked chains while leaving K63-linked polyubiquitination unaffected.

[75]. The ubiquitin mutants are expressed as a 6XHis tagged fusion protein of ubiquitin to EGFP driven by a Ubc promoter. To this end, A549 cells have been co-transfected with a puromycin resistance marker and either 6XHis-UbGFP, 6XHis-UbK48RGFP, or 6XHis-UbK63RGFP. Furthermore, Hela cells were transfected with 6XHis-Ub-puromycin and 6XHis-K63R-puromycin constructs. Cells were selected in puromycin for 2 weeks and examined for fluorescence by a GFP dissecting microscope or probed with an anti-his antibody for expression of mutant ubiquitin monomers. Highly expressing clones were expanded and pools of puromycin resistant cells were analyzed by flow cytometry for GFP expression. Subsequently, we used fluorescence activated cell sorting (FACS) to collect the highest expressing cells from this population. Following three FACS sorts we acquired a population of greater than 98% of cells expressing high levels of GFP, as indicated by flow cytometry **(Figure 13)**. In mammalian cells ubiquitin is expressed as a fusion protein with ribosomal subunits and is cleaved into its monomeric form by ubiquitin specific proteases and/or ubiquitin carboxyhydrolases [76-79]. Similar cleavage occurs when ubiquitin is expressed as a fusion protein with EGFP or puromycin. Wild type and mutant forms of ubiquitin are readily detected by Western blot in monomeric form and conjugated to protein substrates when probed with anti-His_6 epitope antibody [75].

As shown in a recent publication, our laboratory has been able to achieve high enough levels of mutant ubiquitin in mammalian cells to exert a dominant negative effect. In our report, cells expressing K48R and K63R mutant ubiquitin displayed differential sensitivity to certain protein damaging chemical agents [75]. K48R cells were highly sensitive to the amino acid analog canavanine (which promotes protein

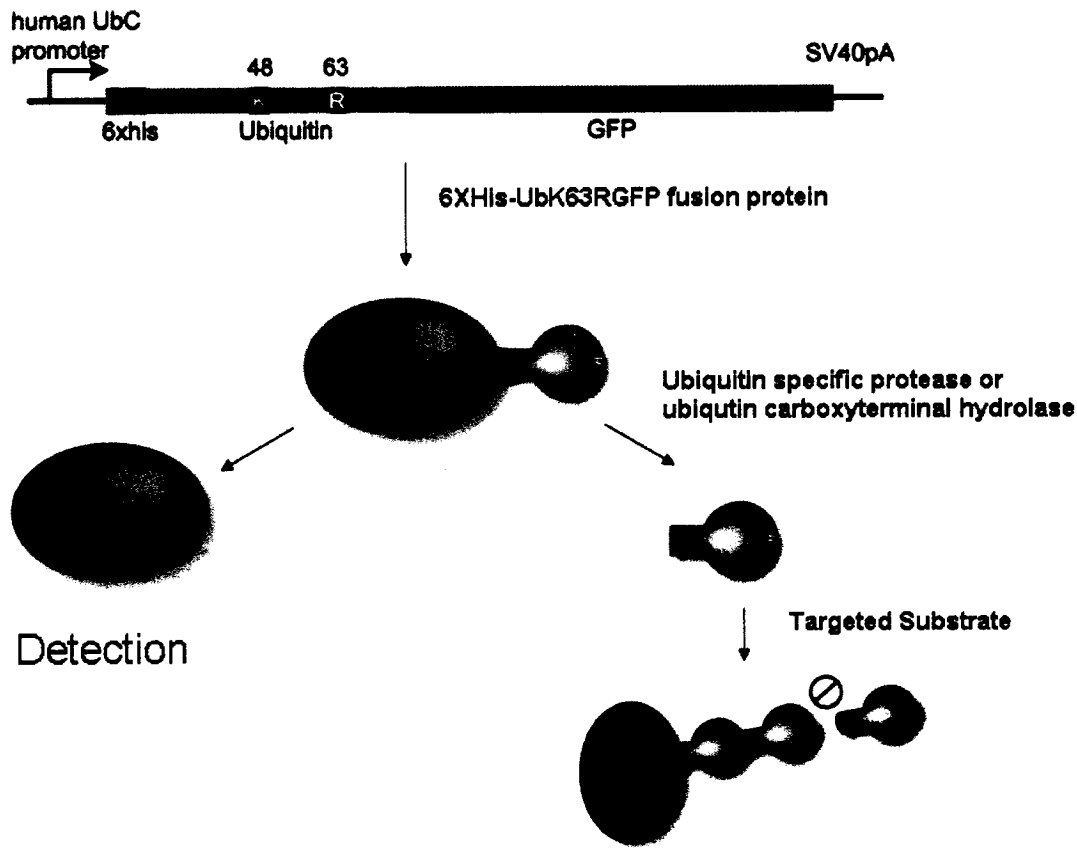

Figure 12. Site-directed mutagenesis of ubiquitin. The expressed fusion protein is processed by ubiquitin specific proteases or ubiquitin carboxyterminal hydrolases generating a mutant ubiquitin monomer and free GFP which can be detected by flow cytometry. Incorporation of this mutant will terminate the elongation of K63-linked chains

Enrichment of Stable Expressors

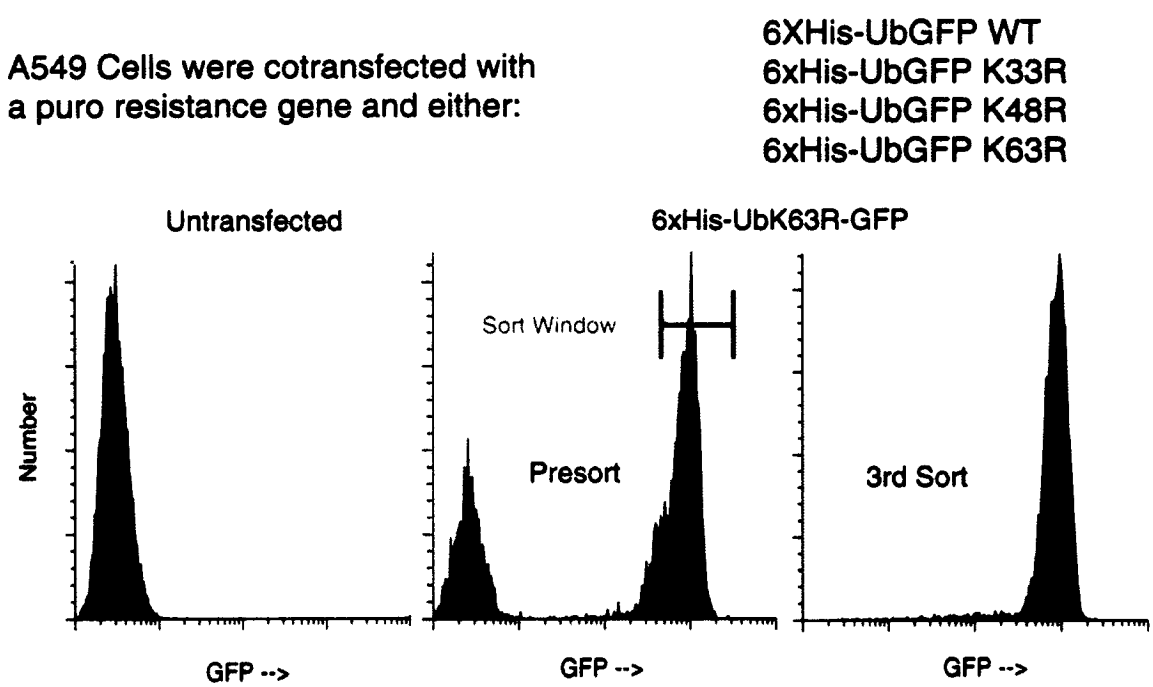

Figure 13. Enrichment of stable expressors. A549 cells were cotransfected with a puromycin resistance gene and wild type ubiquitin or mutant ubiquitin. This was followed by enrichment of stable expressors by sorting cells expressing high levels of GFP. After 3 sorts about 99% of cells had high homogenous expression of GFP.

aggregation) and were able to stabilize GFP^u, an unstable version of GFP that is normally rapidly degraded. Alternatively, cells expressing K63R mutants were highly susceptible to the effects of cadmium suggesting a possible role in DNA repair. Cadmium promotes oxidative DNA damage resulting in the formation of 8-oxodeoxyguanosine, whose elevated levels during S phase impede replication [80]. Repair during S phase involves DDT, a process known to make use of K63-linked ubiquitin chains to recruit error-free repair to the site of the DNA adduct. Therefore, it is conceivable that cadmium-mediated DNA damage is repaired by error-free DDT. However, K63-linked ubiquitin chains are involved in other cellular processes, such as NF-κB activation, for which TRAF2 [81], TRAF6 [13], RIP [82] and NEMO [83-85] are primary substrates. Consequently, NF-κB activation cannot be ruled out as a possible explanation for cadmium sensitivity in K63R expressing cells.

Although mammalian cell culture studies have been and will continue to be highly informative, the more direct measure of the importance of polyubiquitin chain assembly may come from the analysis of its disruption in a mouse model. To this end, the first transgenic mice expressing the tagged wild type ubiquitin (6XHis-UbGFP) construct have recently been published by our laboratory [86]. Since the time of the report, we have successfully established homozygous transgenic mice expressing the mutant isoforms of 6XHis-UbK48RGFP and 6XHis-UbK63RGFP [87]. Due to the establishment of stable mutant ubiquitin mammalian cell lines and the transgenic mouse lines, we hypothesized that we could exploit the role of K48 and K63-linked chains in DNA repair and NF-κB activation in *vitro* and *in vivo*. The role of ubiquitin

in DNA repair is elaborated upon in Chapters 2-7 and Appendix A, and NF-κB activation is addressed in Chapter 8 and Appendix B.

Chapter 2

Lysine 63-polyubiquitination guards against translesion synthesis-induced mutations.

Contribution of collaborators

The contents of this manuscript were written by Jan Brun (JB) and Dr. Roland K. Chiu (RKC) and edited by Dr. Doug Gray (DAG) and Dr. Brad Wouters (BGW). RKC, JB, Chantal Raemakers (CR), Jan Theys (JT), Lin Weng(LW), Philppe Lambin (PL), DAG, and BGW conceived and designed the experiments. A majority of the experiments and figures are the work of Jan Brun with the exception Figure 1A, Figure 2A, B. Figure 3C and 3D, Figure 4, Figure S1B, Figure S2 and Table 1. Overall, RKC, JB, CR, and JT performed the experiments. RKC, JB, CR, JT, LW, PL, DAG, and BGW analyzed the data. RKC and JB contributed reagents/materials/analysis tools. The GFP- POLη construct for visualizing foci was kindly provided by Dr. Alan Lehmann (University of Sussex).

Summary

Genomic instability increases ones predisposition to developing cancer. Therefore, eukaryotes have evolved several mechanisms to maintain the fidelity with which the genome is replicated. Our study involves the examination of a unique DNA damage tolerance pathway which employs the use of the ubiquitin family to rescue stalled replication forks. Studies in lower eukaryotes suggest that novel polyubiquitin chains are essential for this rescue in an error free manner. Here we provide evidence that disruption of these chains leads to UV induced mutagenesis and overt sensitivity to some DNA damaging agents. Several lines of evidence demonstrate that the

mutations are results of increased reliance on lower fidelity polymerases. Finally we demonstrate that the relevant target of ubiquitination is PCNA. Overall this suggests that the PCNA ubiquitination conserved from yeast to human and its polyubiquitination guards against environmental mutagenesis. In the short time since the publication of this chapter, several groups have corroborated and mentioned our work in some high impact publications including that of Lehmann et al., in DNA repair (2007); Chang et al., in the Journal of Biological Chemistry (2006); Unk et al., in Proceedings of the National Academy of Sciences (2006); Motegi et al., in the Journal of Cellular Biology (2006); Langie et al., in DNA repair (2007); and reviewed in DNA repair (2007) and Nature Reviews Cancer (2006) by Bergink et al., and Dikic et al., respectively.

Lysine63 Polyubiquitination Guards Against Translesion Synthesis Induced Mutations

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Running Title: K63-polyUb promotes error-free damage avoidance

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Key words: DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63-polyUb, lysine 63-linked polyubiquitin

Abstract

Eukaryotic cells possess several mechanisms to protect the integrity of their DNA against damage. These include cell-cycle checkpoints, DNA repair pathways, and also a distinct DNA damage tolerance system that allows recovery of replication forks blocked at sites of DNA damage. In both humans and yeast, lesion bypass and restart of DNA synthesis can occur through an error-prone pathway activated following mono-ubiquitination of PCNA and recruitment of specialized translesion synthesis polymerases. In yeast there is evidence for a second, error-free, pathway that requires modification of PCNA with non-proteolytic lysine 63-linked polyubiquitin (K63-polyUb) chains. Here we demonstrate that formation of K63-polyUb chains protects human cells against translesion synthesis-induced mutations by promoting recovery of blocked replication forks through an alternative error-free mechanism. Furthermore we show that polyubiquitination of PCNA occurs in UV-irradiated human cells. Our findings indicate that K63-polyubiquitination guards against environmental carcinogenesis and contributes to genomic stability.

Synopsis

Genome instability is often associated with increased cancer risk and thus considerable effort has been put into unravelling mechanisms underlying genome surveillance. Guarding the integrity of DNA are a number of DNA repair and cell cycle control systems. Insight into how these pathways become activated is crucially important to the understanding of carcinogenesis and in the development of cancer treatments. This study concerns a distinct DNA maintenance pathway that allows for tolerance of DNA damage rather than repair. Previous knowledge from lower organisms has suggested that enzymes that create lysine63 linked chains are required for this pathway which prompted the authors to disrupt the ability to create these specific chains in human cells. Blocking the formation of these ubiquitin chains, results in a significant increase in mutations. The authors provide several lines of evidence to implicate the error prone translesion synthesis polymerases in the formation of these mutations. Furthermore, they provide evidence suggesting that for DNA damage tolerance, PCNA is the relevant target of polyubiquitination in human cells. These findings indicate that lysine63 polyubiquitination guards against environmental carcinogenesis and contributes to genomic stability.

Introduction

In contrast to DNA repair pathways, DNA damage tolerance (DDT) is characterized by bypass of DNA lesions rather than their direct removal or repair. The DDT pathway is likely responsible for the ability of cells to continue to proliferate with tremendous amounts of damage in their genomes [1]. The genetic and

mechanistic basis of DDT is best understood in yeast where it is known to be an extremely important determinant of the toxicity and mutagenicity of many DNA damaging agents [2,3]. Often referred to as RAD6-dependent repair or post-replication repair, DDT requires interaction of the E2 ubiquitin (Ub) conjugase RAD6 and the E3 Ub ligase RAD18 at sites of DNA damage [4]. Here they mediate mono-ubiquitination of PCNA at K164 and subsequent recruitment of a specialized translesion synthesis (TLS) polymerase capable of replication past the lesion [5,6]. Several yeast and mammalian TLS polymerases have been identified including POLη (RAD30A), POLι (RAD30b), REV1, REV3 and POLκ [7]. These are highly error-prone polymerases that allow replication past a variety of DNA lesions [7]. POLη plays a uniquely important role in the repair of UV damage as it mediates error-free bypass of thymine-thymine dimers, the most common UV-induced lesion [8]. *Saccharomyces cerevisiae* Rad6 and Rad18 mutants that are unable to carry out DDT are highly sensitive to various genotoxic agents including UV and MMS [9]. These mutants also show a reduction in UV-induced mutations [10] that arises due to the inability to recruit the error-prone TLS polymerases [11].

Genetic epistasis studies in yeast have established a second arm of the DDT pathway distinct from TLS referred to as damage avoidance [5,12-14]. This pathway is also downstream of RAD6/RAD18, but in contrast to the error-prone TLS pathway resolves blocked replication forks through an error-free manner. Its mechanism is not fully understood, but may involve fork reversal and recombination with the undamaged replicated sister chromatid [5]. This damage avoidance pathway requires a second ubiquitination complex composed of RAD5 and the UBC13/MMS2

heterodimer [5]. UBC13/MMS2 is a unique Ub conjugase that synthesizes polyUb chains linked through K63-G76 bonds rather than the typical K48-G76 bonds [13]. Although K63-polyUb chains can serve as competent proteolytic signals they are less efficient at targeting substrates to the proteasome than K48-linked chains [15], and the proteolytic activity of the proteasome may not be required for error-free repair [13]. In yeast, a model has emerged in which error-free damage avoidance occurs when mono-ubiquitinated PCNA becomes further modified by K63-polyUb via RAD5 and MMS2/UBC13. Interestingly, modification of K164 in PCNA by sumoylation rather than ubiquitination reduces the error-prone pathway by suppression of homologous recombination [16,17].

There is convincing evidence that the DDT pathway and especially the TLS arm is also important in higher eukaryotes including humans. Mouse and human homologs of RAD6, RAD18, PCNA and many of the TLS polymerases have been identified [18]. The TLS polymerases form foci at sites of DNA damage following UV irradiation and are associated with other proteins in the replication machinery [19]. As in yeast, Rad6 and Rad18 mediate mono-ubiquitination of PCNA at K164 in UV-irradiated mammalian cells in a dose and time dependent manner [11]. Mono-ubiquitination of human PCNA has been suggested to provide a signal for polymerase switching since it leads to its increased association with POLη via its ubiquitin binding domain (UBD) or the UBZ (Ub-binding zinc-finger) in this TLS polymerase [20]. In vitro studies have also demonstrated that mono-ubiquitination of PCNA in yeast can stimulate the activities of both POLη and REV1 [21]. Recently, the deubiquitinating enzyme USP1 was shown to directly remove the monoUb from

PCNA leading to the suggestion that USP1 is required to suppress the error prone activity of TLS [22]. The functional importance of TLS is exemplified by the fact that mutations in POLη are responsible for the variant form of Xeroderma Pigmentosum (XPV), a disease characterized by a 2000-fold increased risk of developing skin cancer [8]. In contrast to other XP patients, those with XPV have no defect in excision repair [8] but are deficient in postreplication repair [23]. Furthermore, they display enhanced mutation at TT sites due to usage of an alternative error-prone TLS polymerase [24].

In contrast to TLS, the importance of the damage avoidance arm of DDT in mammalian cells is not yet firmly established. Perhaps the strongest evidence supporting a role for this pathway comes from Li et al, who showed that antisense inhibition of hMMS2 resulted in an increase in mutation frequency [25]. Nonetheless, several open questions remain to be resolved. First, a human homolog of RAD5 has not yet been identified. This may be due to the fact that yeast RAD5 contains a helicase activity required for its function in DNA double strand break repair, but is unimportant for DDT [26]. These authors speculated that RAD5 in higher organisms may have evolved to lose this domain. Second, although homologs of MMS2 exist (hMMS2 and hCroc1) and are able to functionally complement loss of yeast MMS2 [27], they are additionally required for polyubiquitination of proteins in pathways unrelated to DDT [28]. Third, although evidence for human PCNA mono-ubiquitination is strong [11,29], there is less evidence for its polyubiquitination. High molecular weight bands in PCNA Westerns were noted in mouse fibroblasts following UV irradiation [11]. However, Kannouche and colleagues found no

evidence for polyubiquitination in human fibroblasts [29]. They concluded that polyUb forms of PCNA were either insignificant, occurred only at low levels or were rapidly turned over [29]. Thus, whether polyubiquitination of PCNA and subsequent activation of an error-free damage avoidance pathway is evolutionarily conserved in humans is a source of uncertainty that we sought to resolve.

Here, we provide evidence that the ability to create K63 based polyUb chains is required for an error-free damage response pathway in human cells. We implicate this ubiquitination step in a pathway that contributes to genomic stability by suppressing translesion polymerase mediated mutagenesis. Moreover, we show that DNA damage induced PCNA polyubiquitination is indeed conserved in human cells suggesting that this ubiquitin based molecular switch plays a decision role in directing repair in either an error free or error prone manner.

Results

Dominant negative approach to disrupt K63-polyUb chain assembly

In order to directly investigate the functional importance of K63 linked polyUb chains in DDT, we employed a strategy similar to that first described in yeast, to specifically inhibit assembly of these chains. In yeast, replacement of Ub with a mutant in which lysine 63 is mutated to arginine (K63R) disrupts the error-free arm of DDT and results in a phenotype equivalent to loss of UBC13 or MMS2 [14]. The K63R mutation disrupts K63-polyUb chain assembly, but has no effect on K48-linked chains that mediate proteasomal based protein turnover [14]. In human cells, an equivalent knock-in approach is not feasible because Ub is expressed from multiple

genes. The UBA52 and UBA80 genes encode a Ub monomer fused in frame with ribosomal subunits while the UBB and UBC genes encode variable length linear polymers of (typically 3-4 and 9 Ub proteins respectively) [30]. The fusion proteins are cleaved by DUBs to release individual Ub monomers. Our approach was to express the K63R-Ub mutant in trans so that it competed with wild-type (WT) Ub for inclusion into polyUb chains. Its incorporation blocks further ubiquitination through K63 and thus acts in a dominant way. In a previous study we validated and used this approach to specifically suppress K48-linked Ub chains by expressing a K48R-Ub mutant [31]. This same construct has also been used to inhibit K48 polyubiquitination in transgenic mice [32]. Here, we expressed 6xhis-tagged *K63R-Ub* or *WT-Ub* fused in frame with *GFP* from the UbC promoter (Figure 1). <insert Figure 1 here> Expression yields a fusion protein that is cleaved, releasing a 6xhis-tagged Ub monomer and free GFP (Figure 1B). GFP was used to sort pools of cells with stable high expression of the transgene. Both WT-Ub and K63R-Ub monomers were efficiently incorporated into polyUb chains as evidenced by their detection in high molecular weight smears characteristic of the heterogeneity of ubiquitinated proteins (Figure 1B). The K63R-Ub mutant did not affect normal cell proliferation as demonstrated by the identical growth rates in the sorted stable high K63R-Ub GFP expressing pools and in the similarly sorted WT-Ub and the untransfected cells (Figure 1C). Furthermore, disrupting K63-polyUb chain formation did not alter normal proteasome-mediated protein degradation of p53 or HIF1α (data not shown). These data indicate that the K63R-Ub fusion protein is properly processed into K63R Ub monomers, incorporates normally into chains, and does not alter the ability of the

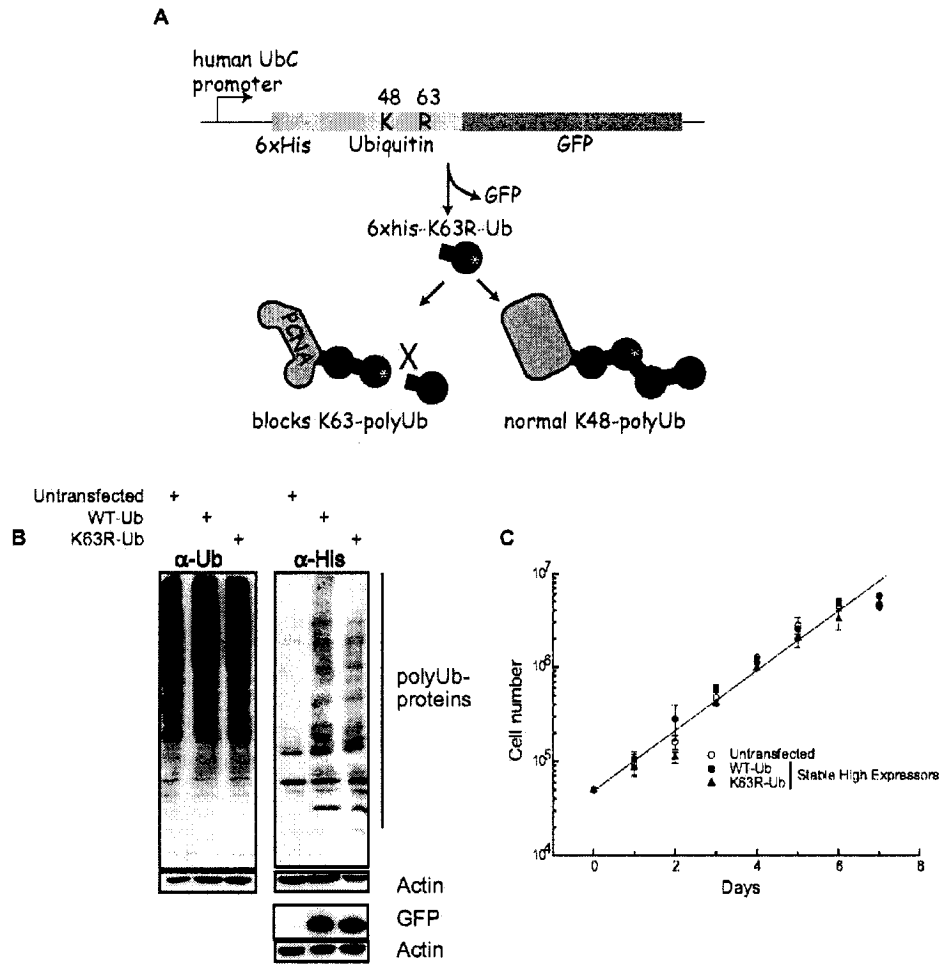

Figure 1: **Disruption of K63polyUb chain assembly**. **(A)** Cartoon depicting the dominant negative *K63R-Ub-GFP* construct. The expressed fusion protein is processed by endogenous ubiquitin proteases generating free GFP used for detection on a flow cytometer and mono-K63R-Ub. Incorporation of this mutant will terminate K63-polyUb chains while not affecting canonical K48-polyUb chain assembly. **(B)** Whole cell lysates were isolated from untransfected, *WT-Ub*, and *K63R-Ub* stably expressing cells followed by immunoblot analysis with antibodies directed against Ub, His and GFP. **(C)** The growth of untransfected, WT-Ub, or K63R-Ub cells was followed by cell counting over the course of 7 days.

proteasome to recognize polyubiquitinated substrates targeted for degradation.

Disruption of K63-polyUb chain assembly sensitizes cells to cisplatin- but not UV-induced cell death

Creation of stable cell lines expressing *WT-Ub* or *K63R-Ub* allowed us to examine the role of K63-polyUb chain assembly during recovery from DNA damage. We first investigated if inhibition of K63 polyubiquitination would sensitize cells to agents known to sensitize yeast mutants in the damage avoidance error-free arm of DDT [2,3]. We found that cisplatin, a chemotherapeutic agent highly toxic to yeast mutants in this pathway [2,3], is also significantly more toxic to A549 cells expressing *K63R-Ub* (Figure 2). <insert Figure 2 here> This sensitivity is specific to expression of *K63R-Ub* since the response of cells expressing either *WT-Ub* or *K33R-Ub* is identical to that of untransfected controls (Figure 2A,B). This effect was not mediated by a general inhibition of ubiquitination since A549 cells expressing the *K48R-Ub* mutant are not sensitized (data not shown). Furthermore, a K63R-Ub clone that lost expression of the transgene (as evidenced by a low GFP signal) returned to normal sensitivity (Figure 2B). These data imply that K63-polyUb chain assembly is absolutely required for recovery from at least a subset of cisplatin induced lesions.

We also examined the functional importance of K63 polyubiquitination in the recovery from UV-induced damage. In contrast to the data with cisplatin, the cell line with stable expression of *K63R-Ub* exhibited a dose response to UV irradiation that was identical to the parental cells or to cells expressing *WT-Ub* (Fig. 2D). Thus, despite evidence that K63-polyUb chains are required for cisplatin tolerance, we

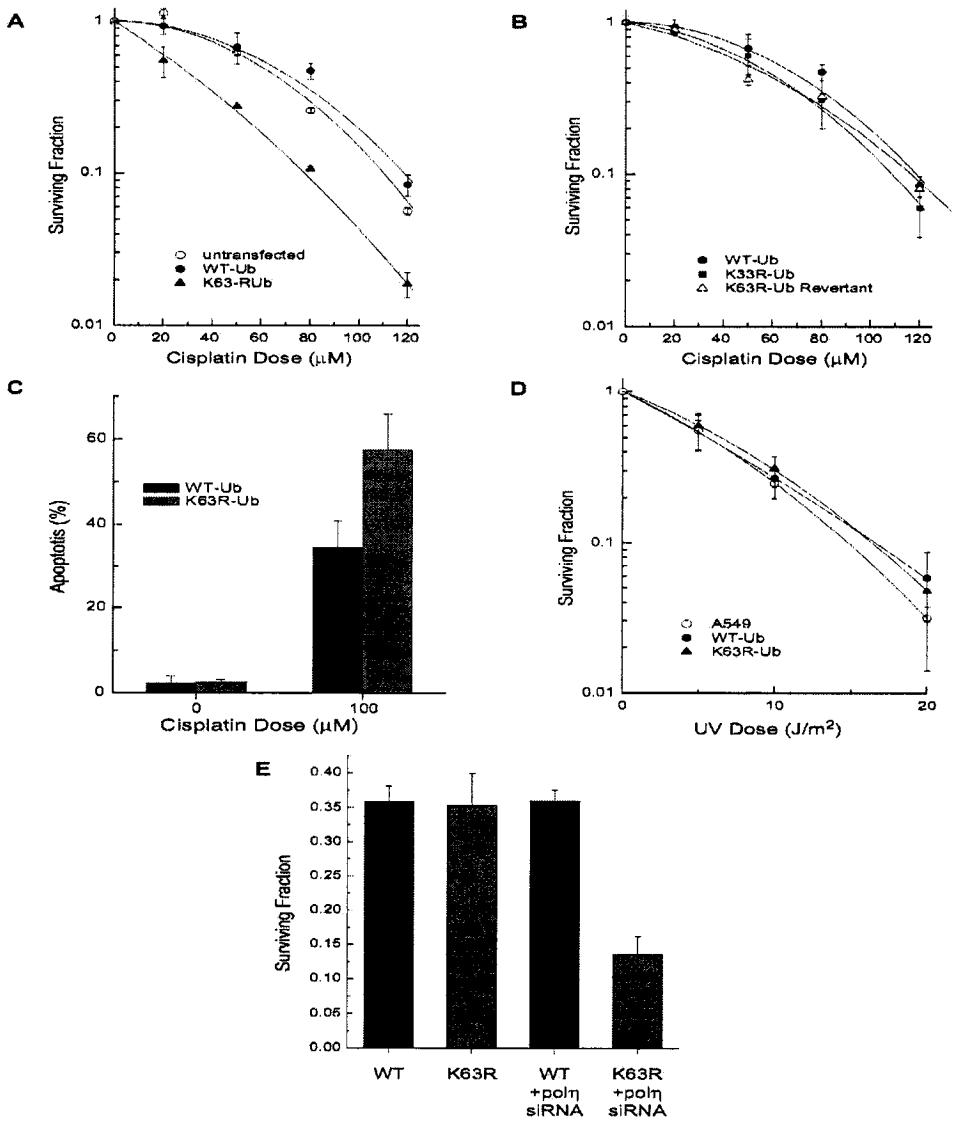

Figure 2: Cells deficient in K63-Ub chain formation are sensitized to cisplatin treatment while UV sensitivity is revealed only upon POLη knockdown. (A-B) Clonogenic survival assays were used to determine sensitivity to 1h acute treatment with cisplatin in untransfected A549 cells or A549 cells stably expressing *WT-Ub* or *K63R-Ub*. The mean values of 3 independent experiments are shown with standard error of the mean (error bars). Cells expressing *K33R-Ub* or cells that lost *K63R-Ub* expression revert to WT-Ub cisplatin sensitivity. **(C)** Cells were treated for 24h with 100μM cisplatin followed by Hoechst staining to detect apoptosis. The mean values of 3 independent experiments are shown with standard deviation. **(D)** Clonogenic survival assays were used to determine sensitivity to UV-irradiation in untransfected A549 cells or A549 cells stably expressing *WT-Ub* or *K63R-Ub*. **(E)** Clonogenic survival of A549 cells stably expressing *WT-Ub* or *K63R-Ub* with or without polη RNAi following 10J/m^2 UV treatment.

found no evidence that disruption of K63-polyUb chain assembly on its own influences UV toxicity. A possible explanation for this lack of sensitivity to UV is that cells can compensate for loss of K63 polyUb dependent repair through increased utilization of the error-prone TLS arm of the pathway. A similar situation occurs in yeast where inhibition of the error-free damage avoidance arm of DDT results in a much milder UV sensitivity than mutations in RAD6 or RAD18 which additionally prevent TLS [33]. Using siRNA, we were able to knock down expression of POLη by ~13-fold (Supplementary Information Figure S1). Similar to inhibition of K63 polyubiquitination, knockdown of POLη had no effect on UV sensitivity on its own. This observation is not unexpected since XPV cells (defective in POLη) are not sensitive to killing by UV. In contrast, knockdown of POLη in cells also expressing *K63R-Ub* did cause increased cell kill after UV treatment (Figure 2E). This increase in UV sensitivity suggests that K63-polyUb and POLη function in distinct, complementary pathways that mediate recovery from UV induced damage.

Disruption of K63-polyUb chain assembly increases UV-induced mutations

Disruption of the error-free arm in yeast is also known to result in a dramatic increase in UV-induced mutations that is synergistic with the TLS mutant, REV3 [34]. If playing a similar role in mammalian cells, inhibition of K63 polyubiquitination should also increase UV-induced mutations. We thus analyzed mutation induction at the HPRT locus after UV and cisplatin exposure in these same cell lines as well as in normal human fibroblasts expressing *WT-Ub* or *K63R-Ub*

(Figure 3). <insert Figure 3 here> Consistent with this hypothesis, A549 cells expressing *K63R-Ub* show a 2.5-fold increase in UV-induced mutations compared to cells expressing *WT-Ub* (Figure 3B) and a similar increase (2.2-fold) is observed in normal fibroblasts (Figure 3C). Untransfected and WT-Ub expressing cells have similar mutation frequencies (data not shown). The increase in mutations upon inhibition of K63 polyubiquitination is consistent with a recent report that used antisense to suppress the expression of MMS2 in human cells. Similar to the cells expressing *Ub-K63R*, loss of MMS2 led to ~2-fold increase in UV-induced mutations without increasing UV-induced cell death [25]. Thus both the enzyme that is implicated in the synthesis of K63-polyUb chains, and the chains themselves are required for recovery from UV damage through a pathway that prevents mutations.

Increases in UV-induced mutations are due to increased utilization of TLS

Many of the TLS polymerases are known to be important contributors to UV-induced mutagenesis as is illustrated by a reduction in mutation frequency when inactivated in yeast [35-38]. The data presented thus far are consistent with a model in which inhibition of K63 polyubiquitination increases UV-induced mutations due to increased use of the error-prone branch of the TLS pathway. However, the possibility that *K63R-Ub* expression in some way increases mutations by affecting the function of one or more TLS polymerases cannot be ruled out. In fact, the phenotype of cells expressing *K63R-Ub* is similar to that described for XPV cells. Both cell types display an increase in UV-induced mutations with no significant change in UV-

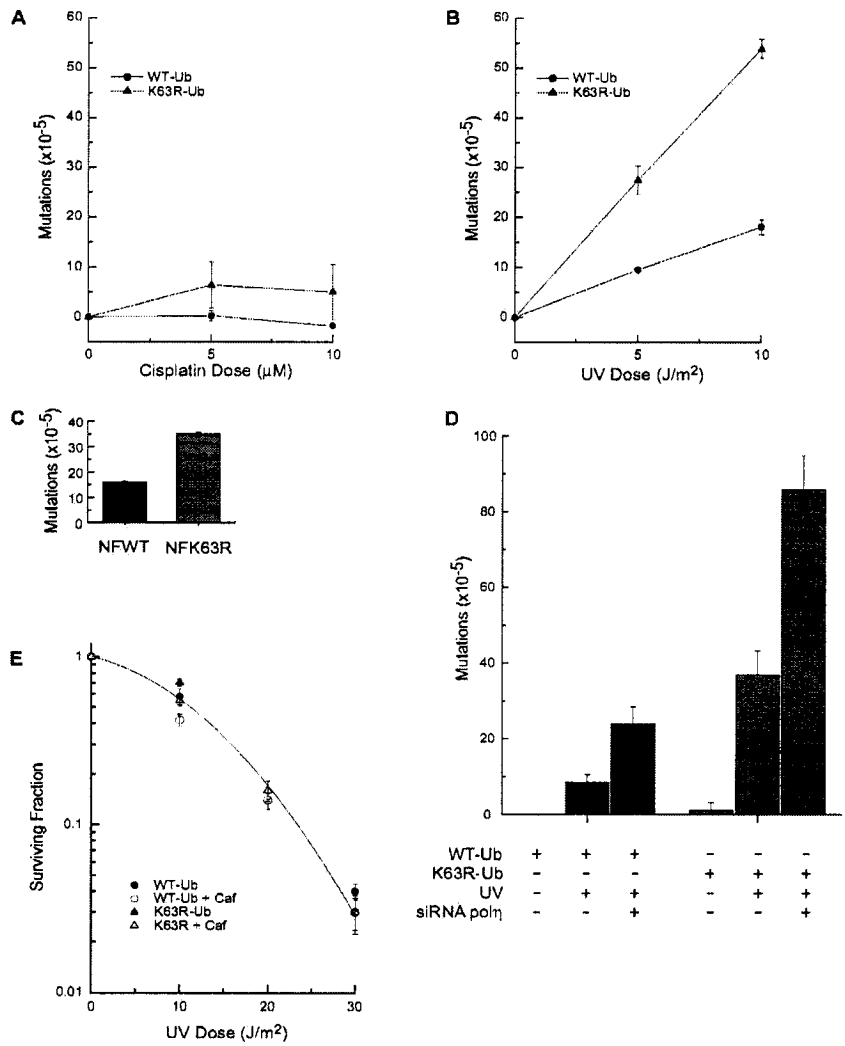

Figure 3: **Cells deficient in K63-Ub chain formation are mutagenic in response to UV treatment. (A-B)** Cells were treated with cisplatin for 1h or UV and subcultured for 7 days. Cells were then plated and grown in 6-TG to select for *HPRT* mutants. The mean values of 3 independent experiments are shown with standard deviation. **(C)** Normal fibroblast(NF) stably expressing *WT-Ub* or *K63R-Ub* were UV-irradiated ($10J/m^2$) and cultured for 5 days. Cells were then plated and grown in 6-TG to select for *HPRT* mutants. **(D)** The number of HPRT mutants were quantitated for A549 cells stably expressing *WT-Ub* or *K63R-Ub* with or without POLη RNAi. Cells were treated as in 3C. **(E)** Cells were UV-irradiated and plated in the absence or presence of 0.4mM caffeine. The mean value of 3 independent experiments are shown with standard error of the mean (error bars).

induced cell death. In XPV cells this is due to loss of POLη, which replicates past T-T dimers in an error-free manner [39]. Defects in POLη can be revealed by a significant increase in UV-sensitivity when irradiated in the presence of caffeine, an assay used to establish the XPV phenotype [40]. However, we found that cells expressing K63R-Ub are not similarly hypersensitive to this combined treatment (Figure 3E) suggesting no overt defect in POLη function in these cells.

In contrast, our data suggest that POLη and K63-polyUb chains participate in separate, alternative pathways for recovery from UV-induced DNA damage. Consistent with this idea, knockdown of POLη in combination with the inhibition of K63-polyUb chain assembly resulted in both an increased toxicity to UV (Figure 2E), and to a further increase in UV induced mutations (Figure 3D). Interestingly, the number of mutations in cells following knockdown of POLη in combination with inhibition of K63-polyUb chain assembly was far greater than additive. As expected, loss of POLη (which replicates past T-T dimers with high fidelity) resulted in a large induction in UV-induced mutations in *WT-Ub* expressing cells (Figure 3D). These mutations are likely due to the activity of alternative TLS polymerases that can substitute for POLη, but which are error prone across T-T dimers [41]. Additional suppression of K63-polyUb chain assembly increased the number of UV induced mutations by 3.5 fold. This synergistic increase in mutations strongly suggests that the inability to form K63-polyUb chains places a larger requirement on the TLS pathway, and thus POLη and likely other lesion bypass polymerases such as POLζ [42,43] for recovery from UV damage. Moreover, the synergistic increase in

mutations suggests that a significant proportion of the repair is normally carried out by the error free component of the damage avoidance pathway.

To further investigate the relationship between inhibition of K63R-polyUb chain assembly and TLS we examined the spatial dynamics of the TLS polymerase POLη. This polymerase is recruited to sites of damage and can be visualized in discrete foci that co-localize with PCNA [44]. We analyzed the effects of *K63R-Ub* expression on POLη foci formation in live cells using a *POLη-GFP* fusion construct [44] (Figure 4A,B,D). Since our original cells co-expressed GFP we generated new stable lines from both A549 and HeLa cells expressing WT-Ub or K63R-Ub fused with the puromycin resistance gene. These cell lines are phenotypically equivalent to the original GFP expressing cells (~3-fold increase in HPRT mutants in K63R-Ub compared to WT-Ub). Similar to previous observations [44], the majority of non-irradiated cells show homogenous nuclear distribution of the tagged polymerases (Figure 4 and Supplementary Information Figure S2). <insert Figure 4 here> Foci were observed in ~11-12% of cells and likely represent sites of ongoing replication [44]. When treated with $10J/m^2$ UV, the percentage of cells with foci increased to 30% in cells expressing *WT-Ub* and to 49% in cells expressing *K63R-Ub* 6 hr post treatment (Figure 4B,D). This corresponds to a ~2-fold increase in UV induced foci as a consequence of inhibition of K63 polyubiquitination (2.4-fold increase over background for WT-Ub vs. 4.6-fold for K63R-Ub, $p<0.007$). We also analyzed the co-localization of these foci with sites of DNA replication as revealed by positive PCNA foci. We found that in both *WT-Ub* and *K63R-Ub* expressing cells, 100% of the UV-induced POLη foci co-localized with PCNA foci (figure 4A). This suggests

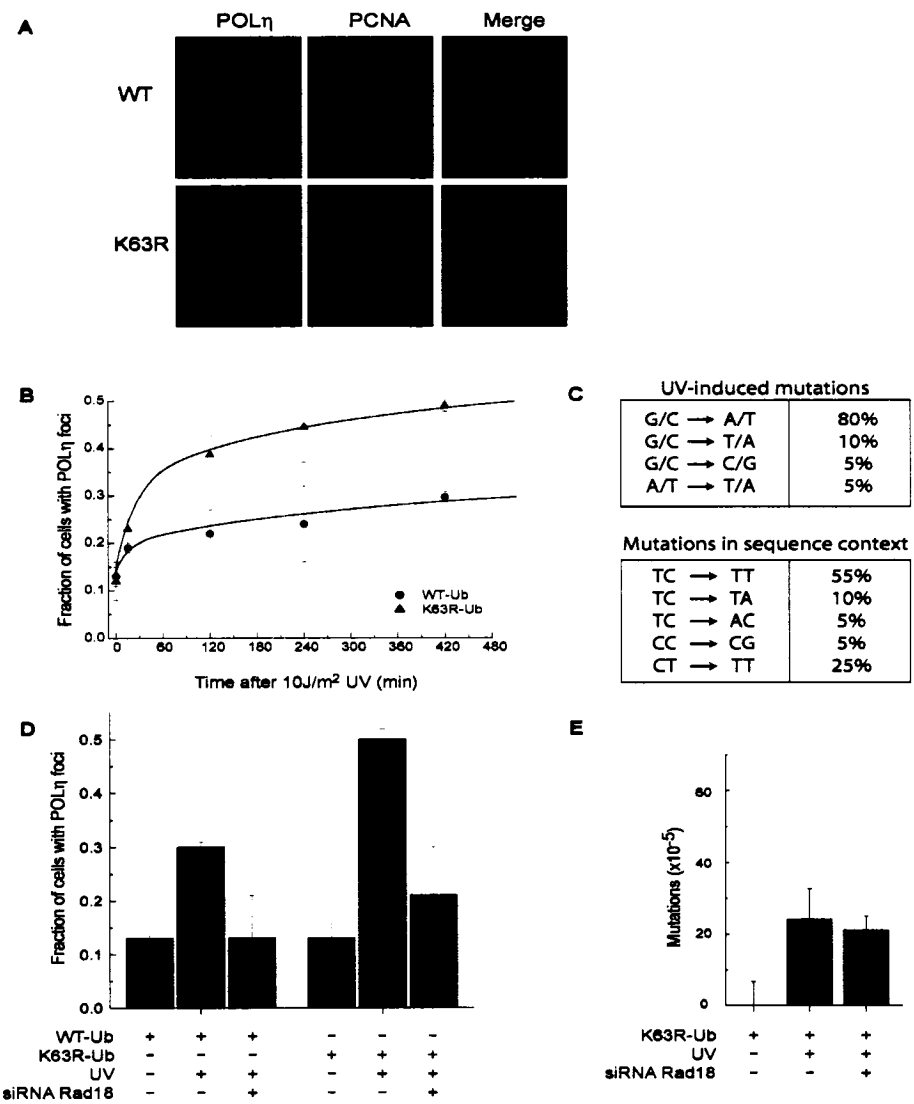

Figure 4: Disrupting K63-polyUb chain formation increases reliance of cells on the error-prone TLS pathway. **(A)** HeLa cells stably expressing *WT-Ub-puro* or *K63R-Ub-puro* were transiently transfected with a plasmid expressing a *POLη-GFP* fusion. 24 hours post-transfection, cells were UV-irradiated (10J/m^2). POLη (green) and PCNA (red) were detected using antibodies. Shown are representative confocal photographs of cells 6h post UV treatment. **(B)** Kinetics of POLη foci formation in *WT-Ub* and *K63R-Ub* expressing HeLa cell lines. **(C)** *HPRT* mutation spectra. RNA was isolated from 6-TG resistant 10J/m^2 UV-treated clones followed by RT-PCR and sequence analysis of the *HPRT* locus. The UV induced mutations are shown in the upper table. Most of the point mutations were G→A or C→T transitions indicated as G/C→A/T. The lower table shows the same mutants in sequence context. **(D)** Foci were quantitated 6h post-UV treatment using a live-cell imaging fluorescent microscope. **(E)** The number of *HPRT* mutants were quantitated for A549 cells stably expressing *K63R-Ub* with or without Rad18 RNAi. Cells were treated as in 4B.

that the foci produced in the *K63R-Ub* expressing cells are typical of those previously reported to occur at sites of blocked replication [44]. To rule out the possibility that UV differentially affects the cell cycle in the two cell lines (and thus the number of cells in S phase) we measured cell-cycle distributions before and after UV treatment and found no significant differences (Supplementary Information Figure S3). In both cell lines the percentage of cells with foci after UV treatment increased rapidly during the first 30 min and then reached a plateau after 3-4 hours (Figure 4B). Thus, although the percentage of positive cells was consistently higher in cells expressing *K63R-Ub*, the kinetics of foci formation are similar. This argues that the K63R-Ub mutant is not interfering in some way with TLS polymerase recruitment dynamics. Interestingly, the magnitude of the increase in foci formation in the K63R-Ub mutant cells is similar to the increase in UV-induced mutation frequency in these cells (Figure 3B and 4B).

We also looked for possible changes in the types of mutations induced by UV after inhibition of K63 polyubiquitination. The two predominant UV-induced lesions are the cis-syn cyclobutane pyrimidine dimer (CPD) and the pyrimidine-6/4-pyrimidone (6-4PP) photoproduct [45,46]. The most common lesion is the thymine-thymine CPD (represented by T-T) followed by T-C and T(6,4)C [47]. Levels of T(6,4)T, C-T and C-C dimers are comparatively much lower. However, the normal spectrum of UV-induced mutations does not match this pattern of damage induction. Mutations are primarily C to T transitions at TC and CC sites that arise due to mis-incorporation of adenine opposite the 3'C [48,49]. The weak contribution of the TT dimer to mutation may be explained by the activities of POLη and POLι, which accurately bypass T-T

and T(6,4)T lesions respectively [50,51]. To further probe for possible changes in the function of these polymerases upon inhibition of K63 polyubiquitination we examined the spectrum of UV-induced mutations in cells expressing *K63R-Ub.* By sequence analysis of the expressed *HPRT* transcript we found that the increase in mutations noted in figure 3 can be accounted for entirely by additional point mutations. We sequenced 20 of these mutations and found that they were all located at dipyrimidine sites (Table I). The majority of mutations were C to T transitions (80%), with most of these being TC to TT (55%) (Figure 4C). These data are consistent with the mutation spectrum of other normal cell lines and contrast with that reported for cells with disruptions in TLS polymerases [48-50,52]. Importantly, inhibition of K63 polyubiquitination did not cause any mutations at TT sites suggesting normal function of both POLη and POLι in these cells.

Collectively, these data suggest that inhibition of K63-polyUb chain assembly results in an increased requirement for TLS after UV-irradiation and consequently increased numbers of visible TLS foci, and an increase in TLS associated mutations. To further support this assertion, we examined the dependence of the observed phenotype on RAD18 function. We transfected our WT-Ub and K63R-Ub stable cell lines with siRNA directed against RAD18 using conditions which consistently showed >10-fold reduction in expression (Supplementary Information Figure S1). In both cell lines, UV induced POLη foci formation was abrogated by RAD18 knockdown implying that the recruitment of TLS polymerases to sites of damage are RAD18 dependent (Figure 4D). This is similar to previous reports showing the requirement of RAD18 for POLη foci formation [11]. Significantly, the UV induced foci formation in K63R-

Mutation Type	Pyrimidine Dimer	Sequence Change	Position (Strand)	Exon	Amino Acid	Mutant
	TC → TT	TATT(C>T)ATTA	674 (–)	8	Glu → Lys	U26-2 27
	TC → TT	TATT(C>T)ATTA	674 (–)	8	Glu → Lys	U26-2 26
	TC → TT	TATT(C>T)ATTA	674 (–)	8	Glu → Lys	U26-2 10
	TC → TT	ACGT(C>T)TTGC	230 (+)	3	Leu → Phe	U5G1
	TC → TT	TAAT(C>T)CAAA	548 (+)	6	Pro → Ser	U26-2 28
	TC → TT	GGTT(C>T)ATCA	125 (–)	2	Glu → Lys	U8B3
G/C → A/T	TC → TT	ATGT(C>T)TTGA	475 (+)	5	Val → Val	U26-2 17
or	TC → TT	TAAT(C>T)CAAA	548 (+)	6	Pro → Ser	U26-2 17
C/G → T/A	TC → TT	TTGT(C>T)TGGA	638 (–)	8	Asp → Asn	U26-2 20
	TC → TT	TATT(C>T)ATTA	674 (–)	8	Glu → Lys	U26-2 20
	TC → TT	TGCT(C>T)GAGA	236 (+)	3	Arg → STOP	U26-2 1
	CT → TT	ATTC(C>T)TATG	366 (+)	3	Pro → Leu	U4E2
	CT → TT	CTTG(C>T)TCGA	234 (+)	3	Ala → Val	U26-2 1.1
	CT → TT	CTTG(C>T)TCGA	234 (+)	3	Ala → Val	U26-2 18.1
	CT → TT	CTTG(C>T)TCGA	234 (+)	3	Ala → Val	U26-2 36.1
	CT → TT	CTTG(C>T)TCGA	234 (+)	3	Ala → Val	U26-2 5.1
G/C → T/A	TC → TA	TTGT(C>A)TGGA	638 (–)	8	Asp → Tyr	U26-2 33
or	TC → TA	TATT(C>A)ATTA	674 (–)	8	Glu → STOP	U1G11
C/G → A/T						
G/C → C/G						
or	CC → CG	CCAC(C>G)AATT	437 (–)	4	Gly → Arg	U6E6
C/G → G/C						
A/T → T/A						
or	TC → AC	CAAA(T>A)CCAA	625 (–)	8	Gly → Gly	U26-2 35
T/A → A/T						

Table I. Disrupting K63-polyUb chain assembly induces a characteristic UV mutation signature. A549 cells stably expressing *K63R-Ub* were treated with 10J/m^2 UV, further cultured for 7 days and then seeded in media containing 6-TG. Single colonies were picked from each dish to ensure no sister clones will be analyzed. The *HPRT* gene was then amplified by RT-PCR followed by sequencing using 4 overlapping primers. The mutants are grouped by observed point mutations and inferred pyrimidine dimers.

Ub was also reduced to non-irradiated levels suggesting that the increased number of foci that are found in cells expressing the K63R mutant is also downstream of RAD18. RAD18 has been previously shown to be important for recombinational repair and RAD18-knockout mouse embryonic stem cells exhibit more sister chromatid exchanges in response to DNA damage [53]. The combination of disrupting K63R-polyUb chain formation and RAD18 knockdown did not show an increase in mutations, in fact a modest but non-significant decrease was observed (Figure 4E). The lack of an additive mutation effect supports the foci data implicating a role for K63-polyUb chain formation downstream of RAD18.

PCNA is polyubiquitinated

Our data support a role for the formation of K63-polyUb chains in promoting the recovery of human cells from DNA damage through an error-free pathway that is distinct from TLS. A likely target of this polyubiquitination is PCNA, which in yeast is modified by K63-polyUb by RAD5/MMS2. However, similar modification of PCNA has not been observed in UV irradiated human cells [29]. We investigated PCNA modification after UV irradiation in 3 separate human cell lines: A549 lung cancer cells; 293T embryonic kidney cells; and Hela cervical cancer cells. 6 hours following a dose of $30J/m^2$ we observed the appearance of a prominent band consistent with mono-ubiquitinated PCNA and overexposure of this blot revealed additional PCNA-immunoreactive bands of higher molecular weight consistent with PCNA modified with 2, 3 and 4 ubiquitin molecules (Figure 5). <insert Figure 5 here> As it has been previously demonstrated that RAD18 is required for the mono-

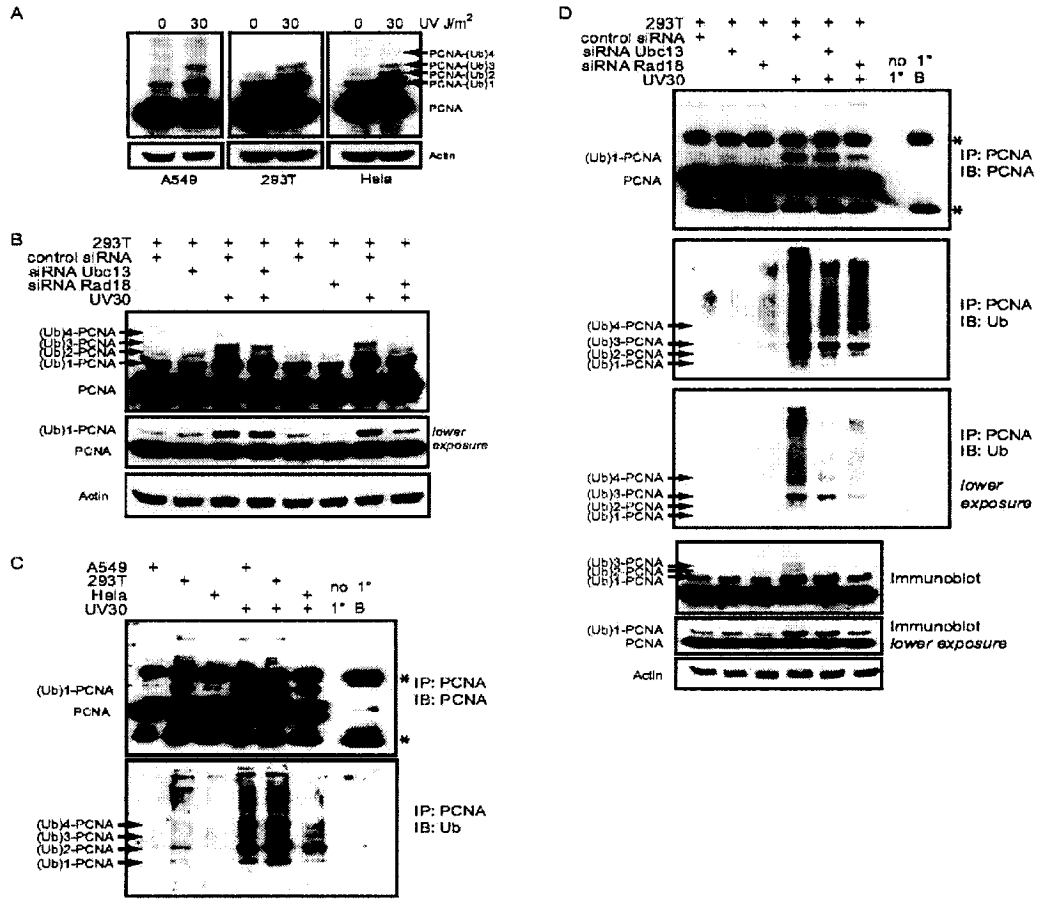

Figure 5: Modification of PCNA by polyubiquitin in human cells after DNA damage. **(A)** A549, 293T and Hela cells were irradiated with 0 or 30J/m^2 UV and lysed 6 hours post-treatment followed by immunoblotting for PCNA. **(B)** 293T cells were transfected with 100nM of either control siRNA, siRNA Ubc13 or siRNA Rad18. 72hours posttransfection cells were treated as in 1A. A darker and lighter exposure of the PCNA immunoblot is shown. **(C)** A549, 293T and Hela cells were irradiated with 30J/m^2 UV and lysed in boiling SDS, diluted in lysis buffer and subjected to immunoprecipitation with a PCNA antibody and detected with PCNA or Ub antibodies. The controls in the immunoprecipitations were 'no 1°' in which lysates were incubated with beads but no PCNA antibody and '1°B' in which PCNA antibody was incubated with beads alone. **(D)** 293T cells were transfected the same as in Figure 5B. 72 hours post transfection cells were irradiated with 30J/m^2 of UV and lysed 6 hours later in boiling SDS, diluted in lysis buffer and subjected to immunoprecipitation with a PCNA antibody and immunoblotted for PCNA (upper panel) and ubiquitin (lower panel). A lighter exposure of the PCNA IP immunoblotted for ubiquitin is also shown. A PCNA immunoblot with darker and lighter exposure performed on protein lysates from the same samples used in the immunoprecipitations is also shown. * denotes immunoglobulin heavy and light chains as detected on the immunoprecipitations.

ubiquitination of PCNA in human cells and that this monoUb PCNA species is required as a substrate for UBC13 mediated K63-polyubiquitination in yeast, we sought to determine whether the observed higher molecular weight bands are dependent on either RAD18 or UBC13. To this end, the expression of *RAD18* or *UBC13* was knocked down using the appropriate siRNAs (Supplementary Information Figure S1). As expected, the band corresponding with monoUb PCNA was substantially reduced in lysates from RAD18 siRNA transfected cells and this also resulted in suppression of the higher molecular weight (polyUb) forms of PCNA presumably modified with 2, 3, or 4 Ub molecules (Figure 5B and Supplementary Information Figure S4A). In contrast, knock down of the E2 ubiquitin ligase responsible for K63 polyubiquitination had no effect on the formation of monoUb PCNA after UV irradiation, but did effectively reduce the di, tri, and quad polyUb PCNA bands to levels similar to those in the RAD18 knockdowns (Figure 5B and Supplementary Information Figure S4A). Together these data suggest that in both cell lines tested, UV induces modification of PCNA by both monoUb (in a RAD18 dependent manner) and by K63 polyUb chains of length 2, 3 and 4 (in a RAD18 and UBC13 dependent manner).

