NOVEL MECHANISMS THROUGH WHICH TRANSLESION SYNTHESIS PROTECTS GENOME INTEGRITY

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ABSTRACT

MICHAEL DURANDO: Novel Mechanisms through which Translesion Synthesis Protects Genome Integrity
(Under the direction of Cyrus Vaziri, Ph.D.)

Exposure to ubiquitous environmental carcinogens, such as polycyclic aromatic hydrocarbons and UV light, is a major cause of human disease. It is well accepted that genetic mutations are an important step in the development of cancer. It has become clear that such mutations are introduced in part by error-prone DNA polymerases. In response to many environmental genotoxins, eukaryotic cells have evolved alternative methods of replicating damaged DNA via the Translesion DNA synthesis (TLS) Polymerases, consisting of DNA Polymerase eta (Polη), DNA Polymerase kappa (Polκ), DNA Polymerase iota (Polι), and Rev1. TLS is a DNA damage tolerance mechanism that uses low-fidelity DNA polymerases to replicate damaged DNA. The inherited cancer-propensity syndrome Xeroderma Pigmentosum Variant (XPV) results from error-prone TLS of UV-damaged DNA. TLS is initiated when the Rad6/Rad18 complex monoubiquitinates PCNA, but the basis for recruitment of Rad18 to PCNA is poorly understood. This dissertation studies several aspects of regulatory mechanisms that contribute to the damage-induced activation of Rad18 E3 ligase activity at PCNA. First, we report a novel role for Pol η, the XPV gene product that is mutated in XPV, in targeting Rad18 to PCNA to initiate TLS. Using structure-function analyses and immunofluorescence microscopy, we identified a C-terminal domain of Polη that physically bridges Rad18 and PCNA to facilitate redistribution of Rad18 to

stalled replication forks and promote PCNA monoubiquitination. This scaffold function is unique to Polη among Y-family TLS polymerases and dissociable from its catalytic activity. Importantly, XPV cells expressing full-length, catalytically inactive Polη exhibit increased recruitment of error-prone TLS Polymerases after UV irradiation, indicating that maintaining the bridging function of Polη in the absence of its catalytic activity greatly predisposes to mutagenesis. These findings define a molecular basis for TLS pathway activation and provide a new mechanism for mutagenesis and genomic instability in XPV individuals. Next, this dissertation reports novel mechanisms of regulating TLS via stress-activated protein kinase (SAPK) phosphorylation of Rad18 and via Chk1-dependent phosphorylation events. Finally, this dissertation presents data indicating that TLS is involved in the tolerance of oncogene-induced replication stresses and potentially oncogene-induced mutagenesis.

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LIST OF ABBREVIATIONS

Polη	DNA Polymerase η
Polκ	DNA Polymerase κ
Polι	DNA Polymerase ι
XP	Xeroderma Pigmentosum
XPV	Xeroderma Pigmentosum Variant
RPA	Replication Protein A
ssDNA	single stranded DNA
DSB	Double Strand Breaks
DDR	DNA Damage Response
RF	Replication Fork
OIS	Oncogene Induced Senescence
PCNA	Proliferating Cell Nuclear Antigen
PIP box	PCNA Interacting Peptide domain
IF	Immunofluorescence
PAH	Polycyclic Aromatic Hydrocarbon
B[a]P	Benzo[a]pyrene
BPDE	Benzo[a]pyrene-Dihydrodiol Epoxide
ROS	Reactive Oxygen Species

TLS	Translesion DNA Synthesis
NER	Nucleotide Excision Repair
BER	Base Excision Repair
HR	Homologous Recombination
NHEJ	Non-homologous End-joining
CPD	Cyclobutane Pyrimidine Dimer
6-4-PP	6-4 Pyrimidine-pyrimidone Photoproduct
RNR	Ribonucleotide Reductase
MCM	Minichromosome Maintenance Complex