To further demonstrate that these higher molecular weight species are indeed ubiquitinated forms of PCNA, we immunoprecipitated PCNA from A549, 293T and Hela cells and probed using an antibody directed against Ub or PCNA (Figure 5C). In addition, we excluded the possibility that an ubiquitinated protein was co-immunoprecipitated with PCNA by lysing cells in boiling 0.5% SDS to ensure dissociation of PCNA complexes. Following a 5-fold dilution (0.1% SDS), the lysates

were immunoprecipitated. Under these conditions we reproducibly observed several higher molecular weight bands consistent with polyubiquitination of PCNA in each of the three cell lines (Figure 5C, D and Supplementary Information Figure S4B, C). These Ub immunoreactive bands correspond well with the predicted molecular weights for di-, tri- and quad-ubiquitinated PCNA. The antibody against Ub reproducibly demonstrated less affinity for the mono-ubiquitinated form of PCNA, although this was clearly the most abundant form as shown by PCNA immunoblots (Figure 5C, D and Supplementary Information Figure S4B, C). This appears to be a characteristic of the antibody, as we have seen this reproducibly for other ubiquitinated proteins (data not shown). Interestingly, each of the cell lines, especially 293T cells, also show low levels of PCNA polyubiquitination in the absence of UV. However, in all cases the Ub immunoreactive bands are significantly increased upon irradiation in a manner consistent with the increase in mono-ubiquitinated PCNA (Figure 5C, D and Supplementary Information Figure S4B, C). Similar to previous reports [29], mono-ubiquitinated PCNA was readily visible 1.5h after UV treatment and remained present for up to 24h as detected by the PCNA antibody (Supplementary Information Figure S4B). Similarly, bands consistent with di, tri, and quad polyUb forms of PCNA became visible within 1.5h following UV, and remained present up to 24h after exposure (Supplementary Information Figure S4B). Importantly, consistent with the PCNA Western blots (Figure 5B and Supplementary Information Figure S4A) the Ub immunoreactive bands following PCNA IP in both Hela (Supplementary Information Figure S4C) and 293T (Figure 5D) cells were substantially reduced following knockdown of either RAD18 or UBC13. As

expected, RAD18 knockdown blocked both mono-ubiquitination and polyubiquitination of PCNA whereas UBC13 knockdown inhibited only the di, tri and quad polyUb forms (Figure 5B, D and Supplementary Information Figure S4A, C).

Collectively, these data show that PCNA is indeed modified by polyUb chains in human cell lines. Similar modification was observed in primary skin and lung fibroblasts (data not shown) and in response to other forms of damage such as cisplatin (Supplementary Information Figure S5). We speculate that the lack of PCNA polyubiquitination reported earlier [29] may be explained by technical difficulties in detecting Ub due to its strong tertiary structure [54], the low abundance of polyubiquitinated PCNA, or perhaps differences in cell types. In our studies, Ub blots were autoclaved to overcome detection problems due to the strong tertiary structure [54]. We also excluded the possibility that deubiquitinating (DUB) enzymes in cell lysates may have activity against ubiquitinated PCNA by repeating the immunoprecipitation in the presence of N-ethylmaleimide (NEM), a non-specific inhibitor of DUBs (Supplementary Information Figure S6). Under these conditions, no change in PCNA polyubiquitination was observed.

Discussion

The highly conserved ubiquitin protein serves as a pleiotropic covalent tag for many cellular proteins. It has essential proteolytic and nonproteolytic functions that are based on the length and topology of the chain formed. The pathway in which Ub is most commonly associated is the proteasome pathway, a system for targeting

protein substrates via K48-linked polyUb chains for degradation in the 26S proteasome [55]. However, there is increasing evidence that Ub plays an important role in a number of nonproteolytic pathways including receptor internalization [56], translation [57], signal transduction [28], gene regulation [58] and DNA repair [5,6,14,25,29,59]. These roles appear to be mediated in part by the non-canonical polyUb chains. Much less is known about this aspect of ubiquitination compared with the role of K48-polyUb in protein degradation. Of particular interest are chains linked through K63 as genetic studies in *S. cerevisiae* have shown that the enzymatic complex (RAD5, UBC13/MMS2) that assembles these chains are required to protect cells from the harmful effects of genotoxic agents by allowing the replication machinery to bypass DNA lesions in a faithful manner [14]. In fact, ubiquitination of the DNA polymerase processivity factor PCNA is emerging as a key 'molecular switch' for DDT [5,6,29]. Mono-ubiquitination of PCNA promotes error-prone TLS while K63-polyUb activates error-free damage avoidance. The body of evidence supporting the requirement of PCNA post-translational modifications for DDT in mammalian cells is only now emerging.

In this report we provide evidence to support a model (Figure 6) in which ubiquitination of PCNA acts at a central decision point to direct the recovery of blocked replication forks towards one of two alternative pathways in mammalian cells. Recent reports have confirmed that RAD6 dependent mono-ubiquitination of PCNA also stimulates TLS in human cells. This stimulation appears to result through direct binding of the TLS polymerases to mono-ubiquitinated PCNA [11,29].

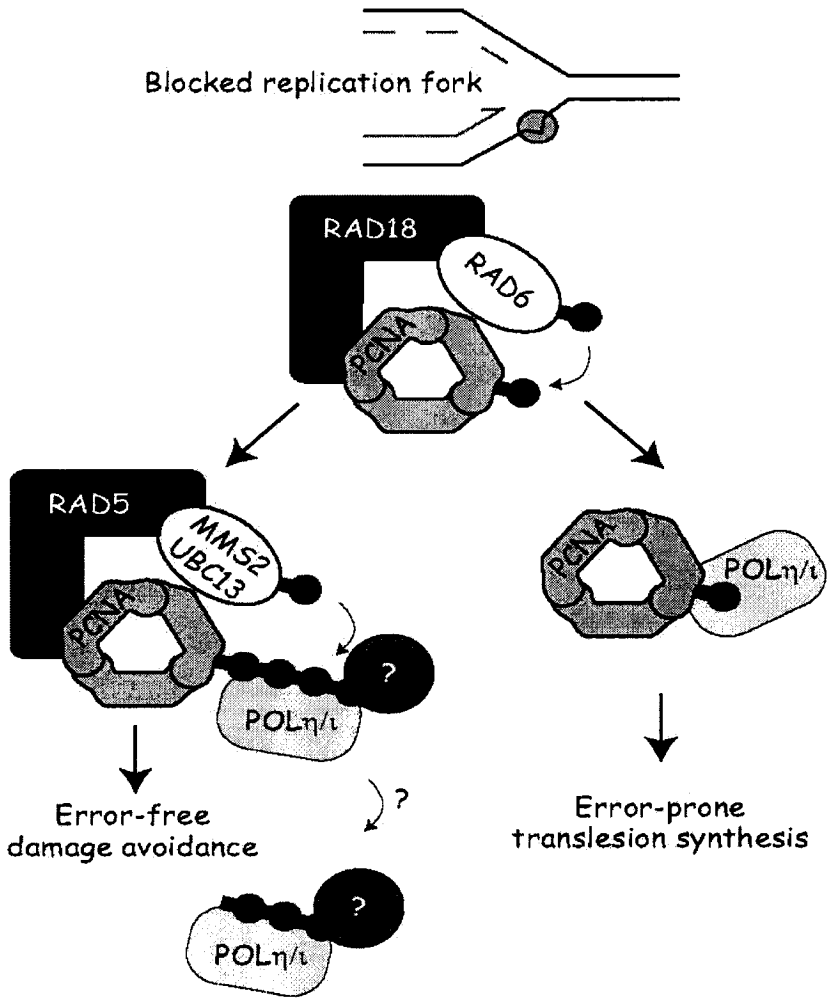

Figure 6: Model of the DNA damage tolerance pathway in mammalian cells. Recovery from a stalled replication fork at sites of DNA damage can occur by one of two alternative pathways. Previous work has shown that PCNA mono-ubiquitination by the RAD6/RAD18 complex stimulates lesion bypass through recruitment of the error-prone TLS polymerases. Here we show that an alternative error-free pathway requires formation of K63-polyUb chains. Blockade of this error-free pathway results in increased use of the TLS polymerases after DNA damage and a corresponding increase in mutations. As the TLS polymerases, POLη and POLι both bind directly and avidly to polyUb chains [20], it is hypothesized that the interaction with K63-polyUb causes a disengagement of the polymerase from the DNA and allowing other proteins to migrate to the site of damage to perform error-free repair. This model predicts that K63-polyubiquitination acts to suppress environmental carcinogenesis by preventing genomic instability that would otherwise be introduced by the TLS polymerases.

Our data indicate that formation of K63-polyUb chains is required to utilize an error-free pathway distinct from TLS. For cisplatin-induced damage this pathway is required for cell survival from at least some types of damage as its inhibition cannot be compensated for by the alternative TLS pathway. For UV-induced damage, inhibition of K63 polyubiquitination does not affect overall cell survival but instead causes an increase in mutations arising from an apparent increased requirement for the error prone branch of TLS. This is supported by several lines of evidence. First, blockade of K63-polyUb chain formation led to a 2.4 fold increase in RAD18 dependent TLS foci after UV-irradiation. Second, we found that the number of UV induced mutations increased by a similar factor in these cells, and that the spectra of these mutations is consistent with that produced normally by error-prone TLS polymerases after UV. Third, POLη knockdown in combination with blockade of K63-polyUb chain formation led to increased toxicity to UV, although no change was seen with either individually. And fourth, an increased reliance on the TLS arm upon blockade of K63-polyUb chain assembly was revealed by a synergistic increase in UV induced mutations when expressed in POLη knockdown cells. POLη knockdown cells showed a high mutation rate as expected, but this rate increased by a factor of 3.5 when K63-polyUb chain assembly was inhibited Together, these data imply that formation of K63-polyUb chains can activate an error-free mechanism to protect cells against mutations that would otherwise be induced by the error-prone TLS polymerases. It will be of interest to determine if K63-polyUb chain formation also plays a role in the protection against sunlight-induced skin cancer.

An obvious question that emerges is how formation of K63-polyUb acts to suppress TLS. Recent reports have demonstrated that the TLS polymerases, POLη and POLι both bind directly and avidly to polyUb chains through newly discovered binding domains [20] [Plosky et al, *in press*]. A C-terminal zinc finger domain of POLη and the proline residue at position 692 of POLι are required for the respective interaction with ubiquitin [20]. Together with our data, this suggests a possible mechanism whereby differential ubiquitination of PCNA could act as a switch between TLS and an alternative error free pathway (Figure 6). In this model, the TLS polymerases are recruited to the sites of replication through interaction with mono-ubiquitinated PCNA and subsequently mediate TLS across DNA lesions. Extension of the Ub chain through K63 linked polyubiquitination in some way suppresses TLS activity and promotes recovery through an alternative error-free pathway. This suppression may be mediated also through the recently discovered binding of POLη and POLι to the K63-polyUb chains and preventing access to the site of damage. An intriguing possibility is that K63-polyUb chains are cleaved upon binding to TLS polymerases thereby functionally removing them from the site of the lesion. This possibility is supported by the low detectable levels polyubiquitinated PCNA as well as by the observed increase in POLη foci in *K63R-Ub* expressing cells.

Although our data suggest that PCNA is indeed a target for K63 polyubiquitination, they do not exclude the possibility that other key proteins in this pathway are also important substrates for these chains. Indeed, K63 polyubiquitination occurs on at least 3 proteins (RIP, NEMO and TRAF6) in an unrelated pathway that activates NF-κB [28,60,61]. In this pathway K63-polyUb

chains on multiple proteins may facilitate their assembly into an active complex [61]. It is therefore intriguing to speculate that K63-polyUb chains may not only uncouple the TLS polymerases from the site of damage, but also provide a mechanism for recruitment of other proteins required for error-free repair.

Non-proteolytic roles for Ub have also been implicated in other DNA repair pathways that may interact with DDT, most notably that involving the Fanconi's anemia (FA) gene products [18]. FANCD2 becomes mono-ubiquitinated after DNA damage and localizes to nuclear foci [62]. FANCC has been associated with the TLS polymerases REV1 and REV3 [63] and may also interact with the BLM helicase [64], a candidate for promoting fork reversal in the error-free damage avoidance pathway [65]. A challenge for future investigations will be to understand how K63-polyUb chain assembly is regulated and how these chains promote interaction with other pathways to mediate error-free recovery from DNA damage.

Materials and methods

Cell culture and treatments

The construction of the Ub expressing plasmids has been described elsewhere [31]. The *POLη-GFP* plasmid was a gift of Dr. Alan R. Lehmann, (Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton U.K.). All cell lines were cultured in DMEM (Sigma, St. Louis, Missouri, United States) supplemented with 10% FBS (Sigma). A549 cells were co-transfected with *WT-Ub-GFP* or *K63R-Ub-GFP* plasmids and pBabePuro using FuGene 6 (Roche, Basal Switzerland). HeLa

cells were transfected with *WT-Ub-puro* or *K63R-Ub-puro* constructs using lipofectamine (Invitrogen, Carlsbad, California, United States). Stable transfectants were selected in 1μg/ml puromycin (Sigma) and/or by flow cytometry (FACSAria, BDBiosciences Pharmingen, San Diego, California, United States).

The sensitivity to UV-irradiation alone, UV combined with caffeine, and cisplatin alone was evaluated using clonogenic survival assays. UV-irradiation was performed on 80% confluent cells in 6cm dishes using a UVC germicidal lamp at a fluence of $1J/m^2/s$. UV and caffeine combination studies were done as above but cells were plated in 0.4mM caffeine immediately after UV irradiation. Cells were treated for 1h in cisplatin diluted in culture media. Cells were plated in 6 cm dishes in triplicate and incubated for 2 weeks to obtain colony-formation. Colonies were fixed, stained with 2% bromophenol blue in 70% ethanol and colonies containing ≥50 cells counted. All experiments were normalized for plating efficiency.

The sensitivity to UV in POLη knockdown cells was performed as above with the exception that cells were transfected twice with siGENOME SMARTpool reagent specific for human POLH (Dharmacon Research, Lafayette, Colorodo, United States) using oligofectamine (Invitrogen). The transfections were done 72 and 24h before UV treatment to achieve optimal long term knockdown as determined by quantitative RT-PCR.

Quantitation of gene expression was performed using an Applied Biosystems (Foster City, California, United States) 7500 Real Time PCR system. Applied Biosystems "assay on demand" were used to measure gene expression. For Rad18 Hs00220119_m1, POLη Hs00197814 and for 18S Hs99999901_s1 was used.

Reactions were performed using Taqman Universal PCR Master Mix from Applied Biosystems.

Immunoblotting

Following the indicated treatments with either UV, cisplatin and/or siGENOME SMARTpool reagent specific for human UBC13 or human RAD18 (Dharmacon), cells were harvested in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton-X-100, 2mM EDTA, and 5% glycerol with 200μg/ml phenylmethylsulfonyl fluoride, 2mM $NaVO_4$, 2 mM NaF, and 2mM NaPPi, 1x protease inhibitor cocktail). Samples were sonicated, soluble fractions recovered and proteins quantified using the Bradford protein assay (Bio-Rad). Proteins were resolved on either a one or two phase SDS-polyacrylamide gel (10% and 15% or 10%) and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States). The membrane was stained with Ponceau S (Sigma) prior to Western blotting with the indicated primary antibody. The following antibodies were used: rabbit polyclonal Ub (Dako, Glostrup, Denmark), mouse monoclonal RGS-His (Qiagen, Valencia, California, United States), mouse monoclonal PCNA PC10 (Chemicon, http://www.chemicon.com), rabbit polyclonal GFP (Santa Cruz Biotechnology, Santa Cruz, California, United States) and mouse monoclonal actin (Sigma). Proteins were visualized by a horseradish peroxidase method using ECL (Kirkegaard & Perry Laboratories, http://www.kpl.com)).

Immunoprecipitation

Cells were UV-irradiated with $30J/m^2$ as described above and either left untreated or transfected with siGENOME SMARTpool reagent specific for human UBC13 or human RAD18 (Dharmacon). Cells were lysed (6h after irradiation) in lysis buffer supplemented with 0.5% SDS. Lysates were sonicated and boiled for 5min followed by dilution to 0.1% SDS. After protein quantitation 500µg of protein was incubated overnight at 4°C with anti-PCNA (1/200). The following day, lysates were incubated for 48h at 4°C with 100µl of Gamma bound Sepharose Beads (Pharmacia Amersham Biotech). Beads were washed extensively in lysis buffer and proteins were eluted by boiling in 1x Laemmli's SDS sample buffer. Immunoblotting was performed as described above except the membranes were autoclaved for 20min in ddH_2O after protein transfer, and proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, Illinois, United States).

Mutation spectrum

To eliminate background *HPRT* mutations, cells were cultured in hypoxanthine, aminopterin, and thymidine (HAT) supplemented culture medium for one week. UV-induced *HPRT* mutants were obtained by seeding $1.5x10^4$ cells in 24 well plates, followed by $10J/m^2$ UV irradiation 24h later. Cells were subcultured for 7 days, and re-seeded at $5.0x10^4$ cells on 35 mm dishes in medium containing 30µM 6-thioguanine (6-TG). Individual colonies were picked and grown until enough cells were obtained for RNA-isolation using RNA-aqueous kit (Ambion, Austin, Texas,

United States). The *HPRT* gene was subjected to RT-PCR followed by sequencing using the following overlapping primers: HPRT1 - 5'CTTCCTCCTCCTGAGCAGTC3'; HPRT2 - 5'AAGCAGATGGCCACAGAACT3'; HPRT3 - 5'CCTGGCGTCGTGATTAGTG3'; HPRT4- 5'TTTACTGGCGATGTCAATAGGA3';HPRT5-5'GACCAGTCAACAG-GGGACAT3'; and HPRT6 5'ATGTCCCCTGTTGACTGGTC3'.

Mutation frequency

$1.0x10^6$ *HPRT*-mutant free cells were seeded and treated the following day with either UV-irradiated (0, 5, 10 J/m^2) or cisplatin (0, 5, 10 μM for 1 hour). After subculturing the treated cells for one week, $4.0x10^5$ cells were seeded in selective medium containing 6-TG (as above) and incubated until colonies were formed. Colonies were counted and HPRT mutation frequency was defined after correcting for plating efficiency.

Mutation frequency in response to UV in POLη and RAD18 knockdown cells was performed as above with the exception that cells were transfected twice with siGENOME SMARTpool reagent specific for human POLH or human RAD18 (Dharmacon) using oligofectamine. The transfections were done 72 and 24h before UV treatment to achieve optimal long term knockdown as determined by quantitative PCR.

Foci

A549 and Hela cells stably expressing *WT-Ub-puro* and *K63R-Ub-puro* were transiently transfected with a *Polη-GFP* plasmid. 24 hours post-transfection, cells were UV-irradiated at a dose of $10J/m^2$. To observe living cells, cells were cultured in 35mm glass bottom dishes (MatTek Corporation, http://www.mattek.com) with coverslips. Real time excitation measurements to monitor fluorescent signals in transfected cells were subsequently performed using a live cell microscopy unit mounted on a Leica DR IRBE inverted microscope (Wetzlar, Germany), equipped with a polychromator that allows generation of light of the required wavelength, using a 63x objective. Both the polychromator and filterwheel were controlled via the PC using specialized Openlab software from Improvision (http://www.improvision.com/products/openlab). At least 100 cells were counted for each cell line at each time point per experiment by a blinded independent observer.

The recruitment of POLη to foci was determined in response to UV in RAD18 knockdown cells was performed as above with the exception that cells were transfected twice with siGENOME SMARTpool reagent specific for human RAD18 using oligofectamine. The transfections were done 72 and 24h before UV treatment to achieve optimal long term knockdown as determined by quantitative PCR.

For colocalization studies, Hela cells stably expressing *WT-Ub-puro* and *K63R-Ub-puro* were transiently transfected with a *Polη-GFP* plasmid in a chamber slide (BD Biosciences Pharmingen). 24 hours post-transfection, cells were UV-irradiated at a dose of $10J/m^2$. For detection of PCNA and POLη, cells were fixed in cold methanol for 20 min at -20°C followed by 30 sec in cold acetone. Cells were washed twice

with PBS and then incubated at room temperature with both anti-PCNA and anti-POLη. After 1 h, cells were washed with PBS and then incubated with FITC-conjugated goat antimouse IgG (Invitrogen) and Texas red-conjugated goat antirabbit (Invitrogen), for 45 min. After washing in PBS, cells were dehydrated for 1 min in 70% ethanol followed by twice 1 min in 100% ethanol. Cells were then mounted with Fluorescent Mounting Media (Dako) and visualized by confocal microscopy.

Gene identification numbers

The Gene I.D numbers for the gene and gene products discussed in the paper were obtained from Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene)

UBC13 (7334), RAD18 (56852), PCNA (5111), POLH (5429), MMS2 (7336), RAD5 (850719), HPRT (3251), BLM (641), FANCD2 (2177), FANCC (2176), REV3 (5980), UBA52 (7311), UBA80 (6233), UBB (7314), UBC (7316), NEMO (8517), TRAF6 (7189), NF-kappaB (4790), POLI (11201), CROC1 (7335)

Acknowledgements

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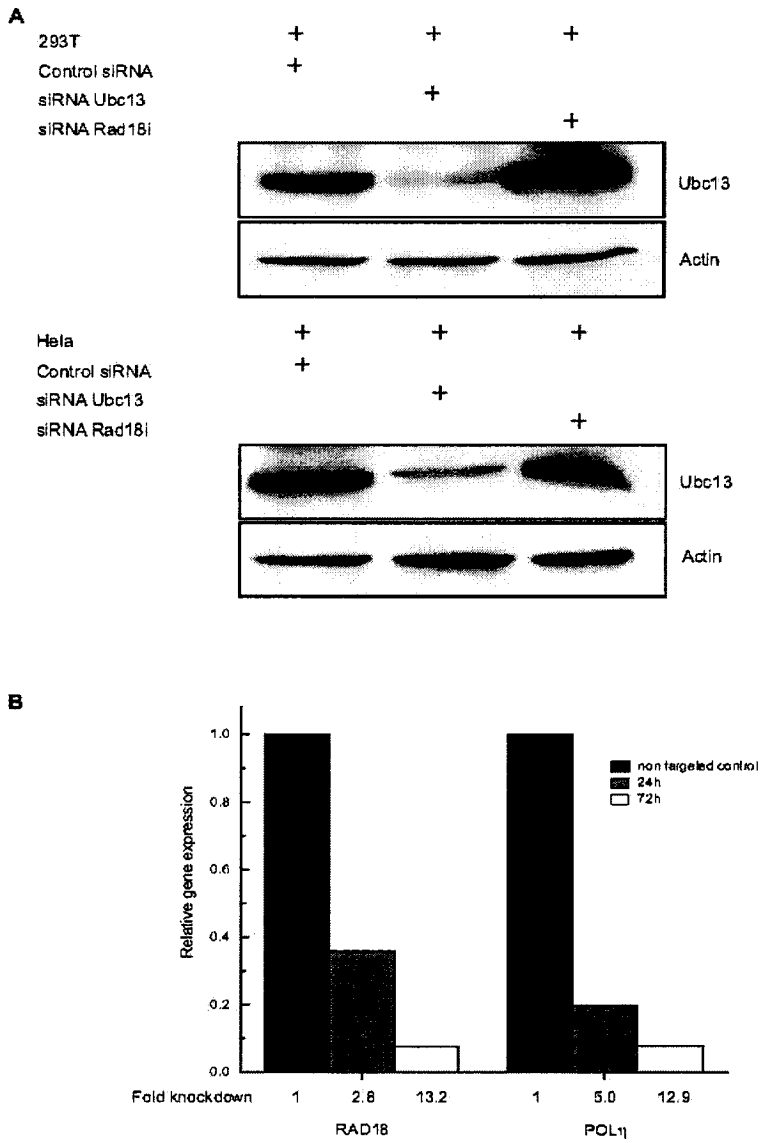
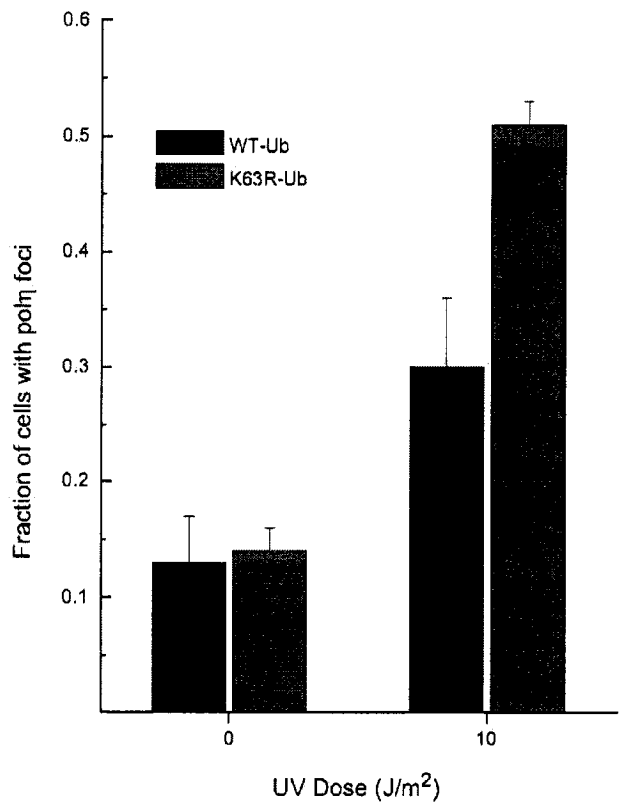
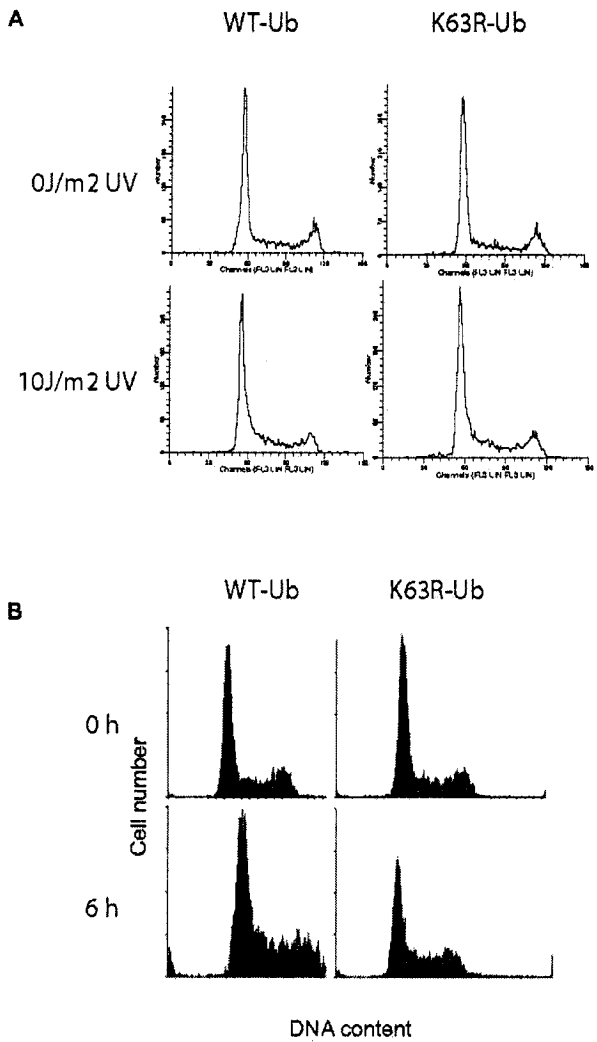
Supporting Information



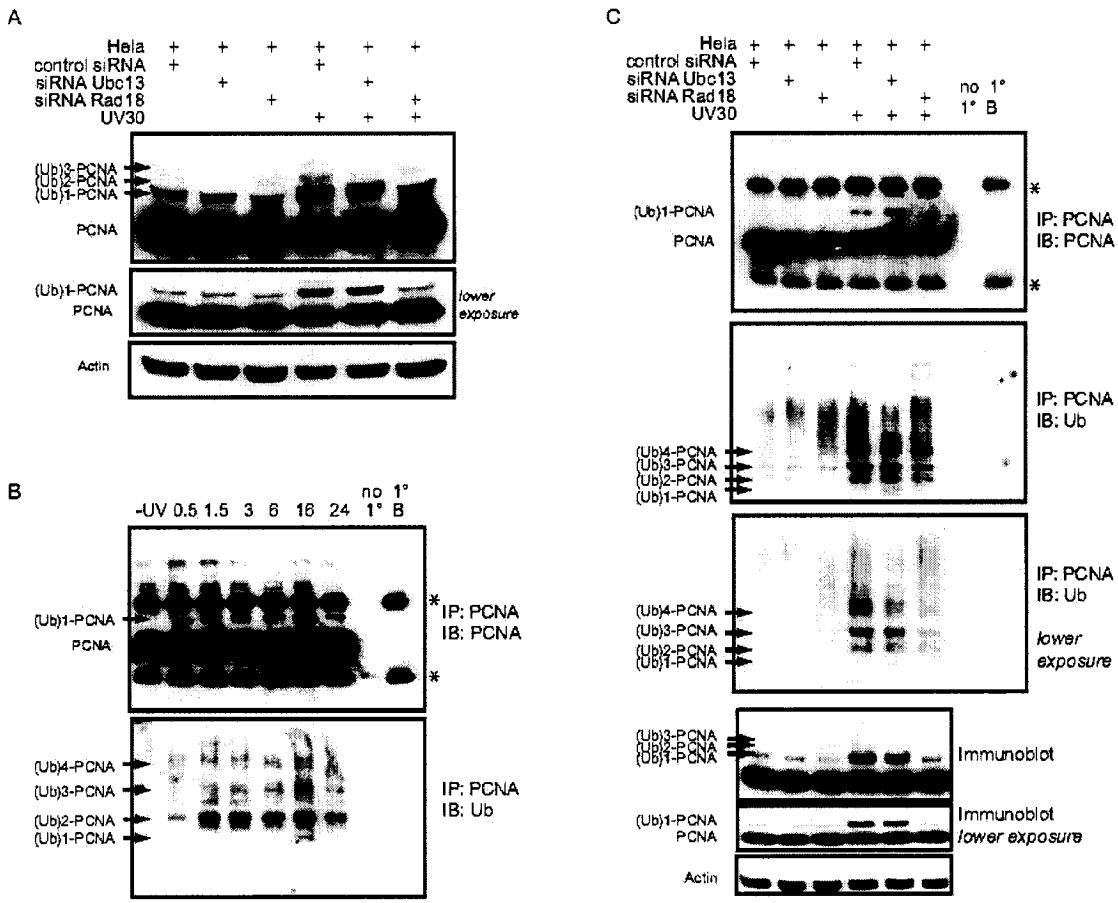
Supplementary Figure S1. Knock down of *UBC13, RAD18* and *POLη*. **(A)** Hela and 293T cells were transfected with siRNA against *UBC13* and analyzed by Western blot. **(B)** A549 cells were transfected twice (48h apart) with siRNA against RAD18 or POLη. Knockdown was analyzed 24 and 72h post 2nd transfection for mRNA expression relative to 18S rRNA using quantitative RT-PCR.



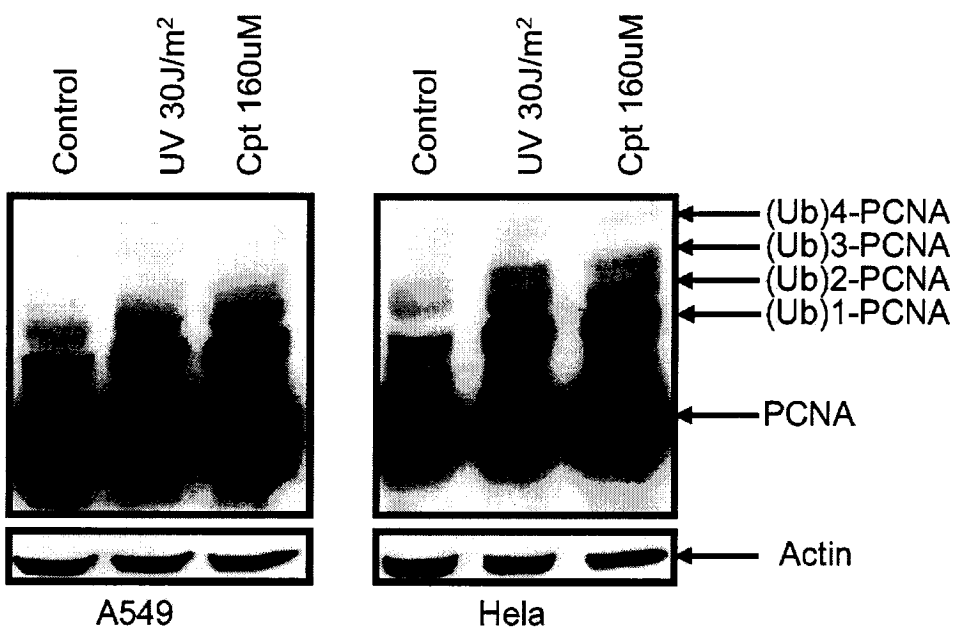
Supplementary Figure S2. Increase in POLη foci are also observed in A549 cells. A549 cells stably expressing *WT-Ub* or *K63R-Ub* were treated as in Figure 4. Two independent experiments were performed.



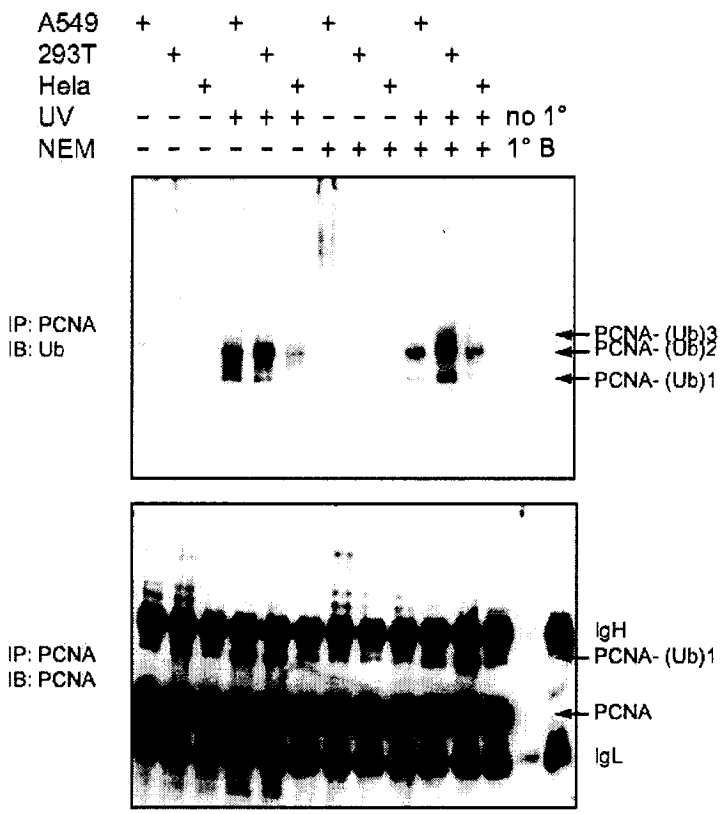
Supplementary Figure S3. Cell cycle profile following UV treatment. (A) A549 cells expressing WT-Ub-GFP or K63R-Ub-GFP were treated with the indicated dose of UV (B) Hela cells expressing WT-Ub-puro or K63R-Ub-puro were treated with 10J/m^2 UV and fixed either immediately or 6h post-treatment. Following PI staining, cells were analyzed for DNA content using a FACSAria flow cytometer (BD Bioscience).



Supplementary Figure S4. Modification of PCNA by polyubiquitin in human cells after DNA damage. (A) Hela cells were subjected to the same procedure as in 5B. A darker (upper panel) and lighter exposure (lower panel) of the PCNA immunoblot is shown. **(B)** A549 cells were UV-irradiated as in Figure 5A and lysed at the indicated times posttreatment. Whole cell lysates were subjected to immunoprecipitations with an anti-PCNA antibody followed by immunoblotting for PCNA (upper panel) and Ub (lower panel). The controls in the immunoprecipitations are the same as in 5C. –UV indicates no UV treatment. **(C)** Hela cells were subjected to the same procedure as in Figure 5D. A lighter exposure of the PCNA IP immunoblotted for ubiquitin is shown. A PCNA immunoblot with darker and lighter exposure performed on protein lysates from the same samples used in the immunoprecipitations is also shown.



Supplementary Figure S5. Cisplatin treatment also induces modification of PCNA by polyubiquitin in human cells. Untreated, 30J/m^2 UV irradiated and 160μM cisplatin treated A549, and Hela cells were lysed 6 hours post-treatment followed by immunoblotting for PCNA.



Supplementary Figure S6. Inhibition of deubiquitinating enzymes does not affect appearance of polyUb-PCNA. A549, 293T and Hela cells were treated with 30J/m^2 UV and lysed in the presence or absence of the general thiol protease inhibitor NEM. Immunoprecipitation and Western blots were done as described in materials and methods. The controls in the immunoprecipitations were 'no 1°' in which lysates were incubated with beads but no PCNA antibody and '1°B' in which PCNA antibody was incubated with beads alone.

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Appendix A

Formation of lysine 63-linked poly-ubiquitin chains protects human lung cells against benzo(a)pyrene-diol-epoxide-induced mutagenicity.

Contribution to Collaboration,

Our contribution to this manuscript includes providing data demonstrating that the environmental mutagen BPDE induces the polyubiquitination of PCNA similarly to UV light. The written contribution of Jan Brun to this manuscript includes the appropriate result section, materials and methods, and figure legends. RKC edited the manuscript.

Summary

The authors of this paper demonstrate that disruption of K63-linked polyubiquitination on PCNA leads to a significant increase in BPDE induced mutations. This is an important finding since BPDE is a common environmental mutagen that is a product of the incomplete combustion of organic materials such as charbroiled foods, car exhaust, asphalt processing and cigarette smoke. Significantly, Langie et al., show that a majority of mutations are G→T transversions which is a result of recruiting POLη to the sites of BPDE damage. Interestingly, most pulmonary lung cancers have been associated with decreased expression of hRAD6 (marker of DDT function) and the common G→T signature. This suggests an important role for K63-linked chains in protecting against carcinogenesis of the lung.

Formation of Lysine 63-linked poly-ubiquitin chains protects human lung cells against benzo(a)pyrene-diol-epoxide-induced mutagenicity.[#]

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Key words: benzo(a)pyrene, DNA damage tolerance, mutagenesis, translesion synthesis, ubiquitin

Abstract

Benzo(a)pyrene exerts its mutagenic effects via induction of benzo(a)pyrene-diol-epoxide (BPDE)-DNA adducts. Such helix-distorting adducts are not always successfully repaired prior to DNA replication, which may result in a blocked replication fork. To alleviate this stall, cells utilize DNA damage tolerance systems involving either error-free damage avoidance or error-prone translesion synthesis. Studies in yeast suggest the modification of PCNA by lysine 63-linked poly-ubiquitin (K63-polyUb) chains as a key mediator of the error-free damage avoidance pathway. Recently, we extended this observation to human cells, showing the occurrence of poly-ubiquitination of PCNA in UV-irradiated human cells. In the present study, we hypothesized that disrupting the formation of K63-polyUb chains inhibits damage avoidance and favors error-prone repair involving low-fidelity polymerases (e.g. POLη), causing increased BPDE-induced mutagenicity. To test this hypothesis, we generated A549 cells expressing either a mutant ubiquitin (*K63R-Ub*) which blocks further ubiquitination through K63, or the wild type ubiquitin (*WT-Ub*). We show that PCNA is poly-ubiquitinated in these cells upon BPDE-exposure and that disruption of K63-polyUb chain formation has no effect on BPDE-induced toxicity. In contrast, significantly higher frequencies of BPDE-induced *HPRT* mutations were observed in *K63R-Ub* expressing cells, of which the majority (74%) was G→T transversions. BPDE treatment caused an enhanced recruitment of POLη to the replication machinery of the *K63R-Ub expressing cells*, where it co-localized with PCNA. Suppression of POLη expression by using siRNA resulted in a 50% reduction of

BPDE-induced mutations in the K63R cells. In conclusion, we demonstrated that formation of K63-polyUb chains protects BPDE-exposed human cells against translesion synthesis-mediated mutagenesis. These findings indicate that K63-polyubiquitination guards against chemical carcinogenesis by preventing mutagenesis and thus contributing to genomic stability.

1. Introduction

Humans are continuously exposed to a wide variety of DNA-damaging environmental carcinogens such as polycyclic aromatic hydrocarbons (PAHs). The most studied PAH is benzo[a]pyrene (B[a]P) whose mutagenic and carcinogenic potential has been demonstrated in both humans and animals [1]. After uptake, the chemically unreactive B[a]P is metabolically activated into its predominant reactive form, (±)-*anti*-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE). This metabolite can covalently bind to the DNA, preferably to guanine, resulting in the formation of (+)-*trans-anti*-BPDE-N^2-dG being the most predominant adduct (>90%) [1-3]. The formation of these (±)-*anti*-BPDE-N^2-dG DNA adducts is one of the key factors of B[a]P-induced mutagenesis, producing mostly G → T transversions upon error-prone replication of adducted DNA templates [4].

Generally, nucleotide excision repair (NER) is the primary pathway to remove large helix-distorting DNA lesion, such as BPDE-DNA adducts. Nevertheless, despite NER activity and the presence of cell-cycle checkpoints, many of such bulky lesions may persist until replication, thereby stalling the replication fork. To avoid an aberrant cessation of the cell-cycle caused by such a blockage, cells have evolved DNA damage tolerance (DDT) systems (also known as post-replication repair) to circumvent the damage and continue replication in the presence of lesions [5,6]. Studies in bacteria (E.coli), yeast as well as mammalian cells [5-8], revealed that DDT can be accomplished through the action of error-prone low-fidelity translesion synthesis (TLS) polymerases (e.g. Y-family DNA polymerases). Studies in mammalian cells have revealed TLS to be an important mechanism in the response to

BPDE-DNA adducts [8,9]. In general, TLS can either be error-free or error-prone, depending on the type of DNA damage, as well as the specific polymerase that is involved. Candidates for performing TLS bypassing bulky-DNA adducts are POLη, POLκ, POLι, REV1 (Y-family polymerases) and POLζ (B-family) [6,10]. In contrast to its anti-mutagenic role in response to UV radiation, bypass of (±)-anti-BPDE-DNA adducts by POLη will promote mutagenesis by predominantly inserting the incorrect nucleotide opposite the lesion [9,11].

There is now evidence showing that yeast have a second DDT pathway distinct from TLS, referred to as error-free damage avoidance. Although its mechanism is yet unclear, it may involve replication fork reversal, thereby using the information of the undamaged sister duplex at the replication fork [12,13]. Studies in yeast have shown that this pathway requires modification of proliferating cell nuclear antigen (PCNA) with lysine 63-linked polyUb (K63-polyUb) chains [12-14]. Specifically, upon exposure to DNA-damaging agents PCNA becomes mono-ubiquitinated by the ubiquitin conjugase–ubiquitin ligase (E2-E3) complex RAD6-RAD18 at its Lys164 residue, promoting its interaction with TLS-polymerases [12,15]. Subsequent Lys63-linked poly-ubiquitination of PCNA by the E2-E3 complex UBC13/MMS2-RAD5 then facilitates the error-free damage avoidance pathway. As far as we know, only studies in yeast and metazoans (i.e. X. laevis) confirmed this K63-linked poly-ubiquitination [12,14,16], and there is still debate on the importance of such damage avoidance mechanism in mammalian cells [17]. However, we recently demonstrated the formation of PCNA-linked polyUb polymers in UV-exposed human pulmonary epithelial cells (A549 cells) [18]. In addition, in the

same study it was shown that formation of K63-polyUb chains protected against UV-induced mutations. The aim of the present study was to investigate the effect of lysine 63-linked poly-ubiquitination processes on BPDE-induced mutagenesis in mammalian cells. To this end, we applied previously established A549 cell lines stably expressing ubiquitin either in its WT-form (*WT-Ub*) or as Lys63-mutants (*K63R-Ub*) which blocks further ubiquitination through K63 [18]. We show that PCNA is poly-ubiquitinated after BPDE treatment and that disruption of K63-polyUb chain formation enhances BPDE-induced mutagenicity, likely involving increased recruitment of the Y-family polymerase POLη. Therefore, we implicate the formation of these specific polyUb chains in the protection against chemical carcinogenesis.

2. Materials and Methods

2.1 Cell strains and culture.

As previously described, A549 cells were stably transfected with 6xhis-tagged wild-type ubiquitin-GFP (*WT-Ub-GFP*) or K63R mutant ubiquitin-GFP (*K63R-Ub-GFP*) constructs using FuGene 6 (Roche Molecular Biochemicals, Mannheim, Germany) [19]. Plasmids were co-transfected with a plasmid conferring resistance to puromycin.

To study POLη-GFP foci formation, a set of A549 and HeLa cells were transfected with *WT-Ub-puro* or *K63R-Ub-puro* constructs using lipofectamin (Invitrogen Life Technologies, Carlsbad, California). These Ub-puro cell lines were phenotypically comparable to the original *Ub-GFP* expressing cells as demonstrated by Chiu *et al.* [18].

To assess the role of POLη in the mutagenicity of BPDE, *K63R-Ub* expressing A549 cells were transfected twice with siGENOME SMARTpool reagent specific for human POLH (Dharmacon) using oligofectamine (Invitrogen). The transfections were done 72 and 24h before treatment to achieve optimal long term knockdown of POLη of approximately 13 fold as determined by quantitative RT-PCR, as described previously [18].

Cells were cultured in a humidified atmosphere at 37°C (5% CO_2), in DMEM (Sigma, St. Louis, Missouri, United States) supplemented with 10% heat inactivated FCS (Gibco Invitrogen, Scotland, UK) and 0.11% filtered puromycin (1 μg/mL) to establish stable transfectants. For experiments, cells were cultured in DMEM supplemented with 10% heat inactivated FCS and 0.01% gentamicin, and grown until confluency.

2.2 Cell treatments.

Before BPDE-exposure, medium was aspirated and cells were washed with sterile HBSS (-Ca/-Mg) (Gibco Invitrogen, Scotland, UK). BPDE (NCI Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) was dissolved in anhydrous dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) at a concentration of 20 mM. This stock solution was diluted in culture medium to the desired concentrations of 0.1 – 1 μM and immediately added to the cells. The final DMSO concentration was always 0.5% [20]. After 30 min of incubation at 37°C, medium containing BPDE was replaced by fresh culture medium. To study PCNA poly-ubiquitination, UV-irradiated cells were used as a positive

control. This was performed on 80% confluent cells in 6-cm dishes using a UVC (254-nm) germicidal lamp at a dose rate of 1 $J/m^2/s$.

2.3 Clonogenic cell survival.

The sensitivity of A549 cells expressing *K63R-Ub* or *WT-Ub* to BPDE was assayed using a clonogenic survival assay. Therefore, cells were seeded at a density of $4x10^5$ cells per 60 mm dish (Falcon, Le Pont De Claix, France) and grown for 24h before exposure to BPDE (0 – 1.0 μM) or DMSO. Cells were treated for 30 min with the indicated dosages after which they were harvested and seeded at least in duplicate for each concentration. After ~12 days of growth the medium was removed and colonies were fixed and stained with 2% bromophenol blue in 70% ethanol. Any groupings of cells containing 50 or more cells were counted as a colony. For each dose, survival was calculated from the relative colony forming ability of the BPDE-exposed cells compared to the DMSO-treated controls. All experiments were normalized for the plating efficiency. LC_{50} values were estimated from the obtained concentration-response curves.

2.4 HPRT-mutation analysis.

For the determination of *HPRT*-mutant frequencies in *K63R-Ub* and *WT-Ub* expressing cells, cells were seeded at $1x10^6$ cells per 100 mm dish (Falcon, Le Pont De Claix, France) and cultured in hypoxanthine, aminopterin, and thymidine (HAT) supplemented culture medium for one week, to eliminate background *HPRT* mutations. Subsequently, cells were exposed to the indicated concentrations of BPDE (30 min, 37°C) and were maintained for one week to allow for phenotypic expression

of the acquired mutations. Cells carrying mutated *HPRT* were then selected in gentamicin-containing medium supplemented with 30 μM 6-thioguanine (6-TG). After ~14 days of culture, cells were fixed and stained with 2% bromophenol blue in 70% ethanol. 6-TG resistant colonies consisting of 50 or more cells were counted. In parallel, cells were plated (200 cells/100 mm dish) in medium lacking 6-TG to determine the plating efficiency at the time of selection. Mutation frequency for each treatment was calculated as follows; mutation frequency = # colonies/(plating efficiency x # cells seeded).

2.5 Mutation spectrum

BPDE-induced *HPRT* mutants were obtained as described above. To exclude sister clones only a single colony per treated population was isolated and grown in 6 well plates until enough cells were obtained for RNA-isolation. Each sample was lysed in 1 ml Trizol and stored at -20°C until use for RNA isolation. Total RNA was isolated from Trizol according to the manufacturer's protocol. Isolated RNA was purified using the RNeasy® Mini Kit (Qiagen) together with DNAse treatment (RNAse-free DNAse provided by Qiagen) according to the manufacturer's recommendations. Quantity and purity of the RNA was determined spectrophotometrically. cDNA was prepared using the iScript™ cDNA Synthesis kit (BioRad, CA, USA). The HPRT cDNA was amplified by PCR, followed by DNA sequencing on a ABI 3700 using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosciences) using the following overlapping primers: *HPRT1* 5'-CTT CCT CCT CCT GAG CAG TC-3'; *HPRT2* 5'-AAG CAG ATG GCC ACA GAA CT-3'; *HPRT3* 5'-CCT GGC GTC

GTG ATT AGT G-3'; and *HPRT4* 5'-TTT ACT GGC GAT GTC AAT AGG A-3' [18].

2.6 DNA isolation and ^{32}P-postlabeling of BPDE-DNA adducts.

To study possible differences in BPDE-DNA adduct removal, A549 cells stably expressing *K63R-Ub* or *WT-Ub* were exposed to 0.1 μM BPDE. After 30 min of incubation at 37°C, medium containing BPDE was replaced by fresh culture medium and cells were harvested after 0, 1, 8, and 24h of recovery. Cells were centrifuged at 1000 g and pellets were stored at –20°C until DNA isolation. Standard phenol extraction was used to obtain genomic DNA [21]. ^{32}P-postlabeling was carried out using the nuclease P1 enrichment technique as described by Reddy and Randerath with some modifications [21]. Briefly, an aliquot containing 10 μg DNA was digested using micrococcal endonuclease (0.25 U/μL) and spleen phosphodiesterase (2 μg/μL) for 3.5 hours at 37°C. Subsequently, DNA-digests were treated with nuclease P1 (2.5 μg/μL) for 30 min at 37°C. To stop the NP1-reaction, 1 M Tris (pH 9.6) was added. BPDE-modified nucleotides were subsequently labeled with (γ-^{32}P)ATP (50 μCi/sample; ICN, Indianapolis) using T4-polynucleotide kinase (10 U/μL) for 30 min at 37°C. The radiolabelled adducted nucleotide biphosphates were separated on PEI-cellulose sheets (Machery Nagel, Düren, Germany) by multi-directional thin layer chromatography (TLC). In all experiments two BPDE-DNA standards with known adduct levels (1 adduct/10^7 and 1 adduct/10^8 nucleotides) were analyzed in parallel for quantification purposes. Quantification was performed using Phosphor-Imaging technology (Fujifilm FLA-3000).

To asses the amount of DNA in the reaction, an aliquot of DNA-digest was diluted and labeled with (γ-^{32}P)ATP. Nucleotides were separated on a PEI-cellulose sheet by one directional TLC in 0.12 M NaH_2PO_4 (pH 6) for 5-6h. A dAP-standard were analyzed along with the other samples for quantitation purposes.

2.7 Western Blots

To study PCNA poly-ubiquitination upon BPDE-exposure, immunoblotting techniques were carried out as described previously [18]. Briefly, A549 and HeLa cells were treated with BPDE for 1h and lysed 5h post-treatment (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton-X-100, 2 mM EDTA, and 5% glycerol with 200 µg/ml phenylmethylsulfonyl fluoride, 2 mM NaVO4, 2 mM NaF, and 2 mM NaPPi protease-inhibitor cocktail). Since, in our previous study UV was shown to induce poly-ubiquitination of PCNA [18], UV-irradiated cells (30J/m^2) were used as a positive control. Samples were sonicated, soluble fractions were recovered, and proteins were quantified. Proteins were resolved on a single-phase 10% SDS-polyacrylamide gel and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States). The following antibodies were used: rabbit polyclonal anti-Ub (Dako, Glostrup, Denmark), mouse monoclonal anti-PCNA PC10 (Chemicon, http://www.chemicon.com), and mouse monoclonal anti-actin as reference (Sigma). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, Illinois, United States).