CHAPTER 1

INTRODUCTION

1. UV light, benzo[a]pyrene, and environmental carcinogenesis

UV light and polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental mutagens and carcinogens. Solar UV light is omnipresent and is a major cause of morbidity worldwide.[1,2] Solar UV radiation is divided into three types: UVC, UVB, and UVA (**Table 1-1**).[3] UV radiation causes DNA damage in a manner inversely proportional to wavelength.[4] Solar radiation below 290 nm carries the highest energy and is most detrimental to DNA and other biomolecules but is primarily absorbed by stratospheric ozone. Approximately 90% of the UV radiation that traverses atmospheric ozone is relatively low-energy UVA.[3]

Table 1.1. Properties of solar ultraviolet (UV) radiation

UV radiation	Wavelength (nm)	Energy (eV)	% reaching earth surface
UVC	(<280)	>4.4	0
UVB	(280-315)	3.9-4.4	10
UVA	(315-400)	3.1-3.9	90

UV radiation causes DNA damage through different mechanisms depending on wavelength.[5] The genotoxic effects of UVC and UVB are predominantly due to absorption by adjacent pyrimidine moieties in DNA, causing fusion of their double bonds to form cyclobutane pyrimidine dimers (CPDs) and also to a lesser extent 6-4 pyrimidine-pyrimidone photoproducts (6-4-PPs) (**Figure 1-1**).[6] UV-induced cell killing correlates directly with the induction of DNA lesions up to about 313 nm, but

at greater wavelengths (UVA), cell killing remains high while the relative number of DNA lesions decreases. In fact, irradiation at 385 nm and higher still induces high relative cytotoxicity, even when CPDs are virtually undetectable,[7] indicating that DNA damage mechanisms other than CPDs or 6-4PPs contribute to UVA-induced killing at these wavelengths. Consistently, depletion of glutathione to cause oxidative stress leads to a many-fold increase in UVA-induced DNA mutations but no change in UVB-induced mutations, indicating that the genotoxic effects of UVA are predominantly due to production of Reactive Oxygen Species (ROS), such as hydrogen peroxides and hydroxyl radicals.[8] Thus, UVC and UVB induce mutations primarily via direct modification of DNA whereas UVA is mutagenic primarily via ROS.

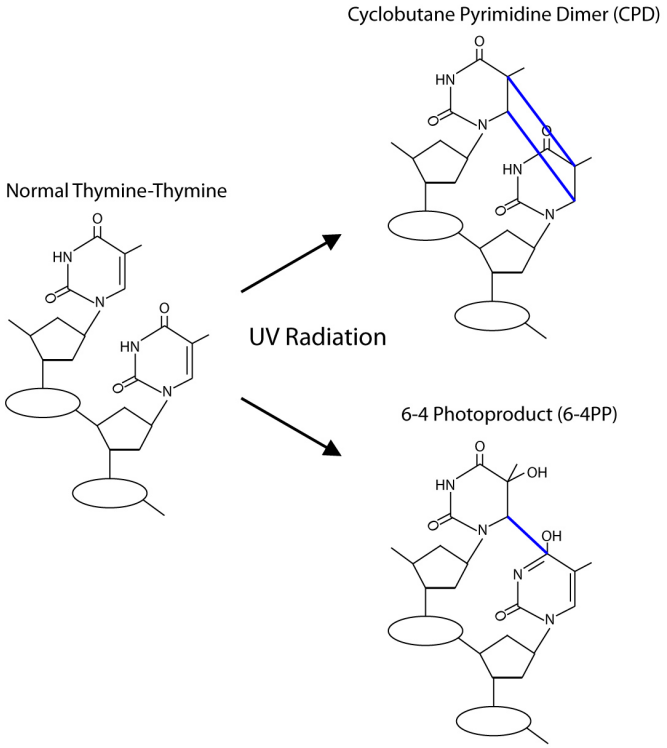


Figure 1-1. Schematic representation of major UV-induced DNA lesions.