2.8 Immunoprecipitations

Immunoprecipitation of PCNA was performed as described previously [18]. In brief, cells were treated with BPDE as described above and harvested in lysis buffer. An anti-PCNA antibody was incubated overnight with 500 μg of protein lysate. The following day, lysates were incubated with 100 μl of Gamma-Bound Sepharose Beads (Amersham Pharmacia Biotech). After 48h beads were washed extensively in lysis buffer, and proteins were eluted by boiling in Laemmli's SDS sample buffer. Immunoblotting was performed as described above except that the membranes were autoclaved for 20 min in ddH_2O after protein transfer, and proteins were visualized as described above.

2.9 GFP-POLη foci formation.

A549 and HeLa cells stably expressing *WT-Ub-puro* and *K63R-Ub-puro* were grown on 35 mm glass bottom dishes (MatTek Corporation, Ashland, U.S.A) until 80% confluency. Plasmids expressing POLη-GFP fusion protein (a generous gift of Dr. Alan R. Lehmann, Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton U.K.) were transfected into the cells using lipofectamine according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, California). After 24h of incubation, POLη-GFP transfection efficiency was checked by fluorescence microscopy, and cells were exposed to 0.1 and 0.5 μM BPDE for 30 min at 37°C. Then 6h post-incubation, cells expressing GFP-POLη proteins were visualized using a live-cell microscopy unit mounted on a Leica DR IRBE inverted microscope, equipped with a polychromator that allows generation of light of the required wavelength, using a 63x magnification level. Both the polychromator and

filterwheel were controlled via the PC using specialized Openlab software. To determine the percentage of cells with POLη foci, only the POLη expressing cells were evaluated. At least 100 cells were counted for each experimental condition by an experienced and blinded observer.

For co-localization studies, *WT-Ub-puro* and *K63R-Ub-puro* expressing A549 cells were transiently transfected with a POLη-GFP plasmid in a chamber slide (BD Biosciences Pharmingen). Cells were exposed to 0.1 and 0.5 μM BPDE (30 min at 37°C), 24 hours post-transfection. For detection of PCNA and POLη, cells were fixed in cold methanol for 20 min at -20°C followed by 30 sec in cold acetone to allow mild permeabilisation of the cells. Cells were washed twice with PBS and then incubated at room temperature with both anti-PCNA and anti-POLη antibodies. After 1h, cells were washed with PBS and then incubated with Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) and Texas red–conjugated goat anti-mouse (Invitrogen) secondary antibodies for 45 min. After washing in PBS, cells were dehydrated for 1 min in 70% ethanol followed by two 1-min incubations in 100% ethanol. Cells were then mounted with Fluorescent Mounting Media (Dako) and visualized by two photon microscopy.

2.10 Statistical analysis.

All data are presented as the mean ± standard error of at least 2 independent experiments, unless stated otherwise. Differences in BPDE-DNA adduct removal and mutation frequencies between the different cell strains were analyzed by T-test. To examine differences in *HPRT*-mutation frequencies after exposure to various

concentrations of BPDE versus the control (0 μM BPDE), one-way ANOVA was used with subsequent Dunnett's-correction for multiple comparisons. A non-parametric Mann-Whitney test was performed to analyze differences in Polη foci formation between the two cell lines. Statistical analysis was performed using SPSS v.12.0.1. In each case, a difference was considered significant at $p < 0.05$.

3. Results

3.1 Disruption of K63-polyUb chain formation has no effect on the sensitivity of transfected A549 cells to BPDE.

To test the effect of disruption of K63-polyubiquitination on the sensitivity of transfected cells to BPDE, clonogenic survival assays were performed using a dose range from 0 to 1 μM BPDE (Fig. 1). We observed no statistical difference in the sensitivity of both cell lines to acute (30 min) treatment with BPDE. Similar observations were seen in mammalian [18] and yeast [15] cells demonstrating that expression of *K63R-Ub* does not affect the sensitivity towards UV. A possible explanation for the absence of a difference in sensitivity to acute BPDE exposure is that cells that are unable to form K63-polyUb chains can compensate for the loss of this pathway by utilizing the error-prone TLS arm of the DDT pathway. Therefore, these results suggest that inhibition of K63-polyUb chain formation does not sensitize cells to the toxic effect of BPDE indicating that *WT-Ub* and *K63R-Ub* expressing cells bypass BPDE-induced replication fork blockage with equal efficiency.

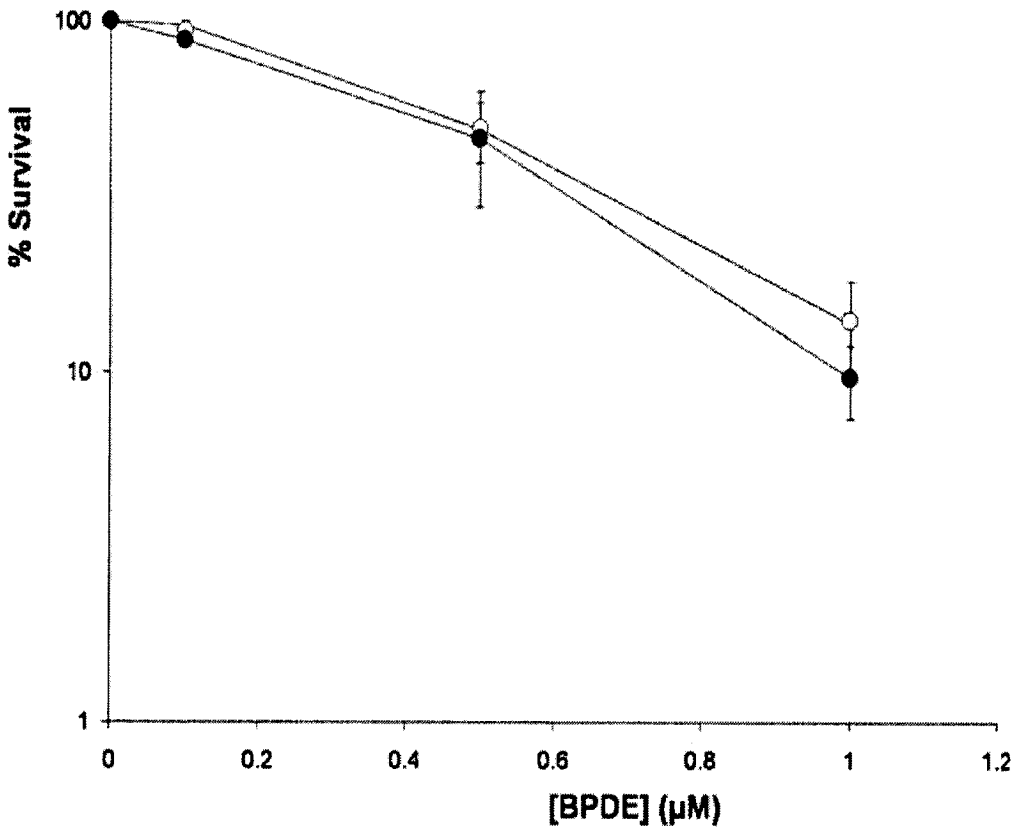

Fig. 1. BPDE-induced cytotoxicity in A549 cells stably expressing *K63R-Ub* (○) or *WT-Ub* (●). Cells were exposed for 30' to increasing concentrations of BPDE. Survival was evaluated by means of a clonogenic survival assay and expressed as the mean of three independent experiments (± standard error).

3.2 Disruption of K63-polyUb chain formation enhances BPDE-induced HPRT-mutations and induces a characteristic mutation spectrum.

To evaluate whether K63-polyUb chain disruption affects BPDE-induced mutagenesis, we determined the mutation frequency at the *HPRT* locus upon BPDE exposure. Following the correction for cell survival, A549 cells stably expressing *K63R-Ub* showed a dose dependent and significant increase in the frequency of *HPRT*-mutations (~54, 87, 19 fold increase at exposure levels of 0.1, 0.5 and 1 μM BPDE, respectively) as compared to their *WT-Ub* expressing counterparts (Figure 2). This increased BPDE-induced mutagenicity in the *K63R-Ub* expressing cells suggests that the formation of K63-polyUb chains is indeed a prerequisite for the error-free bypass of BPDE-DNA adducts.

Furthermore, we examined the spectrum of BPDE-induced mutations in individual clones of *K63R-Ub* expressing cells. Sister clones were excluded as only a single colony per treated population was isolated, as described above. Upon examination of the four overlapping sequences from each *HPRT* cDNA and removal of background mutations, 19 BPDE-induced mutations were found. 74% of these mutations were G→T transversions, while G→C transitions accounted for 21% (Table 1). The large number of G→T mutations suggests that POLη is the relevant mutagenic TLS polymerase (see Discussion). A single T→G mutation was unexpectedly observed suggesting that either the adenosine on the minus strand contained the BPDE-adduct, or more likely, the mutation occurred as a result of misincorporation directly following a guanine adduct (see Table 1). Interestingly and in contrast to the previously reported UV-induced mutation spectra [18], the BPDE-

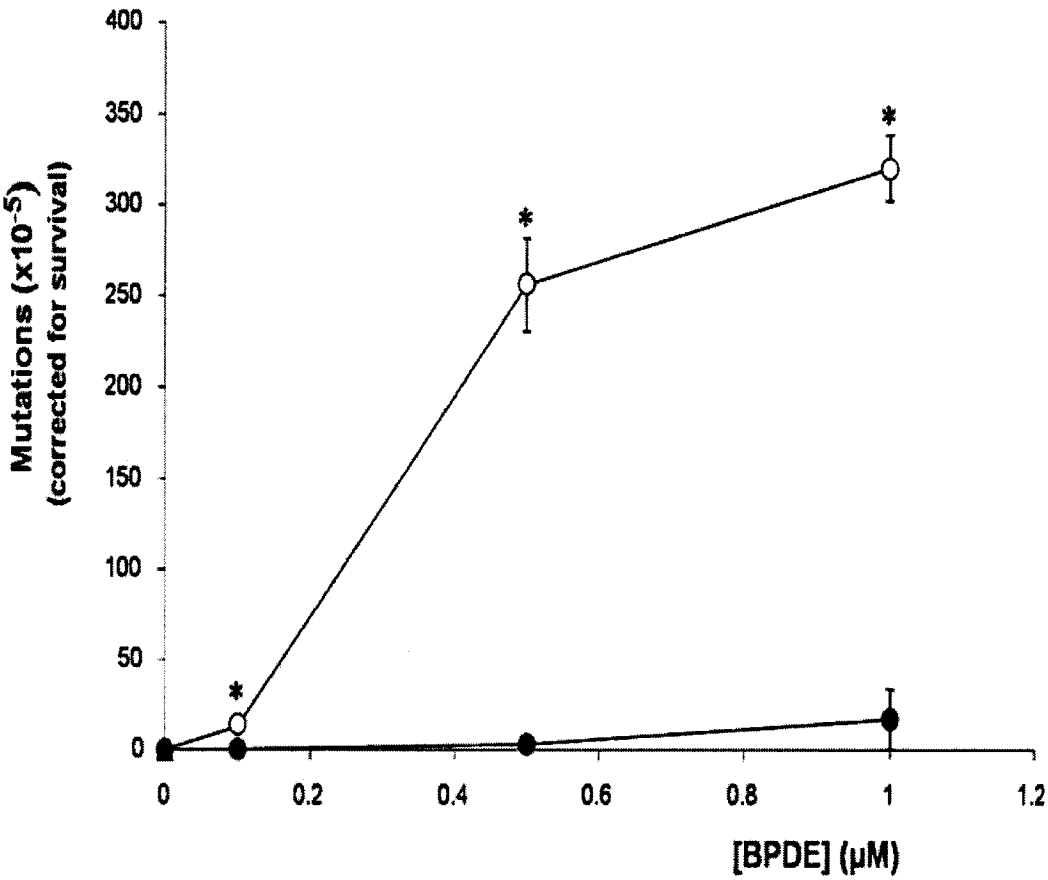

Fig. 2. Effect of *K63R-Ub* expression on BPDE-induced mutagenicity. After exposure to BPDE, mutation frequencies at the *HPRT* gene were determined via selection in medium containing 6-TG. Mutations were corrected for survival and BPDE-induced mutation frequencies were obtained by subtracting the background frequencies, which were 0.59 and 10.37 $x10^{-5}$ clonable cells for *WT-Ub* (●) and *K63R-Ub* (○) expressing cells respectively. The mean values of two independent experiments are shown with standard errors. (*$p<0.001$, *K63R-Ub vs. WT-Ub*)

Point mutations	Sequence change	Position (strand)	Amino acid	Mutant	% of Total
G>T	GATG(G>T)TCAA	472 (+)	Val → Phe	1	
G>T	GACA(G>T)GACT	134 (+)	Arg → Met	2	
G>T	GGGG(G>T)GCTA	212 (+)	Gly → Cys	5	
G>T	ACAG(G->T)ACTG	135 (+)	Arg → Ser	6	
G>T	TGAT(G>T)AAGG	162 (+)	Met → Ile	7	
G>T	GATG(G>T)TCAA	472 (+)	Val → Phe	12	
G>T	GTTG(G>T)ATTT	539 (+)	Gly → Val	13,2	
G>T	TCAA(G>T)GGGG	207 (+)	Lys → Asn	14	
G>T	GACT(G>T)AACG	139 (+)	Glu → STOP	17	
G>T	CAGG(G>T)ATTT	601 (+)	Asp→ Tyr	19	
G>T	ACTG(G>T)AAAG	380 (+)	Gly → Val	20	
G>T	ACAG(G>T)GGAC	335 (+)	Gly → Val	21,2	
G>T	TCAA(G>T)GGGG	207 (+)	Lys → Asn	24	
G>T	AAGT(G>T)TTGG	514 (+)	Val → Phe	28	74
G>C	GAAC(G>C)TCTT	143 (+)	Arg → Pro	10	
G>C	TCAA(G>C)GGGG	207 (+)	Lys → Asn	11	
G>C	TAAT(G>C)ACCA	322 (+)	Asp→ His	13,1	
G>C	GACT(G>C)AACG	139 (+)	Glu → Gln	21,1	21
T>G	AAGG(T>G)CGCA	479 (+)	Val → Gly	4	5

Tabel 1. BPDE-induced mutation spectrum in *K63R-Ub* expressing cells.
A549 cells stably expressing *K63R-Ub* were exposed to 0.5 μM BPDE, further cultured for 7 days and then seeded in medium containing 6-TG. Single colonies were picked from each dish to ensure that no sister clones would be analyzed. The *HPRT* gene was then amplified by PCR followed by sequencing using four overlapping primers. The mutants are grouped by the type of observed point mutations.

induced point mutations were all located at the non-transcribed (+) strand. These data are consistent with the mutation spectrum of BPDE-treated human fibroblasts or T-lymphocyte clones that were conducted in other studies [22,23]. Collectively, these data suggest that inhibition of K63-polyUb chain assembly results in an increased mutagenic effect of BPDE-exposure, indicating the involvement of error-prone TLS.

3.3 Disruption of K63-polyUb chain formation does not affect the occurrence and repair of BPDE DNA-adducts.

Mutagenicity of BPDE is directly related to the number of BPDE-DNA adducts in the cell [22,24]. Therefore, to exclude the possibility that the distinct mutagenic effects of BPDE in *K63R-Ub* versus *WT-Ub* transfected cells as described above, could be explained by either different initial BPDE-DNA adduct levels, or differences in time-dependent adduct removal, we evaluated the kinetics of BPDE-DNA adduct levels over a period of 24 hours by ^{32}P-postlabelling. Representative DNA adduct profiles of both A549 cell lines, acutely exposed to 0.1 μM BPDE, are shown in Fig. 3 (insert). For both the *WT-Ub* and *K63R-Ub* expressing cells, clear BPDE-dG spots were observed after a 30 min incubation period (t = 0h). The intensity of the (±)-*anti*-BPDE-dG spots rapidly reduced in time, indicating effective removal of the BPDE-DNA adducts. Quantitative analysis revealed that the initial adduct level caused by acute treatment with 0.1 μM was similar between both cell lines (32.9±4.8 and 31.6±6.9 adducts/10^8 nucleotides for *WT-Ub* and *K63R-Ub* expressing cells, respectively). Moreover, no significant differences ($P>0.5$) in removal of BPDE-DNA adducts were observed between A549 cells stably expressing *K63R-Ub* or *WT-Ub*

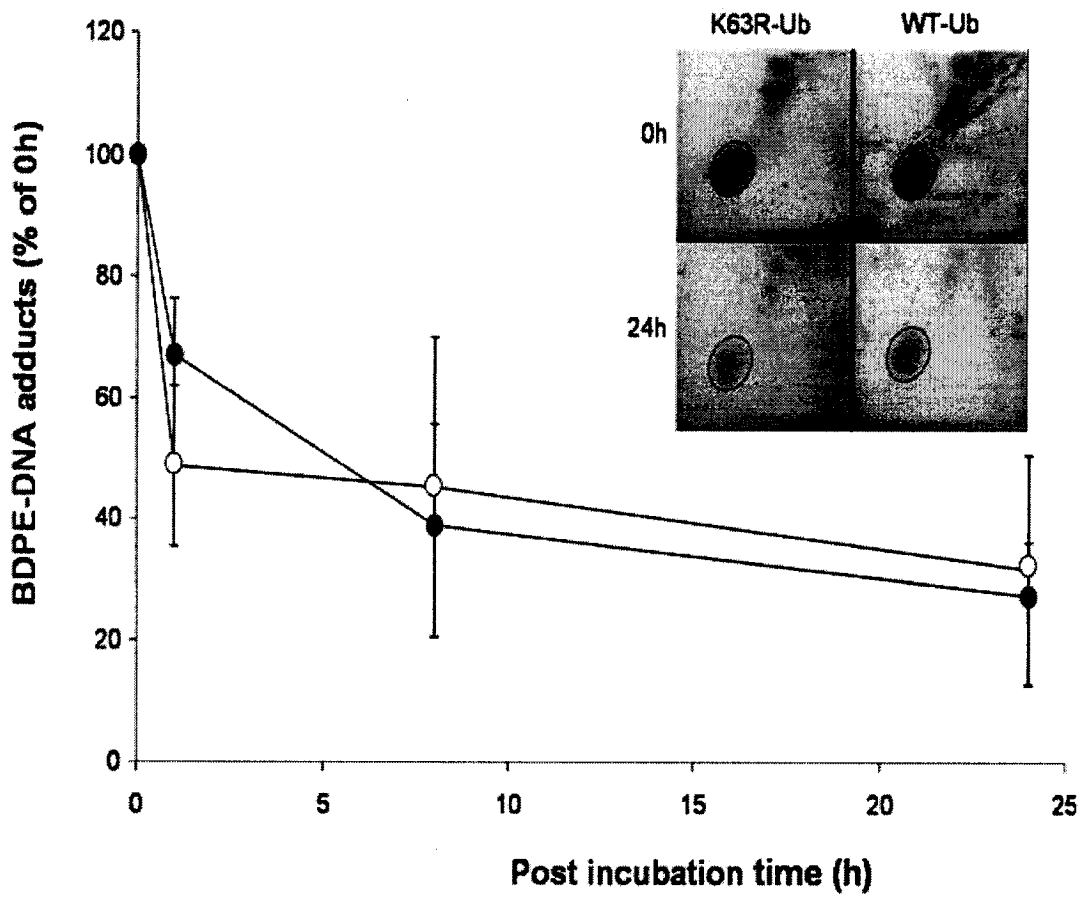

Fig. 3. Removal of (±)-*anti*-BPDE-N^2-dG adducts in *WT-Ub* (●) and *K63R-Ub* (○) expressing A549 cells after acute exposure to 0.1 μM BPDE. Chromatograms of ^{32}P-labeled BPDE-DNA adducts. A549 cell strains stably expressing *K63R-Ub* and *WT-Ub* show clear spots immediately after exposure to BPDE **(Insert)**. After recovery of 24h spot-intensities were reduced. Quantification was performed on the circled spots, which represent the (±)-*anti*-BPDE-N^2-dG adducts. (±)-*anti*-BPDE-DNA adduct removal in A549 cells strains at various time intervals. Data are expressed as the mean adduct levels of two independent experiments (± standard error).

(Fig. 3). In both cell lines, DNA-adduct levels are reduced by 50% within 8h. To correct for possible dilution effects on DNA-adduct levels due to DNA synthesis during cell replication, adduct levels were corrected for cell proliferation for each time point (#cells at t = recovery / #cells at t = 0h). Overall, these data suggest that the NER capacities of both A549 cell strains are comparable, and that NER is not affected by genetic modification of the Lys63-poly-ubiquitination process.

3.4 BPDE induces PCNA poly-ubiquitination.

Thus far our data support the idea that K63-linked ubiquitin chains are critical in the recovery of replication forks after the production of BPDE induced DNA damage. Based on our previous study [18], we postulated that the likely substrate of these chains is PCNA. Other studies have shown that BPDE exposure leads to mono-ubiquitination of PCNA [25] while PCNA poly-ubiquitination has not previously been reported. We investigated whether PCNA is poly-ubiquitinated in mammalian cells after BPDE treatment. Both A549 and HeLa cells were treated with 0.5 or 1 μM BPDE or 30 J/m^2 UVB as a positive control. Five hours post-treatment, a prominent mono-ubiquitinated and a faint di-ubiquitinated band was observed in both UV and BPDE treated cells (Fig. 4A). In addition, over-exposure of this blot revealed a stronger higher molecular weight band corresponding to di-ubiquitinated PCNA. Similar to our previous report using UV light as a mode of DNA damage, it is often difficult to see more than a di-ubiquitinated form of PCNA on an immunoblot after DNA damage, which is a likely limitation of the antibody. In addition, we also see

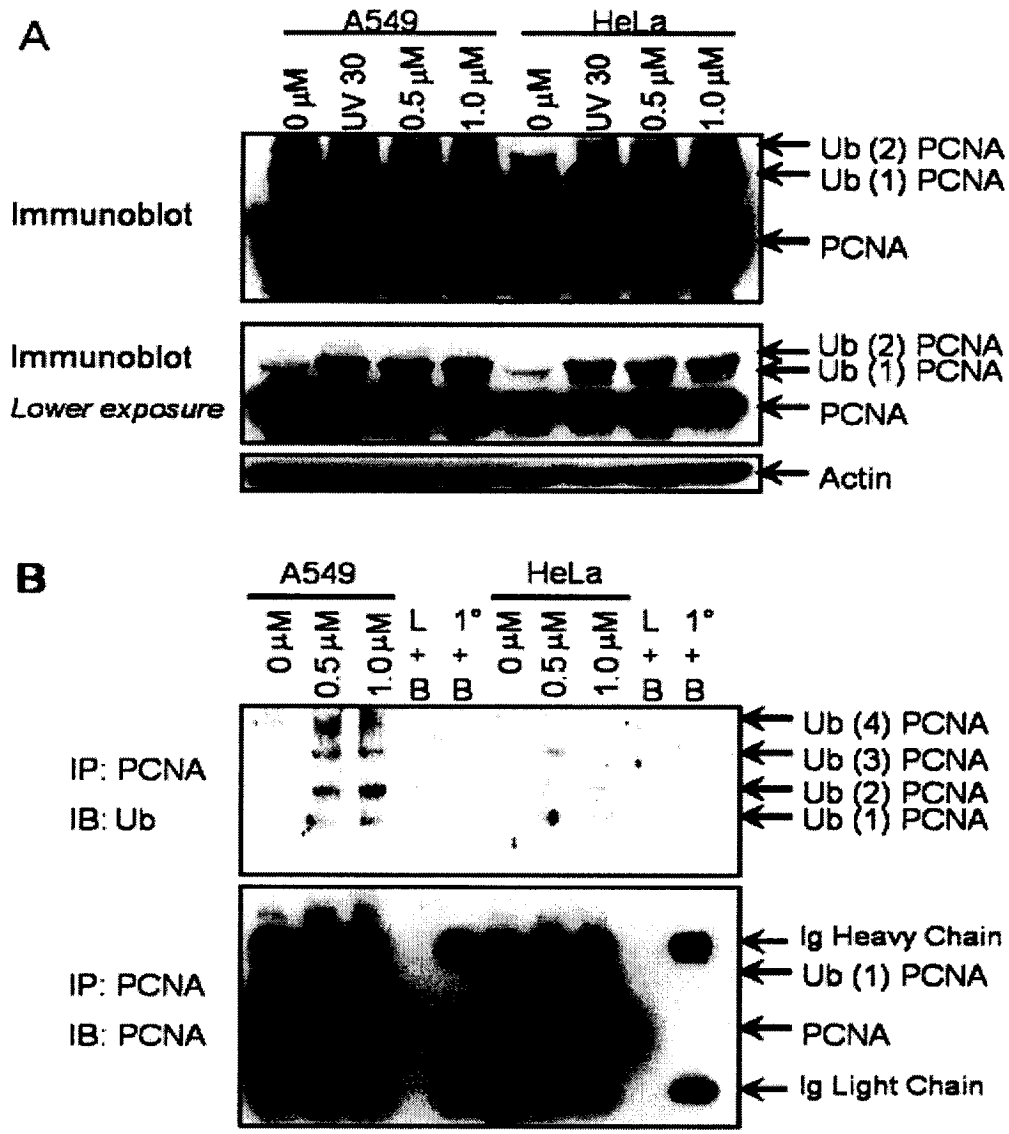

Fig. 4. Poly-ubiquitination of PCNA upon UV- and BPDE-exposure. (A) Immunoblot form A549 and HeLa cells that were irradiated with 30 J/m^2 UV or exposed to 0.5 and 1 μM BPDE. Cells were lysed 6h post-treatment followed by immunoblotting for PCNA. A high and lower exposure of the PCNA immunoblot is shown. **(B)** A549 cells were exposed to BPDE and lysed in boiling SDS, diluted in lysis buffer and subjected to immunoprecipitation with a PCNA antibody and detected with PCNA or Ub antibodies. The controls in the immunoprecipitations were "L+B", in which lysates were incubated with beads but no PCNA antibody, and "1°+B" in which PCNA antibody was incubated with beads alone. In the later case only the immunoglobulin heavy and light chains are detected on the immunoprecipitations.

lower levels of mono- and di-Ub PCNA in our untreated cells. We postulate that this modification is a response to endogenous DNA damage.

Nevertheless, to confirm that these bands are indeed ubiquitinated PCNA, immunoprecipitation using an antibody directed against PCNA followed by immunoblotting with anti-ubiquitin was performed (Fig. 4B). Several ubiquitinated species were observed corresponding to mono-, di-, tri- and tetra-ubiquitinated PCNA. This laddering pattern was similar to the banding pattern observed after UV irradiation from a previous study [18]. Taken together, our data suggest that PCNA is a target for poly-ubiquitination after BPDE-induced DNA damage, further supplementing the accumulating evidence that PCNA poly-ubiquitination is an important process in response to a variety of DNA lesions.

3.5 Increased recruitment of POLη to the replication fork upon blockage by BPDE.

To support our suggestion that the increased BPDE mutagenesis in the K63R-Ub expressing cells is brought about by the enhanced recruitment of error-prone Y-family polymerases, we analyzed the effects of *K63R-Ub* expression on POLη foci formation. We selected this particular TLS polymerase as previous reports suggest that it is the relevant polymerase responsible for the error-prone bypass of BPDE-DNA adducts [9,11]. The previously described HeLa and A549 cells, expressing *WT-Ub* or *K63R-Ub* fused with the puromycin resistance gene were used and transfected with the POLη-GFP plasmid. No differences in transfection efficiency with POLη-GFP in both cell lines were observed (data not shown). Importantly, we previously

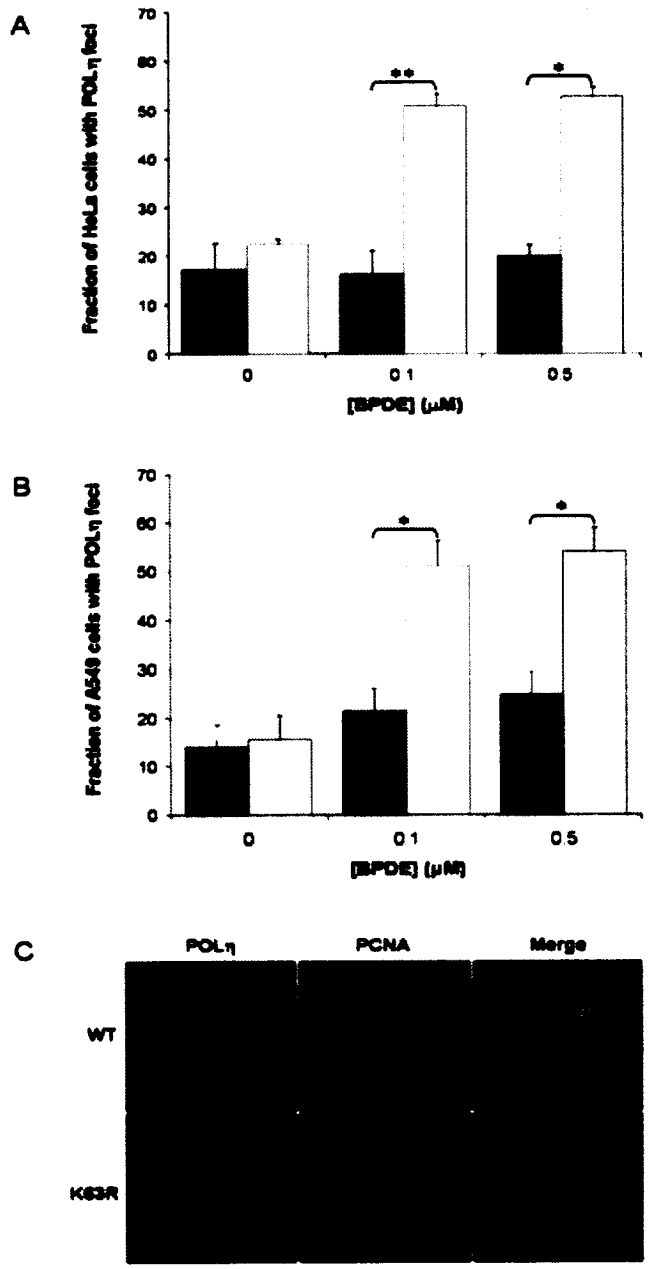

Fig. 5. Relocalization of POLη into intranuclear foci after BPDE-exposure. (A) HeLa cells (n=4) and **(B)** A549 cells (n=2) stably expressing *WT-Ub-puro* (■) and *K63R-Ub-puro* (□) were transiently transfected with plasmids encoding GFP-POLη. 24h post-transfection, cells were exposed to 0.1 and 0.5 μM BPDE for 30 min at 37°C. Foci were quantified 6h after treatment using a live-cell imaging microscope and percentages of cells with POLη foci were calculated. The mean values of at least two independent experiments are shown with standard error (**p=0.02, *p<0.05 *K63R-Ub* vs. *WT-Ub* for 0.1 and 0.5 μM BPDE, respectively). **(C)** Representative confocal photographs showing a 100% co-localization of POLη with PCNA in these A549 cells, 6 h after BPDE-exposure. POLη (green) and PCNA (red) were detected using antibodies.

showed that these cells behave identically to the ubiquitin GFP fusion expressing cells and that there were no overt defects in POLη function [18]. In line with other similar studies [26], we observed homogenous nuclear expression of the POLη protein in all transfected cells. Similar to previous reports [18,26-28], in the absence of BPDE, foci were observed in 17 and 22% of the HeLa cells (Fig. 5A) and 14 and 15% of the A549 cells (Fig. 5B) expressing *WT-Ub* or *K63R-Ub* respectively, showing no statistical differences between both cell lines. Within 6h of exposure to 0.1 and 0.5 μM BPDE, POLη relocalized in distinct focal patterns in the nucleus (Fig. 5C left). Upon exposure to 0.5 μM BPDE, the percentage of cells with foci increased to 52.5% and 53.9% in HeLa and A549 cells expressing *K63R-Ub*, respectively (Fig. 5A and B). In HeLa and A549 cells stably expressing *WT-Ub*, the induction of POLη foci was significantly less after BPDE-exposure (20% and 25% at 0.5 μM of BPDE for HeLa and A549 cells, respectively). Overall, our data show that inhibition of K63-linked poly-ubiquitination causes a ~3-fold increase in POLη foci ($p<0.05$) after acute exposure to BPDE. This increase parallels the enhanced mutation frequency in BPDE-treated K63R-Ub expressing cells (Fig. 2) suggesting the involvement of POLη in BPDE-induced mutagenesis in the K63R-Ub expressing cells. Furthermore, we also analyzed the co-localization of these foci with sites of DNA replication as revealed by positive PCNA foci. In both the *WT-Ub* and *K63R-Ub* expressing A549 cells, 100% of the BPDE-induced POLη foci co-localized with PCNA foci (Fig. 5C). This indicates that the foci produced in the *K63R-Ub* expressing cells are typical of those previously reported to occur at sites of blocked replication [18,26].

3.6 Knockdown of POLη diminishes the mutagenicity of BPDE.

Collectively, our data suggest that inhibition of K63-polyUb chain assembly results in an increased requirement for TLS to bypass BPDE-DNA adducts (cf. Fig. 5). To establish a possible causal role of POLη in the increased mutagenicity of BPDE as observed in the *K63R-Ub expressing cells*, we suppressed the expression of POLη in these cells by using siRNA. The combination of disrupting K63R-polyUb chain formation and POLη knockdown resulted in a 50% reduction in BPDE-induced *HPRT* mutations (Figure 6), indicating a predominant role of POLη in error-prone bypass of BPDE lesions in the K63R-Ub expressing cells.

4. Discussion.

In the present study we showed that lysine 63-linked poly-ubiquitination is an important process to protect mammalian cells against BPDE-induced mutagenicity. This protective effect is distinct from TLS and is likely caused by directing DDT mechanisms into an error-free pathway, in which the information on the daughter strand is used to correctly circumvent the DNA lesion. This would suggest that K63-poly-ubiquitination is a general requirement for physiological protection against mutagenesis in mammalian cells.

Stelter and Ulrich have shown that disruption of the error-free arm of DDT in yeast, as a consequence of a UBC13 mutation, results in a dramatic increase in UV-induced mutations [15]. Moreover, we recently reported that K63-linked poly-ubiquitination is required for error-free DDT in human cells as disruption of this specific chain formation leads to increased UV mutagenicity [18,29]. In the present

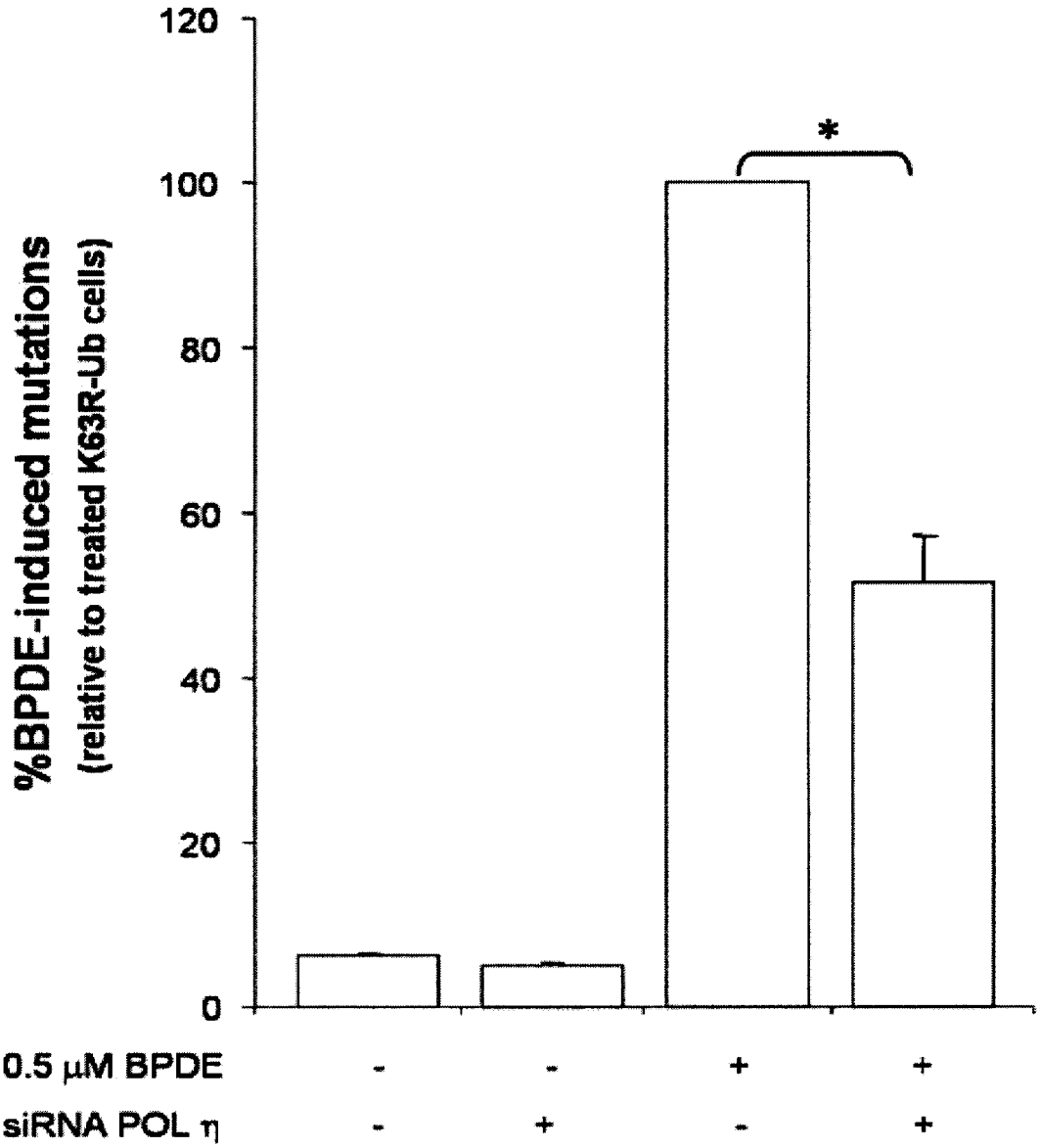

Fig. 6. Effect of POLη knockdown on BPDE-induced mutagenicity. The number of *HPRT* mutants was quantitated upon exposure to 0.5 μM BPDE for A549 cells stably expressing *K63R-Ub* with or without POLη RNAi. BPDE-induced mutations were corrected for the plating efficiency and presented as the percentages of the mutation frequency in the BPDE treated *K63R-Ub* expressing cells. The mean values of two independent experiments are shown with standard error (*$p<0.03$, BPDE exposed cells with vs. without POLη RNAi).

study, K63-linked poly-ubiquitination was observed upon exposure to BPDE and inhibition of this polyUb chain formation resulted in up to 87-fold increase in BPDE-induced mutations in *K63R-Ub* compared to the *WT-Ub* expressing cells.

In a previous study we proposed a model in which K63-polyUb chains are suggested to activate an error-free mechanism that protects cells against UV-induced mutations that would otherwise be induced by error-prone TLS polymerases [18]. In this present study, we extend this model to include the bypass of BPDE-DNA lesions implying that the observed increased BPDE-induced mutation frequency in the *K63R-Ub* expressing cells would likely be caused by the bypass by low-fidelity and error-prone DNA polymerases. Previous reports have suggested that POLη is the main polymerase responsible for the mutagenicity of BPDE-DNA adducts by predominantly inserting an adenosine opposite the BPDE-N^2-dG lesion, causing the characteristic G → T transversions [9]. Appropriately, the majority (74%) of mutations observed in our model is also this G→T mutation supporting the idea that POLη is the relevant enzyme for the bypass of BPDE-DNA adducts. Furthermore, intra-nuclear POLη foci formation was ~3-fold increased in BPDE-treated *K63R-Ub* expressing cells, indicating the involvement of POLη in the increased BPDE-induced mutagenic effects in absence of K63-linked poly-ubiquitination. In the HeLa and A549 cells stably expressing *WT-Ub* there was no clear induction of cells with POLη foci after BPDE-exposure. This is in contrast with studies from Ogi *et al.* observing POLη foci recruitment in ~90% of the human lung fibroblast exposed to 20 μM of B[a]P [27]. These contrasting observations are most likely explained by a significant difference in dosing strategies. In the present study POLη foci were assessed 6h post

acute (30 min) BPDE treatment, while Ogi *et al.* quantitated foci following 16h of continuous exposure to complete medium containing B[a]P and S9 mix.

We observed POLη foci to co-localize with the DNA polymerase clamp, PCNA. As this protein is required for replication, this suggests that POLη is tightly associated with the replication machinery and is therefore locally available to carry out TLS past the blocking lesions. Similar POLη recruitment and co-localizations were observed in human fibroblasts [26,30] and human cancer cells lines [18] upon exposure to UV irradiation. Furthermore, we studied the effect of POLη knockdown on the BPDE-induced mutation frequencies in the *K63R-Ub* expressing A549 cells, and found a 50% reduction in *HPRT* mutations. This further confirms that POLη is a crucial component of the error-prone bypassing of replication stalling BPDE-DNA adducts in cells with suppressed K63-linked poly-ubiquitination.

The preferential insertion of adenosine by POLη opposite (±)-*anti*-BPDE-N^2-dG adducts will lead to the induction of G:C-to-T:A transversions [9]. This was indeed confirmed by analyzing the mutation spectrum in the BPDE-treated *K63R-Ub* expressing cells, showing predominantly G→T point mutations. An intriguing explanation for the increase in mutation frequency and the high proportion of G:C-to-T:A transversions is the relative resistance of especially the (+)-*trans-anti*-BPDE-N^2-dG adducts to NER due to its minimal distortion of the helix [31]. As a consequence of this adduct being silent to NER, we predict that these adducts will persist over time and thus present as a substrate for POLη mediated mutagenesis. Intriguingly, this would suggest that known helix deforming lesions such as those produced by UV light would be less mutagenic than BPDE. Indeed, UV induced mutation frequency in

this model system was previously reported to be 2-fold increased [18] compared to 87-fold for 0.5 μM BPDE. Since, UV-induced DNA lesions cause severe DNA-helix distortions that will be repaired faster by NER [31,32], reliance on potentially mutagenic TLS-bypass processes during replication will be attenuated.

Interestingly, most of the observed BPDE-induced point mutations occurred in the non-transcribed (+) strand of the *HPRT* gene. As previously reported, this strand specificity of the BPDE-induced mutations indicates preferential repair of BPDE DNA-adducts in the transcribed strand, which is consistently faster than the repair of adducts in the non-transcribed strand [23,33]. The transcription coupled repair sub-pathway of NER is highly selective for adducts in the transcribed DNA strand [34,35], whereas repair of the non-transcribed strand will be dependent on DNA replication across these 'bulky' DNA adducts by TLS. This suggests that cells that contain lesions that are less amenable to NER would rely more on bypass by TLS as a means for survival. Interestingly, this would predict that lesions on the non-transcribed strand would persist and remain in the genome.

G:C-to-T:A transversions are also found in higher frequencies in lung cancers from smokers compared to non-smokers [36]. Moreover, others have shown a good correlation between hotspots of DNA adduct formation by PAHs, such as B[a]P, found in tobacco smoke and G:C-to-T:A transversion hotspots in lung cancer [37]. This higher prevalence of G→T transversions in lung cancer is generally interpreted as the primary mutagenic signature of PAH-DNA damage. As such, it would be of great interest to determine whether Lys63-polyUb chain formation has an etiological role in tobacco smoke-induced and PAH-mediated lung cancer. Studies of Sasaki et

al. for instance [38], showed a link between lung cancer and the RAD6-dependent DDT mechanisms. They observed a decreased expression of the human homologue of yeast RAD6 (hRAD6B) in lung cancer, which might be a biomarker for decreased DDT capacity. Since, RAD6 is one of the key players in the enzymatic complex (RAD18, RAD5, UBC13/MMS2) that assembles the K63R-polyUb chains [14], this may suggest a link between pulmonary carcinogenesis and K63R-polyUb chain formation.

In conclusion, our data show that inhibition of Lys63-linked poly-ubiquitination of PCNA significantly enhances BPDE-induced mutations involving recruitment of the error-prone Y-family polymerase POLη. We therefore propose that formation of K63-polyUb chains protects (BPDE-exposed) human cells against translesion synthesis-mediated mutagenesis, implying that ubiquitination guards against chemical carcinogenesis. This predicts that alterations in ubiquitination and the genes that control it may influence the susceptibility of individuals to environmental mutagenesis, and possibly carcinogenesis. For example, it has been demonstrated that oxidative stress modulates the ubiquitination process via reversible S thiolation/dethiolation of E1 and E2 enzymes [39-41]. Such an effect could have major consequences for functioning of ubiquitin-conjugating enzymes (E2s), such as RAD6 and UBC13, and thus Lys63-linked poly-ubiquitination. Finally, a major challenge for future studies will be to further elucidate DDT pathways in humans and to appreciate their role in cancer development upon exposure to environmental xenobiotics. Such studies should reveal whether K63-polyUb chain formation could serve as a target for chemopreventive strategies.

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Chapter 3

hMMS2 serves a redundant role in human PCNA polyubiquitination

Contribution of collaborators

The contents of this manuscript were written by Jan Brun and edited by the supervisor Dr. Doug Gray, and our collaborators Dr. Roland Chiu, Dr. Brad Wouters. JB performed the experiments. JB, RKC, BGW and DAG analyzed the data; JB provided the necessary analysis tools, reagents and materials. All the figures are the work of JB. The MMS2 knockout embryonic fibroblasts were a kind donation of Dr. Wei Xiao (University of Saskatchewan).

Summary

The manuscript of Brun et al., extends previous work from our laboratories on the polyubiquitination of PCNA in mammalian cells (first reported by us in 2006). We have attempted to confirm that MMS2 is a component of the complex that builds the polyubiquitin chains, but have discovered through a variety of techniques that MMS2 can be eliminated without abrogating chain elongation. These data clearly demonstrate the existence of a functionally redundant protein in mammalian cells, and in the manuscript we discuss possibilities for what the redundant entity might be. We believe the manuscript is suitable in scope and format for publication as a brief report and has been submitted and currently being reviewed for the journal of BMC Molecular Biology June 2007.

hMMS2 serves a redundant role in human PCNA polyubiquitination

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Running Title: PCNA is ubiquitinated in the presence or absence of hMMS2

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Key words: RAD18, MMS2, UBC13, PCNA, DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63, lysine 63

1 Abstract

In yeast, DNA damage leads to the mono and polyubiquitination of the sliding clamp PCNA. Monoubiquitination of PCNA is controlled by RAD18 (E3 ligase) and RAD6 (E2 conjugating enzyme), while the extension of the monoubiquitinated PCNA into a polyubiquitinated substrate is governed by RAD5, and the heterodimer of UBC13/MMS2. Each modification directs a different branch of the DNA damage tolerance pathway (DDT). While PCNA monoubiquitination leads to error-prone repair via TLS, biochemical studies have identified MMS2 along with its heteromeric partner UBC13 to govern the error-free repair of DNA lesions by catalyzing the formation of lysine 63-linked polyubiquitin chains (K63-polyUb). Recently, it was shown that PCNA polyubiquitination is conserved in human cells and that this modification is dependent on RAD18, UBC13 and SHPRH. However, the role of hMMS2 in this process was not specifically addressed. In this report we show that mammalian cells null for MMS2 or cells in which MMS2 and/or UEV1A were reduced by siRNA-mediated knockdown maintain PCNA polyubiquitination, suggesting existence of another hMMS2 variant (hMMSv) or complex that can compensate for its loss.

2 Introduction

Protecting the integrity of the DNA genome is important for the long-term survival of higher eukaryotes [1]. Given the importance of genomic integrity, it is not surprising that an elaborate system of cell cycle checkpoints and DNA repair systems has evolved in higher vertebrates. However, the failure to remove DNA lesions in a timely and efficient manner often forces a cell to bypass the damage in order avoid

replication stalling. This is accomplished through DNA damage tolerance (DDT) an important component of the DNA damage response. DDT allows replication machinery stalled at sites of DNA damage to continue with DNA synthesis by allowing bypass of such sites in either an error-prone or error-free manner [2]. The control of such a pathway is dependent on the modification status of the sliding clamp PCNA.

In the absence of DNA damage, yeast PCNA is typically sumoylated on K164 which promotes normal S phase progression by preventing unwanted recombination, while replication stress results in PCNA ubiquitination on the same lysine residue [3,4]. There is a central role for RAD6 (ubiquitin conjugating E2 enzyme) and RAD18 ubiquitin ligase (E3 ligase) in PCNA ubiquitination. During genotoxic stress, RAD6 is recruited by RAD18 to sites of DNA damage where it monoubiquitinates PCNA[4]. Monoubiquitinated PCNA facilitates the recruitment of error-prone translesion polymerases including polymerase η, ι, κ and rev1 [5,6]. While undesirable, the mutations induced by this error-prone mechanism may be less deleterious than catastrophic blockage of replication forks. However, there is also an alternate complex (UBC13/MMS2/RAD5) that forms and mediates error-free bypass by extending the monoubiquitinated PCNA via K63-polyubiquitin chains [4]. The outcome of this polyubiquitination has yet to be fully elucidated; it clearly does not involve the proteasome but more likely involves fork reversal, recombination with an undamaged sister chromatid either at or behind the replication fork [4].

A majority of the genes of the DDT pathway have been conserved from yeast to human including RAD18, RAD6, MMS2, UBC13, PCNA and most of the

translesion polymerases [7,8]. As such, Kannouche et al., and Watanabe et al., have shown that the error-prone arm of DDT involving PCNA monoubiquitination and concordant polymerase switching is fully conserved in mammalian cells [9,10]. Recently, we reported that PCNA is polyubiquitinated after DNA damage [11-13]. Accordingly, PCNA polyUb was shown to be dependent on RAD18 (suggesting that monoubiquitination of PCNA is required), and also on UBC13 (the ubiquitin conjugase previously shown to be involved in K63-polyubiquitin chain formation) [13]. Recently, Motegi et al., and Unk et al., have confirmed the existence of polyubiquitinated PCNA and have suggested that SHPRH (human ortholog of yeast RAD5) is the E3 ligase involved this process [11,12]. However, it has yet to be determined whether hMMS2 (heteromeric partner of hUBC13) participates significantly in PCNA polyubiquitination in human cells.

MMS2 is a ubiquitin conjugating enzyme variant (UEV) protein that resembles ubiquitin conjugating enzymes (E2s) but lacks a conserved active cysteine site [14]. Based on this original observation, it was hypothesized that UEV proteins behaved as negative regulators of ubiquitination. However, biochemical evidence showed that MMS2 forms a heteromeric complex with UBC13 (an E2 conjugating enzyme with active catalytic cysteine site) to catalyze the formation of K63-polyubiquitin chains [15]. In addition, loss of MMS2 in yeast resulted in overt sensitivity to a variety of genotoxic agents as well as increased spontaneous and UV induced mutagenesis confirming its role in the error-free damage avoidance arm of DDT [16,17]. Similar to the findings in yeast, the expression of anti-sense MMS2 in human cells resulted in increased UV induced mutagenesis [18]. These data provide

strong evidence of the importance of MMS2 in the assembly of polyubiquitin chains linked through K63 in the error free damage avoidance arm of the DDT pathway in both yeast and humans.

Since MMS2 serves such an important role in error free DNA repair in both humans and yeast and has been shown to be indispensable in yeast PCNA polyubiquitination, we predicted that it would be equally important in PCNA ubiquitination in mammalian cells. Therefore, we examined the state of PCNA ubiquitination in mouse embryonic MMS2 knock-out embryonic stem cells and human cells in which hMMS2 had been depleted by siRNA. Here we report that PCNA polyubiquitination proceeds with normal kinetics in the presence or absence of MMS2 whereas UBC13 or RAD18 knockdown abrogates PCNA polyubiquitination. Additionally, depletion of UEV1a does not disrupt PCNA polyubiquitination after DNA damage. This suggests that there is a high degree of redundancy built into mammalian systems and that an MMS2 variant exists that can compensate for the loss of hMMS2.

3 Materials and Methods

3.1 Cell culture and treatments

The mouse embryonic $MMS^{+/+}$ or $MMS^{-/-}$ stem cells (kindly provided by Dr. Wei Xiao, University of Saskatchewan) were cultured in DMEM supplemented with 15% FBS, 1X pen/strep, LIF (kindly provided by Dr. M. McBurney, Ottawa Health Research Institute) and B-mercaptoethanol (EMD Chemicals, Omnipur, Gibbstown New Jersey, United States). The HEK 293T and Hela cell lines were cultured in DMEM (Gibco, Invitrogen, Carlsbad, California, United States) supplemented with

10% FBS (Gibco, Invitrogen, Carlsbad, California, United States). 293T and Hela cells were transfected with siGENOME SMARTpool reagent specific for human RAD18, UBC13, MMS2 and/or hUEV1a (Dharmacon Research, Lafayette, Colorodo, United States) using oligofectamine (Invitrogen Carlsbad, California, United States). The transfections were performed 72 hours prior to harvesting the cells to achieve optimal long-term knockdown as determined by immunoblotting. UV irradiation ($30J/m^2$) was performed on exponentially growing cells using a UVC germicidal lamp at a fluence rate of $1J/m^2/s$.

3.2 Western blotting

RNA interference and the preparation of proteins lysates have were performed as described previously [13]. Cells transfected with siGENOME SMARTpool reagent specific for human UBC13, human RAD18 human MMS2 or human UEV1A (Dharmacon Research, Lafayette, Colorodo, United States) were UV irradiated and lysed 6h post-treatment. Samples were sonicated, soluble fractions were recovered, and proteins were quantified. Proteins were resolved on either a one phase or two phase SDS-polyacrylamide gel (10% or 10% and 15%) and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States). The following antibodies were used: mouse monoclonal that recognizes both hMMS2 and UEV1a (kindly provided by Dr. Wei Xiao), mouse monoclonal UBC13, mouse monoclonal PCNA PC10 (Millipore, Chemicon, Temecula California), and mouse monoclonal actin (Sigma, St. Louis Missouri, United States). Proteins were visualized using SuperSignal West Pico

Chemiluminescent Substrate (Pierce Biotechnology Rockford, Illinois, United States).

4 Results

4.1 Efficient Knockdown of MMS2 and UEV1a

In yeast, monoubiquitination requires the ubiquitin E2/E3 complex RAD6/RAD18 and further polyubiqutination is dependent on the RAD5/UBC13/MMS2 complex. In mammalian cells, RAD18 has been implicated in monoubiquitination, and SHPRH and UBC13 in polyubiquitination of PCNA [11-13]. To ascertain whether mammalian MMS2 is also required for polyubiquitination we targeted MMS2 in HEK 293T and Hela cells using siRNA. The Mms2 antibody used in our studies recognizes both Mms2 and the other mammalian homolog Uev1a [19]. Therefore to confirm effective knockdown with this antibody we performed a double knockdown with siRNA targeting both hMMS2 and hUEV1a. The abundance of MMS2 and UEV1a was found to be effectively reduced 72 hours post- transfection in both 293T and Hela cells (Figure 1A-C) suggesting that both individual knockdowns were effective.