PAHs are a major source of environmental pollutant that are produced primarily through the processing and consumption of coal and crude oil but also through combustion of wood and tobacco products.[9] Human exposure to PAHs has been associated with increased cancer risk, and many PAHs are tumorigenic in animal models.[9] PAHs such as Benzo[a]pyrene (B[a]P) require activation to electrophilic metabolites to exert mutagenic or carcinogenic effects. In vivo, such PAHs are metabolized in mammalian cells by cytochrome P450s to generate the highly reactive and mutagenic metabolites. B[a]P, for example is metabolized to its highly reactive form BPDE, which binds covalently to genomic DNA to form bulky adducts at the N2 position of guanine (**Figure 1-2**).[10,11] Such bulky adducts resemble UV-induced DNA lesions and induce mutations and cell killing via similar mechanisms, although PAHs illicit distinct cellular responses as well.[12]

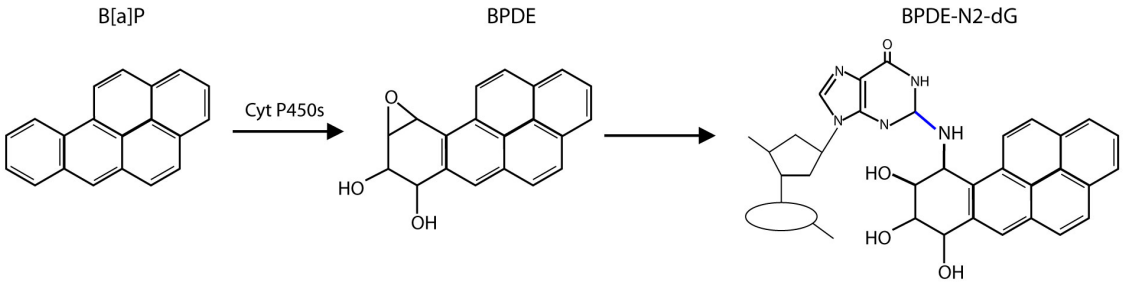


Figure 1-2. Metabolic processing of Benzo[a]pyrene to the genotoxin BPDE, which covalently binds to deoxyguanine.

Mutations are a fundamental step in the progression of many human diseases, including cancer, and propagation of cells that have acquired mutations in oncogenes or tumor suppressor genes contributes to multi-step carcinogenesis. Mutations can be introduced via numerous mechanisms, such as replication and repair errors,

depurination or deamination, and endogenous or environmental mutagens.[13] DNA harboring bulky DNA lesions, such as CPDs or dG-BPDE, adopts a distorted conformation that impedes normal replication. The presence of such damaged DNA is strongly correlated with the acquisition of mutations. For example, the location of UV- or BPDE-induced mutations in the p53 gene correlates strongly with hotspots of CPDs and BPDE target sites, respectively.[14, 15] Thus, replication or repair of DNA containing CPD and dG lesions carries a high risk of introducing mutations.[14]

2. DNA Damage Tolerance and Trans-Lesion Synthesis (TLS)

Living cells are continually exposed to endogenous and environmental DNA damaging agents, such as UV light and BPDE. It has been estimated that cells encounter approximately 10,000 DNA lesions/day[16]. Such an onslaught of DNA damage poses a major threat to genetic instability, and cells have evolved numerous mechanisms of tolerating it. Major DNA repair pathways in human cells include base excision repair (BER), nucleotide excision repair (NER), homologous recombination, non-homologous end-joining, Fanconi Anemia, and translesion synthesis (TLS)[17]. Deficiencies in any of these pathways compromises genetic stability, as evidenced by their associated cancer propensity syndromes (Table 1-2).

Table 1-2. Major DNA repair pathways and associated cancer propensity syndromes.

Pathway	Syndrome	Deficient protein
NER	XP	many
BER	Various cancers	OGG1, XRCC1[18]
HR	Breast, ovary cancer	Brca1, Brca2[19]
NHEJ	LIG4 syndrome	Ligase IV[20]
FA	Fanconi Anamia	15 FANCs[21]
TLS	XPV	Poleta

Each of these DNA repair pathways protect against the deleterious effects of DNA damage. With regard to UV light and PAHs, CPDs and BPDE adducts greatly predispose to mutagenesis. Replicative polymerases replicate undamaged DNA with a high processivity and fidelity, but they are unable to replicate past UV- and BPDE-induced DNA lesions. Instead, replicative polymerases stall after encountering such DNA lesions, as the tight catalytic active site of a replicative polymerase is incapable of accommodating such bulky DNA-distorting adducts (**Figure 1-3**). Stalling of the replicative polymerase causes uncoupling of the replication fork, in which the MCM-containing helicase complex continues unwinding DNA after the polymerase ceases replication (**Figure 1-4**). This leads to long stretches of single-stranded DNA that is rapidly coated by Replication Protein A (RPA), thus initiating a series of signaling cascades that halts replication and turns on DNA repair pathways (see Checkpoints section).