4.2 PCNA is polyubiquitnated in the absence of MMS2 or UEV1a

Cells treated with siRNA against MMS2, UEV1a, or both were exposed to UV light to induce DNA damage and then assessed for ubiquitination of PCNA. As expected, the band corresponding to monoubiquitinated PCNA was unaffected by MMS2 knockdown after UV irradiation (Figure 2). Unexpectedly, knockdown of MMS2 alone did not affect the higher molecular weight bands corresponding to

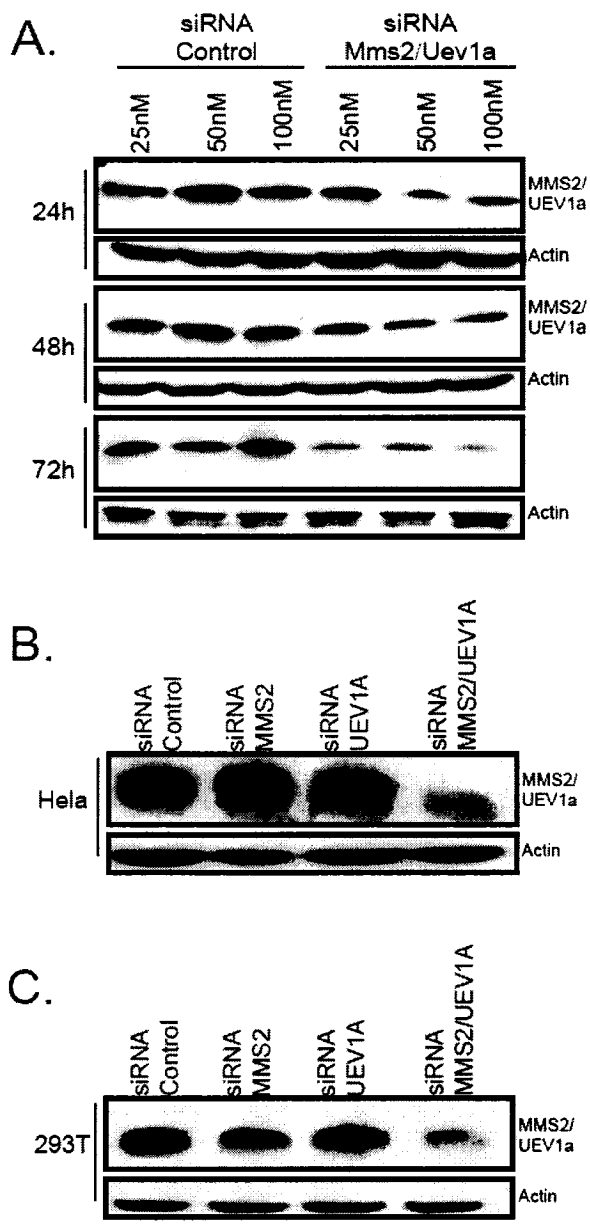

Figure 1: siRNA targeting MMS2 and UEV1a results in an efficient knockdown. **(A)** 293T cells were transfected with 100nM of either control siRNA, siRNA MMS2 and siRNA UEV1A followed by immunoblotting. Cells were lysed at the indicated time points and subject to immunoblotting. **(B)** Hela Cells were treated as above and lysed 72 hours post-transfection. (C) 293T cells were treated as in (B).

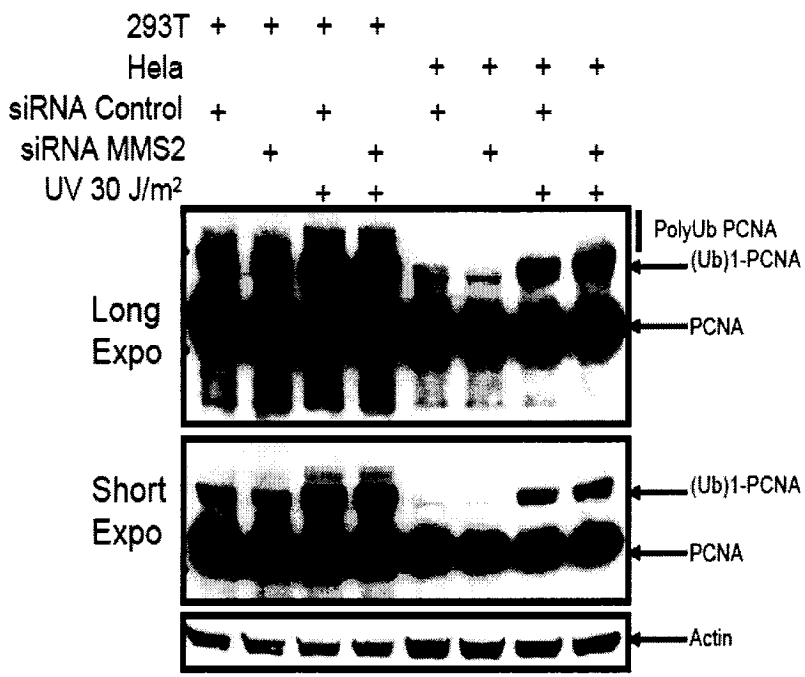

Figure 2: MMS2 Knockdown does not disrupt PCNA polyubiquitination. 293T and Hela cells were transfected with 100nM of either control siRNA or siRNA specific for MMS2. 72 hours later cells were UV irradiated with either 0 or 30J/m^2 and lysed 6 hours post-treatment followed by immunoblotting with an anti-PCNA antibody.

polyubiquitinated PCNA (Figure 2). Since MMS2 belongs to a family of UEVs that have been conserved throughout evolution in higher eukaryotes [14,20-22] we speculated that one of these UEVs could compensate for its loss. Human UEV1a is an obvious candidate since it can bind to UBC13 and has greater than 90% amino acid sequence identity with hMMS2[23]. However, our results demonstrated little or no reduction in polyubiquitinated PCNA after knockdown of UEV1a alone or in combination with MMS2 (Figure 3a and b). This is in contrast to knockdown of UBC13 or RAD18 which effectively blocked PCNA polyubiquitination (Figure 4a, and b). These findings suggest that MMS2 likely serves a redundant role in PCNA polyubiquitination.

4.3 MMS2$^{-/-}$ knockout stem cells maintain PCNA polyubiquitination

In order to confirm the results of our study in human cells and rule out the possibility of off target effects of siRNA, we obtained mouse embryonic stem cells in which the MMS2 gene had been inactivated by gene targeting (a kind gift from the laboratory of Dr. Wei Xiao at the University of Saskatchewan). Loss of MMS2 results in an approximate 50% reduction in the intensity of the MMS2/UEV1a band detected with the antibody described above (Figure 5a), since UEV1a expression is not altered. Monoubiquitination of PCNA after UV treatment occurred normally and with identical kinetics in the MMS2-/- cells compared to wild type. Consistent with our siRNA results (Figure 4B), we did not observe a reduction in PCNA polyubiquitination in the MMS2 knock-out cells.

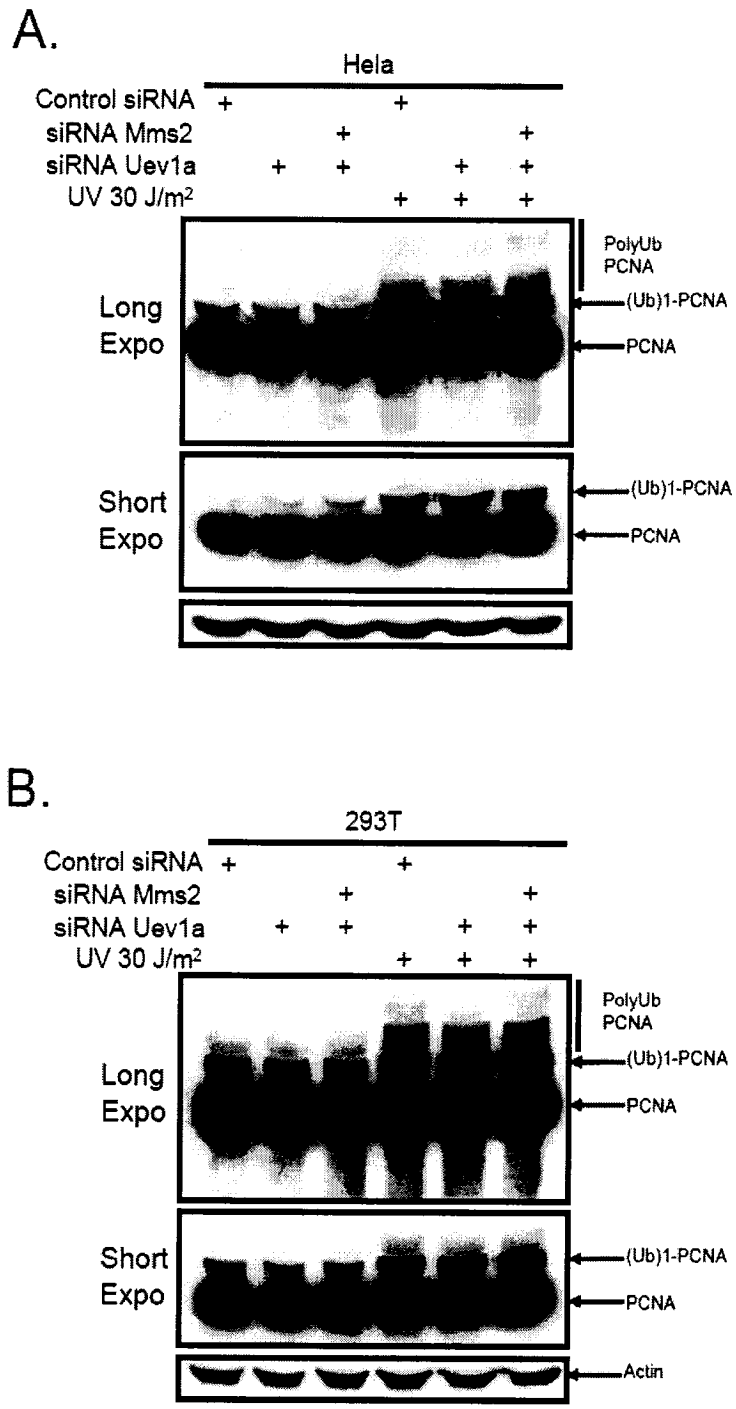

Figure 3: A knockdown of MMS2 and UEV1a does not abrogate PCNA polyubiquitination. **(A)**Hela cells were treated as in Figure 2 except they were additionally transfected with siRNA UEV1A. **(B)** 293T cells were treated as in Figure 3B.

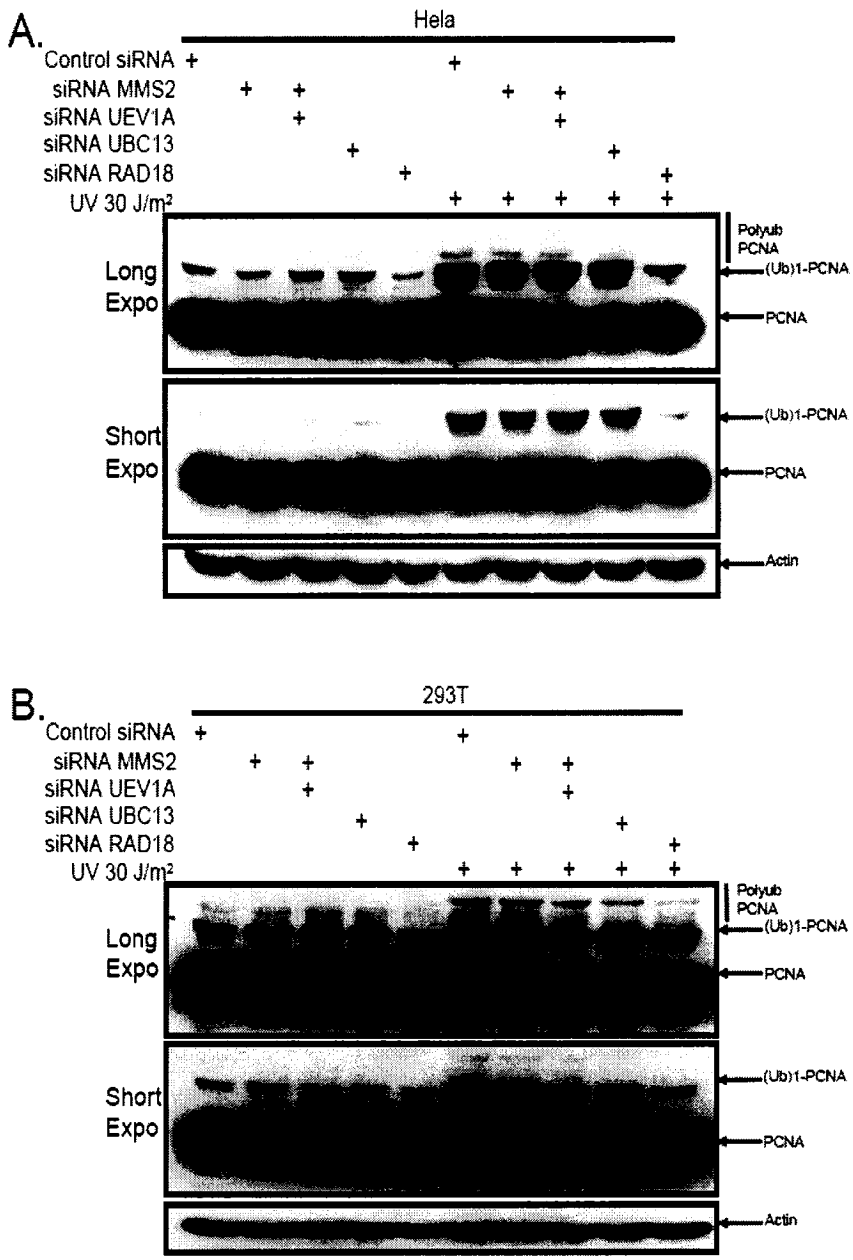

Figure 4: RAD18 or UBC13 knockdown disrupts PCNA polyubiquitination. (A) Hela cells were treated the same as in Figure 3 except that cells were also transfected with siRNA targeting UBC13 and RAD18. **(B)** 293T cells were treated as in Figure 4B.

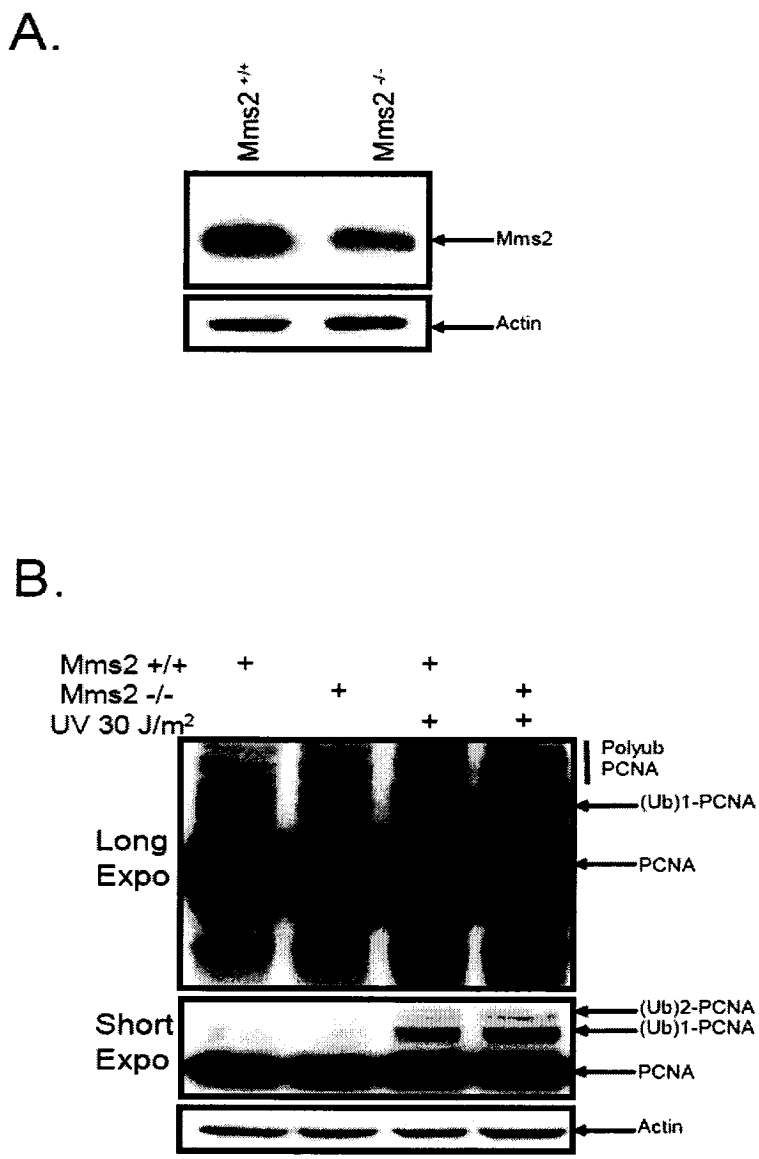

Figure 5: MMS2 null mouse emybronic stem cells show no overt disruption of PCNApolyubiquitination after UV irradiation. (A) Wild type and MMS2 null embryonic stem cells were lysed and subjected to immunoblotting with anti-Mms2/Uev1a antibody. (B) Confluent plates of wild type and MMS2 null embryonic stem cells were split 1:4 on Day 1. On Day 2 media was replaced and followed by UV irradiation with either 0 or 30J/m^2 on Day 3. Cells were harvested 6 hours post-treatment and subjected to immunoblotting with an anti-PCNA antibody.

5 Discussion

UEVs such as MMS2 and UEV1a constitute a highly conserved family of distinct E2 conjugating enzymes devoid of a catalytic cysteine [14,18,22]. Functionally they act in complexes with a heteromeric partner such as UBC13. The MMS2/UBC13 or UEV1a/UBC13 heterodimer catalyzes the formation of non-canonical K63-polyubiquitin chains that are involved in DDT and activation of NF-κB respectively [19]. Since MMS2 has been implicated in the error-free lesion bypass in yeast and humans and in light of the recent data demonstrating PCNA polyubiquitination after DNA damage in human cells [11,13], we sought to determine whether hMMS2 plays an important role in polyubiquitinating PCNA in mammalian cells.

In the present study we demonstrate that cells partially or totally devoid of MMS2 (through siRNA or gene targeting) and cells experiencing knockdown of both MMS2 and UEV1a do not exhibit a significant reduction in PCNA polyubiquitination after UV irradiation. These results are in sharp contrast to those obtained from the knockdown of UBC13 or RAD18. In isolation, the MMS2 findings might beg the question of whether K63-polyubiquitination is important in human DNA repair. There are, however, convincing studies in the recent literature in support of the importance of hRAD18, hUBC13 and SHPRH (the human RAD5 homolog) in K63-polyubiquitination of PCNA and its role in maintaining genomic stability [11-13]. The most parsimonious explanation of our results would therefore invoke a built in redundancy of the MMS2 component in the higher eukaryotes.

Several lines of evidence support our contention that redundancy is the best explanation. First, although hMMS2 was shown to be involved in damage induced mutagenesis, no overt sensitivity to UV light or increased spontaneous mutagenesis was observed in human fibroblasts as it had been observed in the yeast system [18]. Second, Simpson et al., showed that disruption of MMS2 in a chicken cell line, DT 40, did not result in increased sensitivity to DNA damaging agents nor did it promote sister chromatid exchange [24]. Third, in a previous publication we demonstrated that knockdown of UBC13 or RAD18 did not entirely abrogate PCNA ubiquitination [13]. Although this could be due to incomplete knockdown, recent evidence by Simpson et al. have demonstrated that RAD18 independent PCNA ubiquitination occurs in RAD18 null DT40 cells suggesting the presence of compensatory PCNA ubiquitinating enzyme in higher vertebrates [25]. We believe this to be the case in human cells as well. Finally, we show that PCNA is ubiquitinated in the presence or absence of hMMS2. Overall, these data highlight the inherent complexity of the DDT pathway and in particular point to a greater degree of built in redundancy in vertebrates.

Given the importance of MMS2 in yeast and human cells and our current finding we asked whether another UEV gene could complement the loss of hMMS2. To our knowledge yeast contain only a single UEV locus (MMS2) while humans contain at least 4 UEV loci including UBE2V1 (also known as CROC1A, UEV1A, UEV1, CIR1), UBE2V2 (also known as hMMS2 UEV2, DD-VIT, EDAF-1, and EDPF-1), TSG101 and UEV3 [23,26,27]. UBE2V1 has alternatively spliced isoforms including UEV1a (CROC1A), KUA-UEV, and Croc1B which vary in their

5' sequence [22,28,29]. Such UEVs may potentially complement hMMS2. However, based on structural studies TSG101 is an unlikely candidate to complement hMMS2 as it does not bind with UBC13 and to date has not been shown to catalyze K63 polyubiquitination [22]. UEV3 can also be eliminated as its sequence is very similar to TSG101 and it likely does not associate with UBC13 [26,28]. In addition, Thompson et al. show that KUA-UEV is strictly localized to the cytoplasm[28], excluding the protein from the site of DNA repair and thereby excluding it as a functional substitute for MMS2. Furthermore, a yeast two hybrid study eliminates CROC1b as a candidate since it failed to show interaction with UBC13 [19]. hUEV1a would appear to be a strong candidate to complement MMS2 because it can bind to UBC13, localize to the nucleus, and complement the mms2 yeast mutant. It also has greater than 90% amino acid sequence identity with hMMS2 [23,28,29]. Therefore, we used siRNA to target UEV1a along with hMMS2. However, the combined knockdown of UEV1a and hMMS2 did not abrogate PCNA polyubiquitination in human cells. This result is consistent with two distinct cellular roles of hMMS2 (in DNA repair) and UEV1a (in NF-κB activation) as previously shown by Anderson et al. and argues against a joint or collaborative role in DDT[19].

It is possible that complexes other than the heteromeric couple of UBC13/UEV may be able to complement the loss of hMMS2 (an example of such a complex is Np14/UFD1 which is also able to catalyze the formation of K63-polyubiquitin chains [30]) but there exists the intriguing possibility that a previously unreported MMS2 variant (herein designated hMMS2v) functionally complements MMS2 in the assembly of polyubiquitin chains on the PCNA of cells sustaining DNA

damage. We have preliminary data indicating that such a variant is present at low levels in HEK 293 cells (Brun et al., unpublished). If response to DNA damage is compartmentalized then even low levels of the hMMS2 variant could potentially compensate for the loss of MMS2. However whether this variant form is resistant to the siRNA used in this paper or if it is absent in the mouse embryonic knock cells remains to be determined. Further investigations will be required to determine the ubiquity of hMMS2v, its role in DNA repair, whether it contains UBC13 binding domain, and whether siRNA targeting of both hMMS2 and hMMS2v will fully abrogate PCNA ubiquitination.

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Chapter 4

Regulation of PCNA ubiquitination in human cells

Contibution of collaborators

The contents of this manuscript were written by Jan Brun and edited by the supervisor Dr. Doug Gray, and our collaborators Dr. Roland Chiu, Dr. Brad Wouters. JB performed the experiments. JB, RKC, BGW and DAG analyzed the data, JB provided necessary the analysis tools reagents and materials. All the figures are the work of JB. The GFP-PCNA and GFP-K164RPCNA constructs were kindly provided by Roland Chiu (Universtiy of Maastricht).

Summary

The work of Brun et al., extends upon a previous publication which provided the first evidence for K63-linked PCNA polyubiquitination after DNA damage in cancer cell lines. Here we resolve several key issues which include the status of PCNA ubiquitination in normal fibroblasts, agents that induce this modification, and the regulation of this pathway by cell cycle checkpoints and deubiquitinating enzymes. We show that PCNA polyubiquitination on K164 is an important physiological response in all cell lines to a variety of DNA lesions. Importantly, we find that cell cycle checkpoints and PCNA polyubiquitination are independent responses to DNA damage and that the key regulator of PCNA polyubiquitination is the deubiquitinating enzyme USP1.

Regulation of PCNA ubiquitination in human cells

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Running Title: Analysis of PCNA polyUb in human cells

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Key words: RAD18, MMS2, UBC13, PCNA, DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63, lysine 63

Abstract

The ubiquitin-based molecular switch dictating error-free versus error-prone repair has been conserved throughout eukaryotic evolution. A central component of this switch is the homotrimeric clamp PCNA, which is ubiquitinated in response to genotoxic stress allowing recovery of replication forks blocked at sites of DNA damage. The particulars of PCNA ubiquitination have been elucidated in yeast and more so recently in human cells. However gaps in the detailed mechanism of PCNA polyubiquitination still persist in human cells. We expand upon several studies and show that PCNA is polyubiquitinated in normal skin and lung fibroblasts, and that this ubiquitination is dependant on RAD18 and UBC13. Furthermore cisplatin (CPT), methylmethane sulphonate (MMS) and benzo(a)pyrene-diol-epoxide (BPDE) induce the polyubiquitination of PCNA with similar kinetics to UV while little to no polyubiquitination was detected after mitomycin (MMC) or X-ray treatment. Moreover, we show that ubiquitination of PCNA is not regulated by cell cycle checkpoint kinases ATM-Chk2 or ATR-Chk1 or p38-MK2. Significantly, we report that PCNA polyUb is negatively regulated by USP1. Our results add to the accumulating evidence of the importance of PCNA polyubiquitination in humans and define some of the details that regulate its ubiquitination.

1. Introduction

Moving platforms such as DNA sliding clamps are important processivity factors that tether polymerases to DNA and enable rapid and processive replication of vast stretches of DNA without randomly disengaging [1,2]. One of the most extensively studied sliding clamps is the eukaryotic proliferating cell nuclear antigen (PCNA). It is a homotrimeric clamp that is not only involved in tethering replicative polymerase δ and ε to DNA but serves multiple roles in the cell including cell cycle control, DNA repair (such as nucleotide excision repair, mismatch repair, DNA damage tolerance and base excision repair), transcriptional regulation, maintenance of chromosome structure, chromatin modeling and assembly, and possibly apoptosis [3,4]. As the number of new functions and interacting partners increases so does the complexity of PCNA to orchestrate its role based on the stresses the cell encounters [5]. However this may be eased somewhat by its long half-life and by the fact that it exists in both chromatin bound and nucleoplasmic free forms.

PCNA plays a particularly interesting role in many facets of DNA repair. Mammalian cells possess a number of distinct repair systems to maintain the fidelity of the genome in response to endogenously and exogenously produced DNA lesions. Nucleotide excision repair is the primary mechanism by which bulky deforming lesions are removed from DNA including those of UV and other carcinogens; this is a process requiring the services of PCNA [4,6]. However, some lesions such as those generated by UV are repaired more slowly than others, leading to replication stalling at sites of damage [6]. Unrepaired damage and extended stalling can lead to fork collapse and eventual cell death. To minimize the consequences of replication blocks

eukaryotic cells have developed, in addition to NER, a distinct DNA repair system termed DNA damage tolerance (DDT) [7]. In the DDT pathway, PCNA behaves as a sliding molecular toolbelt recruiting a variety of proteins involved in ubiquitination (including ubiquitin E2 conjugases and E3 ubiquitin ligases) that govern two subpathways (1) error-free homologous recombination (HR) repair and (2) error-prone translesion synthesis (TLS). It has been long appreciated that the error-prone subpathway is governed by RAD6 and RAD18 mono-ubiquitination while the error-free subpathway is governed by MMS2/UBC13/RAD5 complex via K63 linked polyubiquitin chains [8,9]. However, the substrate(s) remained at large. Interestingly, a seminal paper by Hoege et al, showed that PCNA not only served as a molecular toolbelt but also as a substrate for ubiquitination in yeast, thus adding the role of "molecular switch" to its repertoire by dictating the error-free and error-prone arms of the DDT pathway after DNA damage [10].

Recently, PCNA mono and polyubiquitination has been identified as an important modification in human cells [11-14]. Similar to yeast, human PCNA is monoubiquitinated by hRAd6/hRAd18 which increases the affinity for polη and enables a polymerase switch in response to DNA damage [14]. Moreover Rad18 also interacts with polη thereby facilitating its localization at sites of DNA damage [15]. PCNA is also polyubiquitinated by hMms2/hUbc13/SHPRH (human rad5 homolog) and disruption of PCNA polyubiquitination leads to genomic instability resulting in increased mutagenesis and gross chromosomal rearrangements [11,12]. The studies by Chiu et al., and Motegi et al., used cancer cell lines to demonstrate PCNA polyubiqutination. However, the use of cancer cells may result in a distortion of the

physiological damage response. Therefore, it remains to be determined whether PCNA polyubiquitination is important in normal human fibroblasts and if this potential modification depends on UBC13 and RAD18.

Another central question is how human cells regulate mono and polyubiquitination of PCNA and hence how they induce or limit the deployment of DNA repair machinery in the presence or absence of damage. One possibility are cell cycle checkpoint kinases ATR-Chk1, ATM-Chk2 and/or p38 SAPK-MK2, given their central role in damage surveillance [16-19]. An alternative possibility is by directly regulating ubiquitination. Similar to phosphorylation, ubiquitination is a reversible process that involves deubiquitinating enzymes (DUBs). DUBs are cysteine proteases that cleave ubiquitin from mono and polyubiquitnated substrates. In humans there are scores of DUBS, some of which are linked to certain pathways such as USP4 with cell cycle control [20,21]. However, very little is known about their substrates and specific biological role. Recently Huang et al., revealed that USP1 negatively regulates monoubiquitination of PCNA in the absence of DNA damage in order to control TLS [22]. To this end, the DUB that regulates PCNA polyubiquitination still remains elusive.

In this study we examined the details of PCNA polyubiqutination in normal human fibroblasts and cancer cell lines. Contrary to previous studies, we found that PCNA is mono and polyubiquitinated in primary human skin and lung fibroblasts after UV irradiation and that this modification is dependant on UBC13 and RAD18. In addition we found that PCNA is polyubiquitinated in response to a variety of DNA damaging agents and that polyubiquitination occurs on K164. Since cell cycle

checkpoint kinases are predominantly activated after DNA damage we also sought to determine whether PCNA ubiquitination is regulated by global sensors such as ATR, ATM and/or p38. Similar to studies in *Xenopus* and *S. pombe*, we report that mono or polyubiquitination of PCNA is not regulated by the cell cycle checkpoint kinases in human cells [23]. Finally, we find that the candidate DUB for negatively regulating PCNA polyubiquitination is USP1.

2. Materials and Methods

2.1 Cell Culture and Treatments

The A549, HEK 293T and Hela, HCT116, WI38 (normal human lung fibroblasts), GM038 (Skin Fibroblasts) cell lines were cultured in DMEM (Gibco, Invitrogen, Carlsbad, California, United States) supplemented with 10% FBS (Gibco, Invitrogen, Carlsbad, California, United States) and 1X Pen/Strep (Gibco, Invitrogen, Carlsbad, California, United States). UV irradiation ($30J/m^2$) was performed using a UVC germicidal lamp at a fluence rate of $1J/m^2/s$. Cisplatin (obtained from the Ottawa Hospital Pharmacy) and MMC (Sigma, St. Louis Missouri, United States) were added to cells for 3 hours at a dose of 160μM and 0.04μg/ml, respectively, after which they were washed with PBS and supplemented with fresh media. Cisplatin and MMC treated cells were lysed 3 hours post treatment. MMS (Sigma, St. Louis Missouri, United States) was added directly to cells up to 0.02% for 45 minutes followed by immediate lysis. Cells were irradiated with either 4 or 10 Gray of X-rays and harvested 6 hours post irradiation. For caffeine treatment, cells were incubated for 1 hour with either 1, 10 or 20mM caffeine (Sigma, St. Louis Missouri, United

States) prior to UV irradiation (30J/m^2). Following UV treatment cells were again supplemented with fresh caffeine containing media and lysed 6 hours post-irradiation. All treatments with genotoxic agents were performed on exponentially growing cells.

2.2 Transfections

WI38, GM038, 293T and Hela cells were transfected with siGENOME SMARTpool reagent specific for either human RAD18, UBC13 or USP1 (Dharmacon Research, Lafayette, Colorodo, United States) using oligofectamine (Invitrogen, Carlsbad, California, United States). The transfections were performed 72 hours prior to harvesting the cells to achieve optimal long-term knockdown as determined by immunoblotting. 293T and Hela cells were also transiently transfected with either a GFP-tagged WT PCNA or K164R PCNA DNA plasmid using GeneJuice transfection reagent (Novagen) as per company protocol. After 48 hours cells were UV irradiated, lysed and used for immunoblotting.

2.3 Immunoblotting

Methods described elsewhere [11]. Briefly cells transfected with siGENOME SMARTpool reagent specific for human UBC13, human RAD18 and human USP1 (Dharmacon Research, Lafayette, Colorodo, United States) were UV irradiated and lysed 6h post-treatment. Samples were sonicated, soluble fractions were recovered, and proteins were quantified. Proteins were resolved on either a one phase or two phase SDS-polyacrylamide gel (10% or 10% and 15%) and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New

Jersey, United States). The following antibodies were used: mouse monoclonal that recognizes UBC13 (kindly provided by Dr. Wei Xiao), rabbit polyclonal that recognizes USP1 (Abgent), mouse monoclonal PCNA PC10 (Millipore, Chemicon, Temecula, California, United States), rabbit polyclonal ubiquitin (Dako, Glostrup, Denmark), mouse monoclonal Ser15 phospo-specific p53 (New England Biolabs, Cell Signaling, Ipswich, Maryland, United States) and mouse monoclonal actin (Sigma, St. Louis Missouri, United States). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Rockford, Illinois, United States).

2.4 Immunoprecipitation

GM038 and WI38 cells were UV-irradiated with $30J/m^2$ as described above. Cells were lysed (6h after irradiation) in lysis buffer supplemented with 0.5% SDS. Lysates were sonicated and boiled for 5min followed by dilution to 0.1% SDS. After protein quantitation 500µg of protein were incubated overnight at 4°C with anti-PCNA (1/200). The following day, lysates were incubated for 48h at 4°C with 100µl of Gamma bound Sepharose Beads (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States). Beads were washed extensively in lysis buffer and proteins were eluted by boiling in 1x Laemmli's SDS sample buffer. Immunoblotting was performed as described above except the membranes were autoclaved for 20 min in ddH_2O after protein transfer, and proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Rockford, Illinois, United States).

3. Results

3.1 UV irradiation induces PCNA polyubiquitination in normal fibroblasts

To date PCNA polyubiquitination has not been reported in normal human fibroblasts [14,23]. However, in a previous study we were the first to demonstrate that K63 linked polyubiquitination is important in human fibroblasts and that PCNA is both mono and polyubiquitinated in cancer cell lines by RAD18 and UBC13 [11]. This was further corroborated by Motegi et al. whose studies showed that PCNA polyUb was also dependant on the human RAD5 homolog SHPRH [12]. However, Kannouche et al. only observed monoubiquitination of PCNA transformed fibroblasts noting that PCNA polyubiquitination was either not important in human cells, occurred at low levels, or quickly turned over [14]. Therefore, we sought to resolve this issue by determining whether PCNA polyubiquitination was physiologically relevant to primary fibroblasts. We chose to investigate primary cell lines of lung (WI38) and skin (GM038) origin. WI38 and GM038 cells were irradiated with $30J/m^2$ of UVC at 3 hours post-treatment a prominent band representing monoubiquitinated PCNA as well as a second prominent band probably corresponding to di-ubiquitinated PCNA appeared (figure 1a and S1a). To confirm that these bands were indeed ubiquitinated PCNA, we immunoprecipitated PCNA followed by immunoblotting with an anti-ubiquitin antibody. A band corresponding to di-ubiquitinated PCNA was detected in WI38 cells and a di-ubiquitinated and potentially tri-ubiqutinated PCNA band were identified in GM038 after UV (Figure 1B and S1B). Similar to previous results the anti-ubiquitin antibody failed to

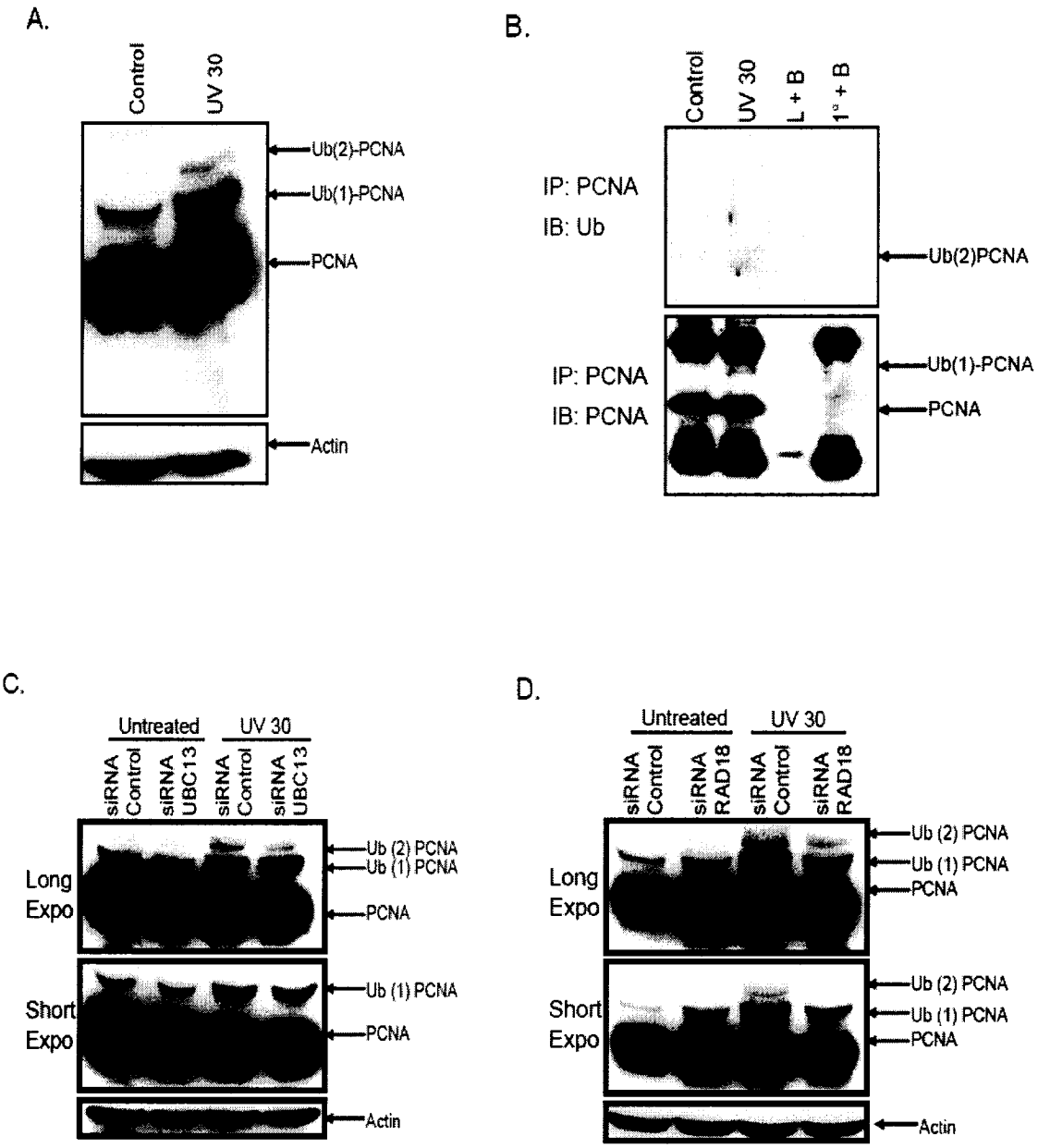


Figure 1. PCNA ubiquitination in primary lung fibroblasts. (A) WI38 cells were either left untreated or UV irradiated with 30 J/m^2 and lysed 3h post-treatment followed by immunoblotting for PCNA. **(B)** WI38 cells were irradiated with 30 J/m^2 UV and lysed in boiling SDS, diluted in lysis buffer and subjected to immunoprecipitation (IP) with a PCNA antibody, and immunobloted (IB) with PCNA (lower panel) or Ub antibodies (upper panel). The controls in the immunoprecipitations were 'B+L' in which lysates were incubated with beads but no PCNA antibody and '1° +B' in which PCNA antibody was incubated with beads alone. **(C)** WI38 cells were transfected with 100nM of either control siRNA, siRNA Ubc13 or siRNA Rad18. 72 hours post-transfection cells were treated as in 1A. A shorter and longer exposure of the PCNA immunoblot is shown. **(D)**WI38 cells were treated the same as in C except that siRNA RAD18 was used instead of siUBC13.

reveal monoubiquitinated PCNA. This seems to be a property of the antibody since it seems to have a lower affinity for monoubiquitinated substrates.

3.2 PCNA polyubiquitination is dependant on UBC13 and RAD18 in primary fibroblasts

In our previous study we demonstrated that disruption of UBC13 or RAD18 abrogates PCNA polyubiquitination in cancer cell lines after UV irradiation [11]. Therefore, we sought to determine whether this was the case in human fibroblasts. WI38 cells were targeted with siRNA against UBC13 and RAD18. As expected RAD18 knockdown resulted in a substantial decrease in the mono and di-ubiquitinated species while UBC13 knockdown resulted in a decrease in the di-ubiquitinated PCNA species (Figure 1C, D). Similar results were observed in GM038 cells (Figure S1C). Therefore, PCNA polyubiquitination seems to be an important physiological response to UV damage in healthy cells.

3.3 PCNA ubiquitination and response to a DNA damaging agents

Thus far, work from our laboratory has shown that UV damage induces PCNA polyubiquitination. We have now examined the ubiquitination status of PCNA after treatment with a variety of DNA damaging agents including cisplatin (CPT), benzo(a)pyrene-diol-epoxide (BPDE), methylmethane sulfonate (MMS), mitomycin C (MMC) and X-irradiation (X-ray). We have been able to detect mono and polyubiquitinated PCNA in 293T, and Hela cells after CPT and MMS treatment appearing with similar kinetics to that of mono and polyubiquitinated PCNA after UV

irradiation (Figure 2 A, B). As in a previous publication, polyubiquitination was dependant on RAD18 (Figure S2A and S2B). Similar results were observed in GM038, WI38 and A549 cells (Figure S3A and S3B). Furthermore, we were able to detect mono and polyubiquitination in BPDE treated A549 and Hela cells (Figure 2C). We were unable to detect an increase in mono or polyubiquitinated PCNA after treatment with MMC and X-ray which may indicate that PCNA ubiquitination is a specific response to certain types of DNA damage.

3.4 PCNA is polyubiquitinated at K164

In yeast, PCNA is mono and polyubiquitinated at K164 [10]. However recent evidence demonstrate that PCNA polyUb occurs on K164 in vitro [13]. To verify that K164 on PCNA is the target for ubiquitination Hela and 293T cells were transiently transfected with a GFP tagged wild type (WT) or mutant K164R PCNA plasmid. GFP PCNA, GFP-mono and di-ubiquitinated forms of PCNA were detected in the cells transfected with WT PCNA, however, they were notably absent in cells transfected with the K164R mutant (Figure 3A, B). This suggests that K164 is the site which is ubiquitinated on PCNA in human cells.

3.5 PCNA polyUb is regulated by USP1 and not by cell cycle checkpoint kinases

A recent study implicating USP1 in negatively regulating monoubiquitinated PCNA lead us to evaluate whether USP-1 is also involved in regulating PCNA polyubiquitination [22]. Using siRNAs targeting USP-1 in human cells, we observed a significant increase in the levels of both monoubiquitinated and polyubiquitinated

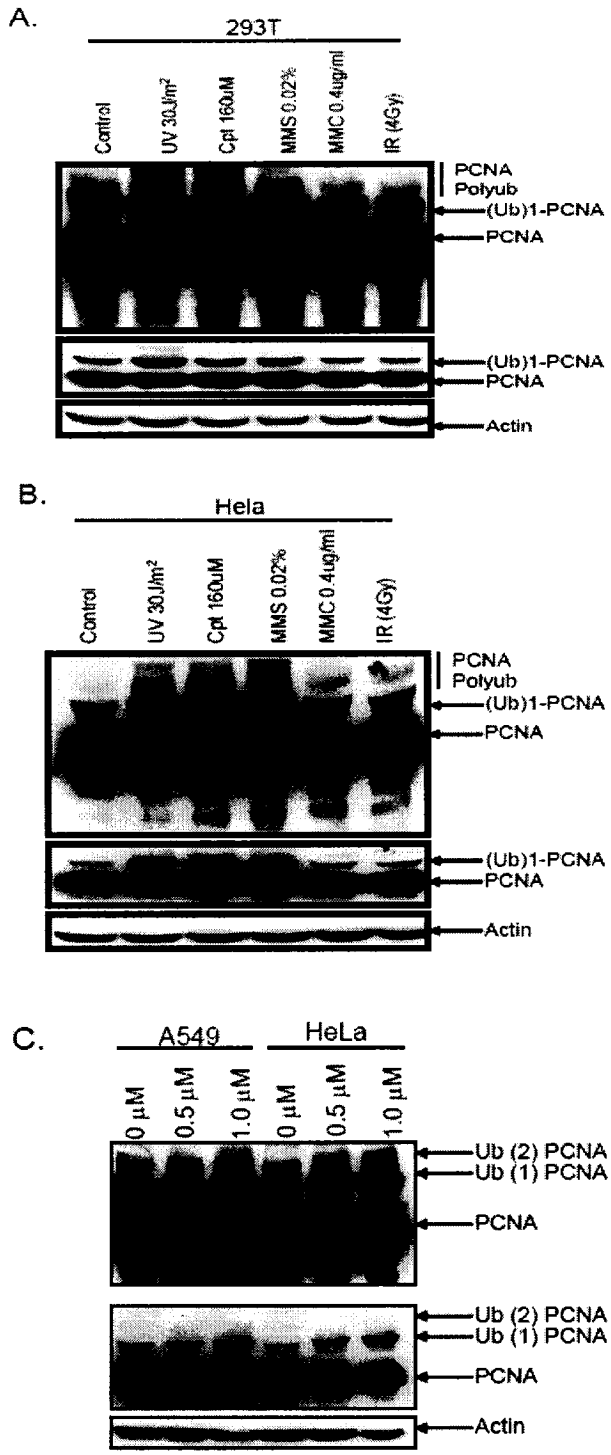


Figure 2. PCNA is polyubiquitinated in response to a variety of replication stressors. (A) 293T cells were untreated or treated with the indicated dose of UV, CPT, MMS, MMC or X-ray according to materials and methods. Both longer and shorter exposures are shown. **(B)** Hela cells were treated the same as in A. **(C)** A549 and Hela cells were treated with 0.5 or 1 μM BPDE for 1h and lysed 5 hours post-treatment following by immunoblotting for PCNA.

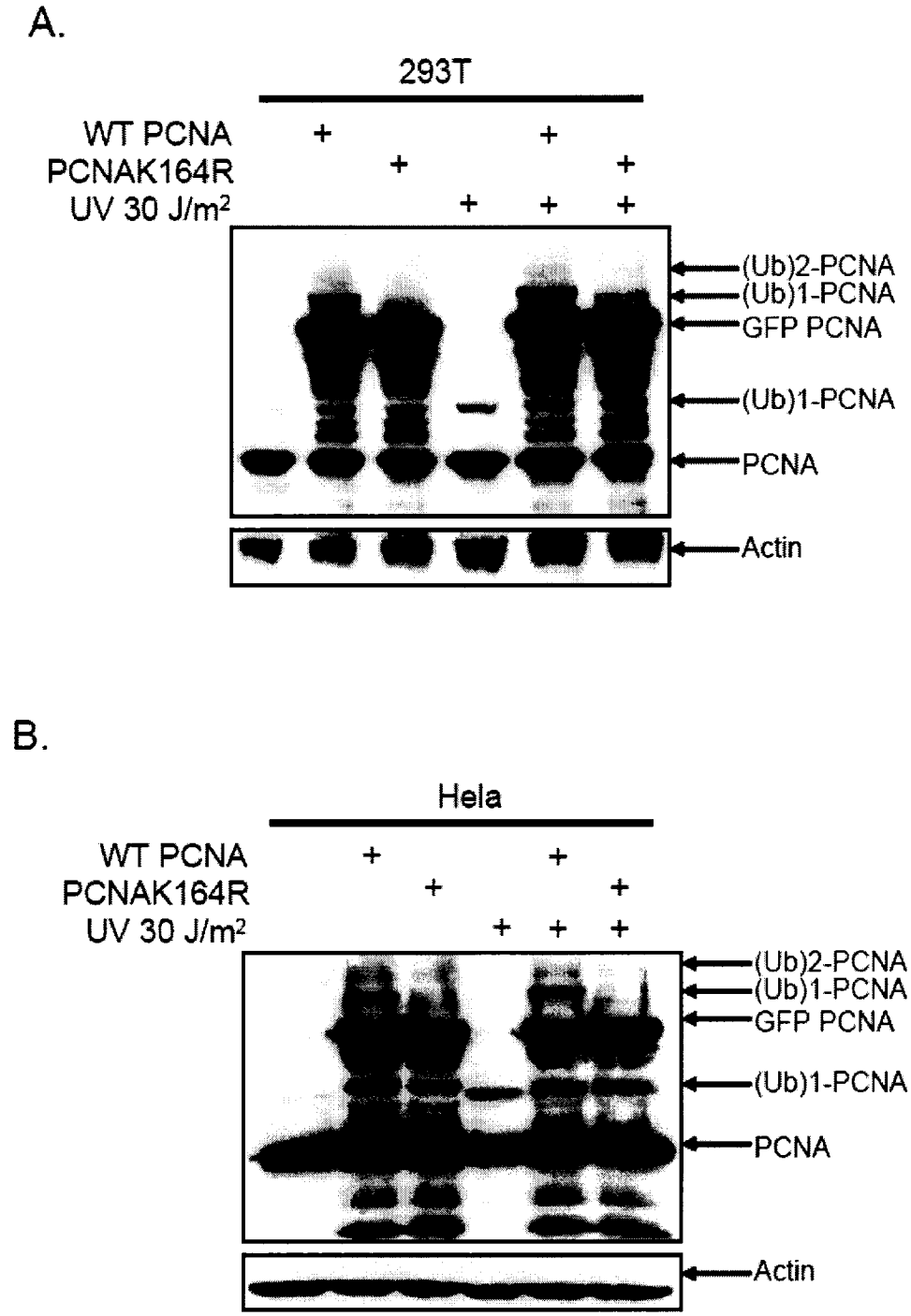


Figure 3. Polyubiquitination of PCNA occurs at K164. (A) 293T cells were transiently transfected with GFP tagged WT or K164R PCNA. 48 hours post-transfection cells were UV irradiated, lysed and immunoblotted for PCNA. **(B)** Hela cells were treated the same as in A.

PCNA in GM038, Hela, A549, 293T, and HCT116 cells (Figure 4 A-E). Surprisingly, we found that USP1 does not negatively regulate PCNA ubiquitination in WI38 cells despite their dependency on UBC13 and RAD18 for building K63-polyUb chains on PCNA (Figure S4).

USP1 also regulates the monoubiquitination of FANCD2, a protein that colocalizes with PCNA in response to DNA damage [22,24]. Interestingly, monoubiquitination of FancD2 is dependent on its prior phosphorylation by ATM or ATR, which are cell cycle checkpoint kinases activated in response to replication stress [25,26]. Therefore, to determine whether ATR or ATM regulate PCNA ubiquitination we analyzed the effect of inhibiting their kinase activity by incubating A549 and Hela cells with 10 or 20mM caffeine 1 hour prior and 6 hours after UV irradiation. We demonstrate that PCNA is mono and polyubiquitinated with the same kinetics in presence or the absence of caffeine (Figure 5A and B). It is interesting to note that after UV irradiation there is a marginal increase in mono and polyubiquitination in the caffeine treated A549 cells (Figure 5A) while levels of PCNA mono and polyubiquitination are similar in untreated or caffeine treated Hela cells (Figure 5B). To confirm that our caffeine doses were inhibiting ATR/ATM we analyzed the p53 phosphorylation at serine 15 after UV irradiation in the A549 cell lines (known to express wildtype p53) [27]. It was observed that 10 or 20mM caffeine abolished the phosphorylation of p53 while a strong phosphorylated ser15 p53 signal was present in the untreated but UV irradiated lane (Figure 5C). Lower doses of caffeine also abolished phosphorylation of p53 after UV irradiation (Figure 6C).

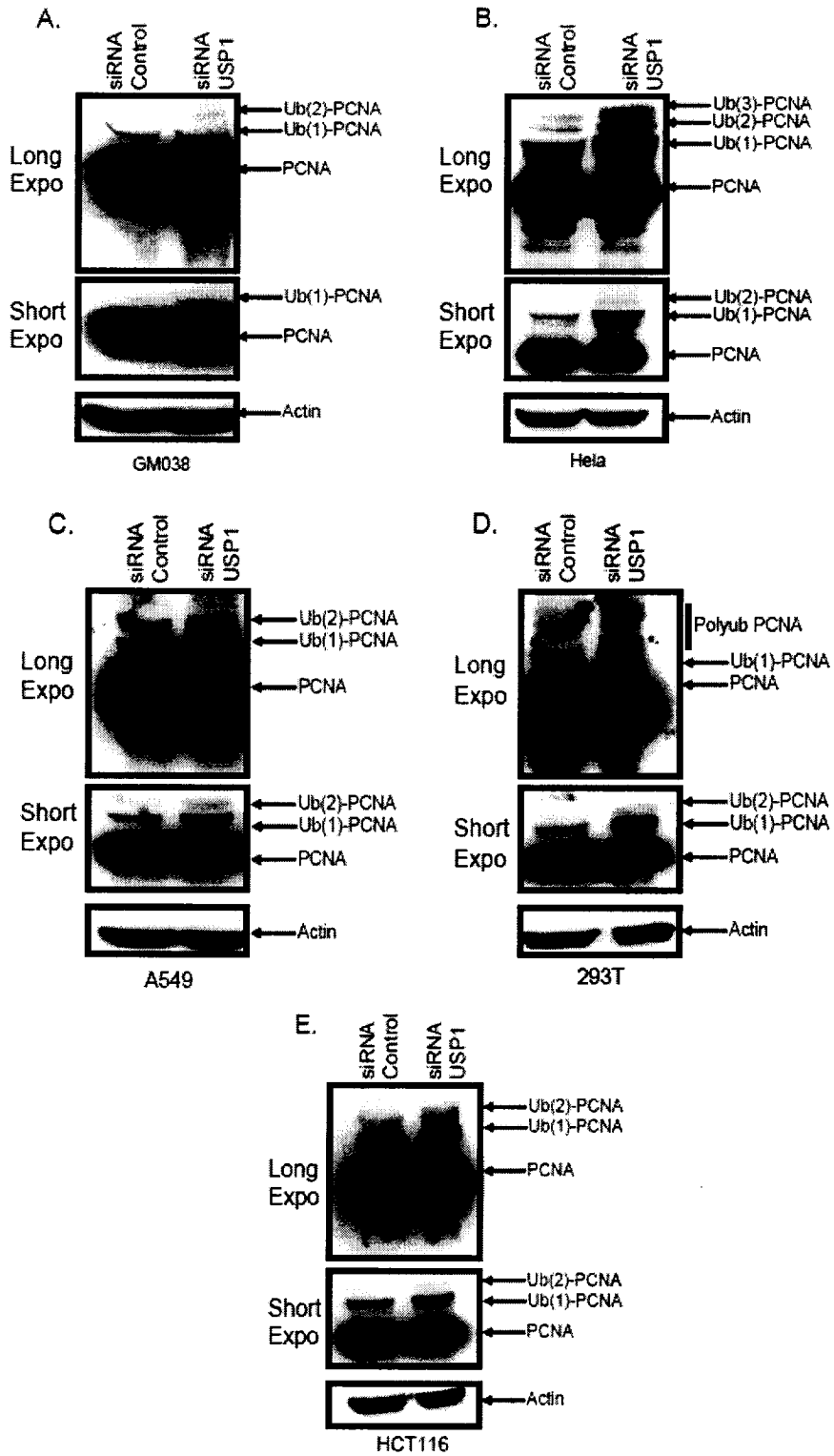


Figure 4. Knockdown of USP1 leads to increase PCNA ubiquitination. (A-E) All cell lines were transfected with 100 nM of either control siRNA or siRNA USP1. 72 hours post-transfection cells were lysed and immunoblotted for PCNA.