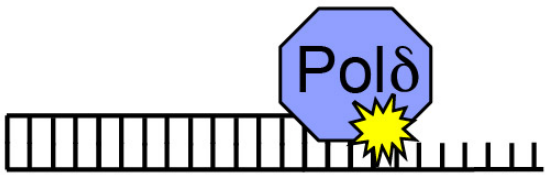


Figure 1-3. Diagram of a processive polymerase that has stalled upon encountering a bulky DNA adduct (yellow) in its active site.

Unlike other DNA repair pathways that do not necessarily distinguish between the nature of DNA damage, the translesion synthesis (TLS) pathway of DNA damage tolerance is specifically directed toward replication-stalling bulky DNA adducts, such as those induced by UV light and BPDE. TLS utilizes a group of low-processivity, low-fidelity polymerases (compared to replicative polymerases) that are capable of replicating past bulky DNA adducts. Bypass of bulky DNA adducts is performed primarily by a specialized group of four DNA polymerases called the Y-family Polymerases (Y Pols),[22] consisting of DNA Polymerase eta (Polη)[23], DNA Polymerase kappa (Polκ),[24] DNA Polymerase iota (Polι),[25] Rev1.[26,27]

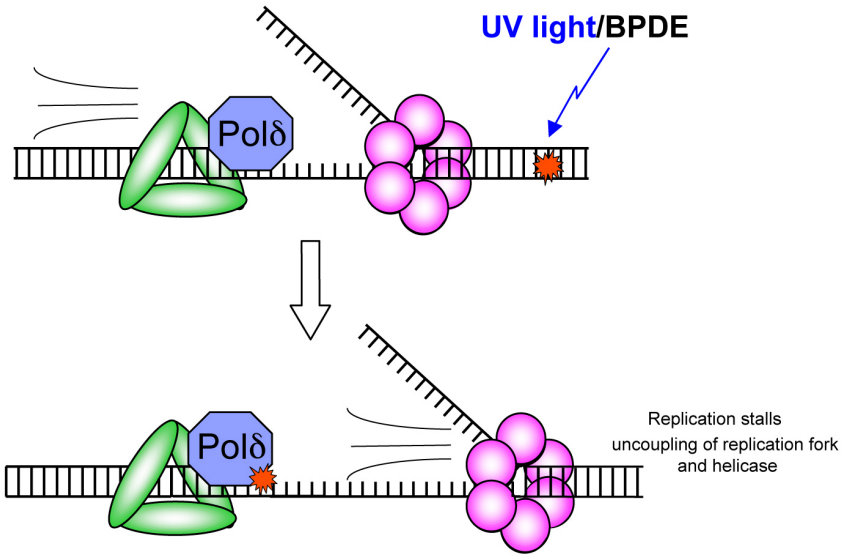


Figure 1-4. Uncoupling of the replication fork and helicase after encountering a bulky adduct DNA lesion.

In response to bulky DNA adducts like CPDs, these polymerases are recruited to replication forks where they interact with proliferating cell nuclear antigen, PCNA, a homotrimeric replication processivity factor to which DNA polymerases bind as they replicate DNA. Polη, Polκ, and Polι contain highly conserved PCNA Interacting Peptide domains (PIP boxes) that facilitate physical interaction with PCNA,[28] whereas Rev1 interacts with PCNA via a BRCT domain.[29] For TLS to occur, a Y-family polymerase must switch places with the replicative polymerase that has stalled upon encountering a bulky adduct **(Figure 1-5a)**.[30,31] The mechanisms dictating this polymerase switch and the selection of the appropriate polymerase are poorly understood, but engagement of the appropriate polymerase with PCNA facilitates replicative bypass of the bulky adduct **(Figure 1-5b)**. Such replicative bypass of bulky adducts is called *TLS* or *lesion bypass*.