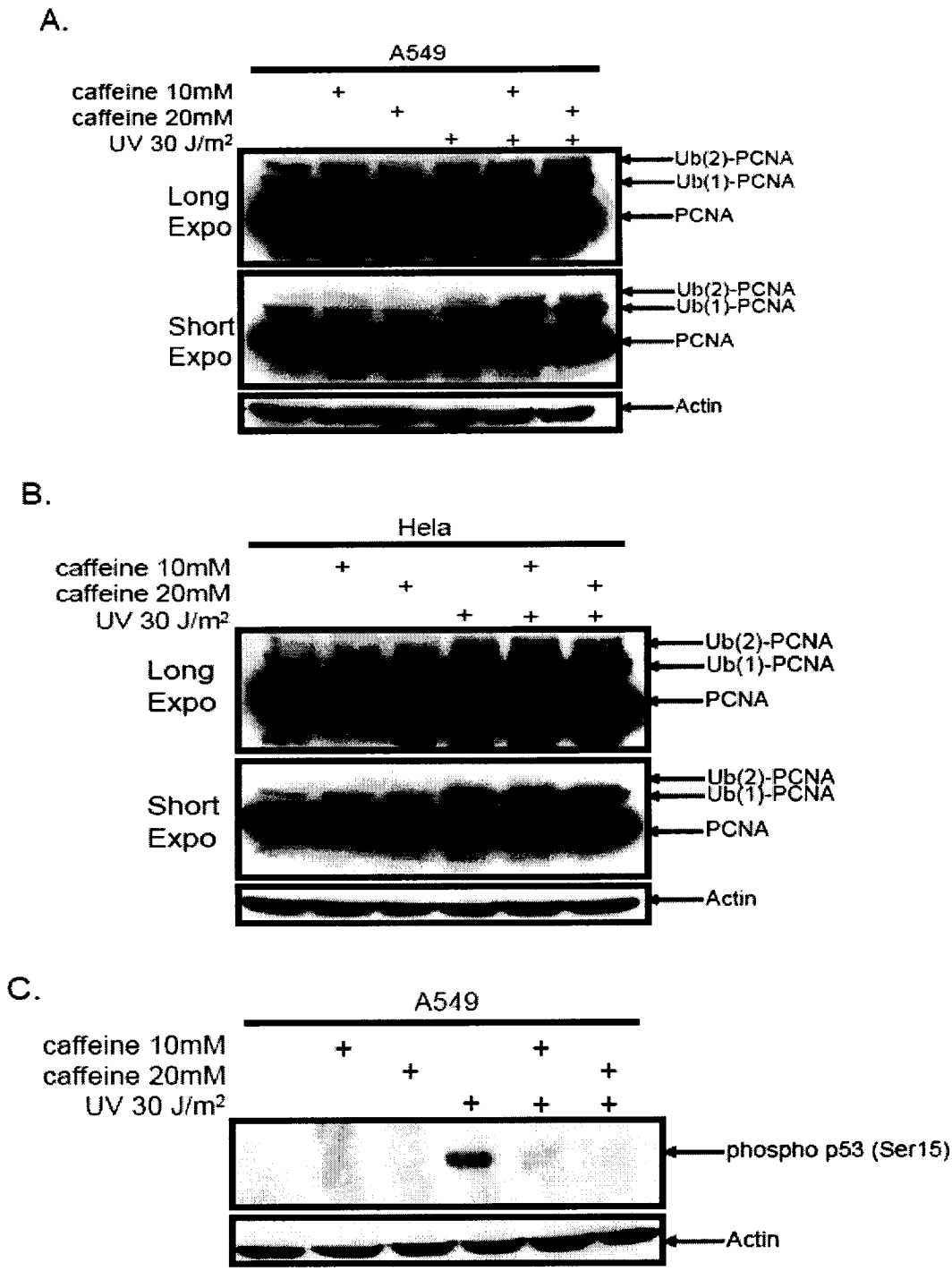


Figure 5. Inhibition of ATR or ATM does not abrogate PCNA ubiquitination. **(A-B)** A549 and Hela cells were incubated with the indicated doses of caffeine 1 h prior and 6 hours post UV irradiation at which point the cells were lysed followed by immunoblotting for PCNA. **(B)** A549 cells were treated as in A followed by immunoblotting for phosphorylated S15 on p53.

Recently it has come to light that p38-MK2 may constitute a third checkpoint kinase that responds to UV or cisplatin induced damage[17]. Therefore we inhibited p38 with 20μM of a specific inhibitor (SB203580) in A549 either alone or in combination with 2.5mM caffeine 1hour prior and 3 hours after a 20 J/m^2 dose of UV irradition. The data show that PCNA ubiquitination is not disrupted (Figure 6A) despite the strong abrogation of ATR/ATM by caffeine (Figure 6C) or of p38 phosphorylation by SB202390 inhibitor (Figure 6B) after UV irradiation. Infact, we observed a mild increase in mono and polyubiquitination in the samples treated with 2.5mM caffeine (Figure 6A). Overall our results suggest that ATR, ATM or p38 do not couple PCNA ubiquitination to the DNA damage response.

4. Discussion

In the present study we demonstrate that there is accumulating evidence that not only PCNA monoubiquitination but that PCNA polyubiquitination is an important physiological response to replication stress in human cells. We believe PCNA polyubiquitination confers a protective effect as it is speculated to be involved in an error-free repair subpathway distinct from TLS. Recently the error-free pathway governed by RAD6/RAD18 was revealed to contribute more significantly to replicating past CPD (6-4) photoproducts than TLS [28]. In fact 70% of error-free replication was dependant on the RAD6/RAD18 error-free component while only 10% was dependant on TLS [28]. Furthermore, several studies demonstrate that disruption of PCNA polyubiquitination results in genomic instability [11,12]. This

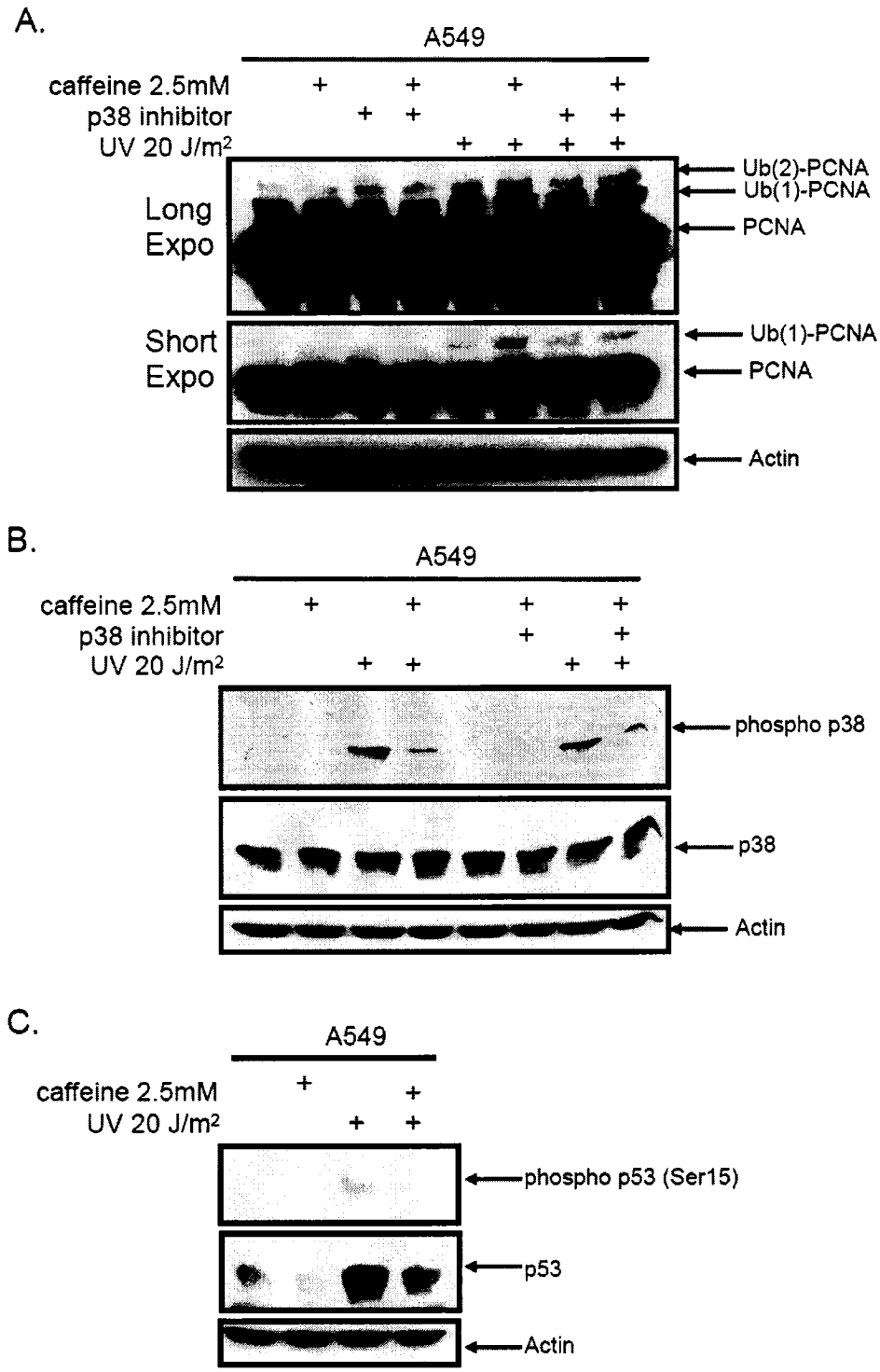


Figure 6. P38 inhibition does not disrupt PCNA ubiquitination. (A) A549 and 293T cells were incubated with caffeine or with the p38 inhibitor SB203580 for 1hour prior and 5 hours after UV irradiation. Cells were then lysed and immunoblotted for PCNA. (B-C) A549, 293T and Hela cell were incubated with caffeine and p38 inhibitor for 1 hour prior and 5 hours after UV irradiation. Cells were then lysed and immunoblotted for PCNA.

would argue that PCNA polyubiquitination is playing a significant role in replication stress.

In a previous study we were the first to show that K63 linked polyubiquitination is important in skin fibroblasts but did not demonstrate the substrate involved in these cells [11]. Here we expand upon that study and demonstrate that PCNA is the substrate for K63 linked polyubiquitination and that PCNA polyubiqutination is a normal physiological response in primary lung and skin fibroblasts dependant on both UBC13 and RAD18. Moreover, we report that PCNA is polyubiquitinated in response to a variety of genotoxic agents that distort DNA including UV, CPT, MMS and BPDE. Our results also show mono and di-ubiquitination of PCNA at K164. This would suggest that PCNA polyubiquitination is a common mechanism used to protect cells against the mutagenic effects of DNA damage.

Interestingly, X-ray and MMC did not induce mono or polyubiquitination of PCNA in any of the cell lines tested. X-rays induce single and double strand breaks and are not expected to induce PCNA ubiquitination above background levels and they do not, even at higher doses (data not shown). The negative result with MMC was surprising as it has been speculated to induce monoubiquitination of PCNA and monoubiquitination of other DNA repair proteins, namely FANCD2. Originally we speculated that perhaps we did not use a high enough dose, so we increased it to 1ug/ml. However, we did not observe a difference (data not shown). MMC induces the formation of interstrand crosslinks, which unlike UV, minimally distorts DNA, thus it is not immediately recognized by DNA repair machinery [29]. Moreover, it

has been reported that after MMC treatment it may take one round of replication to reveal structural distortions that stall replication forks [30]. Therefore, although we have not tested this directly, an attractive explanation may lie in the kinetics of PCNA ubiquitination after MMC treatment. In this study we only checked for PCNA polyubiquitination 3 hours after a 3 hour MMC treatment. Certainly, PCNA mono and polyubiquitination could appear at later time points.

Thus far our data have shown that PCNA polyubiquitination is induced by several DNA damaging agents in a variety of human cell lines. Nevertheless, the regulatory factors involved in PCNA polyubiquitination remain unknown. Interestingly, the same replication stressors used in this study also activate a checkpoint response which is governed by two serine/threonine kinases (ATM and ATR) from the highly conserved phosphoinositide 3-kinase-related kinase (PIKK) family. Typically, replication stalling as a result of UV reveals extensive regions of single stranded DNA which are immediately coated by ssDNA binding protein RPA. RPA coated ssDNA recruits ATR and its heteromeric partner aATRIP which activate a checkpoint response [16]. This stimulates the loading of a unique heteromeric sliding clamp Rad9-Hus1-Rad1, collectively known as 9-1-1 [31]. This then facilitates the recruitment and phosphorylation of other proteins, including Chk1 and p53, which consequently arrests cells at G1 [32,33]. Our interest in this pathway in regulating PCNA ubiquitination stems from studies which demonstrated that ATM and ATR activation are necessary for phosphorylation and subsequent ubiquitination of FANCD2 [26]. Since FANCD2 colocalizes with PCNA after DNA damage we postulated that ATR or ATM may regulate PCNA ubiquitination. Inhibition of

ATR/ATM with caffeine showed no disruption in PCNA ubiquitination. In fact it showed a mild increase in both PCNA mono and polyubiquitination, at least in A549 cells. These results were similar to those recently reported by Chang et al. [34]. However, this does not rule out that PCNA ubiquitination is regulated by cell cycle checkpoint kinases; rather, it may point to alternative pathways that may compensate for inhibition of ATM/ATR. Recently, the p38 SAPK-MK2 pathway was demonstrated to be involved in the response to DNA damage converging on similar substrates activated by ATR/ATM [17]. Since caffeine has been shown to upregulate p38 activity we postulated that p38 could be compensating for the loss of ATR/ATM [35]. However, we show that inhibition of ATR/ATM and/or p38 does not abrogate PCNA ubiquitination. We actually observe no increase of PCNA ubiquitination in A549 cells after p38 inhibition alone or in combination with caffeine. This suggests that PCNA ubiquitination and cell cycle checkpoint kinases may represent two independent responses to DNA damage. These results further corroborate and extend upon studies performed in *S. pombe* and *Xenopus* egg extracts [23,34].

Nevertheless, the remaining question is what regulates PCNA polyubiquitination. USP1 has been implicated in the constitutive deubiquitination of monoubiquitinated PCNA [22]. Furthermore, USP-1 functions independently of the cell cycle checkpoint kinases similar to PCNA polyubiquitination [22]. Here we show for the first time that USP1 is also involved in deubiquitinating polyubiquitinated PCNA, the implications of which are unknown. Huang et al. showed that disruption of USP1 leads to increased spontaneous and UV induced mutagenesis. They postulated that the increase in mutagenesis could be due to the dysregulated function

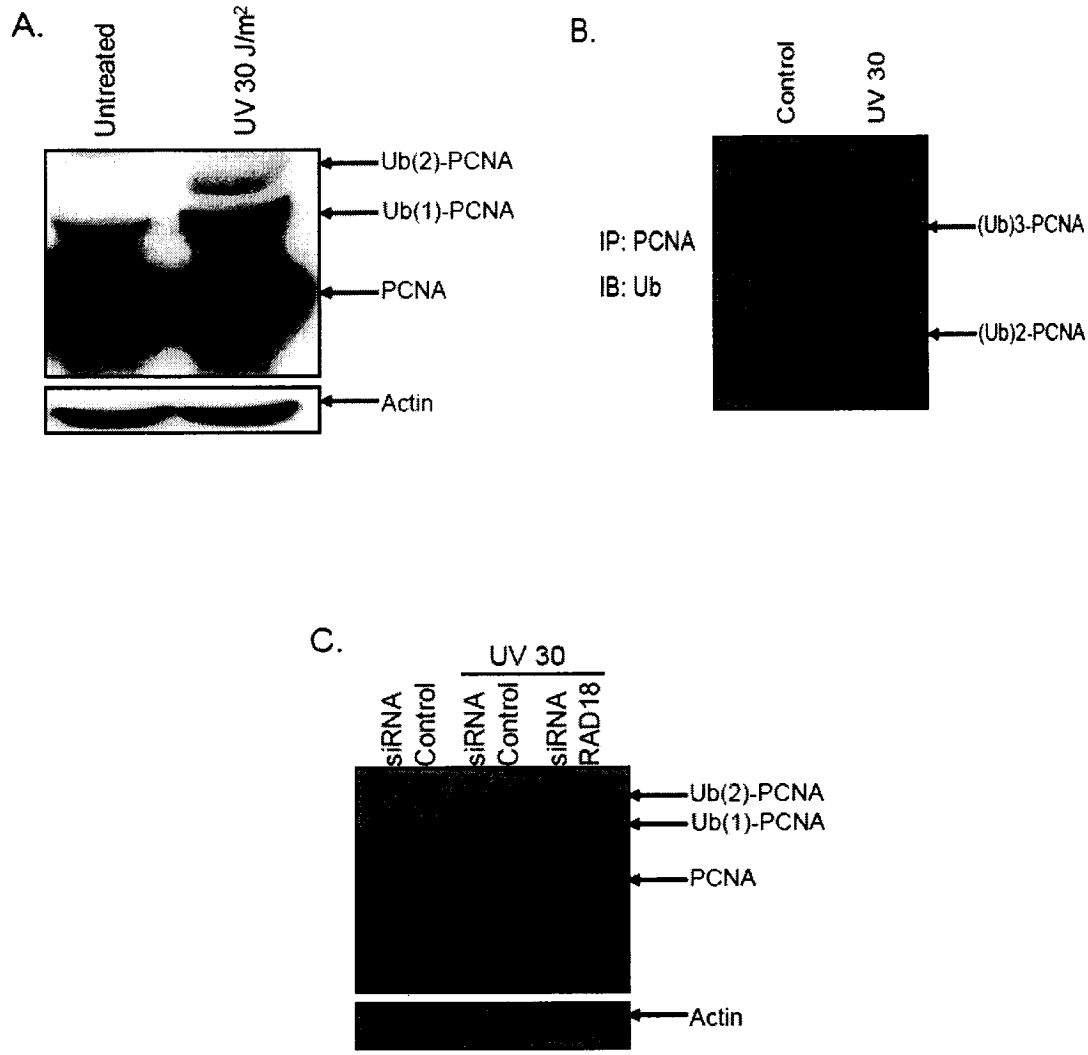
of polη or recruitment of other error-prone polymerases to the damaged sites. In light of our data showing an increase in PCNA polyubiquitination after USP1 disruption, another explanation for the increased mutagenesis could be the contribution of unscheduled, illegitimate and dysregulated homologous recombination.

In conclusion, our data show that PCNA polyubiquitination is a universal response to DNA damage and that it is constitutively regulated by USP1 and not by cell cycle checkpoint kinases. Evolutionarily, this would provide an additional advantage for survival in an environment of replication stressors. A challenge for future investigations will be to elucidate whether PCNA ubiquitination is a compartmentalized response simply regulated by USP1 alone or regulated by pathways other than cell cycle checkpoint kinases.

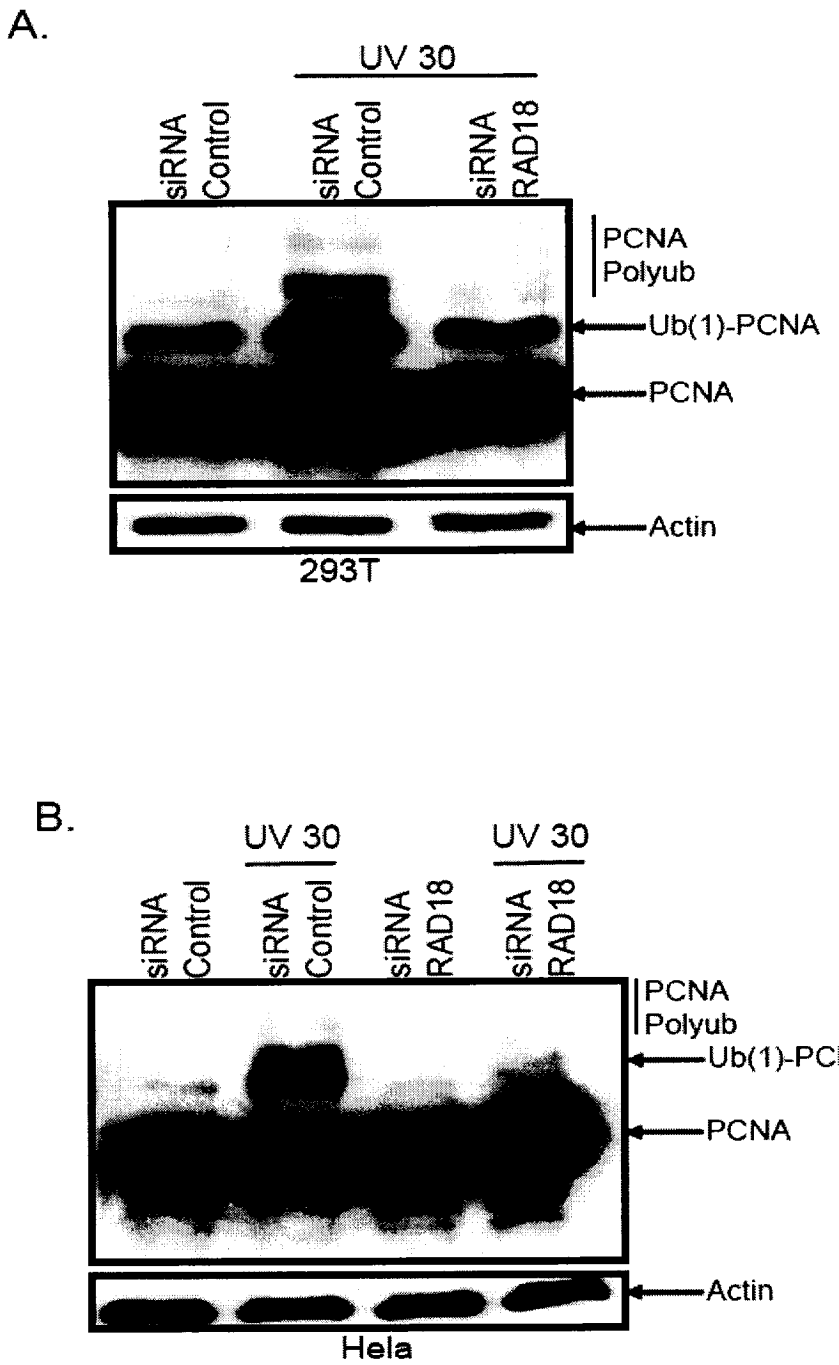
Acknowledgements

We would like to thank Dr. Wei Xiao University of Saskatchewan for the kindly providing us with the UBC13 antibody. This work was funded by the National Cancer Institute of Canada (grant number 014132).

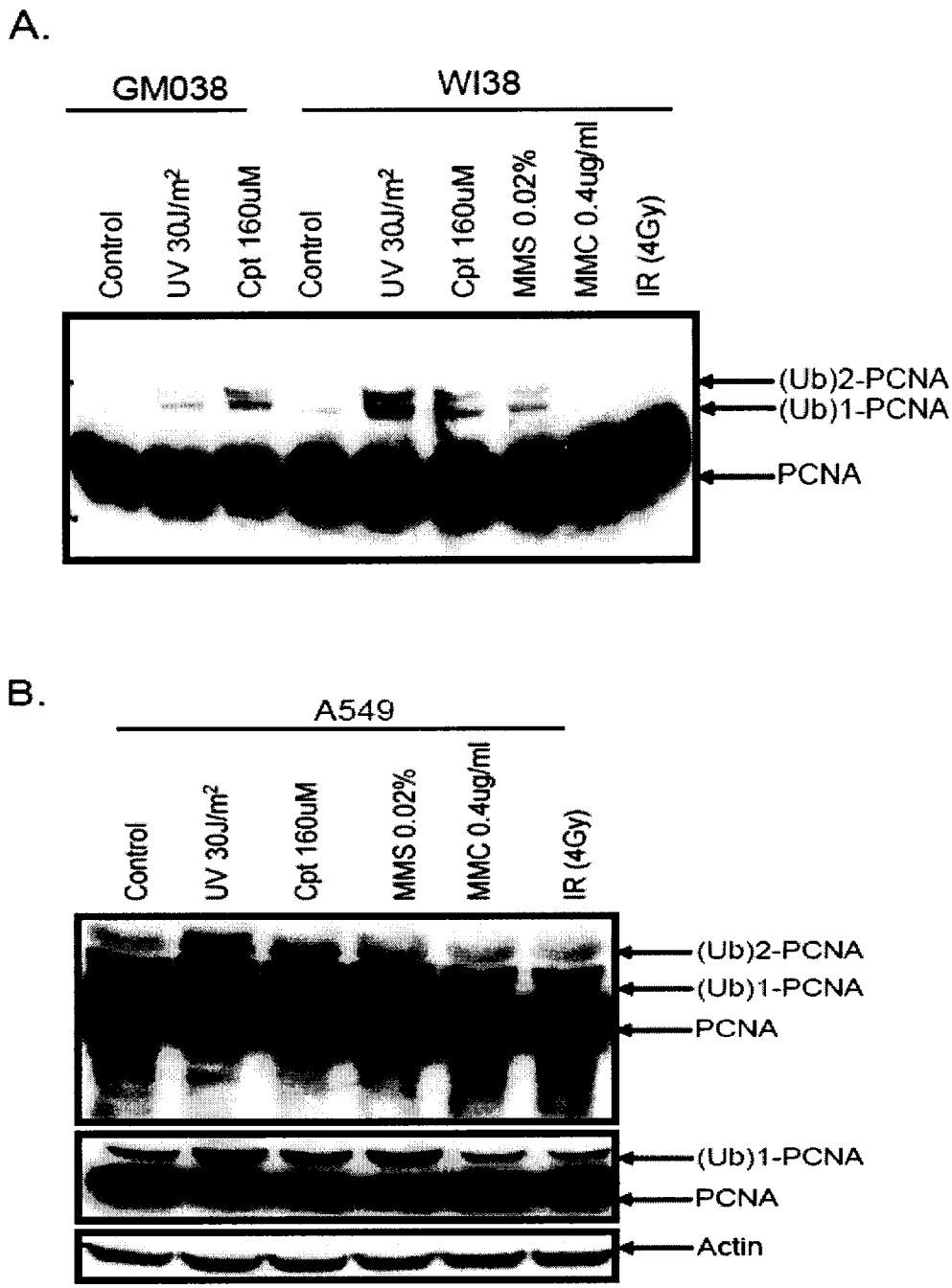
Supporting Information



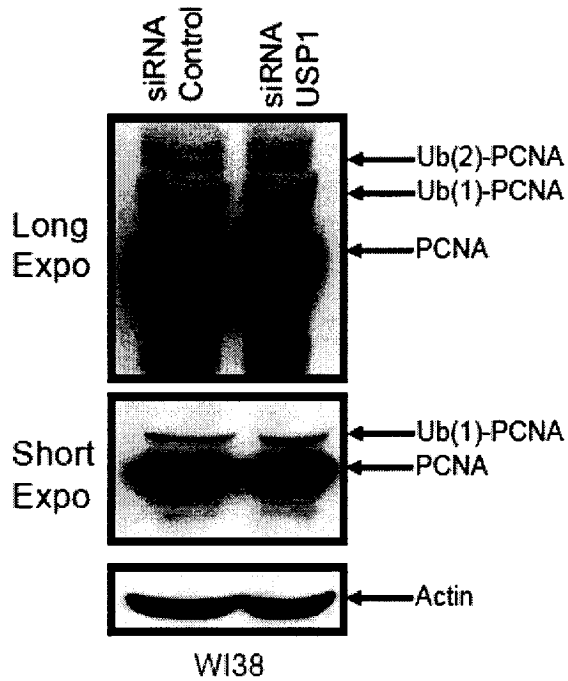
Supplementary Figure 1. PCNA ubiquitination in normal skin fibroblasts. (A) GM038 cells were either left untreated or UV irradiated with 30 J/m^2 and lysed 3 h post-treatment followed by immunoblotting for PCNA. **(B)** GM038 cells were irradiated with 30 J/m^2 UV and lysed in boiling SDS, diluted in lysis buffer and subjected to immunoprecipitation with a PCNA antibody and detected with Ub antibodies. **(C)** GM038 cells were transfected with 100 nM of either control siRNA or siRNA RAD18. 72 hours post-transfection cells were treated as in figure 1A.



Supplementary Figure 2. Polyubiquitination of PCNA is dependant on RAD18 in 293T and Hela cells. (A) 293T cells were transfected with 100 nM of either control siRNA or siRNA RAD18. 72 hours post-transfection cells were treated as in figure 1A. A shorter and longer exposure of the PCNA immunoblot is shown. **(B)** Hela cells were treated the same as in S2A.



Supplementary Figure 3. PCNA is polyubiquitinated in response to a variety of replication stressors. (A) GM038 and WI38 cells were treated with the indicated doses of replication stressor according to materials and methods. (B) A549 cells were treated the same as in S3A.



Supplementary Figure 4. USP1 knockdown does not increase PCNA ubiquitination in WI38 cells. WI38 cells were transfected with 100 nM of either control siRNA or siRNA USP1. 72 hours post-transfection cells were lysed and immunoblotted for PCNA.

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Chapter 5
RAD18 as a potential target for drug development: To Target or not to Target

Contribution of Collaborators

The contents of this drug target assessment were written by Jan Brun and edited by Dr. Doug A. Gray. All figures are the work of JB.

Summary

This mini-review assesses whether hRAD18 is a plausible target for drug development. The article cautions that new roles for RAD18 in cell function continue to be elucidated and the lack of a transgenic or knockout animal model should preclude the targeting of RAD18 in the meantime. This mini-review is currently under peer review and if published will be citable through the Swiss-Prot Targeted Proteins Database (TPdb).

Target Assessments for Swiss-Prot Targeted Proteins Database (TPdb)

RAD18: To Target or Not to Target

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Running Title: RAD18 as a potential target for drug development

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Key words: RAD18, RAD6, PCNA, DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63-polyUb,
lysine 63-linked polyubiquitin; hRAD18, human rad18; hRAD6A/B, HHR6A/B, human rad6 A and/or B; HR6A/B, mouse rad6; MMS, methyl-methane sulfonate; MMC, mitomycin C; PCNA, proliferating cell nuclear antigen; XPV, Xeroderma Pigmentosum variant

RAD18: Function and Target Perspective

Given the importance of genomic integrity, it is not surprising that eukaryotic cells have evolved an elaborate system of DNA damage cell cycle checkpoints and repair mechanisms to counter exogenous and endogenously induced DNA lesions [1]. The bulk of these DNA lesions are removed efficiently by excision repair (ER) pathways. Despite the employment of ER pathways and cell cycle checkpoints, DNA replication machinery often meets the lesion before appropriate repair has taken place on the mother strand resulting in a stalled replication fork. This can result in a gap on the daughter strand that can lead to double-strand breaks and have a catastrophic effect on the cell. To minimize the consequences of stalled replication forks, eukaryotic cells have developed a distinct DNA repair system termed DNA Damage Tolerance (DDT), which maintains the fidelity and integrity of the genome in response to DNA damage [2]. DDT, also referred to as the *RAD6* pathway or post-replication repair (PRR), is involved in a finely orchestrated recruitment of numerous proteins to stalled replication forks to bypass damage while cells are replicating in S phase. In yeast and humans, lesion bypass and restart of DNA synthesis can occur through an error-prone pathway activated by the mono-ubiquitination of proliferating cell nuclear antigen (PCNA), a protein found at sites of replication, followed by recruitment of specialized translesion synthesis (TLS) polymerases [3,4]. Moreover, there is recent evidence for a second, error-free, pathway that requires modification of PCNA with non-proteolytic lysine 63-linked polyubiquitin (K63-polyUb) chains [5,6]. Although, the mechanism of error-free DDT remains unclear, it is speculated that the undamaged sister chromatid is used as a template to avoid damage induction

[6,7]. In contrast to error-free DDT, TLS uses the Y family of DNA polymerases to overcome replication stalling. These TLS polymerases include POLη (RAD30A), POLι (RAD30b), POLζ (REV3 and REV7) and POLκ [8]. Depending on the type of DNA damage, the polymerases work by inserting correct or incorrect nucleotides across from the site of DNA damage [8].

RAD18 (an E3 ring finger ubiquitin ligase) plays a pivotal role in DDT. The mechanistic basis of RAD18 function is best understood in yeast where it is an extremely important determinant of the toxicity and mutagenicity of many DNA damaging agents [9]. Mutations in *RAD18* lead to increased spontaneous mutagenesis, defects in induced mutagenesis and overt sensitivity to DNA damaging agents such as UV light, cisplatin, MMS and MMC [9-14]. Moreover, *RAD18* mutants are compromised in their ability to bind RAD6 (an E2 ubiquitin conjugating enzyme) and single stranded DNA [15-17]. Upon replication stalling, RAD18 localizes to sites of DNA damage and interacts with RAD6 thereby channeling it to the DDT pathway [15]. Here they mediate mono-ubiquitination of PCNA and recruitment of specialized TLS polymerases capable of replication past the lesion (Figure 1). These polymerases can be highly error-prone but allow replication past a variety of DNA lesions [18]. POLη plays a uniquely important role in the repair of UV damage as it mediates error-free bypass of thymine-thymine dimers, the most common UV-induced lesion [18]. Mouse and human homologs of *RAD6*, *RAD18*, *PCNA* and all of the TLS polymerases have been identified [19].

Yeast epistasis studies have established a second arm of the DDT pathway, distinct from TLS, referred to as damage avoidance [3,6]. This pathway is also

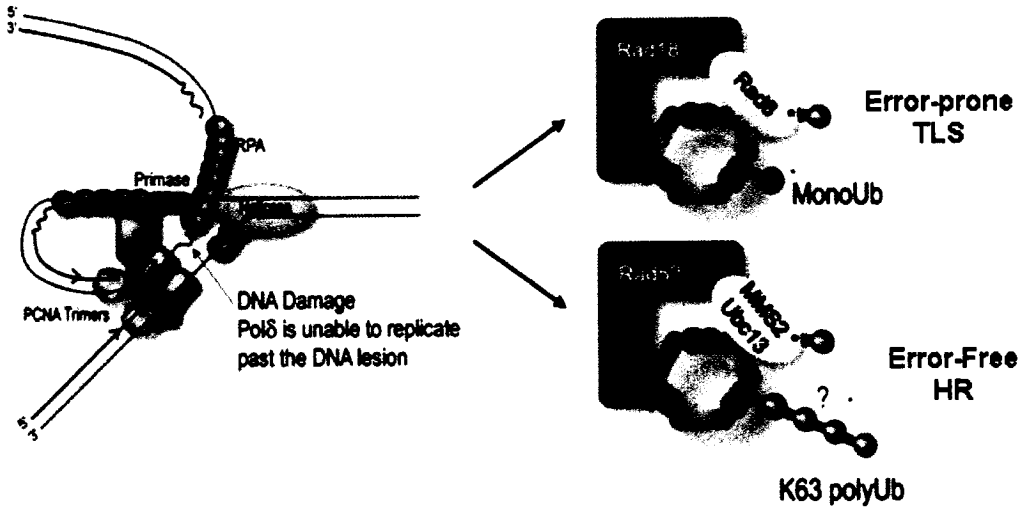

Figure 1: Model of the DNA damage tolerance pathway in mammalian systems. Encountering DNA damage during replication can lead to stalled replication forks and subsequent genomic instability. Cells have evolved two alternative pathways to overcome and bypass this damage and central to these pathways is RAD18. Briefly, RAD18 localizes to the site of damage where it recruits RAD6 and monoubiquitinates PCNA. This increases the binding affinity for the Y family of lesion bypass polymerase, which generally bypasses the damage in an error-prone manner. Alternatively, another ubiquitin conjugating complex consisting of the chromatin associated ring finger protein RAD5 and the E2 ubiquitin conjugating MMS2/UBC13 heterodimer catalyze the formation of K63-linked ubiquitin chains onto PCNA. Polyubiquitinated PCNA appears to be involved in error-free bypass presumably by activating a template switch, however, the exact mechanism still remains unclear.

downstream of *RAD6/RAD18*, but in contrast to TLS it is error-free. Its mechanism is not fully understood, but may involve fork reversal and recombination with the undamaged replicated sister chromatid [20]. This pathway requires a second ubiquitination complex composed of RAD5 and the UBC13/MMS2 heterodimer that polyubiquitinates PCNA (Figure 1) [3].

The absolute requirement of RAD18 in DDT is well established in yeast but much less is known about its role in vertebrate systems. However, there is fast evolving evidence that RAD18 is functional and important in vertebrates. First, there is a significant degree of conservation of RAD18 from yeast to higher eukaryotes. Second, mono-ubiquitination of PCNA occurs in a hRAD6/hRAD18 dependent manner, which results in increased binding to the TLS polymerase POLη through its variant CUE Ub-binding domain [4,21]. Furthermore, mutations in *POLη* (resulting in the inability to form complex with RAD18) are responsible for the variant form of Xeroderma Pigmentosum (XPV), a disease characterized by a 2000 fold increased risk of skin cancer [18]. Third, a recent study clearly demonstrated that K63-polyub PCNA is conserved from yeast to human and is highly dependant not only on *hUBC13* but on *hRAD18* as well [5]. Fourth, *hRAD18* resides on chromosome 3p24-25, a region known to be associated with lung, breast, ovarian, and testicular carcinogenesis [17]. Fifth, a recent report showed that stable expression of a hRad18 (human homolog of yeast RAD18) mutant that was unable to bind HHR6A and HHR6B (human homologs of yeast RAD6) resulted in increased sensitivity to DNA damaging agents [17]. Finally, mouse and chicken *RAD18* knockout cells have been

shown to manifest overt sensitivity to DNA damaging agents and increased genomic instability [22,23].

Clearly, *Rad18* is a major contributor to the integrity and maintenance of genomic stability. However, whether RAD18 is a suitable target for drug development and its consequences on whole organisms such as human and mouse are currently unknown. To date, no known disease is associated with either the overexpression or dysfunction of *hRAD18*. However, recent studies demonstrate that HHR6A is now associated with X-linked mental retardation [24]. Considering that RAD18 is a critical partner of HHR6A, neurological defects could be a consequence if RAD18 is targeted in humans. Furthermore, RAD18 maps to a chromosomal region implicated in cancer development and for that reason it is necessary to explore the role of RAD18 by constructing a transgenic mouse or knockout model. In addition, exploring whether deletions or mutations, polymorphisms, up or down regulation in *hRAD18* correlates with diseases such as cancer or others, is necessary. The use of such models and studies will be critical in providing us with the opportunity to establish the consequences of imbalances in the levels of RAD18 or losing such a critical gene in complex organisms. They will also help in answering whether *RAD18* has functions other than in DDT. To this end, studies in vertebrates have already ascribed other functions to RAD18 including impeding viral transduction, involvement in somatic hypermutation, and a DDT independent function in male meiotic prophase [25-27]. Certainly, studies in these areas will need to be pursued in higher organisms.

Drug targeting RAD18 is a risky proposition. The only approach that is currently available resides in using RNAi, or dominant negative strategies, neither of which has been approved for clinical application. RAD18 is expressed in most tissues and targeting RAD18 could potentially have catastrophic consequences to the patient, such as increased cancer risk, risk of neurological problems and immune dysfunction [24-27]. However, paradoxical observations have been observed with other drugs targeting ubiquitin machinery, such as the successful use of proteasome inhibitor PS-341 in the treatment of cancer [28-31]. Notably, more research is required in higher organisms, such as the mouse, before any drugs targeting RAD18 could be used in humans. Specifically, the role of *RAD18* in cancer, neuronal processes and in the immune system needs to be established before such drugs could be used in patients. Most importantly, we need to determine whether loss of function or overexpression of RAD18 is involved in human disease at all.

Conversely, targeted drug development against RAD18 would be quite useful, particularly in animal experimentation. One could also foresee great use in targeting RAD18 particularly in a variety of cancers, but only under the condition that it can be specifically delivered to the diseased cells. Recently, VSV has been shown to have specific tropism towards a variety of cancer cells while leaving healthy cells unmolested [32-34]. One could envision a scenario where cancer cells could be delivered a double hit by genetically engineering a RAD18 specific peptide into the VSV genome. This would ensure cell killing not only by the virus itself but by further inducing genomic instability. Moreover, targeting RAD18 in the latter scenario may have further use as it has recently been shown that cells null for *RAD18* are more

permissive to viral transduction [27]. However, whether this is specific to all viral infections such as VSV is unknown.

Characteristic Structural Features

Studies in both lower and higher eukaryotes have implicated *RAD18* as a major player in maintaining genome stability in the DDT pathway. Although no human disease has been associated with RAD18, the region it maps to on human chromosome 3 has been associated with cancers that respond to chemotherapies [17]. Therefore, it may be of great use to examine the status of RAD18 in cancer patients in order to determine the likelihood of chemotherapeutic success [17]. Accordingly, drug targeting of RAD18 in cancer could be of therapeutic benefit. In yeast, RAD18 coimmunoprecipitates in a heterodimeric complex with RAD6. This complex has ubiquitin conjugating (ring finger motif) ability, a single strand DNA (ssDNA) binding ability (zinc finger motif) and ssDNA dependant ATPase activity [16,35]. In particular, the DNA binding and ATPase properties belong to RAD18.

Although no crystal data is available concerning hRAD18 structure, older and more recent studies have revealed several important motifs that are critical for its pleiotropic function in the cell. HRAD18 contains a ring finger motif (important for E3 ligase activity and binding HHR6A/B)[36], an HIV-1 integrase binding domain [37], a zinc finger motif (single strand DNA binding activity)[17], SAP box (which mediates binding to A/T rich DNA regions known as SAR (a scaffold attachment region) [37], a RAD6 binding domain[38] and finally a POLη binding domain (Figure 2)[21]. However, the nucleotide binding motif that contains the ATPase activity in yeast RAD18 is notably absent in hRAD18. The latter characteristic may

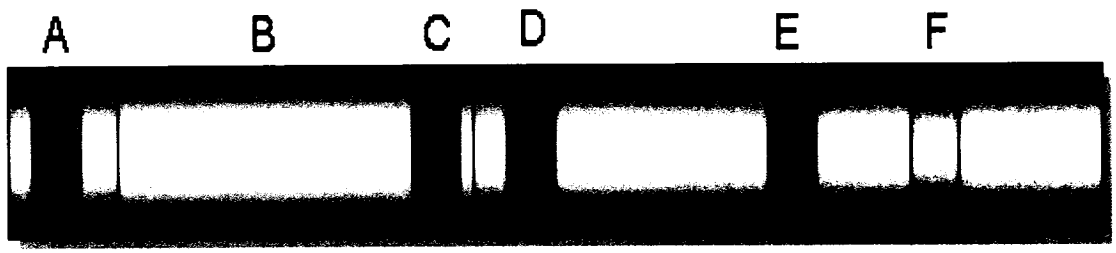

Figure 2: Schematic representation of the functional domains of hRAD18. A) (25-63) Ring Finger Domain, B) HIV-1 Integrase Binding Domain (65-226), C) Zinc Finger Domain(204-223), D)SAP Box(248-282), E) RAD6 binding Domain(340-386), F) POLη Binding Site (402-444)

help explain why *hRAD18* cannot complement the yeast *RAD18* mutants. Human RAD18, a 495 amino acid protein, localizes to the nucleus and physically associates with both human homologs of RAD6, HHR6A and HHR6B[36]. Mutating the ring finger domain (C28F mutant) abrogates interaction with HHR6A/B and sensitizes cells to a variety of DNA damaging agents [17]. The C28F mutant localizes to the nucleus after DNA damage indicating that it may compete with wild type RAD18 for damaged sites [17]. Certainly, targeting the E3 ligase activity in the ring finger could provide a means by which one could attenuate RAD18 activity and increase sensitivity to conventional chemotherapeutics. However, it would be necessary to target the ring finger of RAD18 specifically (using small molecule inhibitors perhaps) in the diseased cells while avoiding healthy cells.

Surprisingly, mutants in the hRAD18 zinc finger domain, in particular mutant C207F, can localize to the nucleus after DNA damage and show similar sensitivity to DNA damage as control cells expressing wildtype *RAD18*. This is interesting since the zinc finger motif is postulated to be necessary for binding ssDNA. However, a recent study by Watanabe et al., demonstrated that amino acid 402-444 of RAD18 interacts with full length and a c-terminal portion of POLη [21]. In the case of the zinc finger mutant (C207F), it still maintains functional E3 ligase activity, and POLη binding ability. Since POLη is able to bind PCNA (trimeric clamp that tethers polymerases and replication machinery to DNA) [21], it brings RAD18 into the vicinity of the damage even though its DNA binding activity is compromised. There, RAD18 interacts with RAD6 and catalyzes PCNA ubiquitination, which increases the affinity of the replication machinery for POLη versus POLδ and allows bypass of the

DNA lesion. Cells expressing hRAD18 lacking the POLη binding domain were overtly sensitive to UV and no colocalization of RAD18 and POLη was observed [21]. However, as predicted, monoubiquitination of PCNA proceeded, as the E3 ligase function of RAD18 was still intact. So, the POLη binding domain would be another interesting drug target as it could potentially sensitize cancer cells to specific chemotherapeutic DNA damaging drugs that are normally bypassed by POLη. However, it remains to be determined whether the RAD18 POLη binding domain can associate with other members of the Y-family of polymerases.

A final target for drug development could be the HIV-1 integrase binding domain of RAD18. In a recent report, RAD18 was able to suppress viral transduction by both retroviruses and adenoviruses [27,37]. Whether this extends to other viruses, such as rhabdoviruses, specifically VSV, is unknown. It will also be important to determine the critical domains that are preventing viral infection. Perhaps the HIV-1 integrase binding domain is a misnomer and could instead be a general foreign DNA binding domain. If such is the case, one could envision a scenario wherein targeted drug development against this RAD18 domain would allow more permissive transduction, which would yield great potential in the world of oncolytic viral therapy. Again, cancer cells would have to be specifically targeted to avoid potentially harmful side effects in healthy cells.

Interestingly, RAD18 is also mono and polyubiquitinated in a RAD6 dependant manner. Non-ubiquinitnated RAD18 is seen predominantly in the nucleus and monoubiquitinated RAD18 forms a homodimer via the zinc finger motif localizing in the cytoplasm. The C207F zinc finger mutant abrogates RAD18 homodimerization as

well as its monoubiquitination[38]. Unfortunately, drug targeting the zinc finger would be of no consequence since a zinc finger mutant does not seem to alter the biological function of RAD18 with respect to DNA damaging agents [17]. To date, the biological function of how and which proteins interact and remove the ubiquitin monomer from RAD18 and the biological consequence of automonoubiqitination of RAD18 still need to be elucidated. It is reasonable to speculate that automonoubiqitination may regulate the amount of RAD18 in the nucleus at one time or serve as a cytoplasmic reservoir, thus controlling the extent to which DDT is activated during genotoxic stress [38].

Drugs and Ligands

To this end, there are no known companies working on targeting the RAD18 protein nor are there any known patents on drugs or ligands that inhibit or promote RAD18 function. Thus far, only gene targeting strategies (homologous recombination) [22,23], using a dominant negative RAD18 ring finger mutant (C28F) [17] and RAD18 RNAi (commercially available from Dharmacon RNA Technologies) have been successfully used to target and abrogate RAD18 expression in a variety of cell lines.

Next Frontiers in Drug Discovery

The main issues being currently addressed are the underlying molecular mechanisms of RAD18 function in the cell. The major unresolved issues that have yet to be addressed are whether or not RAD18 is directly or indirectly involved in disease and the consequences of knocking out or overexpressing RAD18 in mouse models. To date, several roles have been ascribed to RAD18 including a role in

maintaining genome stability, immune function and an indirect hypothetical role in neurological disorders [17,24,26]. Whether these functions will manifest in a mouse model is currently unknown. At this point we are still at the molecular infancy with respect to understanding the complex role RAD18 has in mammalian systems and thus it would be premature to target such a protein in the clinic until our knowledge is furthered with the use of animal models.

Disease models/knockouts

Progress is certainly being made in deciphering the role of RAD18 in vertebrate cells, particularly with the development of two knockout models using both mouse embryonic stem cells and chicken B-lymphocyte DT40 cells [22,23].

In mammals, only a single *RAD18* gene locus was mapped and it interacts with two mammalian homologs of RAD6, HR6A and HR6B [17,39]. This implies that mammalian RAD18 and RAD6 work similarly as they do in yeast. HR6A knockout mice do not display an overt defect in the DDT pathway but females fail to produce offspring [40]. Recently a mutation in HR6A has been associated with a novel X-linked mental retardation syndrome in humans [24]. Finally, HR6B knockout mice do not manifest any DNA repair defect, however, male mice are sterile [41]. Therefore, in order to understand where RAD18 fits in and to further elucidate the molecular mechanism of the DDT pathway Tateishi et al. knocked out the *RAD18* gene in mouse embryonic stem cells. Similar to human cells, *RAD18*$^{-/-}$ mouse embryonic stem cells grew at wildtype rates and were sensitive to a variety of DNA damaging agents including UV, MMS, MMC, and cisplatin. Moreover, the *RAD18*$^{-/-}$ cells showed an increased spontaneous and induced sister chromatid exchange,

elevated transformation efficiencies/illegitimate recombination and significantly smaller newly replicated DNA [22]. These phenotypes are similiar to those of human cells expressing a dominant negative mutant form of RAD18 [17]. Interestingly, there seems to be an attenuation in sensitivity to DNA damaging agents in *RAD18*$^{-/-}$ cells in vertebrates when compared to yeast. However, this may simply be due to the fact that DNA machinery meets DNA lesions at a greater rate due to smaller genome and the higher proliferation rates of yeast as compared to higher eukaryotes [17] . Furthermore, higher eukaryotes have likely evolved several compensatory mechanisms to deal with such damage. Together, these studies clearly show that *RAD18* plays an essential role in DDT and in maintaining genomic stability. However, further studies are required to ascertain the function of RAD18 in other cell types and thus it will be essential to develop a mouse model null for the *RAD18* gene.

The importance of knockout models are undeniable, however, they also do not take into account the potential imbalances of gene expression, which could have profound effects on drug sensitivity. For that reason it will also be vital to develop a transgenic mouse model overexpressing wildtype and a dominant negative form of RAD18. To date, there are no known diseases associated with wildtype RAD18 overexpression, nor have any dominant negative mutant RAD18 been observed in human diseases. However, overexpression of RAD6B has been associated with chemoresistance [42]. Considering that RAD18 interacts with RAD6B, it is plausible that overexpression of RAD18 could potentially confer a similar phenotype, thus modulating drug sensitivity and damage induced mutagenesis. Therefore, by specifically targeting RAD18 (whether overexpressed or not) one could

hypothetically further sensitize certain cancers to conventional chemotherapeutics and thus providing an enormous therapeutic benefit.

Another invaluable RAD18 knockout model is one using the chicken B-lymphocyte line DT40. This model has recapitulated similar findings to those found in mouse embryonic stem cells with respect to RAD18's central role in DDT [22,23]. In addition, it revealed a novel role for RAD18 in somatic hypermutation, a process that leads to antibody diversity in both B and T cells of the immune system ensuring that the binding affinity of antibodies produced by B cells are finely tuned to recognize and eliminate invading pathogens [26]. Several issues have yet to be determined from this study and those include formally demonstrating that ubiquitinated PCNA participates as the main molecular switch between error-free and error-prone lesion bypass in somatic hypermutation and determining the RAD18 dependant polymerases that are involved in the process [26]. Again, whether RAD18 deficiency would lead to general immune dysfunction in a complex organism is a question that could be answered by the development of a knockout or transgenic mouse model. Evidently, more research is required to identify other potential roles of RAD18 in whole organisms, such as the mouse; thus for the moment, drug targeting RAD18 other than for experimental use in the laboratory is very risky and should be not be pursued in the clinic until more is known about its role in human disease.

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Chapter 6
Lysine 63 linked ubiquitin chains protect cells from spontaneous mutagenesis

Contribution to collaboration

The contents of this manuscript were written by Jan Brun and edited by the supervisor Dr. Doug Gray. Experiments for Figures 1, 2 and 4 were performed by Jan Brun, while experiments for Figure 3 and table 1 were performed by RKC and CR. JB, RKC and DAG analyzed the data. JB and RKC provided the analysis tools and reagents for the experiments.

Summary

Thus far K63-linked chains have been implicated in protecting cells against the induced mutagenesis by common environmental mutagens UV light and BPDE. Here we report that these same chains also protect cells from endogenous damage. Although we do not pinpoint the exact mechanism responsible for increased spontaneous mutagenesis we definitively rule out the increased use of TLS in this process.

Lysine 63 linked ubiquitin chains protect cells from spontaneous mutagenesis

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Running Title: Disruption of error-free damage avoidance results in increased mutagenesis

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Key words: RAD18, MMS2, UBC13, PCNA, DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63, lysine 63

Abstract

DNA is bombarded by genotoxic insults on a daily basis. Therefore, a prerequisite for cell survival is the fidelity with which it replicates its DNA. At the cross-roads of replication is the DNA sliding clamp proliferating cell nuclear antigen (PCNA). K63-linked polyubiquitination of PCNA was recently identified for its role in protecting cells against DNA damage induced mutagenesis and gross chromosomal rearrangements in human cells. Here we provide evidence that K63 linked polyubiquitination also protects cells from spontaneous mutagenesis and that one of the substrates involved in this process is PCNA. Moreover, we show that the mechanism leading to spontaneous mutagenesis is not dependant on increased use of error-prone TLS but likely the result of disrupting an alternate error-free mechanism.

1. Introduction

In humans, DNA repair is essential to maintain chromosomal stability in the face of genotoxic stress. A disruption in such a vital process results in a variety of disorders including Fanconi anemia (FA), xeroderma pigmentosa, Bloom's syndrome, ataxia telangiectasia and cockaynes syndrome diseases which lead to genomic instability and an increased predisposition to developing cancer. Interestingly a small gloubular 76 amino acid pleitropic tag is a common element that links these diseases.

In yeast and humans, ubiquitin act as a post-translational modification molecular tag that is covalently bound to a wide range of substrates. While ubiquitin polymerization mediated by lysine 48 is recognized as a signal for degradation, some ubiquitin-conjugating enzymes can assemble polyubiquitin chains via other linkages, notably through K63 [1,2]. Recently, the biological role of K63-linked polyubiquitin chains has been implicated in a variety of cellular processes, most notably DNA repair, as a yeast K63R mutant shows extreme sensitivity towards DNA damaging agents [3]. The heterodimer of MMS2/UBC13 along with RAD5 (ubiquitin ligase) catalyzes the formation of K63-linked chains on PCNA [4-6]. Interestingly, these three proteins are part of the DNA damage tolerance (DDT) pathway which is mediated by another UBC, RAD6. RAD6 along with RAD18 mediate the error-prone TLS arm of DDT while the UBC13/MMS2/RAD5 complex mediates the error-free bypass branch of DDT [4-6].

Recently, the disruption of K63-linked ubiquitin chains in human cells was shown to shunt repair from the error-free pathway towards error-prone TLS after UV

irradiation, which resulted in an increased mutation frequency [7]. This also corroborated work by Li et al who reported that anti-sense knockdown of hMMS2 increased UV induced mutagenesis [8]. Moreover, disruption of SHPRH (human RAD5 homolog) diminished K63 linked ubiquitination of PCNA and lead to elevated levels of gross chromosomal rearrangements after MMS treatment [9]. However, none of these studies comments on the role of K63 polyubiquitin chains in spontaneous mutagenesis. Although spontaneous mutagenesis was not reported in K63R yeast mutants there is indirect evidence that these chains are important in protecting cells against endogenous damage [3]. RAD6, RAD18, UBC13 and MMS2 yeast mutants display a dramatic increase in spontaneous mutagenesis and this effect is largely synergistic with REV3 mutations [4,6,10]. Furthermore, in human cells there are detectable background levels of PCNA mono and polyubiquitin chains suggesting their importance in protecting cells against endogenously induced mutations [7,9,11].

To ascertain whether K63 linked polyubiquitin chains are involved in spontaneous mutagenesis we used a previous strategy of dominant negative ubiquitin mutants to disrupt ubiquitination. Using this system we achieved high enough levels of K48R and K63R mutant ubiquitin to disrupt proteolysis and DNA repair respectively [7,12]. In this study we report detectable levels of mono and polyubiquitinated PCNA suggesting a role in repair of endogenous lesions, and that disruption of K63 linked polyubiquitination leads to a significant increase in spontaneous mutagenesis. Moreover, the mutation spectrum, TLS pol foci studies,

and caffeine treatments suggest that the mechanism responsible for this increase in spontaneous mutagenesis is the disruption of an alternate error-free pathway.

2. Material and Methods

2.1 Cell culture and treatments

The construction of the ubiquitin expressing plasmids has been described elsewhere [7,12]. The *POLη-GFP* plasmid was a gift of Dr. Alan R. Lehmann, (Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton U.K.). All cell lines were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS (Sigma, St. Louis Missouri). A549 cells were co-transfected with *WT-Ub-GFP* or *K63R-Ub-GFP* plasmids and pBabePuro using FuGene 6 (Roche). Stable transfectants were selected in 1μg/ml puromycin (Sigma, St. Louis Missouri, United States) and/or by flow cytometry (FACSAria, BDBiosciences).