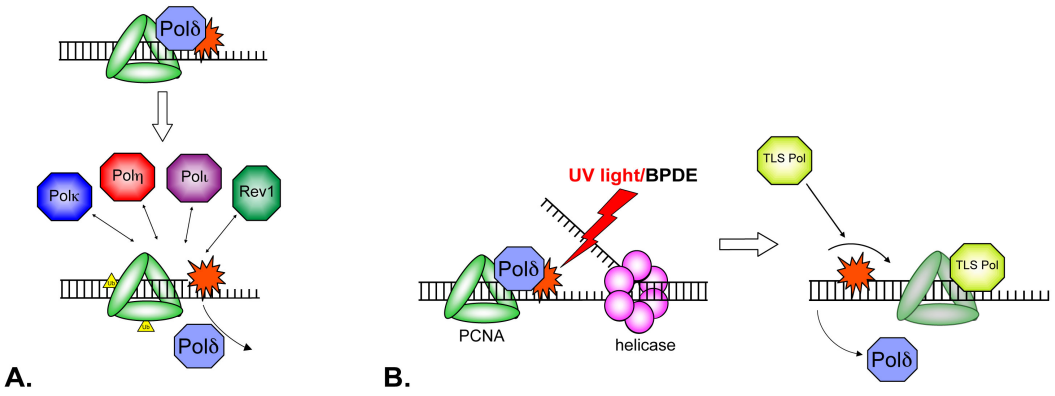


Figure 1-5. High fidelity replicative polymerase is replaced by a low-fidelity Y-family Polymerase (A) to perform TLS across the bulky adduct DNA lesion (B).

TLS across replication-stalling lesions protects against DNA breakage in the wake of uncoupled replication forks. After replicative polymerases stall upon encountering bulky DNA adducts, re-priming of the leading strand downstream of the stalled polymerase allows replication to continue but leaves ssDNA gaps between the site of re-priming and the stalled polymerase.[32] TLS facilitates replication restart at the site of the stalled polymerase and subsequent completion of replication throughout these ssDNA gaps; loss of TLS and checkpoints (see below) increases the number and persistence of such ssDNA gaps,[32, 33] which predisposes to chromosome instability.[34]

TLS is thus a crucial mechanism of preserving genomic integrity in the wake of environmentally induced DNA damage. However, the ability of TLS Pols to replicate past bulky adducts is counterbalanced by their reduced fidelity, incorporating errors at a rate of approximately 1 per 10^3 bp, substantially higher than replicative Pols, such as Polδ.[35,36,37] This relatively low fidelity of TLS Pols leads directly to the introduction of mutations in the genome.

3. TLS and mutagenesis

The existence of error-prone DNA repair pathways was first uncovered in early studies on bacterial mutants that were found to be nearly resistant to UV-induced mutations.[38] The first genes associated with UV-induced mutagenesis were *recA* and *laxA* in *E. coli*, which, when deleted, eliminated a global DNA damage response (SOS response) and also suppressed UV mutability[38]. Over 30 genes have since been linked to the DNA damage-inducible SOS response in bacteria, but the mutagenic portion of SOS is linked almost exclusively to those genes involved in TLS[39]. First discovered in screens of *E. coli* mutants that are not mutable by UV light,[40, 41] members of the UmuC/DinB family were found to exhibit polymerase activity across DNA lesions.[39] Specifically, Pol V in *E. coli*, consisting of UmuC and UmuD'2, is capable of replicating across UV-induced lesions such as CPDs[42] whereas DinB1 (Pol IV) can replicate across bulky adducts at the N2 position of guanine.[43] Eukaryotic homologues of the SOS genes were later discovered. In *S. cerivisiae*, the TLS activities of *E. coli* Pol V are performed by Rad30, which processes UV-induced lesions,[44] and Rev1, which processes abasic sites and drives mutagenesis.[45] In humans, these TLS activities are performed by the Y-family polymerases: Polη,[46] (homologue of E. coli Pol V and yeast Rad30), Rev1[47] (homologue of *E. coli* Pol V and yeast Rev1), Polκ[48] (homologue of E. coli DinB1; no yeast homologue), and Polι[49] (originally called Rad30B, no yeast or bacterial homologues).

Much research has been dedicated to the nature and the significance of low-fidelity replication by TLS polymerases. Important concepts of debate involve (i) whether the infidelity is "beneficial" or "deleterious" for the cell and (ii) whether the infidelity is accurately defined as "error-prone." The following discussion will address both of these points.

The infidelity of TLS Pols is attributed to several structural features of Y-family polymerases that differ substantially from replicative polymerases. Although the Y-family polymerases share almost no sequence homology with replicative DNA polymerases, they maintain structural similarities common to replicative polymerases, such as the classic right-hand polymerase fold that wraps around a DNA template (**Figure 1-6**) and highly conserved aspartate and glutamate residues in the active sites that coordinate magnesium ions and stabilize incoming dNTPs.[50, 51] These similarities, common to nearly all polymerases, are perhaps not surprising considering the replicative capabilities of the TLS polymerases, but several important structural and functional differences from replicative polymerases are responsible for their inaccurate replicative potential and in turn their important roles in error-prone replication, mutagenesis, and genome stability.

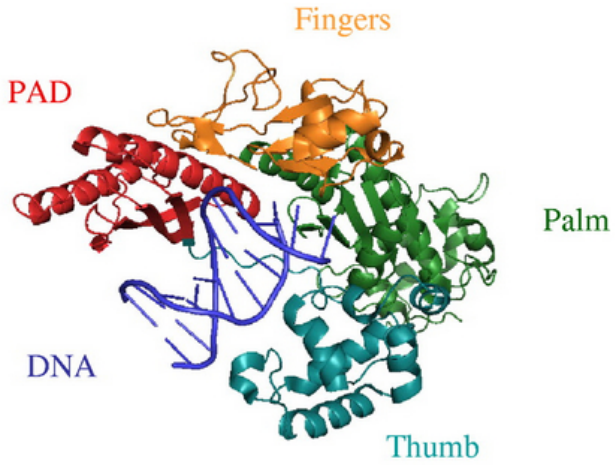


Figure 1-6. Crystal structure of *S. cerevisiae* Rad30 in complex with DNA showing the right-hand conformation consisting of palm, thumb and finger domains common to all DNA polymerases, as well as the little finger domain, or PAD, unique to Y-family polymerases.[50]

Key among these differences is their lack of 3' to 5' exonuclease proofreading activity that is common to all replicative polymerases and that removes mismatched bases.[50] Lack of such proofreading capabilities allows TLS Pols to continue replication even after insertion of the incorrect base, whereas replicative polymerases can remain stalled in futile insertion-excision cycles.[52]

Next, although Y-family polymerases contain palm, thumb, and finger domains analogous to replicative polymerases, structural nuances that are common to the Y-family Pols but different from replicative pols impart unique functional characteristics that directly impact their processivity and accuracy (**Figure 1-6**).[52] The finger domain in Y-family pols uniquely contains a so-called polymerase-associated domain (PAD), also called a little finger domain, which is normally loose and flexible but adopts a stable conformation after engagement with the cognate lesion on DNA.[53] The PAD thus determines the lesion specificity of the Y-family Pols, allowing certain Pols to replicate across their cognate lesion with relative accuracy. Polη and Polκ, for

example, perform TLS across CPDs and dGs, respectively, with relatively higher accuracy compared to replication across undamaged templates or other lesions. The PAD also contributes to catalytic efficiency and the mutational spectra.[53] Compared to replicative Pols, the palm, thumb and finger domains of Y-family Pols hold DNA with a more "open" grip, resulting in a dramatically reduced processivity.[50]

Regarding replicative fidelity, the active sites of Y-family polymerases are larger and more open than in replicative DNA polymerases, they form fewer contacts with template and nascent DNA strands, and they fail to exhibit the "induced-fit" conformational changes that drive dNTP specificity in replicative polymerases.[50] All of these characteristics combined yield a family of polymerases that, compared to replicative polymerases, have (i) reduced fidelity, (ii) reduced processivity, (iii) greater flexibility in accommodating aberrant DNA structures, (iv) unique mutational spectra, and (v) favored cognate lesions (**Table 1-3**). These properties have important implications for both the tolerance of replication-stalling DNA lesions and the genesis of mutations.