The sensitivity to caffeine (Sigma, St. Louis Missouri, United States) alone was evaluated using clonogenic survival assays. Briefly, 200 and 1000 and 10000 cells were plated per 6cm dish in triplicate in either 0, 0.4 or 1mM caffeine. They were incubated for 2 weeks to obtain colony-formation after which colonies were fixed, stained with 2% bromophenol blue in 70% ethanol. Colonies containing ≥50 cells were counted. All experiments were normalized for plating efficiency.

2.2 Immunoblotting

Following the indicated treatments with UV, cells were harvested in lysis buffer, sonicated, soluble fractions recovered, and proteins were quantified using the Bradford protein assay (Bio-Rad). Proteins were resolved on either a one or two

phase SDS-polyacrylamide gel (10% and 15% or 10%) and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was stained with Ponceau S (Sigma) prior to Western blotting with the indicated primary antibody. The following antibodies were used: rabbit polyclonal Ub (Dako), mouse monoclonal RGS-His (Qiagen), mouse monoclonal PCNA PC10 (Chemicon), rabbit polyclonal GFP (Santa Cruz) and mouse monoclonal actin (Sigma). Proteins were visualized by a horseradish peroxidase method using ECL (Kirkegaard & Perry Laboratories).

2.3 Immunoprecipitation

Cells were UV-irradiated with 0 or 30 J/m^2. Cells were lysed 6h post-irradiation in lysis buffer supplemented with 0.5% SDS. Lysates were sonicated and boiled for 5 min followed by dilution to 0.1% SDS. After protein quantitation, 500µg of protein was incubated overnight at 4°C with anti-PCNA (1/200). The following day, lysates were incubated for 48h at 4°C with 100µl of Gamma bound Sepharose Beads (Pharmacia Amersham). Beads were washed extensively in lysis buffer and proteins were eluted by boiling in 1x Laemmli's SDS sample buffer. Immunoblotting was performed as described above except the membranes were autoclaved for 20min in ddH_2O after protein transfer, and proteins were visualized by a horseradish peroxidase method using ECL (Kirkegaard & Perry Laboratories).

2.4 Mutation spectrum

To eliminate background *HPRT* mutations, cells were cultured in hypoxanthine, aminopterin, and thymidine (HAT) supplemented culture medium for one week. Spontaneous *HPRT* mutants were obtained by seeding $1.5x10^4$ cells in 24

well plates. Cells were subcultured for 7 days, and re-seeded at $5.0x10^4$ cells on 35 mm dishes in medium containing 30μM 6-thioguanine (6-TG). Individual colonies were picked and grown until enough cells were obtained for RNA-isolation using an RNA-aqueous kit (Ambion). The *HPRT* gene was subjected to RT-PCR followed by sequencing using the following overlapping primers: HPRT1 - 5'CTTCCTCCTCCTGAGCAGTC3'; HPRT2 - 5'AAGCAGATGGCCACAGAACT3'; HPRT3 - 5'CCTGGCGTCGTGATTAGTG3'; HPRT4 - 5'TTTACTGGCGATGTCAATAGGA3'; HPRT5 - 5'GACCAGTCAACAGGGGACAT3'; and HPRT6 5'ATGTCCCCTGTTGACTGGTC3'.

2.5 Mutation frequency

$1.0x10^6$ *HPRT*-mutant free cells were seeded. After subculturing the cells for one week, $4.0x10^5$ cells were seeded in selective medium containing 6-TG (as above) and incubated until colonies were formed. Colonies were counted and *HPRT* mutation frequency was defined after correcting for plating efficiency.

2.6 Foci

A549 stably expressing *WT-Ub-puro* and *K63R-Ub-puro* were transiently transfected with a *Polη-GFP* plasmid. To observe living cells, cells were cultured in 35mm glass bottom dishes (MatTek Corporation) with coverslips. Real time excitation measurements to monitor fluorescent signals in transfected cells were subsequently performed using a live cell microscopy unit mounted on a Leica DR IRBE inverted microscope, equipped with a polychromator that allows generation of

light of the required wavelength, using a 63x objective. Both the polychromator and filterwheel were controlled via the PC using specialized Openlab software. At least 100 cells were counted for each cell line at each time point per experiment by a blinded independent observer. The recruitment of polη to foci was determined in untreated cells.

3. Results

3.1 PCNA ubiquitination is detected in replicating cells

In a previous study we report the detection of high molecular weight, ubiquitin immunoreactive smears in cancer cell lines exposed to UV irradiation[7]. At the time we also noticed that PCNA ubiquitination was present in untreated cells. This suggested that PCNA ubiquitination was possibly involved in repairing endogenously induced damage. To confirm this we repeated the experiments with our A549 cell lines expressing WT and K63R mutant ubiquitin. WT and K63R cell lines were examined after 0, 5, 10 and 20J/m^2 of UV irradiation. With a higher dose of UV we observed a dramatic increase in band intensities corresponding to mono and polyubiquitinated PCNA (Figure 1A). With these exposures we also observed mono and faint polyubiquitinated species in the untreated cells as well, however at lower levels than those induced by DNA damage (Figure 1A). To confirm that these were in fact ubiquitinated species we performed an immunoprecipitation with and

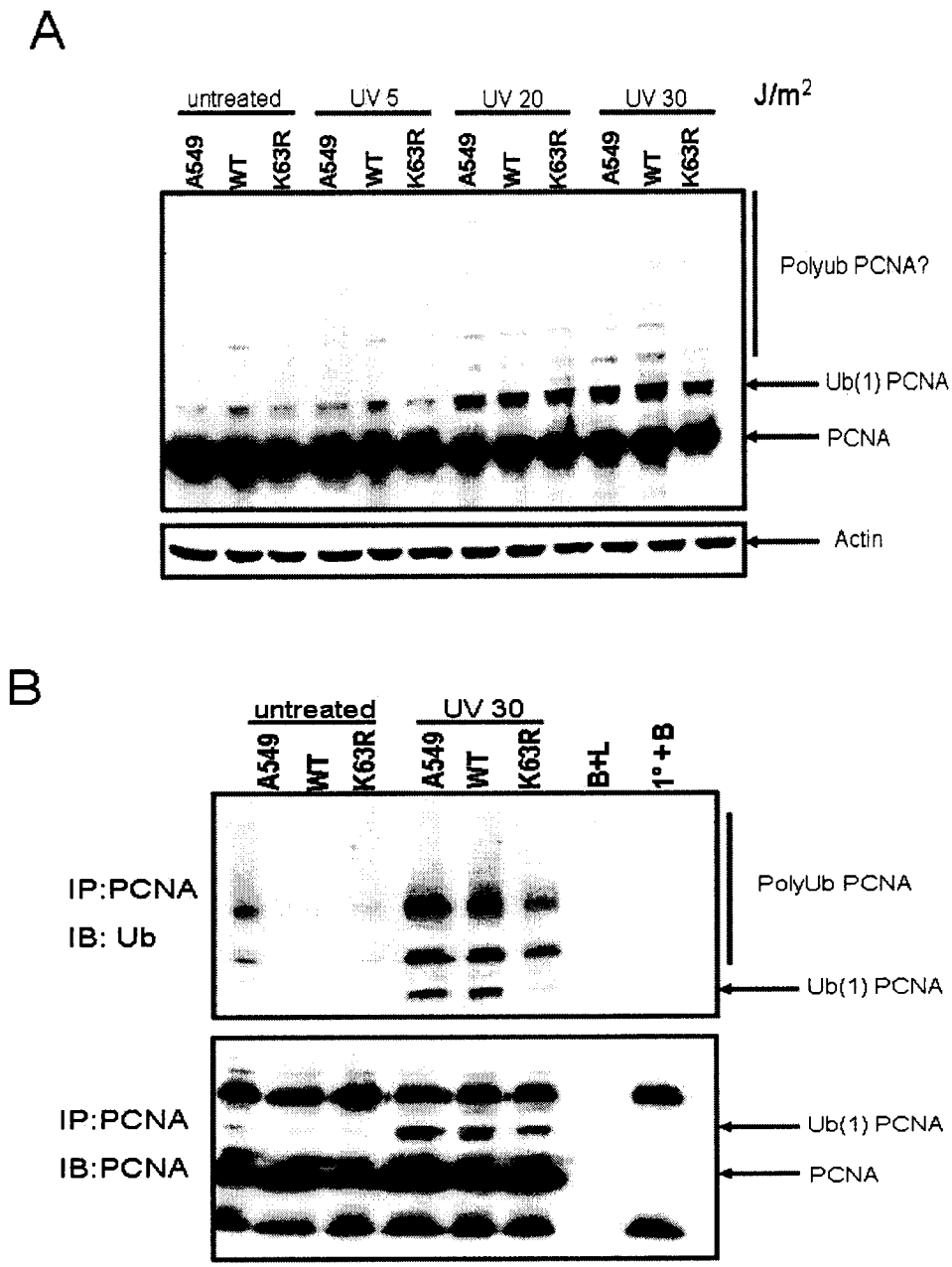

Figure 1. PCNA is polyubuquitinated in untreated cells. (A) A549 cells stably expressing the ubiquitin constructs were UV irradiated with the indicated dosesand lysed 6 hours post-treatment and subjected to immunoblot analysis with an anti-PCNA antibody. (B) A549 cells stably expressing the ubiquitin constructs were treated with 30J/m^2 of UV lysed and immunoprecipitated with an antibody that recognizes PCNA followed by immunoblotting with antibodies against ubiquitin and PCNA. The controls in the immunoprecipitations were 'no 1°' in which the beads were incubated with lysates with no antibody and '1° +B' in which the primary antibody and beads were incubated together.

anti-PCNA antibody followed by immunoblotting for ubiquitin and PCNA. Similar to our previous results, bands corresponding to mono and polyubiquitnated PCNA were revealed with the anti-ubiquitin antibody after UV irradiation (Figure 1B upper and lower panel). Significantly, we also show that PCNA mono and polyubiquitination occurs in untreated cells, however at lower levels than those induced by exogenous damage (Figure 1B upper panel). This suggests that PCNA is ubiquitinated in response to endogenously induced damage in replicating cells.

3.2 Disruption of K63 linked ubiquitination increases spontaneous mutation frequency

Polyubiquitination of PCNA in untreated cells provides preliminary evidence for the conservation of the error-free damage avoidance pathway in response to endogenous damage. To investigate the functional importance of this ubiquitination we examined the frequency of HPRT mutations arising spontaneously in our stable A549 cell lines expressing K63R mutant ubiquitin. Cells were first cultured in HAT supplemented media, a negative selection to eliminate pre-existing HPRT mutations from the population. After 1 week cells were supplemented with normal media and grown for 3 days. Aliquots of cells were then plated in 6-thioguanine, a positive selection for cells bearing loss of function mutations in the HPRT gene. A549 cells expressing K63R mutant ubiquitin were found to have a significantly higher mutation frequency (3-5 fold) than parental cell lines or cells expressing the wild-type version of the transgene (Figure 2).

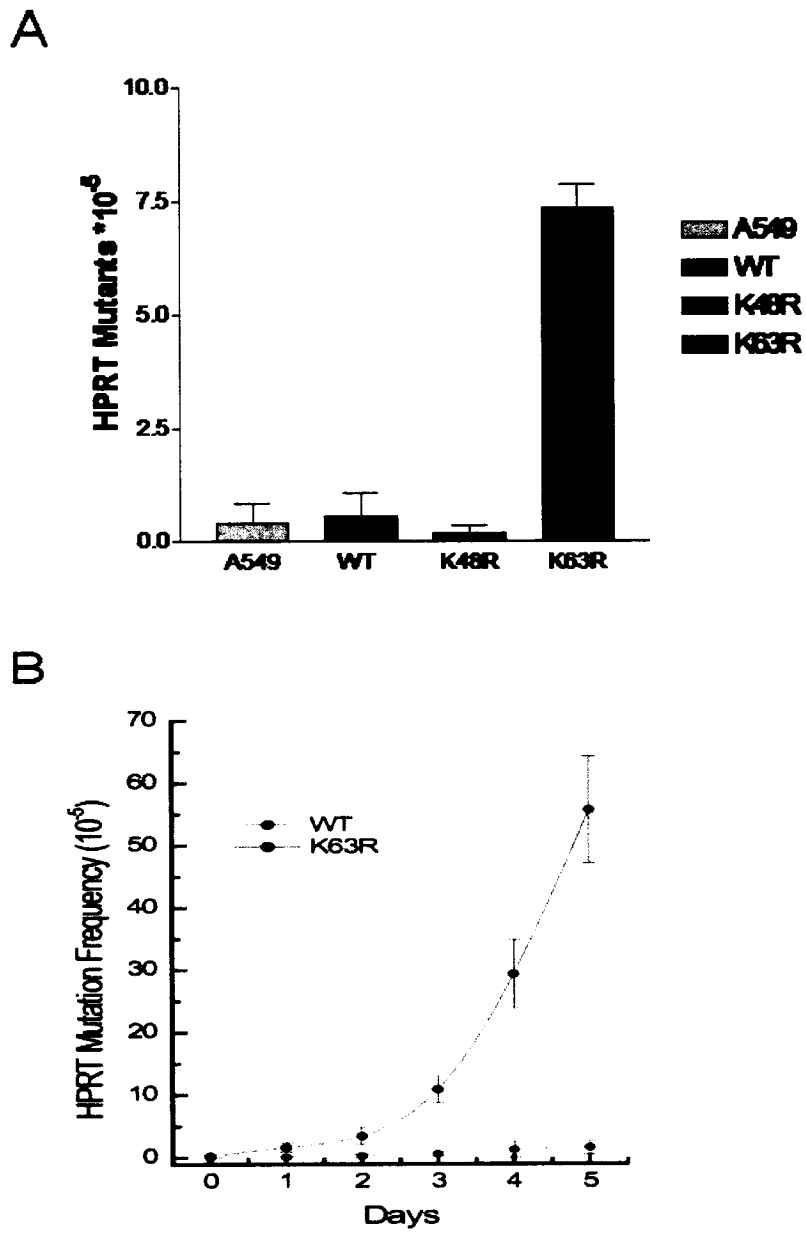

Figure 2. Disruption of K63-linked polyubiquitination leads to increased spontaneous mutation frequency. (A) A549 cells stably expressing the ubiquitin constructs were grown for 3 days post-HAT treatment followed by plating in media containing 6TG. 6TG resistant colonies were stained and counted 14 days after seeding. The mean values of 3 independent experiments are shown with Standard erro of the mean **(B)**A549 cells expressing the WT ubiquitin or the K63R mutant ubiqutin were plated in 6TG selective media at day 0, 1, 2, 3, 4 and 5 days post HAT treatment. At day 14 colonies were fixed, stained and counted. The mean values of 3 independent experiments are shown with standard error of the mean.

3.3 Mutation spectrum reveals no point mutations

In a recent study, we showed that the increase in UV induced mutagenesis in K63R cells was a result of shunting repair from error-free damage avoidance into the error-prone TLS arm of DDT [7]. This assertion was evidenced by the mutation spectrum and a higher number of error-prone polymerase foci in K63R cells as compared to WT after UV irradiation. To begin to understand the possible underlying mechanism for the spontaneous mutagenesis we examined the mutation spectrum of the spontaneous mutants in Figure 2. Interestingly, sequence analysis of the *HPRT* locus in 6-TG resistant clones revealed that none of the spontaneous mutations involved point mutations, but were exclusively large scale deletions (Table 1). About 40% of the mutants consisted of large scale deletions, while 6% consisted of insertions. No mutation was found in 54% of the mutants. This differed from the previously published UV spectrum of K63R cells which consisted mainly of point mutations while no mutation was found in only 5% of the UV induced HPRT mutants. Since no point mutations were identified, we speculated that the K63R mutant could be disrupting TLS polymerases.

3.4 Increased use of TLS polymerase is not responsible for spontaneous mutagenesis

To rule out a disruption in the TLS polymerases themselves we examined the accumulation of GFP tagged polη in untreated WT and K63R cells. Since our original cell lines expressed GFP as well, we generated new A549 cell lines expressing an ubiquitin puromycin resistance gene fusion, which subsequently was cleaved

	Spontaneous mutations
Point-mutations: GC > AT	-
GC > TA	-
GC > CG	-
AT > TA	-
Exon deletions: Exon 2,3,4,5	
Exon 4	2.9%
Exon 5	8.6%
Exon 7	
No PCR-product	28.6%
No mutation found	54.3%
Insertion: A	2.9%
T	2.9%
14 basepairs	

Table 1. Mutation Spectrum of A549 cells expressing K63R mutant ubiquitin. RNA was isolated from 6TG resistant clones followed by RT-PCR and sequence analysis of the *HPRT* locus.

releasing the monomeric ubiquitin and puro resistance enzyme. These cell lines were phenotypically equivalent to the A549 K63R GFP expressing cell lines. Similar to previous studies, unirradiated cells show homogenous nuclear distribution of TLS polymerases. Polη foci were observed in 10-12% of cells and likely represent areas of ongoing replication or response to endogenous damage (Figure 3). However, no difference in foci numbers or distribution was observed between untreated WT and K63R expressing cells. Therefore, an increased use of TLS in not responsible for the high spontaneous mutagenesis we report in the K63R cells.

3.5 K63R cells are sensitive to caffeine

Recently, caffeine has been shown to sensitize cells that are defective in HR [13]. To see whether such a defect was present in our system, we treated the K63R cells with increasing doses of caffeine. We find K63R cells to be acutely sensitive to 0.4mM and 1mM doses of caffeine as compared to cells expressing WT ubiquitin or the parental A549 cell line (Figure 4).

4. Discussion

Coupling DNA repair to replication is an important feature that allows cells to maintain genomic integrity in response to various exogenous and endogenous genotoxic insults. In lower and higher eukaryotes it has become clear that two pivotal branches of the DDT pathway play a critical role, namely error free homologous recombination and error-prone TLS. Here we report that K63-linked ubiquitin chains are important in the recovery of cells from endogenous DNA damage and that when

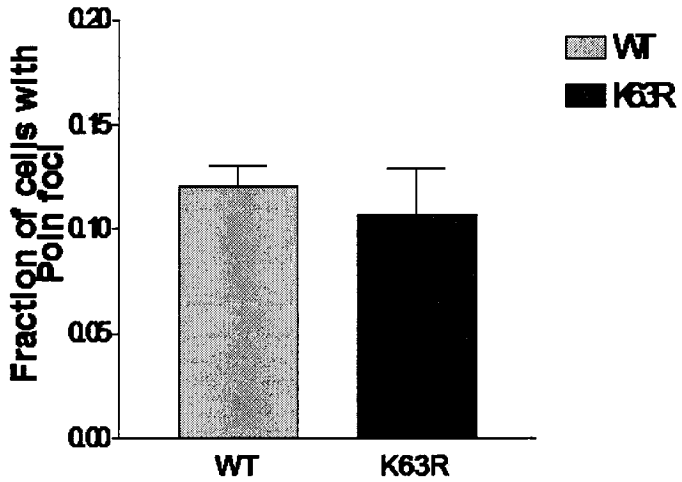

Figure 3. Polη foci foramtion not disrupted in untreated K63R cells. A549 cells stably expressing *WT-Ub-puro* or *K63R-Ub-puro* were transiently transfected with a plasmid expressing a *POLη-GFP* fusion. Polη foci were quantitated 24h transfectio using a live-cell imaging fluorescent microscope. The mean values of 3 independent experiments are shown with standard error of the mean.

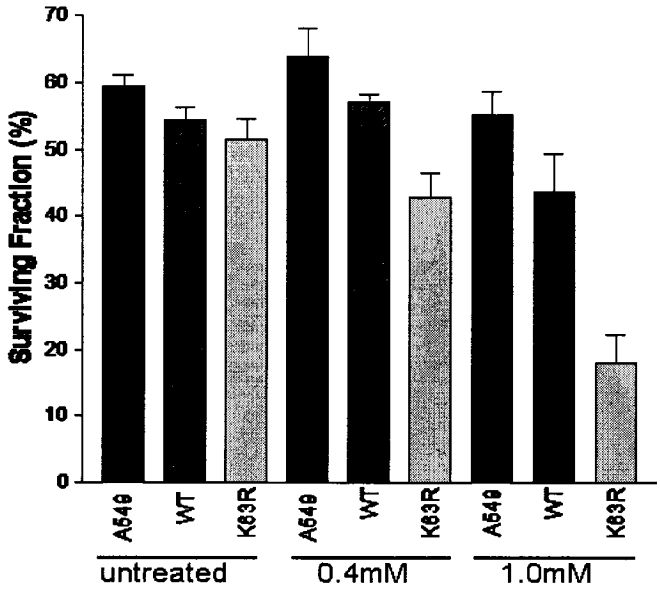

Figure 4: K63R cells are overtly sensitized to caffeine. The parental cell lines, WT and K63R cells were plated at low densities in either 0, 0.4, or 1mM caffeine and assayed for colony formation. At 14 days cells were fixed, stained and counted and surviving fractions determined. The mean values of 3 independent experiments are shown with standard error of the mean

this ubiquitination is impaired dysregulated HR or other systems likely operate with the proclivity to generate a high amount of spontaneous DNA mutations.

Our data support a role for the formation of K63-polyUb chains in promoting the recovery of human cells from endogenous DNA damage through an error-free pathway that is distinct from TLS. One of the possible substrates is PCNA, which has been shown to play a role in spontaneous mutagenesis in yeast [10]. In this study we detect polyubiquitinated PCNA in untreated replicating cells indicating a role for K63-linked polyubiquitination in repair of endogenously induced lesions. While the experiment has been done in our lab, we do not show that the K63R mutant directly disrupts PCNA polyUb. This is due to the fact that the recombinant ubiquitin mutants compete with very high levels of endogenous ubiquitin taking part in this process, thus it is very difficult to conclusively demonstrate this type of ubiquitination in this manner. An alternative approach was the use of siRNA against both RAD18 and UBC13 to illustrate their requirement in the modification of PCNA. We have used this approach in a previous study and showed the process to be dependent on RAD18 and UBC13[7]. Our work was further corroborated *in vivo* by Motegi et al., and *in vitro* by Unk et al[9,14]. These studies provide support for our contention that PCNA is polyubiquitinated via K63 linkages in untreated cells. This data, coupled with the conservation of the enzymes necessary to carry out K63 poly-ubiquitination, have been interpreted by us as evidence that PCNA is modified by K63-linked ubiquitin chians. In fact, this is an issue that has been dealt with previously with both K63 and K48 linked chains. The conclusions were that in either case, the levels of recombinant ubiquitin are too low to resolve this issue by immunoblotting but are sufficient to

reveal a valid biological process [12]. To this end, the data demonstrate that PCNA is one of the likely mediators in resolving endogenously stalled replication forks. However, this does not rule out substrates such as BRCA1, RAD51 or some other unidentified substrates involved in mediating recovery from endogenous DNA damage.

BRCA1 contains a conserved ring domain and heterodimerizes with BARD1, another ring domain protein. Together they autoubiquitinate BRCA1 via K6, K48 and K63 linked polyubiquitin chains and other yet to be identified substrates [15,16]. Although, the exact role of BRCA1 ubiquitination in DNA damage response remains unclear, accumulating evidence suggests that it functions in HR [17]. Moreover, BRCA1's role in HR is potentially related to its function in the Fanconi anemia (FA) pathway where its abrogation affects the nuclear foci formation of many DNA repair factors after genotoxic stress [18]. Interestingly, the FA pathway is speculated to be linked to TLS and HR [19,20]. In humans, the FA pathway is comprised of 8 important proteins (FANCA, -B, -C, -D, -E, -F,-G,-Land –M) all of which lead to the monoubiquitination of FANCD2 [21,22]. A disruption of these genes leads to the rare autosomal recessive disorder termed FA, which results in a dramatic increase in the development of certain cancers including squamous cell carcinomas and leukemias [21,22]. Certainly, our data demonstrating increased genomic instability in cells expressing K63R mutant ubiquitin support the assertion that patients with such a disruption would have increased predisposition to developing cancer similar to those with FA. However it will be important to confirm such an assertion in an *in vivo* mouse model expressing K63R mutant ubiquitin.

To begin to understand the possible mechanism responsible for the increase in spontaneous mutagenesis we examined the mutation spectrum. This revealed no point mutations and indicated that approximately 40% of mutants were large scale deletions and 6% were insertion mutations while no mutation was detected in 54% of the mutants. Interestingly, it has been reported that a majority of spontaneous T lymphocyte mutants from healthy donors consist of point mutations while spontaneous HPRT mutants in T cells from FA patients consist exclusively of deletions (a common feature of the FA phenotype) [23-26]. Since our data show a similar feature of exclusively hosting deletion mutation in K63R cells, it is interesting to speculate on a potential connection between FA, K63-linked ubiquitin chains and the DDT pathway. Of course it will be of importance to examine the mutation spectrum of A549 cells expressing wild type ubiquitin compared to the K63R cells to confirm such an assertion, as well as examining T cells expressing WT and K63R mutant ubiquitin.

Notably, several studies using a variety of cell lines (T lymphocytes, human fibroblasts, V79 Chinese hamster cells, and HL60 cells) have demonstrated that a majority of spontaneously induced HPRT mutants were point mutations (40-70%) and much less were deletions (10-40%), though some reports specifically in bronchial epithelial cells point to high levels of deletions (up to 70%) in the spontaneous HPRT mutants while reporting no point mutations [26-31]. Again these studies point out the importance of identifying the mutation spectrum in parental A549 and WT ubiquitin expressing cells as compared to the K63R spectrum.

Nevertheless, since no point mutations were identified in our study, we speculated that potentially the K63R mutant was disrupting TLS polymerases. However, when we examined POLη foci in WT and K63R cells we found an equivalent distribution of polymerases and no significant differences in foci numbers in either cell line. This suggests that the TLS arm of the pathway remains intact and that an alternate mechanism is at play. To better understand the mechanism responsible for spontaneous mutagenesis, future studies should include (1)karyotyping, (2)examining the distribution of HR proteins RAD51, BRCA1 and γH2AX , SHPRH (involved in PCNA polyUb), (3)recombination assays such as sister chromatid exchange. The latter experiments will help identify whether a disruption of HR or another pathway plays a role. We hypothesize that homologous recombination is the mechanism that is being disrupted in our K63R cells and that maybe its dysregulated function in these cells leads to the large scale deletions observed in the HPRT mutation spectrum.

The claim that HR is likely disrupted is in part supported by the overt sensitization of the K63R cells to increasing doses of caffeine. Caffeine exacerbates DNA repair defects and was also recently shown to increase sensitivity of cells that are defective in HR [13,32]. Therefore, K63R mutants, already predicted to disrupt HR are put under additional stress by caffeine, which sensitizes cells to death by putting more pressure on the HR pathway.

A challenge for future investigations will be to identify the additional K63 modified substrate and their interacting partner and to understand how these chains interact with other pathways such as DDT and FA in mediating error-free

homologous recombination. Moreover, it will be essential to elucidate the exact role of caffeine in HR and its connection to spontaneous mutagenesis.

Acknowledgements

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Chapter 7
The *in vivo* role of K63R mutant ubiquitin in DNA repair

Contribution of Collaborators

The contents of this manuscript were written by Jan Brun and edited by Dr. Doug Gray. All performed experiments and figures presented in this manuscript were the work of Jan Brun. JB and DAG analyzed the data. JB provided the analysis tools and reagents for the experiments. The animal work was done with the technical assistance of Melissa Beyers and Steve Natal. Specifically, Melissa Beyers generated the compound transgenic mice homozygous for wild type or mutant ubiquitin and the LACI gene. Steve Nantal carried out the second round of daily UV exposures to the shaven backs of the transgenic animals.

Summary

Here we report the generation of transgenic mice in which the same expression system previously used to study the role of K63R mutant ubiquitin in stable cell lines provides constitutive expression of the ubiquitin transgene in majority of tissues. This study shows that K63R animals are normal with respect to DNA damage and show no overt increase in spontaneous or induced carcinogenesis which is likely due to compensatory mechanism that allows these animals to tolerate the mutant transgenes. In contrast we report a protective role of K48R and K63R animals in response to cisplatin which is a likely result of differing levels expression levels of GFP or HIS tag. During the course of these studies we unexpectedly found that K63R animals are resistant to the effects of a common anesthetic avertin.

The *in vivo* role of K63R mutant ubiquitin in DNA repair

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Running Title: K63R mutant ubiquitin does not affect DNA repair significantly in vivo

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Key words: RAD18, MMS2, UBC13, PCNA, DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63, lysine 63

Abstract

Although great progress has been made in recent years, the biological consequences of K63-linked chains in mammalian systems are yet to be fully elucidated. In a recent study we demonstrated that the expression of a UbK63R transgene resulted in an increased reliance on translesion synthesis and thereby caused increased mutagenesis. Although mammalian cell culture studies have been informative, the more direct measure of the importance of ubiquitin chain assembly may come from its disruption in an animal model. To this end, we have generated transgenic mice expressing wild-type and mutant ubiquitin isoforms which are expressed in most tissues. Here we report that spontaneously transformed transgenic MEFs demonstrate an enhanced sensitivity to UV; however primary MEFs grown at 3% O_2 do not show such a difference. The skin of K63R animals demonstrate increased erythema following acute UV exposures, however no histological differences were observed between the WT and K63R mice. Furthermore, long term and chronic UV exposures show no histological difference in hyperplasia of the epithelial layer or in the time of tumour formation between WT and K63R animals. Finally, contrary to our studies in A549 cells pointing to an increased spontaneous mutagenesis, there was no increased incidence in carcinogenesis K63R animals as compared to WT. This was further confirmed by using the Big Blue transgenic mouse mutation detection system. In summary, our results along with others suggest an important role for ubiquitination in DNA damage tolerance in human cells which was not exploited in the transgenic animals expressing K63R mutant ubiquitin.

1. Introduction

Ubiquitin is a pleiotropic molecule that is involved in a variety of biological processes concerning the elimination of short-lived or abnormal proteins. The proteolytic pathway is critical for cellular viability as defects are observed in certain cancers and several human diseases [1]. While polymerization mediated through K48 is recognized as a signal for proteolysis, other chains of varying topologies can be assembled via alternative linkages such as K63. The biological consequence of these linkages has been elucidated in yeast and more recently in human cells [2-5]. Notably, K63-linked ubiquitin chains form a linear polymer which plays a non-proteolytic role in signaling DNA repair processes after DNA damage including that of UV irradiation.

UV light induces two major forms of damage: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PP). Nucleotide excision repair (NER) is the primary mechanism which removes most of the UV lesions. However, since CPDs are a poor substrate for NER they often remain unrepaired and block replication [6]. Fortunately CPDs can be efficiently bypassed by a unique protein termed polymerase eta (POLη) which bypasses UV lesions in an error-free manner by inserting adenines across from thymine dimers [7]. Interestingly, POLη is a member of the Y family of polymerases which belong to a unique DNA damage tolerance pathway (DDT) [8].

Most of what we know about DDT has been elucidated in yeast [3]. It is divided into two subpathways: (1) error free damage avoidance, and (2) error prone translesion synthesis. Interestingly, both arms of DDT are governed by members of the ubiquitin family including a series of E2 conjugating enzymes (RAD6, UBC13

and MMS2) and E3 ligases (RAD 18 and RAD5) which utilize ubiquitin to converge upon the common substrate PCNA. Structural and biochemical studies show that the heterodimer of UBC13/MMS2 along with the E3 ligase RAD5 catalyze the formation of K63-linked chains on the replicative sliding clamp PCNA [9-11]. While the complex of RAD5/UBC13/MMS2 governs the error-free arm of DDT via PCNA polyubiquitination, RAD6 and RAD18 activate translesion synthesis (TLS) via PCNA monoubiquitination, a process that recruits error-prone polymerases including POLη, POLι, POLκ and POLζ to bypass damaged DNA [8]. The importance of error-free repair has been exemplified in yeast where a disruption of PCNA mediated ubiquitination via UBC13, MMS2 or K63R mutants shows extreme sensitivity to a DNA damaging agent, such as UV and increased mutagenesis [10-12]. Of late, the role of K63 linked chains has come to light in human cells in which the K63-linked polymers on PCNA protect cells from TLS induced DNA damage and gross chromosomal rearrangements [2,5].

Recently, the development of several animal models, namely of POLη and Polι, revealed the important role of the TLS arm of DDT *in vivo* [13,14]. Ohkumo et al. and Lin et al. demonstrated that a disruption of TLS POLη or POLι in a mouse model leads to increased predisposition of developing skin cancer after exposure to UV light, corroborating similar findings in human subjects. This study along with patients with XPV suggests that the TLS arm of DDT is conserved and functional in animal and human models. However, much less is known about the role of DDT and K63-linked ubiquitin chain in animal models presumably due to functional redundancy[15]. For example, in humans there are two homologs of RAD6, HR6A

and HR6B, which make it difficult to assess their importance in DNA repair [15]. An mRAD6B or mRAD6A knockout mouse is viable and phenotypically normal with respect to DNA damaging agents [16-18]. Thus, it has not been possible to experimentally show a role for error-free DDT in animal models. Although mammalian cell culture and the RAD6 animal studies have been highly informative, we believe that the more direct measure of the importance of K63-linked polyubiquitin chain assembly in DDT may come from the analysis of its disruption in a mouse model. To this end, the first transgenic mice expressing the 6XHisUbGFP (WT), 6XHisUbK48R (K48R) and 6XHisUbK63R (K63R) ubiquitin constructs have recently been published by our laboratory [19,20]. Because of the establishment of stable mutant ubiquitin mammalian cell lines and the transgenic mouse lines, we tried to exploit the role of K48 and K63 lysine linked chains in DNA repair *in vivo*.

2. Materials and Methods

2.1 Transgenic mouse strains and tissue collection

The development and genotyping of wiltype and mutant ubiquitin mice has been described elsewhere. Big blue mice homozygous for the LacI transgene were purchased from Stratagene, crossed with FVB/N ubiquitin transgenic animals, and bred to homozygosity for the ubiquitin mutants and the LacI gene in the FVB/N background. Genotypes for LacI ubiquitin crosses were determined by PCR using specific LacI primers from Stratagene. Compound transgenics homozygous for Wiltype, K48R and K63R ubiquitin, and 'Big Blue' (Lac I transgene) were sacrificed at 3 or 24 months. Whole organs including small intestine, heart, liver, and spleen were snap frozen.

2.2 DNA extraction, Viral packaging and Plating

The standard Big Blue protocol was followed to extract DNA, package DNA and plate the packaged reaction (Stratagene). Briefly, small intestine from various Big Blue ubiquitin crosses were thawed followed by DNA extraction using phenol-chloroform and ethanol precipitation. Genomic DNA was packaged by lambda phage using transpack (Stratagene). The packaged sample was pre-titred using 1-2ul of the packaged phage solution and infecting SCS-8 E. coli cells at an optical density of 0.5 followed by duplicate plating in 10cm dishes. The plaques were counted and used to determine the plating density in the subsequent plating of approximately 4000 plaques per 15cm plate. Standard color controls (CM0 and CM1) from Stratagene served as controls to monitor color sensitivity, and X-gal (Bio Mol) was used as the chromogenic substrate. Blue plaques were counted against a white background and mutation frequency was calculated using the following formula Mutation Frequency=blue plaque # /total # of screened plaques.

2.3 Cells cultures and Cell survival

Mouse embryonic fibroblast cell lines (MEFs) were established from non-transgenic, Wild type, K48R and K63R embryos at day 13.5 of gestation. Briefly, embryos were separated from the placental sac followed by washing in Hanks balanced salt solution (GIBCO). The embryos were minced and incubated in 0.25% trypsin (Gibco) for 30 min at 37°C. The resulting cell suspension was gently vortexed, pelleted by centrifugation and resuspended in fresh medium containing

DMEM and FCS. After passaging the cell lines twice, cells were examined by flow cytometry for GFP fluorescence using the Becton Dickson Fluorescence-activated cell sorting instrument.

Primary MEFs were cultured in a 3% O_2 incubator and exposed to varying doses of UV light followed by counting surviving cells using a coulter counter at 24 and 48 hours. MEFs were also allowed to reach crisis and spontaneously transform in a standard 20% O_2 incubator. The sensitivity of spontaneously transformed MEFs to UV-irradiation or cisplatin was evaluated using clonogenic survival assays. UV-irradiation was performed on 65% confluent cells in 10cm dishes using a UVC germicidal lamp at a fluence of $1J/m^2/s$. Spontaneously transformed MEFs were treated for 1h in cisplatin diluted in culture media. Cells were plated in 6 cm dishes in triplicate and incubated for 2 weeks to obtain colony formation. Colonies were fixed, stained with 2% bromophenol blue in 70% ethanol and colonies containing ≥50 cells counted. All experiments were normalized for plating efficiency.

2.4 Acute effects of UV and cisplatin

A UVB handheld device was used to examine the response of the shaven skin of our mice to acute doses of UV light ranging from $0\text{-}2000J/m^2$ at 48 hours post irradiation. Skin samples were taken and routinely processed for histopathology and examined by hematoxylin and eosin staining. Transgenic mice expressing wild type and mutant ubiquitin received an intraperitoneal injection of cisplatin (15mg/kg) and were monitored for acute toxicity in terms of general appearance, locomotion of mortality and changes in body weight.

2.5 UV induced Carcinogenesis

Long term UV exposure was studied by exposing the shaven back of our WT, K48R and K63R mice to either 100 or 500 J/m^2 of UVB light for 4 consecutive days at a fluence of 3.3 J/m^2. On day 5 mice were euthanized and skin samples were fixed in 10% NBF and processed for routine histology.

UV induced carcinogenesis was studied by chronically exposing the shaven backs of 12 mice from each group to UV light using an incremental dose protocol starting at 80 J/m^2/day to 700 J/m^2/day, gradually increasing the dose by 20 J/m^2/week. Using 4 overhead UVB lamps positioned 3 feet above the cage animals were irradiated with a dose rate of approximately 0.15J/m^2/s. Tumour appearance was checked once a week after cessation of UV exposures and upon establishment of a visible and palpable tumour, tumour size was measured. Once tumours reached a diameter of 7-10mm, animals were euthanized and the tumours were removed followed by routine processing for histological examination.

3. Results

3.1 Dominant Negative Strategy to Disrupt K63 ubiquitin chain formation in vivo

MEF lines were established from the UbGFP, K48R and K63R to assess GFP levels as a measure of transgene expression and their sensitivity to DNA stressors including UV light and the chemotherapeutic agent cisplatin. In previous publications from our laboratory anti-HIS and anti-GFP immunoblots reveal that the ubiquitin

GFP constructs are cleaved into GFP and monomeric ubiquitin which is subsequently incorporated into ubiquitin chains [2,19-22]. Interestingly, these blots reveal that there was slightly more GFP in the K48R animals as compared to WT or K63R which is in agreement with levels of monomeric versions of the transgene in the same tissues [19-21]. As further corroboration of the latter, GFP fluorescence levels were subsequently analyzed by flow cytometry. Flow cytometry data showed that all MEF lines, except for the non-transgenic, were positive for GFP; however, the GFP seemed to be more highly expressed in K48R and K63R MEFs as compared to WT MEFs (Figure 1).

3.2 Sensitivity of K63R mouse embryonic fibroblasts to genotoxic stress

Cells were grown under normoxic conditions (20% O_2) and at a reduced oxygen tension of 3%. We first examined whether inhibition of K63 linked chains

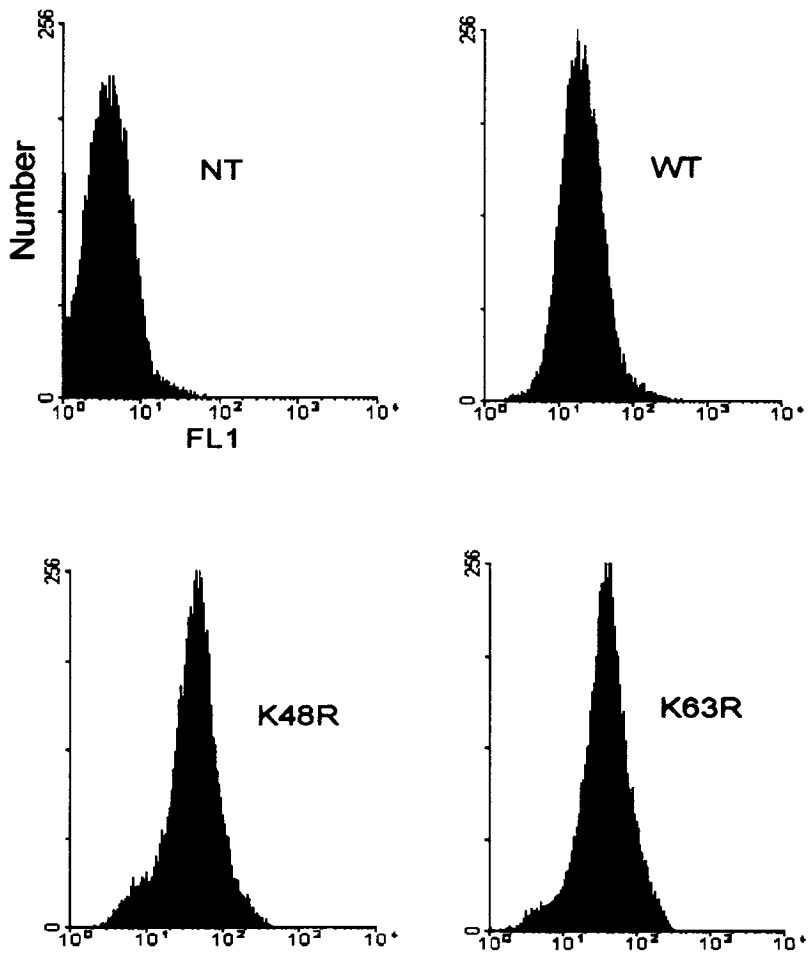

Figure 1. Flow cytometric analysis of GFP expression in transgenic MEFs. GFP levels in the K48R and K63R MEFs are higher as compared to wild type or non-transgenic MEFs .

results in increased sensitivity to agents such as UV light in exponentially growing MEF lines derived under normoxic conditions. We found that UV light, an environmental mutagen highly toxic to yeast mutants, is also significantly more toxic to K48R and K63R MEFs (Figure 2A). This effect seems to be specific to K48R and K63R MEFs as similar toxicity was not observed in WT. This result implies that both K48 and K63 chain assembly are potentially required for UV lesions. In contrast, the highly toxic chemotherapeutic agent cisplatin known to sensitize yeast and human K63R mutants did not sensitize K63R MEFs as compared to WT or K48R (Figure 2B). In fact, K63R exhibited a significant protective effect in response to cisplatin while K48R MEFs displayed a moderate protective effect at the higest dose of 20μM. These results were in stark contrast to those observed in yeast and A549 cells overexpressing the same K63R mutant, and they suggest a potential role for K63 linked chains in the uptake of cisplatin. Furthermore, MEFs grown at 3% O_2 (known to mimic the oxygen tension *in vivo*) were also tested for their sensitivity to UV light. Under these growth conditions no significant differences were observed amongst the MEF lines with respect to the effects of UV light (Figure 2C). This result is markedly different from the one obtained with spontaneously transformed MEFs and may indicate a reduced role of K63-linked chains in an environment with lower levels of oxidative stress.

3.3 Acute Sensitivity of K63R mice to cisplatin

Based on cell culture results in A549 cells we begun studies with cisplatin in our transgenic animals. These initial studies were meant to explore the acute toxicity

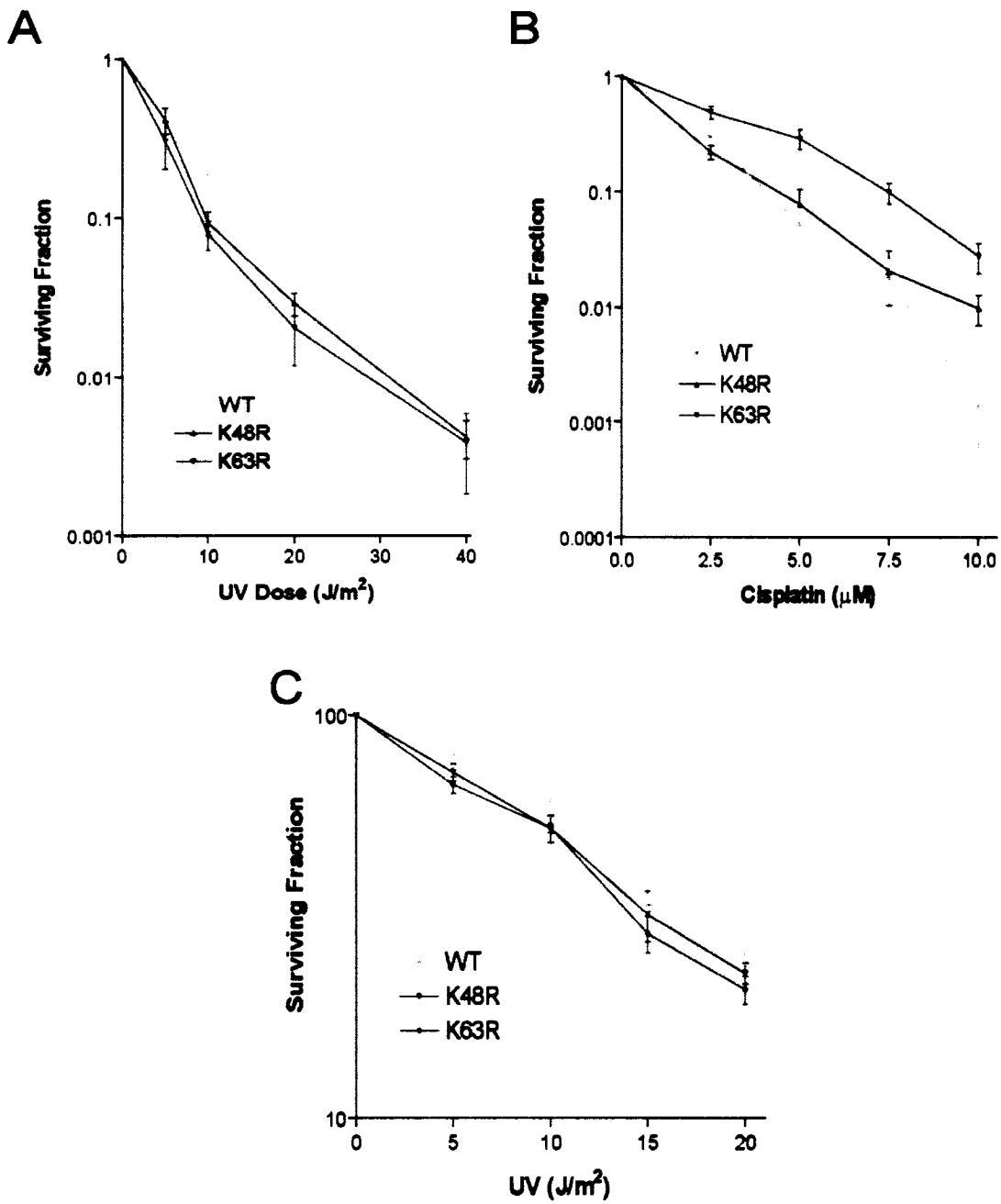

Figure 2. Sensitivity of WT and mutant ubiquitin expressing MEFS to genotoxic stress. Clonogenic survival assays were used to determine sensitivity to **(A)**UV light or **(B)**1h acute treatment with cisplatin in non-transgenic Mefs and MEFs derived form traangenic animals expressing *WT-Ub* or *K63R-Ub*. The mean values of 3 independent experiments are shown with standard error of the mean (error bars). **(C)** Exponentially growing transgenic MEFs grown at 3% O_2 were subjected to UV irradiation followed by coulter counting surviving cells at 24 and 48hours post-treatment. The mean values of 3 independent experiments are shown with standard error of the mean (error bars)

of cisplatin using a one-time dose of 15mg/kg intraperitoneal (IP) injection. Our initial results indicate no overt sensitivity to the chemotherapeutic drug cisplatin in the K63R mice, while K48R mice exhibit a protective effect as compared to UbGFP mice (Figure 3A). A repeat of this experiment revealed a mild protective effect with the K63R and K48R as compared to the UbGFP animals (Figure 3B). We do not yet know the physiological nature of this protective effect. The K48R and K63R mice do corroborate findings in MEF cell lines with respect to cisplatin sensitivity.

3.4 Acute Sensitivity of K63R mice to UV light

UV irradiation has two distinct effects on the skin. Acute exposure causes inflammation characterized by erythema and edema while a long-term exposure causes scaling characterized by hyperkeratosis and hyperplasia. We examined both measures of photosensitivity in K63R mice. Mice exposed to one time acute doses of 500, 1000 and 2000 J/m^2 of UV light showed marked erythema and edmema 48 hours post-irradiation (Figure 4A). K63R mice consistently showed a moderately increased erythema as compared to NT, WT, or K48R mice. However, histopathology showed no difference of any kind in the transgenic mice (data not shown).

Mice were also examined after long-term exposures to UV light. Mice were either irradiated with 100 or $500J/m^2$ for 4 consecutive days. K63R mice showed a modest increased erythema as compared to controls only in the higher dosed group (Figure 4B). However, histopathology demonstrated similar levels of hyperplasia in K63R mice as compared to control animals (Figure 4C). Overall, we found no significant evidence that disruption of K63-linked ubiquitination independently

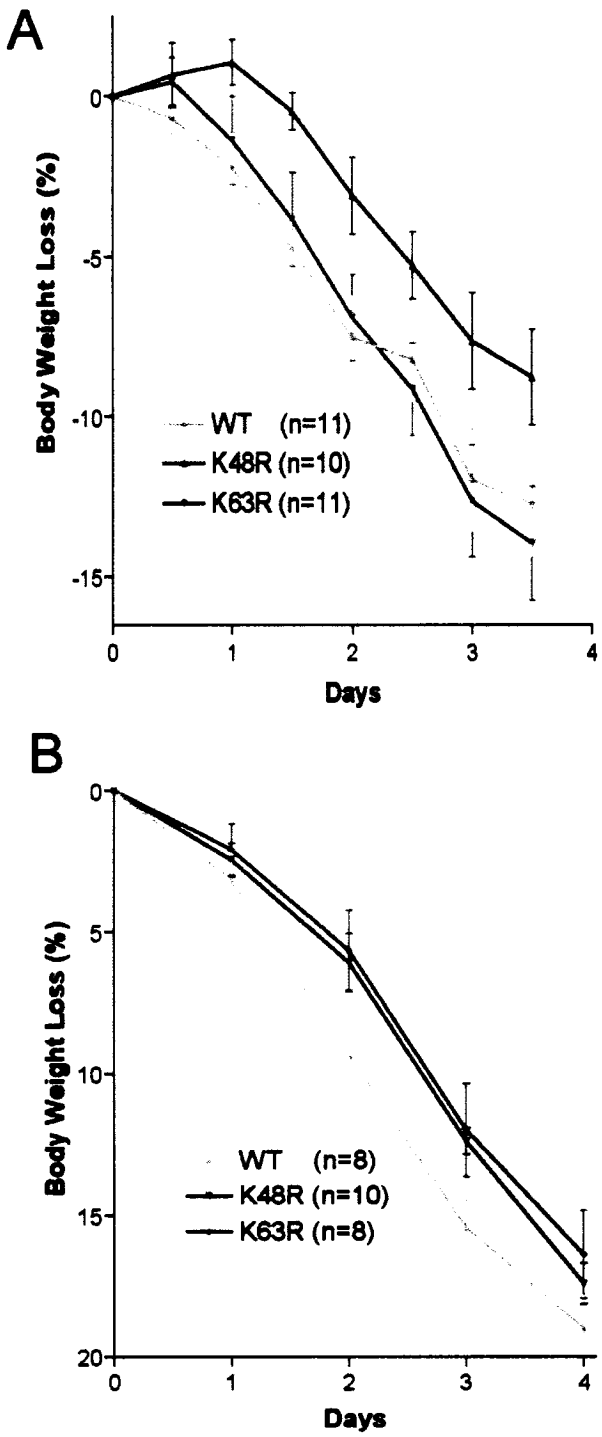

Figure 3. Acute sensitivity of mutant ubiquitin animals to cisplatin. (A) WT, K48R, and K63R transgenic animals received a one time intraperitoneal injection of cisplatin (15mg/kg). Body weight and other symptoms of toxicity were monitered over 72 hours. **(B)** A Repeat of the experiment seen in Figure 3A. The mean values are shown with standard error of the mean

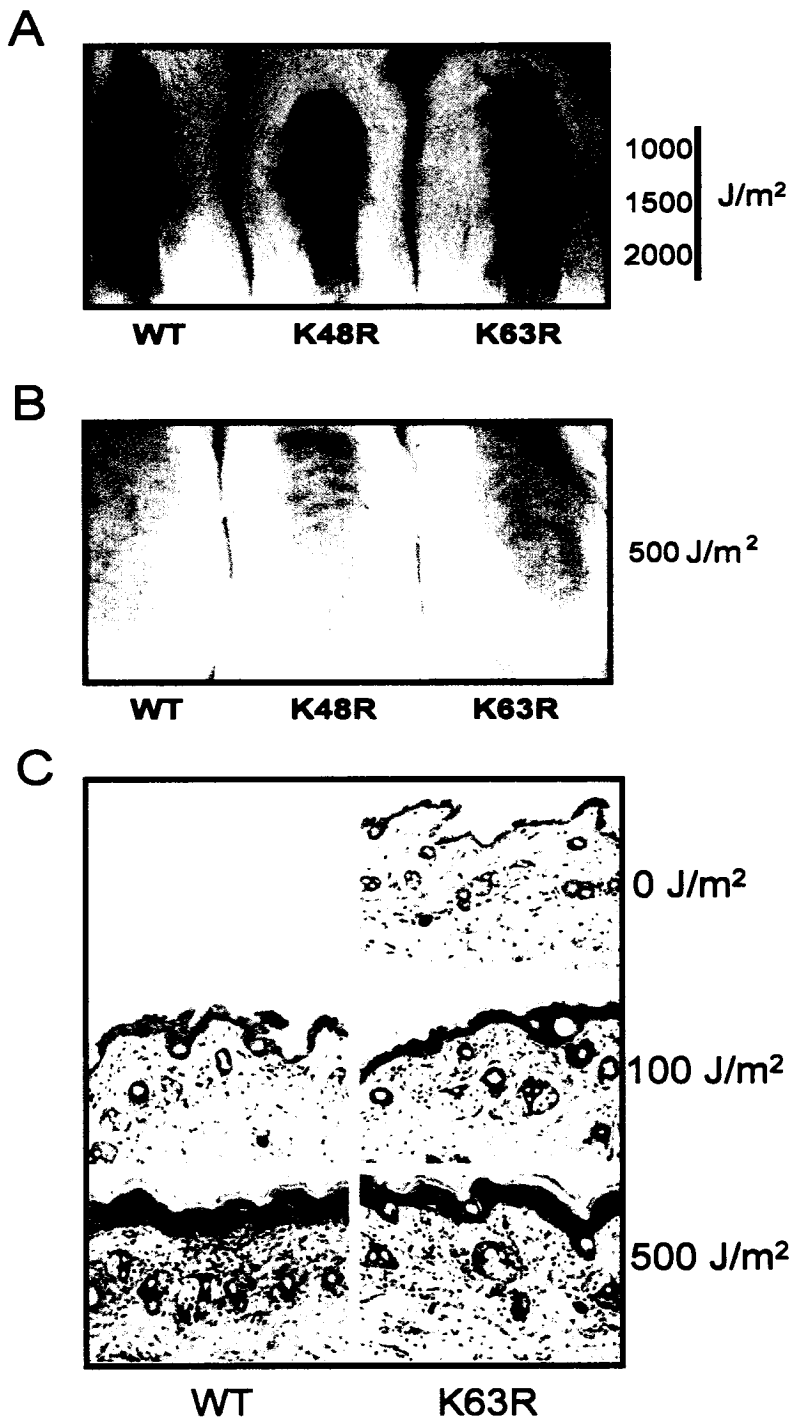

Figure 4. Skin sensitivity due to the effects of UV light in K63R animals. (A) Digital images of WT and K63R animals received various one time acute doses of UV and examined 72 hours post-treatment. **(B)** Digital images of transgenic animals that were treated with 500J/m^2 of UV light for 4 consecutive days. **(C)** Hemotoxylin and eosin stained histological sections of skin samples taken from animals treated with either 100 or 500J/m^2 of UV light for 4 consecutive days.

influences UV toxicity. A possible explanation for this lack in sensitivity maybe be the ability of K63R mice to compensate for the loss of K63-linked polyubiquitination through the increased use of the TLS arm of the DDT pathway. If this assertion is true then chronic UV exposures may translate into early tumour development, a higher frequency of tumour formation, or both.