Table 1-3. Properties of Y-family Pols in comparison to replicative Pols.

Property	Y-family Pols relative to Replicative Pols	Phenotypic outcome
Fidelity	Low	Mutagenesis
Processivity	Low	Short-lived activation
Active site	Loose, non-discriminatory	Mutagenesis
Preferred template	Lesion specific	Mutagenic for non-preferred lesion

Regarding the discussion of whether the Y-family polymerases are *beneficial* or *deleterious* for a cell, one must consider the context in which they are utilized. When replicative polymerases stall after encountering bulky DNA lesions, persistent

uncoupling of replication forks can lead to fork collapse, single-strand DNA breaks, and double-strand breaks. Lesion bypass after replacement of the replicative Pol with a TLS Pol can be considered *beneficial* as it prevents the deleterious effects of DNA breaks by allowing replication to continue. Of note, a single unrepaired double strand break is sufficient to mediate cell death.[54] However, TLS can also be considered *deleterious* if the wrong polymerase is utilized for a specific lesion, thus promoting mutagenesis. For example, use of Polκ, whose preferred lesion is a bulky adduct at dG, to bypass a CPD instead of Polη (whose preferred lesion is a CPD), will be highly mutagenic, whereas use of Polη to bypass a CPD will be relatively accurate. Experiments in human cells have in fact demonstrated Polη-mediated error-free TLS across CPDs and highly mutagenic TLS across CPDs by Polκ.[55] It is critical, therefore, for cells to select the proper polymerase for the proper lesion.

Activation of the TLS Pols in the absence of damage, use of the wrong TLS polymerase, or absence of the proper polymerase after acquisition of DNA damage conferring its preferred lesion will all predispose to mutagenesis. Such scenarios highlight the unique and often confusing aspect of the TLS pathway: the TLS Polymerases are absolutely necessary to prevent genetic instability, but their aberrant activation will unequivocally lead to genetic instability. In other words, the TLS Pols must be activated only in response to DNA damage and the cell must ensure that only the correct polymerase is used for the right type of damage. The consequences of failed TLS regulation is best exemplified by the cancer propensity syndrome Xeroderma Pigmentosum Variant, in which Polη is functionally absent, leading to mutagenic replication of UV-damaged DNA by inappropriate TLS Polymerases (see

XPV section). The properties of TLS Pols summarized in **Table 1-3** and the phenotypic outcome of aberrant TLS activity demonstrate the importance of proper regulation of TLS. The molecular mechanisms that regulate TLS are still poorly understood and are also largely the focus of this dissertation.

One final important distinction of TLS involves the replication past damaged bases without removing the damage. Whereas DNA repair processes such as BER and NER remove damaged bases from the genome, TLS does not actually *repair* damaged DNA. Rather, it promotes *tolerance* of damaged DNA by allowing the continuation of replication without actually removing the damage. TLS thus promotes genomic stability primarily by preventing the catastrophic consequences of replication fork collapse, not by actually repairing or removing damaged DNA.

4. Checkpoints and the DDR

Central to the proper regulation of TLS is a cell's ability to detect and respond to DNA damage. DNA damage responses are activated by a cascade of intracellular signals that are elicited by the recognition of DNA damage. These signals, called checkpoints, utilize DNA damage sensors, signal transducers, and effector proteins to coordinate processes of DNA repair. Generally, sensor proteins (e.g. the MRN and 9-1-1 complexes) detect DNA damage on chromatin and activate signal transducers (e.g. ATM/ATR and Chk1/Ch2), which amplify the checkpoint signal to activate proximal and distal effector proteins (e.g. BRCA1). Such effector proteins are recruited to chromatin, where they facilitate damage repair or tolerance at the site of DNA damage or promote cell cycle stalling via degradation or inactivation of

replication cell cycle proteins such as cyclin-dependent kinases. Checkpoints thus coordinate cell cycle progression with genome maintenance, helping ensure that the cell cycle does not proceed if genome integrity is compromised.