For the UV acute exposures all transgenic lines were anesthetized with avertin to allow high accurate dosing to the exposed skin. Dosing of avertin was based on body weight of the animal and was delivered IP. Interestingly, it was noted that K63R mice were highly resistant to the effects of avertin with respect to the time to sedation and often required an additional dose to anesthetize. We examined this unique phenomenon more closely and found that 50% of K63R mice were protected against sedation with avertin (Figure 5A). This was based on their continued mobility about the cage 5 minutes post injection while 90% of K48R and 100% of WT animals had succumbed to the effects of avertin within 60-90 seconds post injection (Figure 5B).

3.5 Induction of skin cancer by chronic exposure to UV irradiation

Data from A549 cell lines suggest that the frequencies of UV induced mutagenesis maybe higher in K63R transgenic mice as a result of the deficit in error-free DNA damage avoidance. To test this hypothesis we constructed a custom-built cage rack that would deliver a UVB fluence of $1J/m^2/s$. The backs of a cohort of age and sex matched mice were shaven and exposed to UVB light using an incremental protocol wherein the daily dose started at $80J/m^2$ and escalated by 20 J/m^2/week to a final dose of $700J/m^2$, which was maintained until mice developed tumours. Contrary

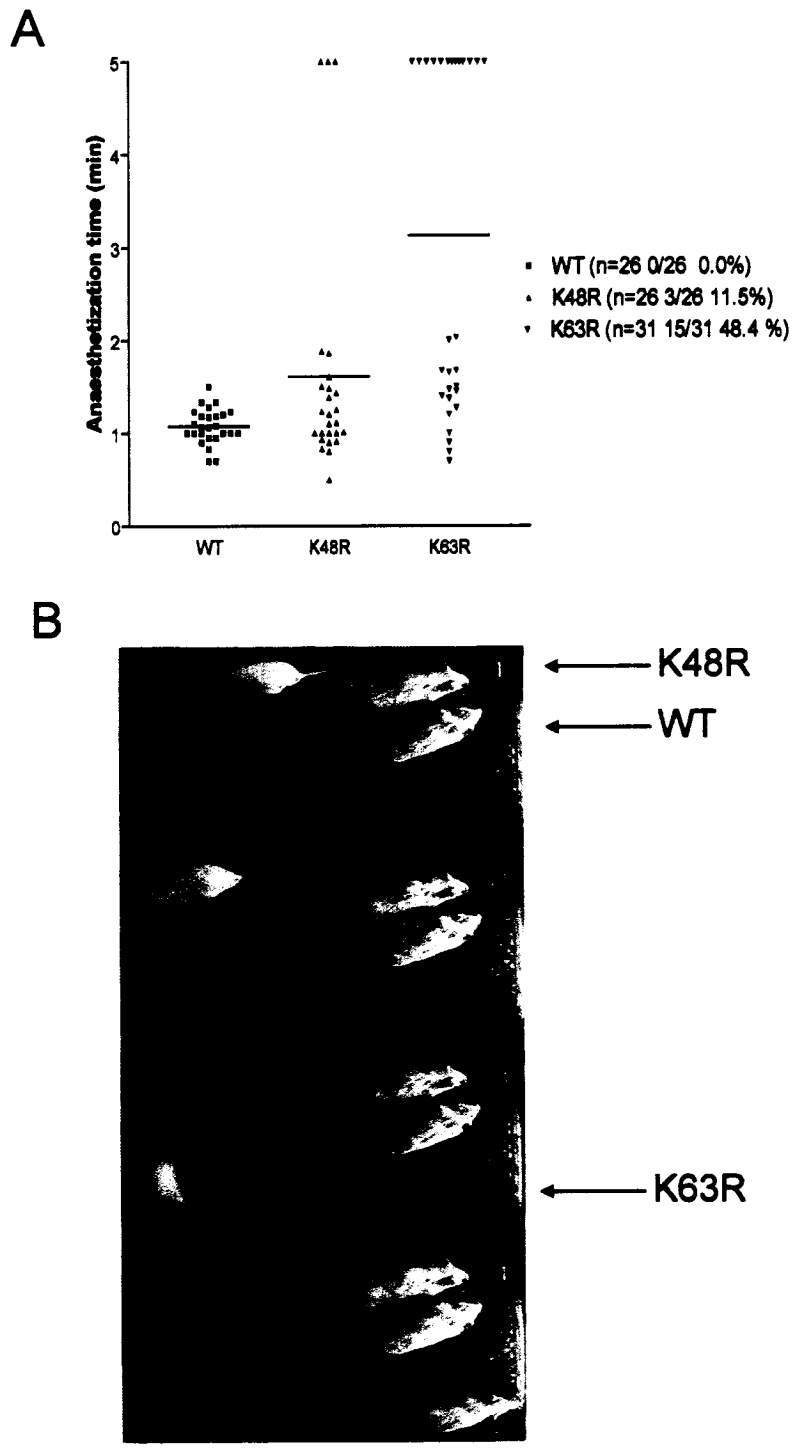

Figure 5. K63R animals are resistant to then effects of avertin. (A) Histogram representing the number of K63R animals that are resistant to the effects of Avertin The mean values are shown **(B)** Digital pictures of a K63R animal that continues to move around the cage after avertin injection as compared to WT and K48R animls which succumb to the anastehtic within 2 minutes.

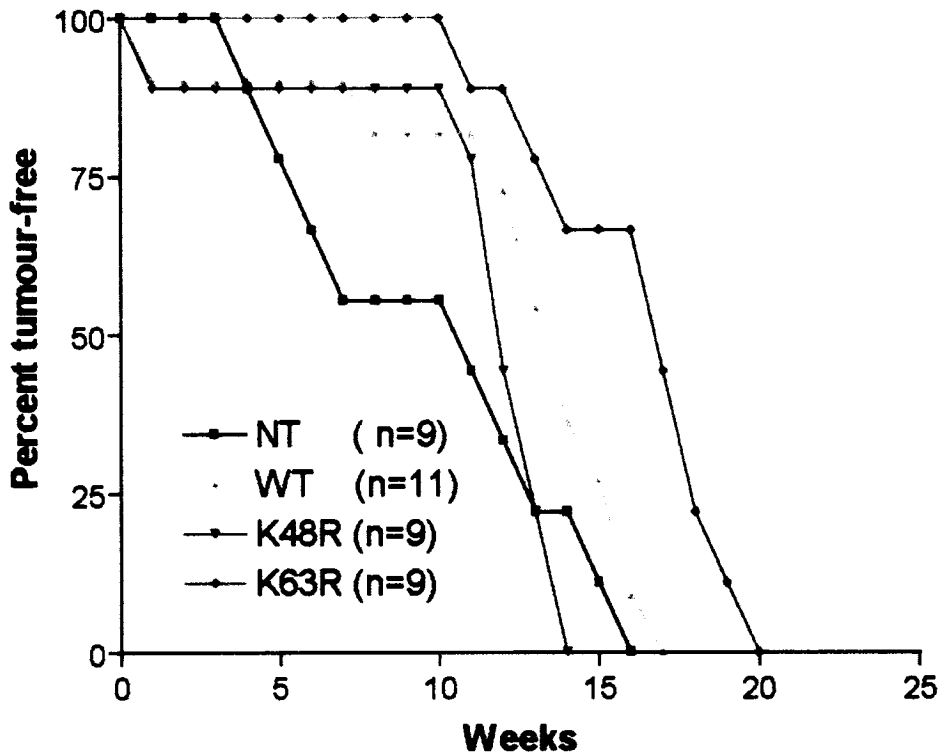

Figure 6. Skin carcinogenesis in K63R mice. Animals were irradiated with UVB at a fluence of $1J/m^2/s$ starting from a total dose of $80\text{-}700J/m^2$/week with increments of $20J/m^2$/week until formation of tumours was observed.

to our prediction, the cohort of mice expressing K63R mutant ubiquitin did not display accelerated tumour development. Rather, depending on which control they are compared to, it would appear that skin tumour development is moderately delayed by 1-2 months (using the time at which 50% of mice had tumours). However, by the end of the experiment when all mice had developed tumours no significant difference was observed (Figure 6).

In order to determine whether the K63R phenotype results in a delay in tumourgenesis we initiated a second series of UV exposures under a modified UV exposure regimen. Mice were exposed at a lower fluence (0.15 $J/m^2/s$ versus 1 $J/m^2/s$) and daily exposures ceased when the dose reached 700 J/m^2. Our expectation was that by spreading the UV dose over a prolonged period of time, the modified regimen would better reveal the subtle difference in tumour development or frequency of tumour formation that may have been masked by the initial concentrated dose of our initial experiment. Ear samples taken from all animals 3 months after the commencement of the second experiment revealed no histological differences in K63R animals as compared to controls. Mice started developing visible and palpable tumours 6 months post UV exposures. Contrary to expectations, no delay in tumour development, tumour type, or frequency of tumours was observed in K63R mice as compared to NT, WT or K48R mice (data not shown).

3.6 Spontaneous mutation frequency *in vivo*

Increased spontaneous and induced mutagenesis was detected in our cell culture studies. Therefore, it is reasonable to assume that we would find an increased mutagenesis *in vivo*. Consequently, we attempted to determine the mutation

frequency directly *in vivo* by crossing our K63R and K48R transgenic mice with the Big Blue transgenic mouse reporter strain. The Big Blue mouse is useful for assaying any cell or organ for spontaneous or induced mutagenesis [30, 31]. It contains a chromosomally integrated lacI repressor gene in tandemly repeated shuttle vectors that are flanked by cos sites. These cos sites allow for the packaging of DNA by λ phage. Phage is then used to infect Escherichia coli delivering lacI reporter gene and form plaques in the lawn of E. coli. Most phage particles will encode a functional lacI repressor and will preclude the expression of the B-galatosidase reporter gene encoded by E. coli. However, if the *LacI* gene suffers an inactivating mutation while resident in our compound transgenic animals it will be unable to repress bacterial B-galactosidase and its plaques will turn blue in the presence of the chromogenic substrate X-gal.

For the initial analysis we chose to determine the frequency of spontaneous mutations in small intestines, a tissue in which mutations are frequent and accumulate with age. Mice were bred to be homozygous for both the lacI gene and ubiquitin transgene. We plated approximately 4000 plaques per 15 cm plates and have scored only those blue plaques with even staining throughout the circumference (disregarding sectored plaques and pinpoint plaques that may reflect mutations occurring during phage replication). The preliminary data are consistent with a spontaneous mutation frequency that is significantly higher (by 4 fold) in 24 month animals versus younger 3 month animals. However, no trends or differences in spontaneous mutagenesis were observed between K63R mice or control animals. In fact the levels of mutations were the same across all 4 transgenic lines of animals

A

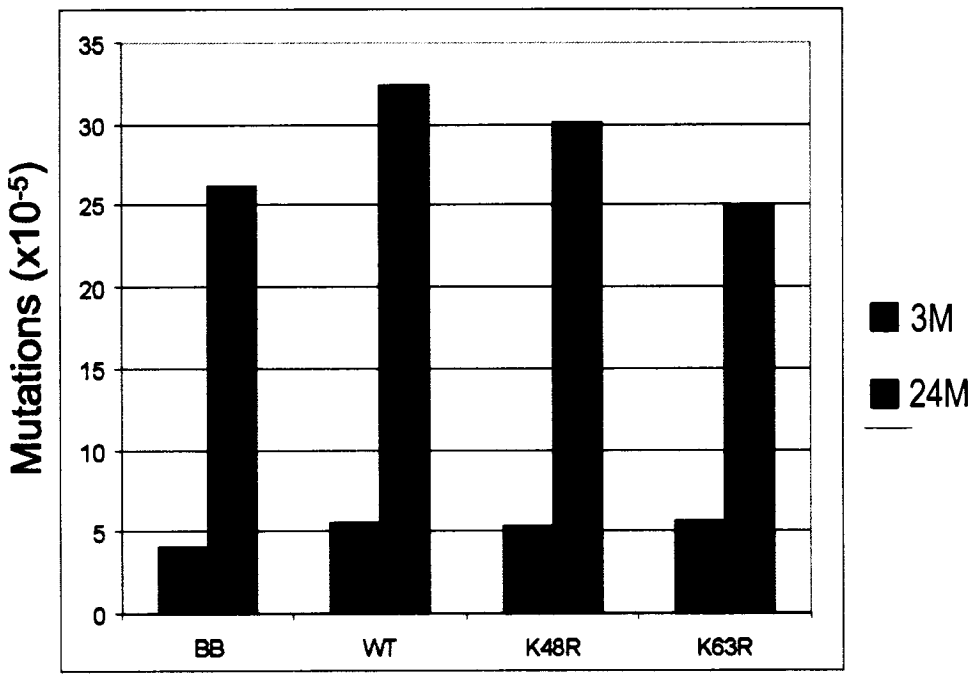

Figure 7. Spontaneous mutation frequency in animals expressing K63R mutant ubiquitin. Compound transgenic animals homozygous for *LACI* and the K63R mutation were generated followed by the examination of sponatenous mutation frequency in small intestines as per protocol (Stratagene). The mutation frequency of 3 month old and 24 month old transgenic mice was compared as was the mutation frequency amongst the transgenic animals of the same age.

(Figure 7). These data are based on an analysis of 300 000 plaques (which has been shown to be adequate to reveal at least a 2 fold difference in mutation frequency) per animal and for only one animal of each strain. Therefore, these results are clearly preliminary.

4. Discussion

Having established the absolute requirement for DDT in yeast and higher eukaryotes, we sought to determine a role for DDT in whole organisms. One could explore the role of error free DDT by generating mutations in UBC13 or MMS2, but as in mammalian cells the same sensitivities should be imparted by expression of K63R mutant ubiquitin. Therefore, we believed that the more relevant test of the importance of error-free DDT would come from its disruption in our transgenic animals expressing WT, K48R, or K63R mutant ubiquitin. Our data from cell culture studies predicts that mice expressing the K63R mutant ubiquitin would demonstrate enhanced sensitivity and mutagenesis upon exposure to DNA damaging agents. To test whether these observations translated into observable phenotypes in whole organisms, we examined the acute, long-term, and chronic effects of UV irradiation on the shaven backs of our ubiquitin transgenic animals. Surprisingly, no significant difference was observed between K63R mice and controls with respect to acute or chronic effects of UV light. Tumours developed with the same latency and frequency across all 3 ubiquitin transgenic lines as compared to non-transgenic animals. We also examined the spontaneous mutation frequency in the small intestine in mice bred to homozygosity for the ubiquitin transgene and the *LACI* gene. As evidenced by the

uptake of tritiated thymidine, the epithelium of the small intestine is the most rapidly proliferating tissue in mice and has been shown to have more frequent mutations that accumulate with age [23,24]. We speculated that the rapid proliferation of intestinal crypt epithelium forces DNA repair systems to work at their capacity and such cells would be particularly sensitive to the overt deficit in repair that cells expressing K63R mutant ubiquitin would impose [25]. Unexpectedly, spontaneous mutation frequency in the gut epithelium did not change in the presence or absence of the K63R transgene in 3 month old or 24 month old animals. This experiment utilizing the Big Blue mice agrees with observations over several years of mouse breeding as the K63R mice never showed an increased predisposition to spontaneously occurring tumours in any tissue as might have been predicted from our mutagenesis studies in A549 cells.

There are several probable explanations for our *in vivo* findings with respect to UV light and spontaneous mutagenesis. First the level of endogenous ubiquitin genes could be upregulated in response to the mutant transgene levels which could compensate for the levels of mutant ubiquitin transgene in the mice. However, the most parsimonious explanation for our findings could simply be that the expression levels of our transgene are too low to demonstrate an interesting biological effect [26]. In fact an experiment was performed by Gilchrist et al. that showed that WT and K48R transgene makes up only 9-11% of the total ubiquitin pool in the cell [26]. It is conceivable that cells need much more mutant ubiquitin to disrupt DNA repair processes requiring K63 linked ubiquitination.

Cisplatin was another genotoxic agent that showed toxic effects in K63R mutants in both yeast and human cells. In contrast our K48R and K63R transgenic mice showed a moderate protective effect against the effects of cisplatin as compared to WT animals. In fact, the K48R mice seem to be resistant to a variety of insults including those of canavanine and LPS [27]. Moreover, our laboratory recently published that the K48R transgenic lines delay the onset of spinocerebral ataxia, an incurable neurodegenerative disease [21]. One potential explanation for these counterintuitive findings may lie in the reprogramming of gene expression by mutant ubiquitin, while another simpler alternative may lie in the expression levels of the ubiquitin transgenes in these animals. In the latter study, anti-his immunoblots show higher levels of K48R monomeric transgene as compared to WT or K63R, which is similar to findings by Zhang et al. in the cerebella of the same transgenic lines [19]. We further confirmed this by examining GFP levels using flow cytometry which showed the highest amount of GFP in K48R mice, with intermediate levels in K63R mice and the lowest levels in WT mice. Interestingly, it has recently been reported that GFP inhibits polyubiquitination which results in the attenuation of several important pathways and stabilization of several genes including the tumour suppressor p53 [28]. Consequently, the moderately elevated levels of GFP in K48R and K63R as compared to WT animals may confer the observed protective effects. Furthermore, the ubiquitin transgene is tagged at the N-terminus with a 6XHis tag. The histidine tag on ubiquitn may inhibit proteasome function which could contribute to increased stability of certain substrates (personal communication of Martin Scheffner, University of Konstanz). Thus, increased levels of His tag and GFP in

K48R mutants could help explain the protective phenotype and why certain substrates are stabilized as compared to WT mice.

Interestingly, we also noted that the K63R transgene conferred protection against the effects of avertin. This protective effect seems to be specific to the disruption of K63-linked polyubiquitination and independent of GFP levels and His tag levels which have been shown to confer protective effects independent of transgene expression. If we were to expect a protective effect from GFP or His tag the expectation would be that K48R animals would be more resistant to the effects of avertin since GFP and subsequent His tag transgene levels are highest in this line of animals. In fact we see that K48R animals succumb to the effect of avertin similarly to WT animals. To date, very little is known about the molecular mechanisms governing the effects of avertin [29]. However, it is interesting to speculate that the drug influx or efflux mechanism may somehow be affected by the K63R ubiquitin mutant or other functions such as synaptic trafficking.

In conclusion we report the successful generation of transgenic animals expressing various forms of mutant ubiquitin most notably K48R and K63R. Under stress free conditions the phenotypes of these K48R and K63R mice were too subtle to detect. However, under stressful conditions that place higher demands on the ubiquitin pools certain phenotypes were revealed. In particular, K48R and K63R animals were resistant to the effects of cisplatin while only K63R animals were resistant to the effects of avertin. The resistance to avertin in K63R animals is not understood but may work through other functions of K63-linked signaling. We attempted to attribute the protective function of these animals to cisplatin increased

levels of GFP and HIS. GFP and HIS effects may contribute to proteolytic deficiencies under conditions where proteolysis is heavily burdened, and this may be most evident in K48R because of the combinatorial effects of the ubiquitin mutation and higer levels of GFP and His tag. However, it is plausible that the effects of GFP and HIS are subtle because they do not affect longevity or fertility in these animals. Moreover, we found all transgenic animals normal with respect to UV damage, presumably due to low expression levels of the mutant ubiquitin transgene. Again the latter argues for subtle effects of GFP and HIS in these animal models. Therefore, it has not yet been possible to experimentally demonstrate the importance of the error-free damage avoidance branch of DDT in an animal model.

In light of our data, we suggest that future investigators target the genes that catalyze the formation of K63-linked ubiquitin chains including the likes of RAD18, UBC13, SHPRH (human ortholog of yeast Rad5) or MMS2/UEV1a. We speculate that animals with such an overt defect would undoubtedly reveal the importance of K63-linked polyubiquitinated PCNA in an animal model.

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Chapter 8

The *in vivo* role of K63R mutant ubiquitin in NF-κB activation

Contribution of collaborators

The contents of this manuscript were written by Jan Brun and edited by Dr. Douglas Gray. All performed experiments and figures presented in this manuscript were the work of Jan Brun. JB and DAG analyzed the data. JB provided the analysis tools and reagents for the experiments. The animal work was done with the technical assistance of Sherry Thurig. Dr. Chris Kennedy provided us with the mouse tail blood pressure monitor.

Summary

The heterodimeric transcription factor NF-κB is activated during innate and adaptive immune responses by proinflammatory such as cytokines bacterial and viruses. Since K48 and K63 linked chains play multiple roles in the eventual activation of NF-κB, we sought to determine whether our mutant ubiquitin cell lines and transgenic animals expressing mutant ubiquitin were deficient in NF-κB activation after stimulation with a bacterial endotoxin LPS and various cytokines. Here we report that mutant ubiquitin mice survive lethal doses of LPS and that this is independent of NF-κB activity. This suggests that other mechanisms are at play *in vivo* that are not well modeled in cell culture.

The *in vivo* role of K63R mutant ubiquitin in NF-κB activation

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Running Title: K63R mutant ubiquitin does not affect DNA repair significantly in vivo

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Key words: RAD18, MMS2, UBC13, PCNA, DNA damage tolerance, translesion synthesis, ubiquitin

Abbreviations: DDT, DNA damage tolerance; TLS, translesion synthesis; K63, lysine 63

Abstract

Besides the important role of K63-linked polyubiquitin chains in DNA repair, recent publications implicate the same novel chains in signal transduction processes, those predominantly involved in the activation of the transcription factor, NF-κB. NF-κB is a tightly regulated heterodimeric (p50 and p65) transcription factor that belongs to the Rel family of proteins. It plays a central role in innate immunity, being responsible for the induction and expression of many important cellular genes after stimulation with LPS and cytokines including TNF-α and IL-1β. Not surprisingly, aberrant regulation of NF-κB results in immune related disorders and cancer. Therefore we hypothesized that the development of transgenic animals expressing dominant negative ubiquitin mutants would lead to an overt phenotype similar to those reported in animals where NF-kB activation was disrupted. We report that transgenic animals expressing K63R mutant ubiquitin do not exhibit any such overt phenotype. In fact NF-κB is activated similarly in K63R MEFs as compared to WT MEFs. Furthermore, high doses of LPS lead to a similar survival in both K63R and WT animals. In contrast we observed a protective effect in K48R transgenic animals which was independent of NF-κB activation. Interestingly, lower doses of LPS lead to protective effect in both K63R and K48R mice as compared to WT. This protective effect was associated with a moderate improvement in blood pressure 24 and 72 hours post-LPS treatment. Our results point to a protective role of mutant ubiquitin *in vivo*.

1. Introduction

The premise of the innate immune response is rapid protein modification in which ubiquitin plays a key role in activating NF-κB [1,2]. Multiple myeloma (MM)

and endotoxic shock are two examples of the functional importance of ubiquitin in the NF-κB pathway [3,4]. MM is a hematological malignancy of the bone marrow characterized by the monoclonal hyper-proliferation of plasma cells. One of the molecular hallmarks of MM is the constitutive activity of NF-κB which is associated with chemoresistance [4]. Endotoxic shock commonly results from a systemic bacterial infection characterized by hypotension, vascular damage, and inadequate tissue perfusion often leading to the failure of many organ systems including liver, kidney, heart and lungs [3,5,6]. The pathogenesis of septic shock seems to be primarily governed by lipopolysaccharide (a component of the outer membrane of gram negative bacteria). It works by stimulating the expression of several proinflammatory proteins such as TNF-α, IL-1β, IL-8, IL-6, cyclooxygenase (COX), and inducible nitric oxide synthase (iNOS)[1,3]. Significantly, NF-κB is stimulated by many of these endogenous mediators in a paracrine and autocrine fashion. Despite available therapies such as volume replacement, antibiotics, and vasopressor support, endotoxic shock remains a common cause of death in intensive care units [6]. Therefore inhibition of NF-κB may be of potential therapeutic benefit in the treatment of both MM and septic shock [7].

Ubiquitin plays a role at several levels of NF-κB activation [2]. Upon extracellular stimulation, adaptor proteins such as TRAF2, TRAF6, IRAK-1, IRAK-4 and MyD88 are recruited to the cytoplasmic domain of the receptor [8]. Subsequently, TRAF2, and TRAF6, shown to act as an E3 ubiquitin ligase, recruits and interacts with the UBC13/UEV1A heterodimer through its ring finger domain [9]. While TRAF2 is critical in mediating TNF-α signaling through itself and RIP

[10,11], TRAF6 mediates CD40, IL-1β and LPS signaling through itself [12,13]. The Ubc13/UEV1A heterodimer is a similar complex that works in DNA post-replication repair catalyzing the synthesis of K63-linked polyubiquitin chains. The blockade of this linkage via a lysine to arginine mutation at position 63 was shown to abrogate activation of IKK by TRAF6 *in vitro* and this blockade is independent of proteasome inhibition [9]. Interestingly, both TRAF2 and TRAF6 are unique E3 ligases that catalyze their own polyubiquitination linked through lysine 63 [13,14]. Moreover, TRAF2 UBC13/UEV1a ubiquitinates RIP via K63 linkages. These linear chains are the primary signal responsible for initiating a kinase cascade that recruits and directly activates the TAK1-TAB2-TAB3 and the IKK complex [15]. Specifically, TAK1-TAB2-TAB3 recognizes K63-linked chains via a highly conserved zinc finger motif which may facilitate the oligermerization of the complex and promote autophosphorylation and activation of TAK1 [15]. Interestingly, NEMO (IKKγ) a member of the IKK complex is also modified by K63-linked chains and also contains a ubiquitin binding domain that specifically binds to these linear chains [16]. It seems that K63 –linked chains are serving scaffold function to form a multi-subunit signalosome which brings TAK1, IKK and TRAF complexes into close proximity. TAK1 then phosphorylates the IκB Kinase (IKK) complex namely IKKα and IKKβ. IKKβ proceeds to phosphorylate IκBα (inhibitor, which sequesters NF-κB in the cytoplasm) at serines 32 and 36. Upon phosphorylation, IκBα is ubiquitinated via a K48 linkage and transported to the 26S proteasome for degradation, a process that can be disrupted by PS-341 [17,18]. NF-κB then translocates to the nucleus where it binds to specific promoters in target genes and stimulates their transcription. This

process is tightly regulated by NF-κB dependant transcription of its direct inhibitor IκB-α. Moreover, ubiquitination is negatively regulated by the zinc finger protein A20, tumour suppressor CYLD, USP31 all of which disassemble K63-linked polyubiquitin chains on TRAF2 and TRAF6 thereby preventing the formation of the signalosome[19-23] . Since K63 linked chains assemble and act at the very earliest of the inflammatory responses we proposed to use our stable cell lines and our transgenic animal model to explore the effects of mutant ubiquitin in this pathway. We hypothesize that a disruption in polyubiquitination will result in a disruption of NF-κB activation.

2. Materials and Methods

2.1 Western Blotting

The preparation of proteins lysates has been described previously. MEFs (passage 3) grown under normoxic conditions were lysed, sonicated, soluble fractions recovered, and quantified. Protein samples were resolved on either a one phase or two phase SDS-polyacrylamide gel (10% or 10% and 15%) and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, United States). The following antibodies were used: anti-ubiqutin (Dako), HIS mouse monoclonal, rabbit polyclonal GFP (Santa Cruz) and mouse monoclonal actin (Sigma, St. Louis Missouri, United States). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Rockford, Illinois, United States).

2.2 LPS Injections

Cohorts of 8 week old mice were injected with either lethal dose (40mg/kg) or sublethal dose of LPS (12.5mg/kg) (Sigma). Animals were monitored at frequent intervals for a variety of physiological parameters including body weight, body temperature, and blood pressure. Animals found to be outside humane endpoints (for example having sustained more than 10% loss of body weight) were euthanized.

2.3 Blood Pressure Measurements

Blood pressure was measured using specialized mouse tail cuffs that are directly attached to a BP monitorBP-2000 Blood Pressure Analysis System™ for Mice and Rats (The laboratory of Dr. Chris Kennedy has kindly provided the use of such a device). Ten mice from each genotype were trained on the BP device for a total of five days before commencement of the experiment. Mice were placed on a plate warmer (37°C) and after 5 preliminary measurements 10 experimental measurements were taken for each mouse and analyzed using BP-2000 Analysis Software.

3. Results

3.1 Effects of mutant ubiquitin on NF-κB activation in cell culture studies

To determine whether expression of mutant ubiquitin would interfere in this pathway we examined the phosphorylation status of IκBα transgenic MEFs lines (Figure 1). Cells were either treated with IL-1β or TNFα for 0, 5 and 10 minutes. In a preliminary analysis we were able to detect an accumulation of phosphorylated IκB-α at time points of 5-8 minutes in the spontaneously transformed transgenic

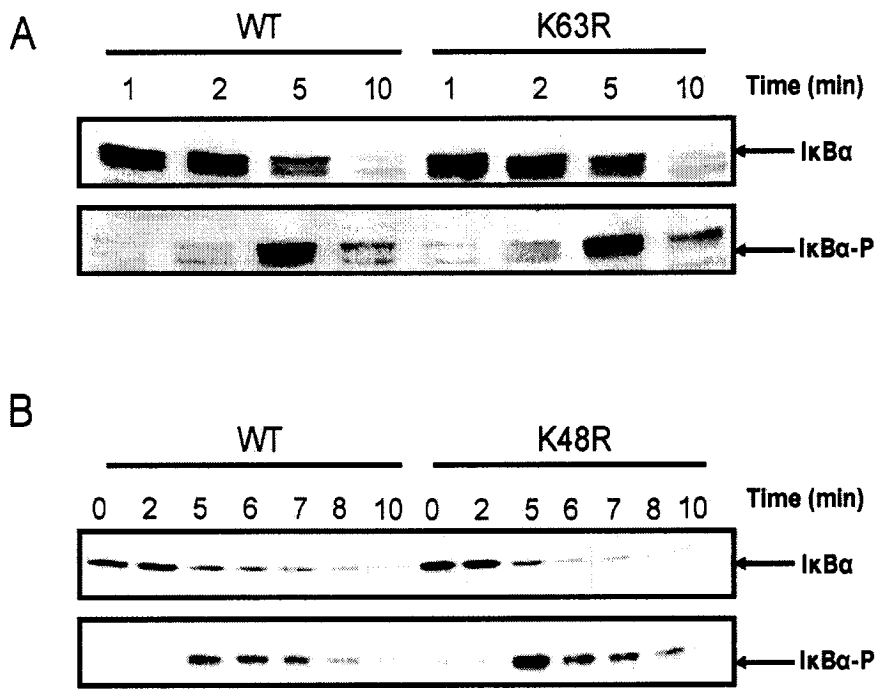

Figure 1. IκBα is phosphorylated similiarly in WT and mutant ubiquitin expressing MEFs (A) IκBα phosphorylation in primary MEFS after IL-1β treatment for various times. **(B)** Spontaneously transformed MEFs after IL-1β stimulation

MEFs expressing the K48R mutant ubiquitin as compared to WT (Figure 1B). However, this delay was transient as by 10 minutes IkB-α was degraded, which is typical in wildtype cells as well. However, primary MEFs indicate that IKB-α was phosphorylated in an equivalent manner across all 4 cell lines and degraded by the 10 minute time poin (Figure 1A).

3.2. *In Vivo* effects of NF-κB Activation in mutant mice

Despite the results in our primary MEFs we were encouraged by our preliminary cell culture data and we proceeded to test the activation of NF-κB in our transgenic mice expressing K48R and K63R mutant ubiquitin. Mice were injected with lethal doses of bacterial LPS (40mg/kg) to induce endotoxic shock, which results in the induction of critical inflammatory genes by activating NF-κB. Whereas all 10 nontransgenic mice succumbed within 10 hours of the peritoneal injection of LPS, at 20 hours 50% of the K63R and K48R mice were still viable. Remarkably, although most of the K63R mice eventually succumbed, 50% of the K48R mutant mice survived for 14 days when the experiment was terminated (Figure 2A).

Moreover, at the lower doses of LPS it was revealed that K63R shared a better survival as compared to WT mice. In fact 66% and 78% of K63R and K48R animals were viable at 48hours respectively, compared to only 33% in the wildtype strain (Figure 2B). This not only confirms our early experiment using lethal doses of LPS but also the protective effect afforded to the mutant ubiquitin strains.

Many of the symptoms of septic shock, especially that of vasodilation and hypotension, have been attributed to the overproduction of nitric oxide (NO) by iNOS [3,24]. Therefore, we decided to monitor blood pressure (BP) after LPS

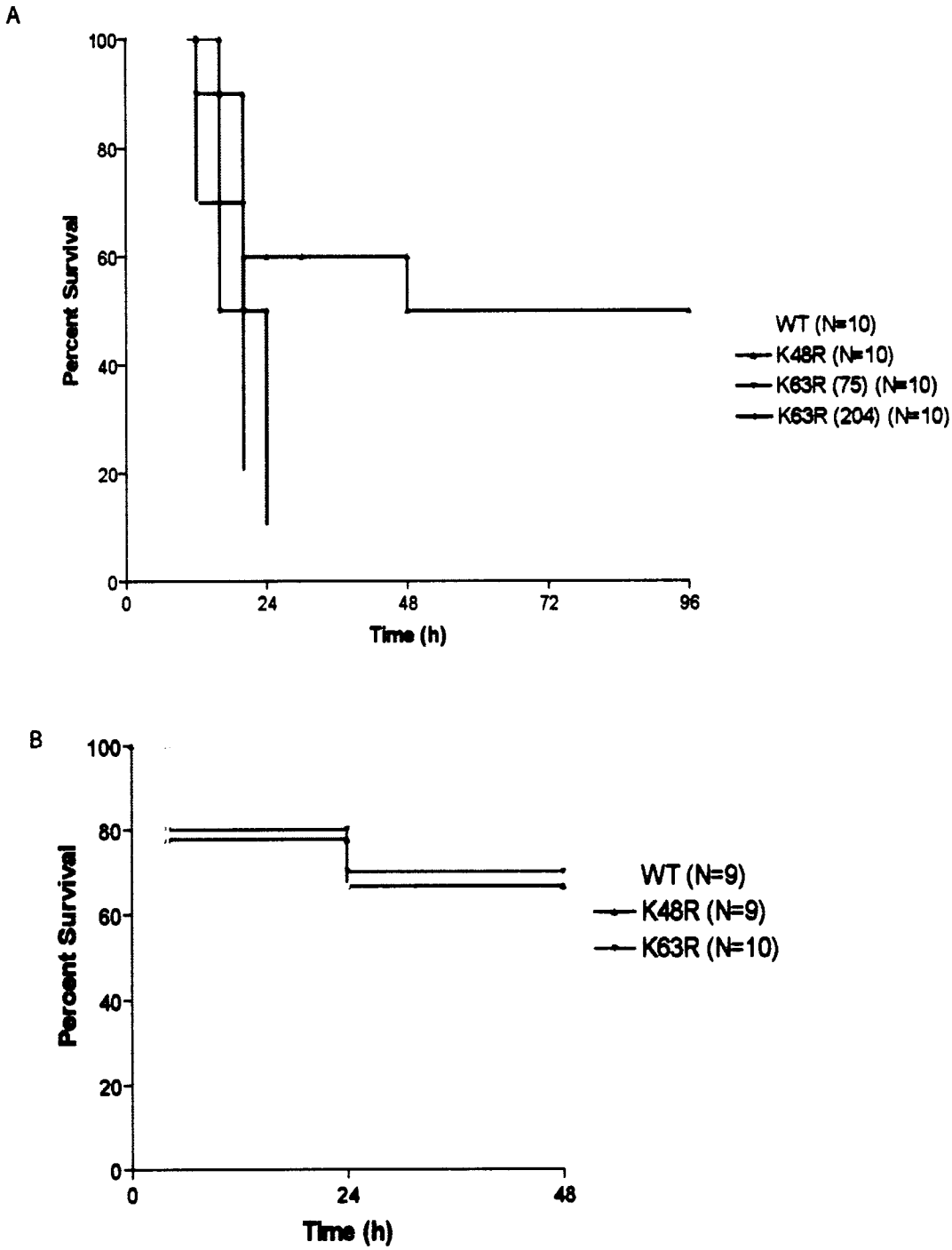

Figure 2. Mutant ubiquitin expressing mice are protected from endotoxic shock. **(A)** Survival of transgenic mice expressing WT and mutant ubiquitin after an IP dose of 40mg/kg of LPS, **(B)** Survival of transgenic mice expressing WT and mutant ubiqutin after low dose IP injection of 12.5mg/kg of LPS

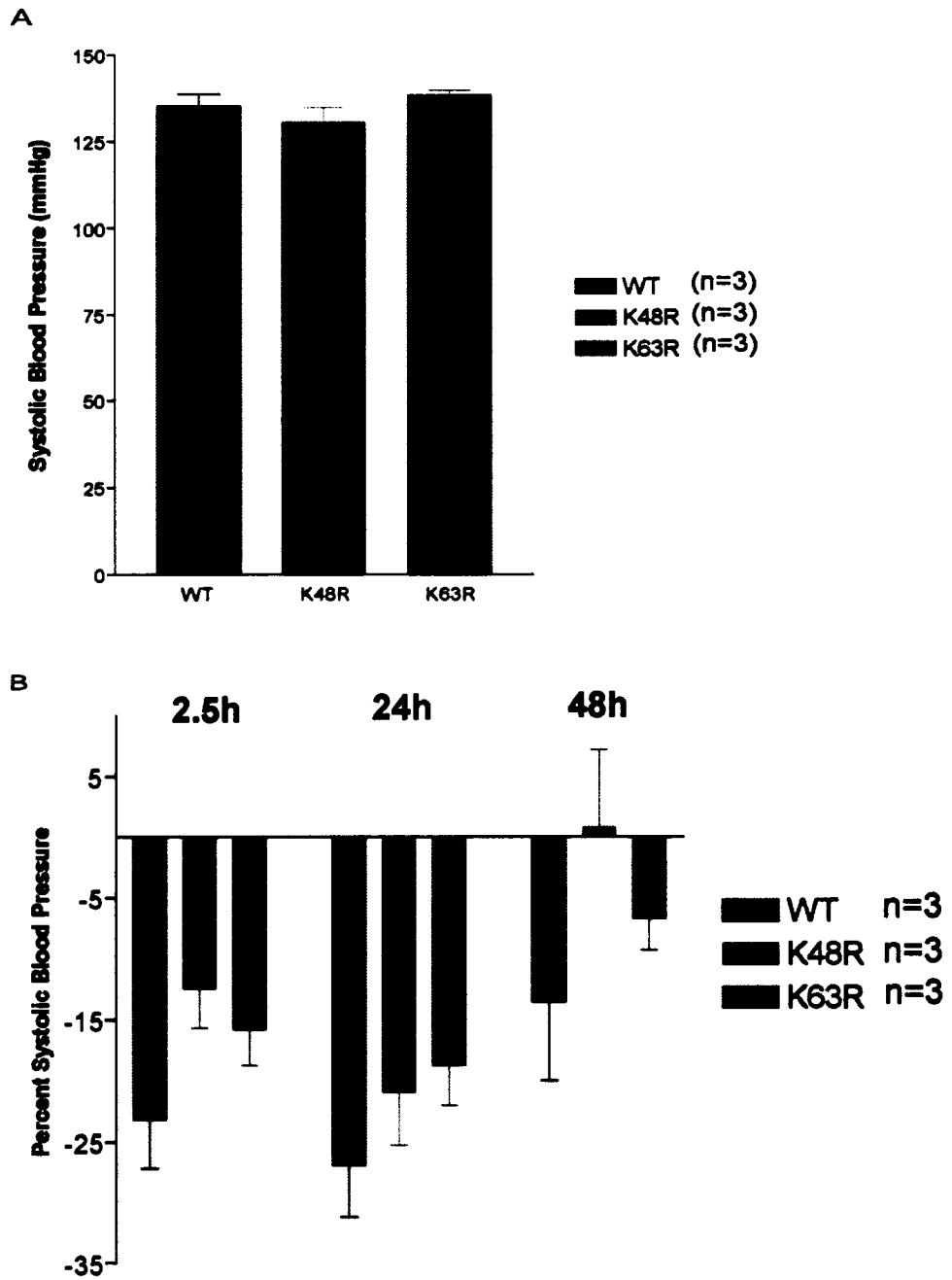

Figure 3. Survival of mutant ubiquitin animals is associated with improved blood pressure during endotoxic shock. (A) Blood pressure was measured over a period of 4 days prior to LPS injection and 10 independent measurements for each animal were taken on the last day and averaged. Error bars represent the standard error of the mean **(B)** Average blood pressure measurements at various time points post injection of 12.5mg/kg of LPS in WT, K48R and K63R transgenic animals. Error bars represent the standard error of the mean

challenge (12.5mg/kg) for 48 hours. Since the induction of iNOS is not expected to be as prominent in the mutant ubiquitin mice as in WT, the BP is predicted to be higher in the mutant mice as compared to WT. The K48R mice had significantly improved blood pressure readings after 2.5 and 48 hours post injection as compared to WT mice. K63R animals also had improved blood pressure readings; however this was not statistically significant (Figure 3). This suggests that the protective effect of these mutant transgenes is associated with normalized blood pressure during toxic shock.

4. Discussion

In this brief study we report that mutant ubiquitin transgenic mice particularly those expressing the K48R transgene are more robust in response to lethal doses of LPS while sublethal doses result in survival of both K48R and K63R as compared to UbGFP animals. Clearly, the robustness of K48R animals may arise from the unexpected consequence of an altered ubiquitin proteasome pathway. For example, LPS experiments feature a direct NF-κB response, and NF-κB activation is regulated by K48-linked polyubiquitination which induces the proteolysis of IκBα. Interestingly, although K63R animals succumbed to the effects of high dose LPS, lower doses revealed a significant protective effect which was comparable to the one observed in similarly dosed K48R animals. This effect was associated with improved blood pressure measurements in the mutant animals. In recent years it has come to light that NF-κB activation is regulated by K63-linked polyubiquitination at various levels [2]. For example K63-linked polyubiquitination occurs on at least 4 proteins,

notably RIP [10], NEMO [16], TRAF2 [14] and TRAF6 [13], which likely facilitate the recruitment of various proteins into an active complex that is able to transduce a phosphorylation signal to the IKK complex that then activates NF-κB. It is intriguing to speculate that these results suggest the proteasome as a better target for anti-NF-κB therapy than at the IKK level. There is clinical relevance to these results and they may help explain why inhibition of proteasome using PS-341 is more effective in blocking proliferation of multiple myeloma cells as compared to the IKK inhibitor PS-1145 [25].

Certainly, the preliminary LPS animal experiments pointed to a potential deficit in NF-κB activation. Therefore, we sought to determine if expression of our K48R and K63R mutant ubiquitin could directly inhibit this pathway in cells. To this end, we were unable to detect a diminution of phosphorylated IκBα in K63R MEFs,. Interestingly, we were also able to detect more phosphorylated IκBα form at 5 minutes in spontaneously transformed K48R MEFs; however, phosphorylated IκBα was degraded by 10 minutes which is similar to degradation of IκBα-P in wild type cells. Therefore, the result may simply be a phenotype of spontaneous transformation and not one indicating a delay in degradation.

Thus far, our results point to a protective effect exerted by mutant ubiquitin in response to LPS, which is associated with improved blood pressure but a seemingly independent role in NF-κB activation. So, what of a potential mechanism? The most parsimonious explanation for our LPS results may lie in the expression of levels of the transgenes themselves. The ubiquitin transgenes express a 6X His tag at the N-terminus and GFP at the C-terminus. GFP is simply used as a reporter for transgene

expression. Based on flow cytometry studies and previous publications from our laboratory, higher levels of GFP are found in K48R, as compared to WT animals. Recently it has been reported that GFP inhibits polyubiquitination a process that has a plethora of important functions in cellular processes. Specifically Baens et al., report that eGFP enhances p53 levels, and attenuates the activation of NF-κB and JNK signaling pathways; processes that require polyubiquitin chains linked through K63 and K48 [26]. To add more complexity, there are also unpublished reports that 6XHIS tags may also inhibit the proteasome. Since GFP levels are higher in K48 animals it is plausible that GFP levels alone, HIS tag levels, K48R levels or all three may account for the protective effect observed in mutant ubiquitin expressing animals. Although we do not show any changes in IκBα phosphorylation or its degradation pointing to NF-κB independent role, more sensitive assays such as NF-κB luciferase reporter assays may reveal the subtle differences between the various lines of animals. However, since polyubiquitination functions in many pathways, it is entirely possible that protective roles in ubiquitin transgenic animals could be independent of NF-kB or JNK signaling pathways. Therefore, it still remains likely that the protective effects observed in the transgenic animals are a result of GFP and 6XHIS levels rather than mutant ubiquitin. Other possible explanations include insertional affects and the potential affects at the transcriptional level or K48R actually stabilizes some critical protein in the organism [27]. However, we cannot rule out other yet to be discovered elements of organismal biology which are at play in the current series of experiments that we simply do not understand and which are not well modeled by cell culture experiments.

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Appendix B

Protective effects of mutant ubiquitin in transgenic mice

Contribution to Manuscript

My contribution to this published manuscript the proposal of the LPS and cisplatin experiments as well as Figure 1. DAG wrote the paper. DAG and JB analyzed the data. JB provided the analysis tools and reagents for the experiments. The animal work was done with the technical assistance of Sherry Thurig.

Summary

This manuscript details the protective effect of K48R mutant ubiquitin *in vivo* to a variety of biological insults such as canavanine, cisplatin and LPS. This contrasts data in yeast and our own cell culture studies which show exquisite sensitivity to such agents. However, since ubiquitin plays important roles in many biological processes including transcription, endocytosis and NF-κB activation, alterations in these pathways may account for this protective effect.

Protective effects of mutant ubiquitin in transgenic mice

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ABSTRACT: The K48R mutant ubiquitin can exert profound *in vivo* protective effects against a variety of insults, including agents of direct clinical relevance. The manipulation of the ubiquitin/proteasome pathway has enormous potential for clinical benefit, and it is not unreasonable to expect that such benefits will include diseases of aging.

KEYWORDS: ubiquitin; mutant; transgenic; mice; life span; K48R

The ubiquitin/proteasome pathway (UPP) is responsible for elimination of damaged and misfolded proteins, and there is reason to believe that a decline in the efficiency of the UPP accompanies, and may contribute to, human aging.[1] For many of its functions ubiquitin must be assembled into chains, and it is the varying topology of these chains that allows ubiquitin to serve apparently separate functions in distinct cellular pathways. In yeast it has been established that mutant isoforms of ubiquitin unable to participate in ubiquitin chain assembly through lysine 48 (K48) linkages exert a dominant negative effect on proteolysis.[2] In contrast, mutant ubiquitin engineered to interfere with K63-linked chain assembly imparts a DNA repair deficit in yeast.[3] To explore the consequences of analogous mutations in the context of mammalian cells *in vivo*, we have created transgenic mouse strains in which wild type or mutant ubiquitin is expressed at high levels from the human UbC promoter. The rationale and details of transgene construction have been described previously.[4, 5]

All the ubiquitin lines we have created to date are viable and fertile. Homozygous individuals from all strains have reached three years of age, arguing against any dramatic reduction in lifespan; with current data we can draw no conclusions about the potential extension of lifespan in these strains. In order to determine if alterations in ubiquitin might predispose to protein or DNA damaging agents as has been observed in yeast we have subjected the mice to various biological and chemical insults. Contrary to our initial expectations, mice expressing K48R mutant ubiquitin insults were remarkably resistant to various stressors that would be expected to provide a burden of misfolded or aberrant protein. A classic yeast methodology for

delivering such a burden is to grow cells in the presence of canavanine, a naturally occurring arginine analog whose incorporation during translation generates structurally aberrant proteins. Yeast cells expressing K48R mutant ubiquitin are sensitive to canavanine [2], as are mammalian neuroblastoma cells.[5] When mouse pups were injected intraperitoneally with canavanine we observed a marked failure to thrive in nontransgenic animals. Expression of K48R mutant ubiquitin resulted in considerable protection against canavanine toxicity, as evidenced by weight gain in the neonatal pups.

*ts*1 is a neuropathogenic retrovirus encoding a envelope protein that misfolds at the body temperature of an adult mouse (the neonatal body temperature is permissive for replication of the virus, allowing systemic infection). Mice infected with *ts*1 as neonates invariably succumb to spongiform degeneration of the spinal cord and hindbrain approximately one month post-infection. Whereas transgenic mice expressing wild-type ubiquitin or K63R mutant ubiquitin developed disease with latency similar to that of nontransgenic control animals, the latency period was doubled in mice expressing K48R mutant ubiquitin. These animals were partially resistant to the *ts*1 virus replication, generating viral titers roughly a log lower at equivalent timepoints.[6]

Bacterial lipopolysaccharide (LPS) injection is a potent means of inducing endotoxic shock, an NF-κB dependent activation of innate immunity in mice. Transgenic lines and nontransgenic control mice of the same genetic background (FVB-N) were injected intraperitoneally with LPS at various dosages. Animals were monitored at frequent intervals for a variety of physiological parameters including

body weight, body temperature, and blood pressure. Animals found to be outside humane endpoints (for example having sustained more than 10% loss of body weight) were euthanized. Whereas LPS given at 40 mg/kg produced profound illness in nontransgenic mice as well as the transgenic animals expressing wild-type or K63R mutant ubiquitin, the K48R mutant transgenics were much healthier both by appearance and by the physiological parameters measured.

Finally, cohorts of mice were treated with an intraperitoneal dosage of cisplatin, a cancer chemotherapeutic agent, at 15 mg/kg body weight. At this dosage cisplatin not only induces DNA damage (the activity thought to be of primary therapeutic importance) but also induces a stress response involving NF-κB and other mediators. Once again mice expressing K48R mutant ubiquitin fared significantly better than animals expressing the wild-type or K63R mutant isoforms of ubiquitin, as evidenced by loss of body weight (Figure 1).

The apparent paradox presented by our in vivo results clearly arises from an unexpected consequence of an altered ubiquitin/proteasome pathway in the mice expressing K48R mutant ubiquitin. These results are also at odds with our previous observations in stable cell lines expressing identical ubiquitin transgenes.[5] It is likely that elements of organismal biology are at play in the current series of experiments that are not well modeled by cell culture experiments. For example, all of the current experiments feature a direct or indirect NF-κB response, and NF-κB activation is regulated by the UPP at various levels.[7, 8] The modulation of NF-κB activation in K48R mice (the sequelae of which would include dampening of cytokine release, etc.) might explain a great deal of what we have observed in our *in vivo* experiments,

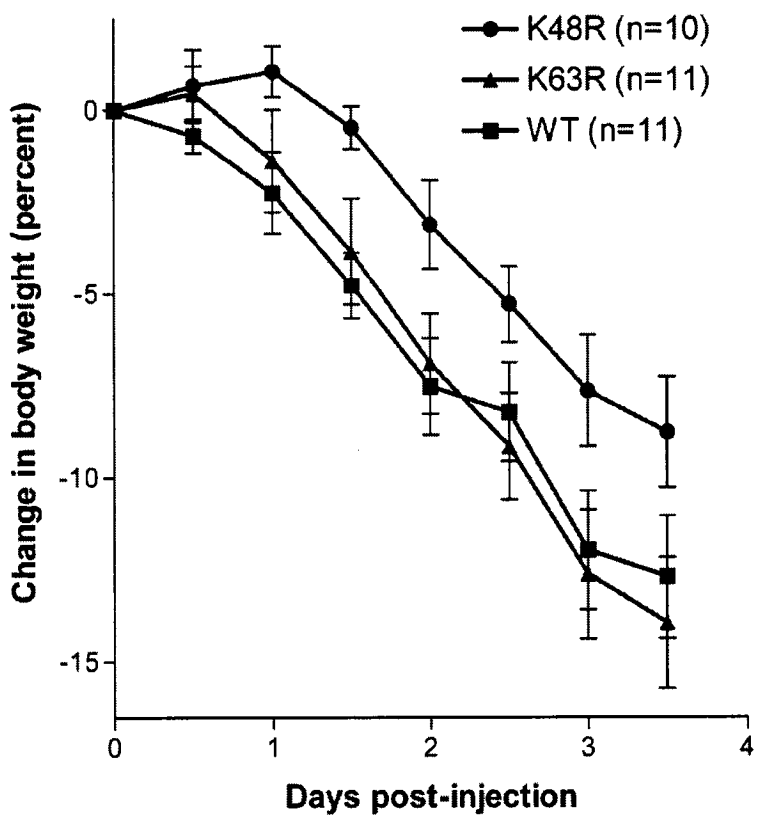

FIGURE 1. Loss of body weight in various cohorts of mice. Mice expressing K48R mutant ubiquitin fared significantly better than animals expressing the wild-type or K63R mutant isoforms of ubiquitin. See text.

and we are actively investigating this possibility. The related issue of nitric oxide and its downstream effects is another common thread in our experiments worthy of further investigation. Altered ubiquitin metabolism can be expected to impinge on the regulation of transcription, and using microarray analyses we are investigating the extent to which transcription is reprogrammed in our ubiquitin transgenics. Ubiquitin also plays an important role in directing trafficking of proteins internalized by endocytosis, and is required for ERAD (endoplasmic reticulum associated degradation), the degradative pathway charged with elimination of misfolded proteins extracted from the ER. There may be important alterations in vesicle trafficking in K48R mice, and these must also be investigated further.

In summary, we have discovered that K48R mutant ubiquitin can exert profound *in vivo* protective effects against a variety of insults, including agents of direct clinical relevance. The manipulation of the UPP has enormous potential for clinical benefit,[9] and it is not unreasonable to expect that such benefits will include diseases of aging. In the coming months we hope to determine if the general robustness of our K48R mice not only protects them against toxic insults, but also extends their lifespan in the absence of such stressors. There is evidence that in at least one tissue aging is delayed in these mice.[10]

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Chapter 9

9. Discussion

In the 3 decades since its discovery, ubiquitin modification has become recognized as a major signaling pathway that rivals that of phosphorylation. Both modifications are rapid responses to a variety of cellular stresses. Analogous to phosphorylation, ubiquitin can be added and removed from substrates by a complex of enzymes, modulate the assembly and disassembly of protein complexes in a timely manner, and control protein stability, interaction, and function. One of the many stressors that cells have had to adapt to during evolution is that of endogenous and exogenous DNA damage. Recent data reveal that ubiquitination plays a key role in mediating the repair of such damage.

Genotoxic stress (UV, cisplatin, BPDE, and MMS) activates a variety of mechanisms to counter DNA lesions, including DNA repair (NER, BER, MMR, HR, NHEJ and TLS) and cell cycle checkpoints (ATR, ATM, and P38) [32,88]. Despite the full function of these systems in healthy cells, DNA lesions often persist, leading to stalled replication forks. This often occurs when cells encounter high amounts of DNA lesions and/or when damage occurs in the S phase of the cell cycle. Replication-coupled repair is critical to protecting the integrity of the DNA genome during S phase by tolerating damage using two important mechanisms, one being error-prone TLS and the other being error-free template switching. In lower eukaryotes, mono and polyubiquitination of PCNA is the molecular switch that mediates the error-prone and error-free components by permitting the assembly of divergent molecular machinery to counter DNA damage.

Recently, several findings have substantially improved our understanding of the ability of human cells to remove, repair or bypass UV-induced DNA damage. In particular, Kannouche et al., and Watanabe et al., found that the TLS arm of DDT was functional and fully conserved in human cells [26,71]. My thesis provides the first evidence that the error-free arm of the DDT pathway is functional and fully conserved from yeast to human [89,90]. Using dominant negative ubiquitin mutants we found interesting biological effects by disrupting K63 polyubiquitin chains in human cells. In yeast, K63 polyubiquitin chains are required for proper resolution of stalled replication forks. Disruption of these chains in human cells sensitized A549 cells to cisplatin but not to UV, BPDE, or UV in combination with caffeine. Significantly, preventing these chains increased BPDE and UV-induced mutations. By examining the mutation spectrum and POLη foci formation we demonstrated that increased reliance on TLS lead to the higher UV and BPDE-induced mutation frequency. Moreover, POLη plays a causal role in BPDE induced mutagenesis. Overall this suggested that K63 polyubiquitin chains direct DNA damage tolerance to a second non-TLS pathway which guards human cells against environmental mutagenesis (**Figure 1**).

In yeast, the error-free pathway requires modification of PCNA with K63-linked polyubiquitin chains, thus we sought to determine whether PCNA was the molecular switch that mediated this pathway in human cells. We again were first to determine that polyubiquitination of PCNA occurred in UV-irradiated, BPDE, CPT, and MMS treated human cells [89,90]. Contrary to the report by Kannouche et al., we also report that PCNA polyubiquitination is a normal physiological response to DNA

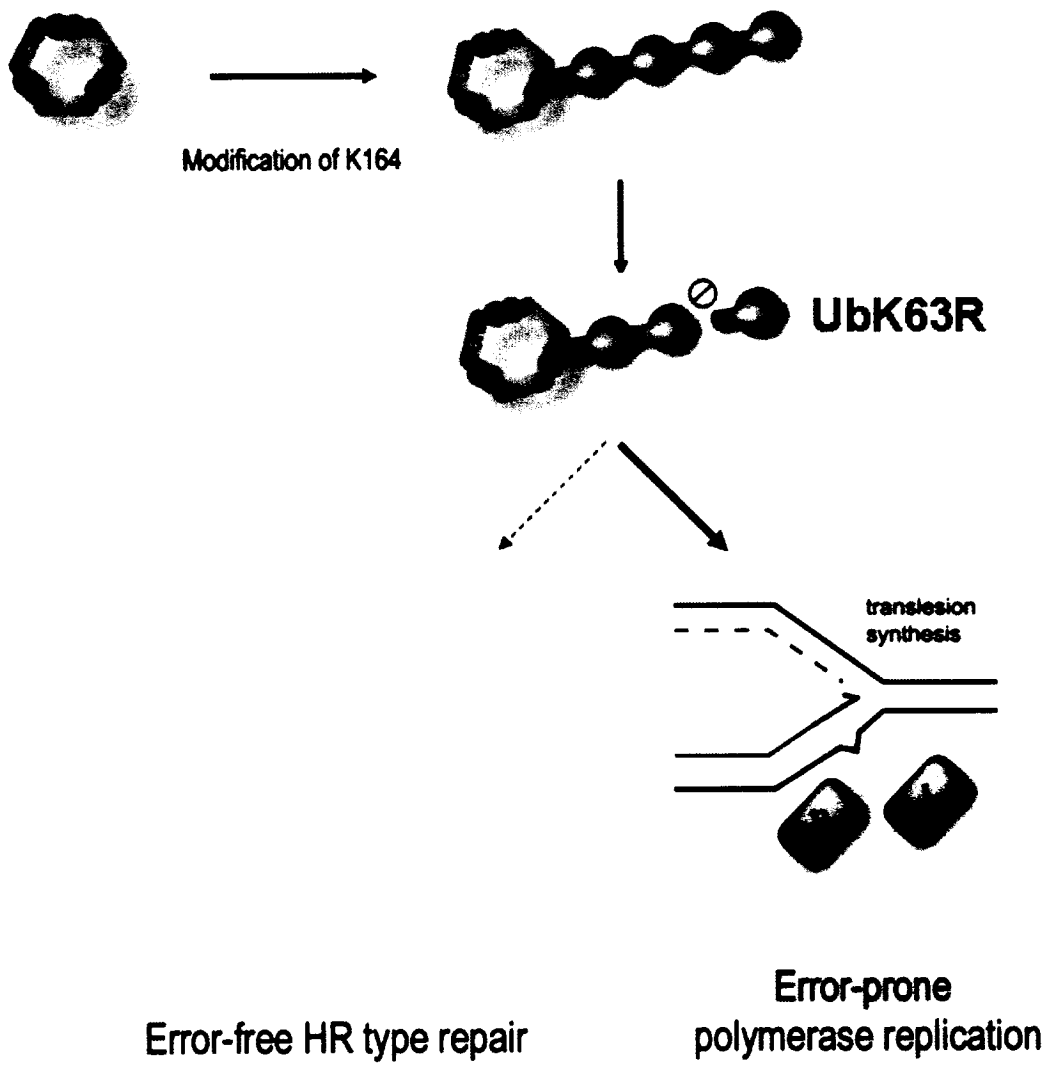

Figure 1. DDT pathway: Overall Model based on results from Chapters 2-5. The disruption of K63 linked ubiquitination on PCNA shunts the DNA repair from an error-free process to a more error-prone process governed by translesion polymerase.

damage not only in cancer cells but in both primary skin and primary lung fibroblasts. Finally, this modification occurred at K164 and was dependent on RAD18 (suggesting that monoubiquitination of PCNA is required), and on UBC13 (the ubiquitin conjugase previously shown to be involved in K63-polyUb chain formation) **(Figure 1)**.

Naturally, we thought that as the heteromeric partner of UBC13, MMS2 would be directly involved in PCNA polyubiquitination. However, to our surprise MMS2 and UEV1a serve a redundant role in PCNA polyubiquitination suggesting a role for an alternative MMS2 variant or novel E2 that can complement the loss of MMS2. Although our work did not identify the redundant protein, we believe the redundancy is in itself an unexpected and important finding that will spur further investigation. Previous work by Simpson et al., in DT40 cells has suggested that MMS2 may be redundant in DNA repair and somatic hypermutation, but our work goes beyond this in providing biochemical data to complement the genetic data of the Simpson paper [91].

Thus far, we and others have identified positive regulators, and redundant of K63-linked chain formation. What of the negative regulators? Clearly, ubiquitination levels are tightly controlled and highly influenced by the opposing activities of ubiquitin E3 ligases and deubiquitinating enzymes (DUB's) [92]. DUB's are cysteine proteases that cleave Ub from specific mono and polyubiquitinated substrates sub-classified into 5 groups [93]. DUBs play key regulatory roles in a multitude of processes from hereditary cancer to neurodegeneration. For example, mutations in the specific DUB's USP1 and CYLD have been implicated in the human disorders

Fanconi Anemia [94] and Cylindromatosis [95-97], respectively. Despite the clear importance of DUBs, our knowledge of their mode of regulation, physiological functions and substrate specificity is limited [93]. Since DUBs comprise of close to 100 enzymes, teasing out the DUB responsible for regulating a particular substrate is a tedious process using *in vitro* experimentation or knockdown strategies in cell culture. Fortunately, Huang et al. simplified this process by identifying USP1 as the negative regulator of monoubiquitinated PCNA [98]. Using RNAi we demonstrate for the first time that USP1 is also a negative regulator of K63-linked PCNA polyubiquitination and quite probably of error-free homologous recombination (Figure 2). Collectively, our data argue that the components for the ubiquitin-based molecular switch dictating error-free versus error-prone repair have been functionally conserved throughout eukaryotic evolution and are important in preventing human disease.

9.1 Prevalence of error-free DDT versus error-prone translesion synthesis

What remains to be determined is which pathway is more prevalent at stalled replication forks. At a recent DNA repair meeting in Noordwijkerhout, Holland, Lehmann et al., argued that TLS is the more prevalent and important pathway involved [99,100]. This was based on the prominence of monoubiquitinated versus polyubiquitnated PCNA species on Western blot analysis from a variety of cell lines (Chapter 2). Moreover, in terms of energetics and bypass efficiency, it is logical to assume that TLS would be the more prevalent process as it is less energetically demanding, requiring only the monoubiquitination of PCNA to bypass DNA lesions. It is also advantageous as one avoids using the ubiquitin pools, leaving them for the

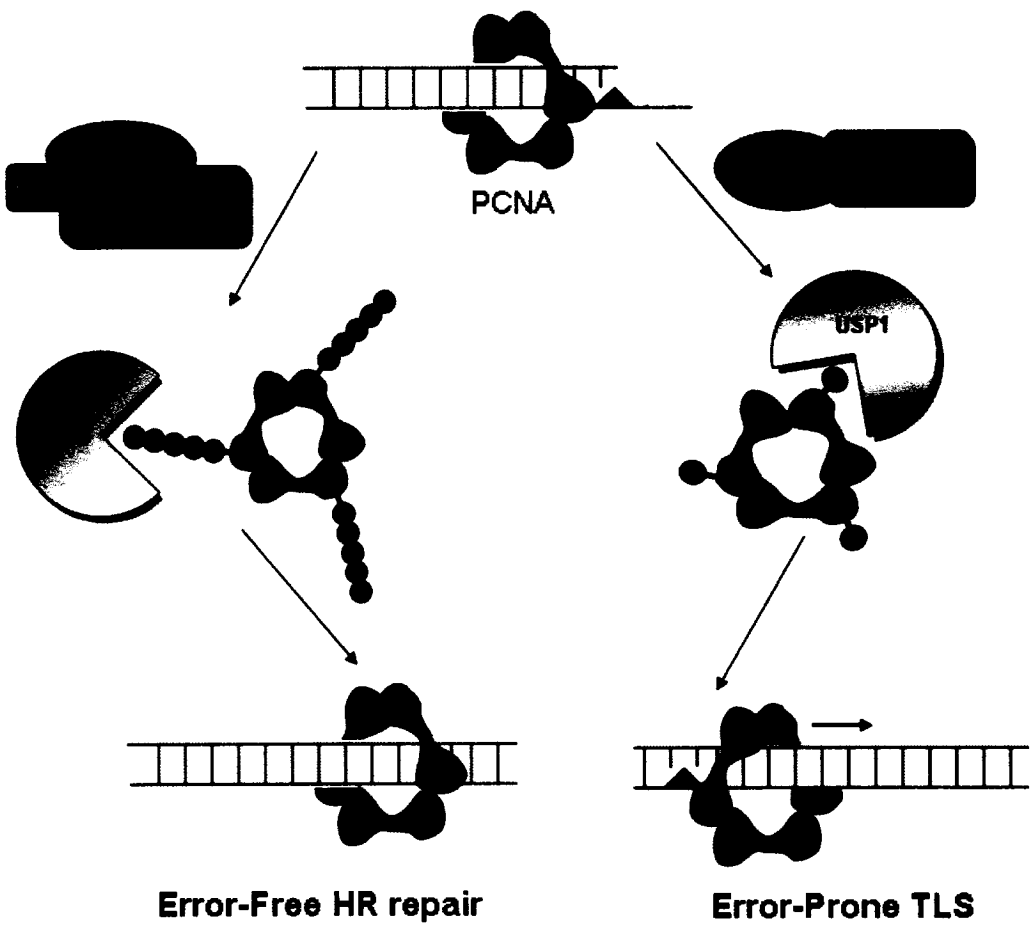

Figure 2. Cartoon depicting USP1 cleavage of K63-linked ubiquitin chains. PCNA ubiquitination is a tightly controlled process involving several positive regulators (ubiquitin conjugases). We show that USP1 is one of the negative regulators of this important process.

use in other pathways such as NF-κB activation during stress response. Of course, the consequence of this mode of bypass is the increased risk for generating potentially deleterious mutations leading to disorders such as cancer. In contrast, mutation could also provide a distinct survival advantage in combating infections through, for example, somatic hypermutation.

While the argument for the prominence of TLS is partially substantiated, intuitively, it is reasonable to suppose that cells in higher vertebrates would have evolved to first repair in an error-free manner to guard against carcinogenesis. Based on several lines of evidence we believe that error-free repair is in fact more prevalent and important at stalled replication forks. First, although PCNA immunoblot data show that PCNA polyubiquitination is less prominent than that of monoubiquitinated PCNA, the contrary is true when we examine PCNA polyubiquitination with the ubiquitin antibody after PCNA immunoprecipitations (IP) (Chapter 2, 3, 4, and appendix A) [89,90]. Specifically the IP's clearly show that di-, tri-, and tetra-ubiquitinated PCNA is more abundant than monoubiquitinated PCNA. Presumably, in the case of the PCNA immunoblots the distinct possibility remains that the PCNA epitope is somehow being affected as additional ubiquitin is being added. Unfortunately, the abundant polyubiquitinated PCNA bands as revealed by the ubiquitin antibody are observed after PCNA IP's, a procedure that is not quantitative. Therefore, although we could measure relative polyubiquitinated PCNA levels with the PCNA antibody by Western blotting, the results would not likely reflect the true levels of monoubiquitinated versus polyubiquitinated PCNA in the cells. Overall, the immunoblot and IP data suggest that the prevalence of TLS as the major player at

stalled replication forks is unclear and certainly points to at least an equivalently important role for error-free repair in bypassing DNA lesions. Moreover, we recently showed that knockdown of POLη increased UV induced mutagenesis, in WT-UB expressing cells. When the K63R mutant was added to this mix, there was a synergistic effect with respect to UV induced mutagenesis suggesting that the error-free pathway is more important and readily used to overcome stalled replication forks [89]. Moreover, while PCNA polyubiquitination is less prominent at lower doses of UV light, BPDE, or aphidicolin, it increases in a dose dependant fashion. This clearly indicates that error-free HR repair is favoured over TLS with higher levels of DNA damage [89,90,101]. Finally, Zhang et al. demonstrated that yeast can efficiently replicate plasmids with UV 6-4PP in a NER deficient background using error-free repair [23]. In the NER deficient yeast, approximately 50% of the UV damaged plasmids were replicated indicating the importance of NER in this process. Interestingly, approximately 90% of the replicated plasmids were repaired by error-free sister strand recombination while only 10% were repaired by TLS. RAD6/RAD18 (70%) error-free repair and RAD52 (20%) accounted for nearly all the recombination events [23]. Overall, the data of Langie et al., Chang et al., Zhang et al., and our recent foray into the field indicate that the error-free arm of DDT seems to be more prevalent and important at sites of stalled replication forks than the error-prone TLS [23,89,90,101].

Thus far, we provide evidence for the prevalent use of error-free arm for tolerance of DNA damage; however, we do not exclude the possibility that the level of DNA injury or energy could have an influence on determining which arm of DDT

is preferentially utilized. Since the level of DNA damage affects ubiquitin and energy pools the supposition would be that low levels of damage favour error-free HR, while increased DNA injury would favour TLS. The likelihood is that both pathways are used equally during various levels of increased replication stress. Certainly the literature shows that high levels of genotoxic stress lead to point mutations and gross chromosomal rearrangements, which suggests that both arms of DDT are being utilized. Moreover, in contradiction to our supposition we have an increase in PCNA polyubiquitination with higher and more persistent damage that suggests that the opposite is true. Therefore, it would be of interest to see if varying levels of DNA damage and/or varying ATP levels via depletion could alter the use of one subpathway over the other. In conjuction, it will be necessary to determine which signal transduction pathways influence PCNA modification by K63polyUb and consequently its role in maintaining genetic stability.

9.2 The molecular pathways that signal for the ubiquitination of PCNA

A challenge for future investigators will be to determine whether PCNA ubiquitination is a compartmentalized response to DNA damage that drives the choice of one pathway over the other, or whether more global regulatory factors, such as cytosolic signal transduction cascades or cell cycle checkpoint kinases, play a role in tipping the balance in favour of TLS over HR based on the modification of PCNA. In order to determine if the latter is plausible we examined whether cell cycle checkpoint kinases play a pivotal role in PCNA ubiquitination. Interestingly, despite strong inhibition of cell cycle checkpoint kinases ATM, ATR and/or P38, PCNA mono- and polyubiquitination preceded uninterrupted (Chapter 4). This suggests that

PCNA ubiquitination and cell cycle checkpoints are independent responses to DNA damage. Interestingly, we found that caffeine lead to a mild increase in PCNA ubiquitination, the biological consequence of which is still unclear. A recent study by Johansson et al., suggests that caffeine facilitates a switch from TLS repair to homologous recombination repair after UV irradiation [102]. Our results certainly provide biochemical evidence for this assertion. Several *in vivo* studies have demonstrated that caffeine significantly delays the onset of tumourgenesis after UV irradiation [103,104]. Furthermore, Johansson et al., show that caffeine results in decreased UV induced mutagenesis and increased recombination, suggesting the inhibition of lesion bypass polymerases and probable promotion of error-free HR [102]. Therefore, caffeine could effectively potentiate the switch from error-prone to error-free repair thus preventing carcinogenesis. It will be of significant interest to see whether live imaging of TLS polymerase foci in human cells will confirm such an event and whether HR is the major pathway that governs error-free DDT.

9.3 K63-linked polybubiquitin chains and homologous recombination

HR is one of two major pathways that respond to double strand breaks (DSBs) [105]. Notably, DSB are produced at collapsed replication forks as a result of replication blockage. Many genes involved in HR are mutated in cancers, strongly suggesting that this pathway is indispensable for the universal maintenance of genetic stability and protection against cancer. Although HR is a relatively slow process it is exceptionally accurate, relying on undamaged homologous stretches of DNA to repair in an error-free manner [106]. The initial steps of HR involve the resection of DSBs to produce 3′ single-strand overhangs. These single strand tails serve as substrates for

RAD51 attachment in a BRCA1 and BRCA2 dependent manner [107-109]. DNA bound RAD51 then performs a homology search and invasion of undamaged homologous stretches of DNA, thereby forming a D loop and initiating faithful repair. Recent evidence points towards a direct role of UBC13, RAD18, and interestingly POLη in HR [105,110-112]. RAD18 seems to faciliate HR by suppressing NHEJ while POLη has been shown to interact with RAD51 and extend the D loop. Moreover, Zhao et al., show that cells deficient in UBC13 are unable to recruit the key HR proteins RPA, BRCA1, and Rad51 to sites of DNA damage [111]. This occurs due to a failure in a very early stage of HR-mediated repair of DNA DSBs where UBC13 is required to process DSBs into a competent HR substrate. Several outstanding questions were left unanswered by Zhao's study, such as: how does UBC13 promote this DSB resection; is this pathway distinct from DNA damage tolerance; is the K63 ubiquitination function of UBC13 required; and what is the identity of the target of ubiquitination.

We and others have recently shown that the modification of PCNA by K63polyUb chains is a critical step in the bypass of replication fork blocking lesions in an error-free manner. In effect, this modification acts as the key decision point between error-free, speculated to be mediated by HR, versus error-prone TLS. Accumulating evidence from our laboratory and from others has implicated K63polyUb chains in HR; however not directly (Chapter 6) [111]. Therefore, it remains our hypothesis that K63-linked ubiquitin chains are central to HR in mammalian systems. In order to confirm such an assertion future studies should focus on using plasmid-based recombination substrates, foci formation (RAD51,

BRCA1, γH2AX), and sister chromatid exchange to assess HR in cell lines that express the K63R dominant negative mutant and are thus deficient in K63-linked polyubiquitination. Undoubtedly, these methods will reveal whether K63polyUb chains play a role in HR and/or the processing of DSB's. To determine if this effect is mediated through UBC13/MMS2, HR assays would be performed in UBC13 and MMS2 depleted cells and compared to those found in cells expressing K164R PCNA mutants. To ensure that the effect is an evaluation of homologous recombination, assessment of these defects in HR mutants and NHEJ mutants as controls will be necessary. Comparing the results with these mutants will help to distinguish effects contributed by the DDT pathway from the repair by way of HR.

9.4 TLS and HR as sequential events in mammalian DNA repair

Thus far, we speculate that ubiquitin pools, energetics, and levels of damage could drive some yet to be identified signal transduction pathway that makes the choice between error-free and error prone repair. Maybe it is presumptuous to think of DDT as a choice between one pathway or the other during genotoxic stress. There remains the distinct possibility that TLS (shown to govern error-prone arm) and HR (thought to govern error-free arm) are sequential events rather than pathways driven by choice (**Figure 3**). Thus, upon encountering a DNA lesion PCNA is monoubiquitinated, facilitating a polymerase switch. This utilizes the Y and B family of polymerases to bypass damage in either an error-prone or error-free manner based on the type of lesion. If, by good fortune, a lesion is bypassed accurately and efficiently such as thymine dimers by POLη, USP1 then facilitates the switch from TLS to normal replication. The supposition is that typically error-pone repair would

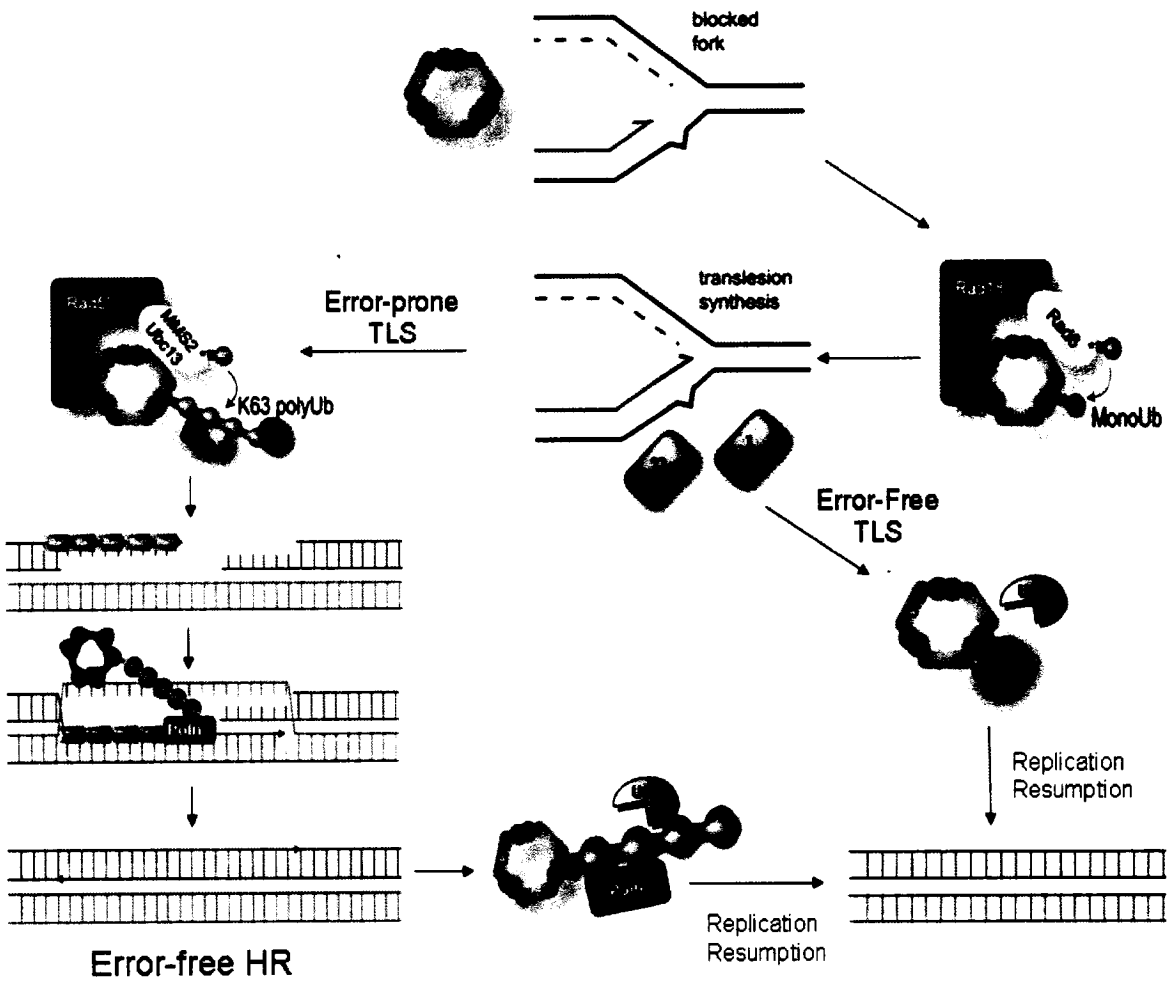

Figure 3. HR and TLS as sequential events. Stalling of replication machinery leads to PCNA monoubiquitination. This modification facilitates a polymerase switch (Polδ & ϵ to TLS polymerases). Depending on the damage TLS polymerases can repair in an error-free or error-prone manner. If they repair error-free, then monoubiqutinated PCNA is cleaved by USP1 and normal replication resumes. However, due to low processivity and lack of exocnuclease activity, TLS polymerases have a high probability to repair in an error-prone manner. In the latter case, we speculate that the resultant DNA distortion leads to PCNA polyubiquitination (sequesters the TLS polymerases), replication collapse, and double strand breaks at the lesion site. DSB's are resected to reveal 3' overhangs which are bound by RAD51. We speculate that K63-linked PCNA acts as a structural instrument aiding RAD51 to initiate invasion and homology search into the sister chromatid. Once the D loop is formed RAD51 recruits TLS POLη sequestered by ubiquitinated PCNA. This is followed by DNA synthesis, resolution of recombination intermediates. Further HR is terminated by cleavage of polyubiquitinated PCNA by USP1 leading to replication resumption.

leave a mismatch that would create a distortion in the DNA helix. This would lead to subsequent replication stalling, double strand breaks and the recruitment of HR repair via polyubiquitination of PCNA or another yet to identified substrate. Interestingly, polyubiquitination of PCNA also increases the affinity for TLS polymerases [56,57]. This is counterintuitive as one would expect disengagement from the complex in order to recruit HR machinery to bypass the lesion in an error-free manner based on our suggested model in chapter 2. However, recent evidence points to the recruitment of TLS polymerases for homologous recombination intermediates by RAD51 [110]. In yeast, sumoylation recruits SRS2 which prevents illegitimate recombination by keeping RAD51 filaments in check [51,113]. Although sumoylation of PCNA has not yet been identified in human cells, genotoxic stress nonthteless leads to PCNA polyubiquitination and recruitment of RAD51 to sites of damage which help initiate HR by promoting "homology search and invasion" in the non-template strand. Coincidently, TLS polymerase eta also colocalizes with RAD51 to sites of DNA damage where it has been shown to extend the D loop and participiate in HR repair [110,114]. Thus, it is conceivable that K63-linked chains on PCNA sequester POLη for use in homologous recombination rather than dislodging the polymerase after lesion bypass. In particular, it is interesting to speculate that K63-linked ubiquitin chains help initiate invasion by structural displacement of the non-template strand. This allows for the recruitment of RAD51 for the coordinated formation of the D loop, promotion of homology search, and continued invasion. Moreover, K63-linked chains likely provide a proximal TLS polymerase for RAD51 to recruit for D loop extension. A challenge for future investigators will be to: (1) determine the spatio-

temporal sequence of events of the DDT pathway, and (2) determine the role of K63-linked ubiquitin chains in homologous recombination to endogenously and exogenously produced DNA lesions.

Despite accumulating evidence for the importance of PCNA ubiquitination in error-free DDT in maintaining genomic stability, several questions still remained unresolved: (1) Are there other DNA repair substrate modified by K63-linked chains? (2) What factors interact with the K63 linked ubiquitin chains on PCNA? (3) What type of overlap and cross-talk exists with other pathways? (4) What is the future of animal studies in DDT? (5) Based on their role in DDT, overlapping roles with HR and role in carcinogenesis, could members of the DDT pathway serve as putative drug targets?

9.5 Identification of DNA repair substrates modified by K63-linked ubiquitin chains

While there are many known substrates for K48 linked polyubiquitin chains, there exist only a handful of reported substrates of K63-linked polyubiquitin chains. These include a number of proteins involved in the inflammatory response such as TRAF2, RIP, NEMO/IKKγ, and TRAF6; all of which lead to NF-κB activation. The K63 polyubiquitin chains formed on these various proteins play an important role in facilitating the formation of multi-subunit complexes that signal downstream effector kinases [82]. In this thesis, we demonstrate that PCNA is a substrate for K63-linked polyubiquitin chains following DNA damage in human cells. Although it is not yet clear how K63-linked polyubiquitin chains signal for error-free HR repair, if

functioning as a potential scaffold like it is in NF-κB activation, it is possible that other DNA damage response substrates are also modified in this manner. For example, in addition to being polyubiquitinated via K6 linked chains, BRCA1 can also be ubiquitinated via K63-linked chains and actively participate in DNA repair processes [33,115-117]. It is attractive to speculate that in addition to PCNA and BRCA1 there are many more yet to be identified substrates of K63-linked ubiquitin chains in DNA repair processes and their identification will undoubtedly further our knowledge of the DDT pathway.

We and others believe that future studies using mass spectrometry-based proteomics will be important for the identification of other protein substrates tagged with K63-linked polyubiquitin chains [118]. Peng et al. used this strategy to identify over 1000 substrates of ubiquitination in yeast and more recently, Kirkpatrick used this strategy to identify 22 ubiquitinated substrates from Human Embryonic Kidney 293 cells expressing our epitope tagged 6XHis-Ub-GFP construct [6,119]. Therefore, it is conceivable to use a 6XHis-tagged K6-48R mutant (which only hosts a wild type K63) to identify K63 ubiquitinated substrates in the DNA repair process. In addition, the method of Kirkpatrick et al. could also be extended into our transgenic mouse models expressing His-Ub or HIS K6-48R as a means of isolating ubiquitinated proteins from mammalian tissues before and after stress.

9.6 UbK63 chain interacting partners

To date the nature of the binding partners for K63-linked ubiquitin chains is limited. However, recent studies examining the role of these chains in NF-κB

activation demonstrate that proteins containing ubiquitin binding domains (UBDs) are essential for binding these non-canonical chains. These proteins include TAB2, TAB3 and NEMO[82,85]. An apparent problem with UBDs is that they are not only limited to K63-linked polyubiquitin chains, but are also potential binding partners for monoubiquitinated substrates, or alternatively for polyubiquitin chains linked through K6, K11, K27, K29, K33 or K48 [120]. Interestingly, NEMO, a central regulator of NF-κB activation, has a higher preference for K63-linked ubiquitin chains as compared to K48-linked polyubiquitin chains [85,121,122]. Similar to the NF-κB field, recent advances in identifying the binding partners of K63-linked chains have provided insights into the regulation of DDT. Recently two UBD (UBM and UBZ) domains were identified in both human POLι and human POLη [56,57]. Additionally these UBDs are essential for binding K48 and K63 polyubiquitin chains and monoubiquitinated PCNA, as well as mediating an appropriate biological response to DNA damage at stalled replication forks [56]. Interestingly, the affinity of POLη for both monoubiquitinated PCNA and K63-linked ubiquitin chains suggests a dual role in TLS and HR [110,112]. The potential certainly exists for other substrates to bind to K63-linked polyubiquitin chains. In order to identify novel binding substrates, one could envision using columns with covalently bound K63-linked ubiquitin chains. One could run nuclear fractions of treated and untreated cells over the K63 column. Presumably proteins with strong binding affinity to K63-linked ubiquitin chains would be bound by the column and subsequently eluted off followed by identification using MS based proteomics. In vitro analysis, RNAi, Western blot analysis, and foci analysis before and after genotoxic stress would be used to validate the identified

binding partner. Alternatively, MS based proteomics could be first used to identify K63 modified substrates from nuclear fractions and then those modified substrates could be tagged and used in pull-down assays for identification of novel partners. Either methodology would help identify novel proteins that bind to K63-linked ubiquitin chains, thus providing further insight into the molecular mechanisms of DDT.

9.7 Overlapping functions of ubiquitin with various DNA repair pathways

Overlapping and non-proteolytic roles for ubiquitin have also been implicated in other DNA repair pathways that may interact with DDT, most notably that involving the Fanconi's anemia (FA) gene products. After DNA damage FANCD2 is phosphorylated by ATM/ATR, followed by mono-ubiquitination and localization to nuclear foci. Although the precise function of this ubiquitination event is unclear, some evidence points to its role in stabilizing the replication fork, and coordinating HR and TLS [123]. Credence to the former is demonstrated by studies that show interaction of ubiquitinated FANCD2 with BRCA2, which helps activate the HR pathway via RAD51 recruitment [36,116], while credence to the latter is demonstrated by the association of FANCC with the TLS polymerases REV1 and REV3 [124]. Interestingly, FANCC may also interact with the BLM helicase, a candidate for promoting fork reversal in the error-free damage avoidance pathway [125]. This advocates the close association of HR and TLS in the FA pathway similar to the one proposed earlier in the discussion for the DDT pathway. Lastly, the FA and DDT pathway share the same DUB that negatively regulates both FANCD2 and

PCNA ubiquitination respectively [98,126]. Therefore, DDT and the FA pathway may have partially overlapping, sequential, or interactive functions in coordinating error-free HR and error-prone TLS.

9.8 Animal Studies of DDT

Currently, there exists a mouse model for the TLS arm of the DDT pathway in which POLη was knocked-out. POLη heterozygous and homozygous null mice had accelerated development of UV induced skin tumours, successfully modeling the human XPV syndrome. In addition, knock-out models for Polι and Polζ have also been developed. While POLι deficient mice are more susceptible to developing mesenchymal tumours after UV irradiation, loss of Polζ in mice leads to embryonic lethality due to gross chromosomal instability [127,128]. This suggests that these TLS polymerases are important in tolerating endogenous and exogenous damage during and post-development. Interestingly, the chromosomal instability observed in POLζ null cells is similar to cells with deficiencies in HR suggesting a dual role for this polymerase in HR and TLS; a role already ascribed to POLη [110,128]. To date, a model for the role of K63 polyubiquitin chains in error-free DNA repair does not yet exist. Therefore, we believed that our mice expressing K63R dominant negative mutant in trans provided us with a unique opportunity to establish the role of K63-linked chain in mammalian DNA repair [87]. However, overall expression levels and the understated importance of GFP and HIS tag levels clouded the results pertaining to the role of K63-linked ubiquitination in carcinogenesis. Consequently, in order to understand the role of DDT in complex organisms, future studies should involve the

development of alternative animal models. Specifically, we believe that the targeted disruption or overexpression of genes that catalyze the formation of such chains including RAD18, UBC13, MMS2/UEV1a and/or SHPRH would help determine and elucidate how various levels of these genes affect the tissues *in vivo*, and whether they corroborate their associated roles in mammalian carcinogenesis.

9.9 Targeting error-free DDT to modulate drug sensitivities in cancer patients

The ubiquitin family regulates many cellular functions including those of NF-κB activation and DNA repair through ubiquitination. Each of these events has an E1 and distinct E2, E3 and DUB enzymes that regulate the addition or removal of ubiquitin to or from specific substrates. Not surprisingly, malfunction of some of these enzymes has been associated with a variety of disorders, such as cancer and rheumatoid arthritis [129,130]. Therefore, members of the ubiquitin family may serve as attractive drug targets for cancer modalities. A drug that has demonstrated great success in this category is the proteasome inhibitor PS-341 (Bortezomide, velcade), which has been approved for clinical use in humans. Due to its selective cytotoxicity towards cancer cells, PS-341 has shown great promise as a therapeutic drug against multiple myeloma (MM) in cell culture, animal models, and in multi-center phase II and phase III human clinical trials [131-138]. Although the selectively of PS-341 toward cancer cells is poorly understood in MM, once internalized it works by blocking ubiquitin-mediated proteolysis, which blocks NF-κB activation, and subsequently triggering a dual apoptotic cascade involving cytochrome C release from the mitochondria, followed by caspase 9 activation, as well as Fas/caspase 8

activation [131,139]. Together, this is thought to mitigate the objective responses and stabilization of disease. One would predict that shutting down an important process, such as proteasome mediated degradation, would wreak havoc within cells and cause severe side-effects in humans. Paradoxically, PS-341 has been well tolerated with minimal side effects, including: low grade fever, diarrhea, fatigue, minor skin rashes, and thrombocytopenia [135,140]. Moreover, it has also been used successfully to quell inflammatory response in the streptococcal cell wall-induced polyarthritis animal model [141]. This expands the repertoire of potential diseases it may treat to include immune disorders, such as rheumatoid arthritis, in humans. Overall the paradoxical success of PS-341 in clinical trials has spurred the search for other viable drug targets in the ubiquitin family.

Based on genetic studies in yeast and human cells, we certainly believe that the ubiquitin family members of the DDT pathway including RAD18, RAD6, SHPRH, and UBD proteins like POLζ, and POLη could be potential and viable disease targets. First, disruption of RAD18 in yeast leads to increased mutagenesis and overt sensitivity to a variety of DNA damaging agents. Second, RAD18 mouse embryonic stem cells lead to increased genomic instability via increased sister chromatid exchange, homologous recombination and illegitimate recombination [142]. Third, targeted disruption of RAD18 in Chicken DT40 leads to a similar phenotype to that found in mouse embryonic stem cells [143]. Fourth, XPV, a syndrome that results in increased skin cancer in humans, results from loss of POLη, a polymerase that binds to RAD18 and is recruited to sites of damage via RAD18 dependant PCNA monoubiquitination [71]. Fourth, RAD18 maps to chromosome

3p24–25, which is a region often associated with a variety of cancers, most notably lung cancer [72]. Fifth, human POLζ (particularly the REV3L encoded gene) maps to chromosome 6q21, a region that is frequently found to be deleted in a variety of hematological malgnancies [144-146]. Recently we demonstrated that disruption of K63-linked chains (chains that are also catalyzed by RAD18) in lung cancer cells leads to a dramatic increase in BPDE (common environmental carcinogen in car exhaust) induced mutagenesis. Interestingly, the decreased expression of hRAD6B (the binding partner of hRAD18) was also associated with lung cancer. Finally, SHPRH, a gene that maps to chromosome 6q24-27, has recently been shown to be associated with a variety of cancers including those of the breast, prostate, pancreas and ovaries [147]. *SHPRH* is the supposed human ortholog of yeast RAD5. It functions as an E3 ligase that forms a complex with RAD6, RAD18, and UBC13/MMS2 and extends RAD18 monoubiquitinated PCNA via K63-linked polyubiquitination [67,148]. Overall, there is accumulating evidence which suggests that members of the DDT pathway are directly and indirectly involved in genomic stability and carcinogenesis.

Certainly there is precedence in determining the exact role of RAD18 and its partners in cancer biology from studies of hRAD6 and disruption of K63-linked polyubiquitin chains. For example, overexpression of hRAD6b is associated with chemoresistance, thus targeting RAD6 in cancer may sensitize tumour cells to chemotherapy. Moreover, we showed that disruption of K63-linked polyubiquitination (chains formed by the orchestrated action of RAD6, RAD18, UBC13/MMS2 and SHPRH) leads to increased sensitivity to chemotherapeutic agent

cisplatin. Therefore, targeting of RAD18 and its binding partners posseses the potential to sensitize cancer cells to chemotherapy. The validity of the latter statement has been partially substantiated in that loss of heterozygosity in the chromosome region of RAD18 is involved in chemosensitivity. Certainly these lines of evidence support the assertion of RAD18 and its partners as a putative drug target. Previous targeting of the UPP pathway, such as the proteasomal inhibition (PS-341), have yielded great results in combating cancer and there is no reason why targeting, for example, RAD18 or its partners could not provide the same benefit. However, since new roles for these proteins are still being elucidated, it is wise to urge caution concerning the use of these inhibitors in the clinic before vigorous testing in cell culture and animal studies is performed. In brief, our understanding of the complex biological functions and interplay of these enzymes with various pathways is at its molecular infancy. However, the success of drugs like PS-341 offer great hope that targeting such members of this pathway will offer therapeutic benefit for patients in the future.

9.10 Conclusions

From its discovery in 1974 as a thymopoeietic hormone, ubiquitin [149-151] has risen from the depths of obscurity into the new millennium as an essential post-translational modification that rivals that of phosphorylation. Significantly, its discovery as a covalent tag involved in targeting proteins to the proteasome (in the 1980's) made its discoverers Aaron Ciechanover, Avram Hershko and Irwin Rose worthy of the Nobel prize in 2004 [152-154]. Their work revolutionized our present understanding of many molecular processes including, translation, transcription,

endocytosis, cellular trafficking, cell cycle, apoptosis, and DNA repair. While some proteins in these processes require a single ubiquitin monomer, others require "ubiquitin in chains" [2]. Of particular interest to us was the role of non-canonical chains linked through lysine 63. Interestingly, ubiquitin chains formed via K63 can serve as competent proteolytic signals but they are much less efficient at targeting substrates to the proteasome in comparison to K48-linked chains [155,156]. Instead, these polymeric signals are implicated in altering protein function/structure and/or serve as structural scaffolds that build multisubunit signalosomes involved in a variety of processes, most notably DNA damage tolerance repair.

The work presented in this thesis reports the functional importance of K63-linked polyubiquitin chains on PCNA in guarding against carcinogenesis in mammalian cells. Furthermore, we show that the functional conservation of this pathway from yeast to humans, coupled with its implied role in a variety of cancers, demonstrates the potential to exploit the DDT family members as novel targets to modulate chemosensitivity of tumours. Thus far, knowledge of ubiquitin mediated proteolysis pioneered the way towards the development of proteasome inhibitors which have shown great promise in treating refractory cancers in human clinical trials [138]. Certainly the success of such therapies will drive further development of drugs that target ubiquitin mediated processes. Finally this thesis also highlights the additional complexity inherent in whole organisms as opposed to cell culture models, as evidenced by the unexpected findings in the transgenic mice

A challenge for future investigations will be to: (1) understand whether PCNA ubiquitination is regulated through broad signal transduction pathways or through an

independent compartmentalized response, (2) identify binding partners and other targets of this novel ubiquitination, (3) understand how these chains promote interaction with other pathways (FA, NF-κB) to mediate error-free recovery from DNA damage, (4) understand the cross-talk with other post-translation modifications (5) understand the role of these chains in animal models, (6) identify the spatio-temporal sequence of ubiquitination in DDT pathway and (7) understand the interaction between TLS and HR and the overlap of DDT genes with HR. It is our hope that the answers to these questions will help further unmask the molecular mechanisms of DDT in carcinogenesis in response to environmental mutagens and should reveal whether DDT repair proteins could serve as putative drug targets to maximize the effects of DNA damaging agents, such as cisplatin, during chemotherapy.

Chapter 10

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for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen, Proc Natl Acad Sci U S A 103 (2006) 18107-18112.

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- Meeting on DNA Repair: From Molecular Mechanism to Human Disease; Noordwijkerhout, Holland (Apr. 2006; **Finalist for Overall Poster Competition**)
- Poster Session A III: Replication of DNA Damage; Noordwijkerhout, Holland (Apr. 2006; **1st Place**)
- Ontario Graduate Scholarship in Science and Technology (2004-2006; **$15000/annum**)
- University of Ottawa Excellence Award Scholarship for Doctoral Studies (2003-2004; **$10000/annum**)
- CIHR National Health Research Poster Competition University of Manitoba (May 2003 **2nd Place**; **$250**)
- CIHR National Health Research Poster Competition Travel Award (May 2003; **$250**)
- University of Ottawa Biochemistry Graduate Student Poster Competition (Apr. 2003; **1st Place** PhD; **$125**)
- University of Ottawa Biochemistry Graduate Travel Award to Reno, Nevada (Jan. 2002; **$900**)
- University of Ottawa Excellence Award Scholarship for Doctoral Studies (2001-2003; **$10000/annum**)
- University of Ottawa Entrance Scholarship for Graduate Studies (1998-1999; **$5000**)
- Dean's Honor List (1992-1996; GPA 3.65)
- Arizona State University Golden Key National Honors Award (1993)
- Arizona State University National Academic Honors Award for Freshmen (1992)

PROFESSIONAL EXPERIENCE

2001-2007: **Centre for Cancer Therapeutics,** Ottawa, Canada
Ph.D Thesis
Supervisor: Dr. Douglas A. Gray
Project: Unmasking the role of ubiquitin in mammalian systems

2000-2001: **Ottawa Regional Cancer Centre,** Ottawa, Canada
Technician
Supervisor: Dr. B. Wouters
Project: Defining the role of ubiquitin in stress response pathways

1998-2000: **Ottawa General Hospital**, Ottawa, Canada
Master of Science Thesis
Supervisor: Dr. R. Goldstein and Dr. H.C. Birnboim
Thesis Title: Pathogenesis of rheumatoid arthritis: Insights into mechanisms of synovial hyperplasia and of reactive nitrogen species mediated inflammatory damage

1994: **Hradec Kralove Hospital Faculty of Medicine,** Hradec Kralove, Czech Republic

Summer Student Research: Department of Internal Medicine
Project: The effect of beta-blockers on the QT interval of the heart

1990-1991: **Amberwood Village Golf and Country Club,** Stittsville, ON, Canada
Sales & Maintenance Assistant
Supervisor: Mr. K. Hossack

1990-1991: **Amberwood Tennis Club**, Stittsville, ON, Canada
Tennis Instructor
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RESEARCH SKILLS

Expertise in Various Molecular and Biochemical Techniques including:
Tissue culture of mammalian tumor, primary human and murine cell lines
Mammalian cell transformation, transduction and transfection
Mouse experimentation, MACs Hypoxic Chamber
Immunohistochemistry , Immunocytochemistry, Immunoprecipitation
Western blot analysis, Flow Cytometry
Enzyme-linked immunosorbent assay (ELISA)
In situ nick end labeling (ISNEL/TUNEL)
DNA Isolation, Cloning, Sub-cloning, Gel Electrophoresis, PCR,
Clonogenic Survival Assay, HPRT Mutagenesis Assay

ORAL PRESENTATIONS

1. **Brun, J.,** Sandhu, J.K., Goldstein R., and Birnboim, H.C. The localization of protein nitrotyrosine in rheumatoid and osteoarthritic synovium. University of Ottawa, Department of Biochemistry, Microbiology and Immunology Seminar Series, Ottawa, Canada (**November 1999**).

2. Chiu, R.K., **Brun, J.,** Klippenstein, J.R., Gray, D.A., and Wouters, B.G. Mammalian DNA repair and the involvement of ubiquitin conjugating enzymes. (Abstract/Oral Presentation) Annual meeting Dutch Radiation Oncology Society, Utrecht, The Netherlands (**March 2001**).

3. **Brun, J.,** Chiu, R.K., Gray, D.A., and Wouters, B.G. The role of novel polyubiquitin chains in mammalian post-replication repair. University of Ottawa, Department of Human and Molecular Genetics Seminar Series, Ottawa, Canada (**January 2002**).

4. **Brun, J.,** Chiu, R.K., and Wouters, B.G., Gray, D.A. Unmasking the role of ubiquitin in mammalian systems. OHRI 3rd Annual Research Day, Hellenic Centre Ottawa, Canada (**January 2003**).

5. **Brun, J.,** Chiu, R.K., Wouters, B.G., Gray, D.A. The role of K63-linked ubiquitin

chains in post-replication repair. OHRI 4th Annual Research Day, Hellenic Centre Ottawa, Canada (**November 2004**).

6. **Brun, J.,** Chiu, R.K., Wouters, B.G., Gray, D.A. Conservation of DNA damage induced polyubiquitination of PCNA in human cells. Poster Discussion Session III: Replication of DNA Damage. Noordwijkerhout, Netherlands (**April 2006**).

7. **Brun, J.,** Chiu, R.K., Wouters, B.G., Gray, D.A. K63-linked polyubiquitination promotes error-free damage avoidance. National Insititute of Health, Bethesda, Maryland. **(October 2006)**

PUBLICATIONS

1. Sabine A.S. Langie, Ad M. Knaapen, Chantal H.M.A. Ramaekers, **Jan Brun**, Jan Theys, Roger W.L. Godschalk1, Frederik-Jan van Schooten, Doug A. Gray, Philippe Lambin, Bradly G. Wouters and Roland K. Chiu. Formation of Lysine 63-linked poly-ubiquitin chains protects human cells against benzo[a]pyrene-diol-epoxide-induced mutagenicity. DNA Repair (Amst). 2007 Mar 27; [Epub ahead of print]

2. **Jan Brun**[#] and Roland K. Chiu[#], Chantal Ramaekers, Jan Theys, Lin Weng, Philippe Lambin, Douglas A. Gray, and Bradly G. Wouters. K63 polyubiquitination protects cells against mutations induced by DNA lesion bypass polymerases. PLoS Genet. 2006 Jul;2(7):e116D.

3. A. Gray, M. Tsirigotis, **J. Brun,** M. Tang, M. Zhang, M. Beyers, and J. Woulfe. Protective Effects of mutant ubiquitin in transgenic mice. Ann N Y Acad Sci. 2004 Jun;1019:215-8.

4. **Jan Brun** and Douglas A. Gray. RAD18 as a potential target for drug development: To Target or not to Target. **(In Press for Swiss-Prot Targeted Proteins Database (TPdb))**

5. **Jan Brun**, Roland K. Chiu, Bradly G. Wouters and Douglas A. Gray. MMS2 serves a redundant role in mammalian PCNA ubiquitination. ***(Submitted to DNA repair)***

6. **Jan Brun**, Roland K. Chiu, Bradly G. Wouters and Douglas A. Gray. Regulation of PCNA ubiquitination in human cells. ***(Submitted to JBC)***

7. Jan Brun. Proteasome inhibition as a novel therapy in treating rheumatoid arthritis. **(Submitted to Medical Hypotheses)**

8. Chantal H.M.A. Ramaekers [#], **Jan Brun**[#], Douglas A. Gray, and Bradly G. Wouters, Roland K. Chiu. K63-linked ubiquitin chains protect cells from spontaneous mutagenesis. (*Manuscript in Preparation*)

9. **Jan Brun,** Jagdeep Sandhu, Rosie Golstein, Chaim Birnboim. Synovial hyperplasia is associated with mild increase in proliferation and inadequate apoptosis in the arthritic synovial layer. (*Manuscript in preparation*)
[#] **Authors Contributed Equally to this Work.**

PUBLISHED ABSTRACTS

1. Sandhu, J., **Brun, J.,** Roberstson, S.J., Goldstein R., Karsh J, and Birnboim, H.C. Evidence of protein nitration in the synovium of rheumatoid arthritis and osteoarthritis patients. ACR Meeting, Philadelphia, Arthritis Rheumatism 43 (suppl): S69, October 2000.

ABSTRACTS

1. Sandhu, J., **Brun, J.,** Robertson, S.J., Goldstein R., and Birnboim, H.C. Widespread Distribution of Protein Nitrotyrosine in the Synovial Tissue of Patients with Osteoarthritis and Rheumatoid Arthritis. (Abstract F070) 43rd Annual Meeting of the Canadian Federation of Biological Societies, Ottawa, Canada. (June 2000).

2. **Brun, J.,** Sandhu, J., Roberstson, S.J., Goldstein R., and Birnboim, H.C. Proliferation and Apoptosis in the Synovial Lining of Patients with Rheumatoid Arthritis and Osteoarthritis. (Abstract F055) 43RD Annual Meeting of the Canadian Federation of Biological Societies, Ottawa, Canada. (June 2000).

3. Chiu, R.K., **Brun, J.,** Klippenstein, J.R., Gray, D.A., and Wouters, B.G. Mammalian DNA repair and the involvement of ubiquitin conjugating enzymes(Abstract/Oral Presentation). Annual meeting Dutch Radiation Oncology Society, Utrecht, The Netherlands (March 2001).

4. Chiu, R.K., **Brun, J.,** Klippenstein, J.R., Gray, D.A., and Wouters, B.G. Sensitivity of mammalian cells expressing mutant ubiquitin to DNA damaging agents. (Abstract) 8th Euregional Oncology Meeting, Maastricht, The Netherlands (April 2001).

5. Chiu, R.K., **Brun, J.,** Klippenstein, J.R., Gray, D.A., and Wouters, B.G. The role of novel ubiquitin chains in mammalian post-replication repair and UV hypersensitive disorders. (Abstract) 31st Annual Meeting of the European Society for Radiation Biology, Dresden, Germany (September 2001).

6. Goldstein R., Sandhu, J., **Brun, J.,** Robertson, S.J and Birnboim, H.C. Proliferation and Apoptosis in the Synovial Lining of Patients with Rheumatoid Arthritis and Osteoarthritis. (Abstract) Canadian Rheumatology Association. Annual Meeting, Lake Louise, Alberta (February 2002).

7. Chiu, R.K., **Brun, J.,** Klippenstein, J.R., Gray, D.A., and Wouters, B.G. Ubiquitin mediated signal in post-replication repair and UV hypersensitive disorders. (Abstract) 49th Annual Meeting of the Radiation Research Society, Reno, Nevada (April 2002).

8. **Brun, J.,** Chiu, R.K., Gray, D.A., and Wouters, B.G. The role of novel polyubiquitin chains in post-replication repair of mammalian cells. (Abstract) 49th Annual Meeting of the Radiation Research Society, Reno, Nevada (April 2002).

9. Chiu, R.K., Theys, J., **Brun, J.,** Gray, D.A., and Wouters, B.G. The role of ubiquitin in DNA damage repair. ASM Conference on DNA Repair & Mutagenesis: From Molecular Structure to Biological Consequences. Southampton Bermuda (November 2004).

10. **Brun, J.,** Chiu, R.K., Wouters, B.G., Gray, D.A. Conservation of DNA damage induced polyubiquitination of PCNA in human cells. DNA Repair: From Molecular Mechanism to Human Disease. Noordwijkerhout, Netherlands (April 2006).

NEWSPAPER ARTICLES

1. Ottawa Scientists find molecule that repairs sun-damage DNA: Breakthrough could help in bid to find way to kill cancer cells. By Tom Spears. The Ottawa Citizen. Ottawa, Canada. July 21st 2006. (http://www.canada.com/ottawacitizen/news/city/story.html?id=6ffa9808-aef2-4c77-8509-89c9972b903e)

2. Eiwit herstelt schade aan DNA door straling. door René Steenhorst. Binneland. Maastricht Netherlands. July 27th 2006. (http://www.telegraaf.nl/binnenland/47197241/Eiwit_herstelt_schade_aan_DNA_door_straling.html)

ACKNOWLEDGEMENTS

1. Sandhu JK, Robertson S, Birnboim HC, Goldstein R. Distribution of protein nitrotyrosine in synovial tissues of patients with rheumatoid arthritis and osteoarthritis. J Rheumatol. 2003 Jun; 30(6):1173-81.

VOLUNTEER ACTIVITIES

1999-present: **Centre for Cancer Therapeutics, Let's Talk Science Program,** Ottawa, Canada
Lecturer for science outreach program for high schools in the Ottawa Carleton District
Co-ordinator: Dr. B. Vanderhyden
Responsibilities: Seminar presentation, demonstrations of molecular techniques.

1996: **Arizona Heart Institute (AHI), Public Relations Dept.,** Phoenix, Arizona
Seminar Coordinator
Supervisor: Denise Frakes
Responsibilities: Arranging public seminar series regarding heart disease

1995: **Hradec Kralove Hospital Faculty of Medicine,** Prague, CR
Student Volunteer for the Cardiology Clinic
Supervisor: Dr. J. Kvasnicka
Responsibilities: Recording EKGs, triage, surgical room assistance during angioplasty and pacemaker implantation.

1993-1994: **Chandler Regional Hospital,** Chandler Arizona
Emergency Room Volunteer

EXTRACURRICULAR ACTIVITIES

Swimming: 1. Arizona State University: A-division Intramural Championship 1993: **1st place**
2. Arizona State University: A-division Intramural Championship 1994: **2nd place**

Triathlons: 1. Olympic Swim-Bike Duathlon 2006 (Ottawa, ON): **3rd Place**
2. Ottawa Athletic Club Team Triathlon 2004 (Gatineau, QC): **3rd/33 Teams**
3.Subaru Triathlon Series: National Team Sprint Triathlon 2002 and 2003 (Ottawa, ON): **1st place**
4. Ottawa Athletic Club Team Triathlon 2003 (Gatineau, QC): **9th/45 Teams**
5. Somersault Team Triathlon Series: Olympic Triathlon 2003 (Brockville, ON): **1st Place**
6. Somersault: Team Sprint Triathlon 2003 (Ottawa, ON): **1st Place**

Tennis: 1. Kiwanis Tennis Club (1996 Tempe, AZ): Advanced League
2. NCTA Junior Tennis Tournaments 1988-1991 (Ottawa, ON)

Cross-country skiing
Alpine skiing
In-line skating
Traveling