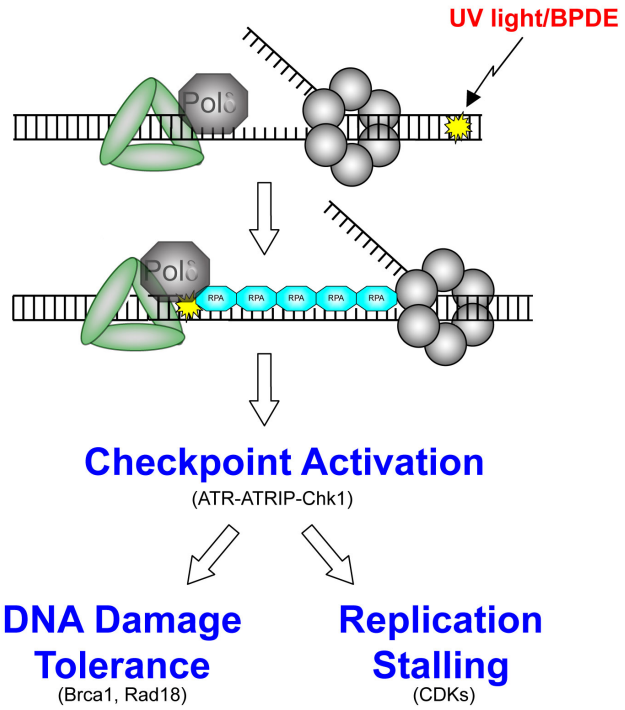


Figure 1-7. Replication-stalling lesions, such as those induced by UV light and BPDE, uncouple the replication fork forming long stretches of single-stranded DNA, which is coated by RPA. RPA recruits ATR, which activates ATRIP to phosphorylate Chk1, which then simultaneously turns on DNA damage tolerance processes and turns off normal replication.

The S-phase checkpoint response to replication stress is typically divided into two main pathways: an ATM-mediated checkpoint that responds to DNA double-strand breaks and an ATR-mediated response to stalling and uncoupling of replication forks.[56, 57] However, much cross-talk exists between the two pathways; over 700 proteins have been identified as targets of damage-induced phosphorylation by ATM and ATR, almost all of which are involved in DNA damage repair or checkpoints and many of which are phosphorylated by both pathways.[58]

Double-strand breaks are processed within cells after recognition by the Mre11-Rad50-Nbs1 (MRN) complex. ATM is rapidly recruited to these DSBs via physical interactions between the HEAT motifs in ATM and the C-terminus of Nbs1.[59] Activated ATM phosphorylates a variety of targets, including KAP1, which induces chromatin relaxation,[60] and Chk2, which promotes cell cycle arrest. Once activated by ATM, Chk2 phosphorylates Cdc25A, a dual-specificity threonine/tyrosine phosphatase that promotes cell cycle progression by activating cyclin dependent kinase complexes, such as CDK2-Cyclin A, via dephosphorylation.[61] Chk2-mediated phosphorylation of Cdc25A targets Cdc25A for ubiquitin-dependent degradation, thus decreasing activation of CDKs and inhibiting cell cycle progression.[62]

Regarding DNA damage from UV light and PAHs, current models depict that damage-induced stalling of replication forks leads to uncoupling of the helicase and polymerase, yielding long stretches of single-stranded DNA (ssDNA)[63, 64] (**Figure 1-7**). ssDNA is rapidly coated by RPA, a heterotrimeric complex consisting of 70, 30, and 14 kDa monomers, RPA1, RPA2, and RPA3, respectively. In addition to its high affinity to ssDNA, RPA also binds to many other proteins involved in DNA repair, including the checkpoint signal transducer ATR-interacting protein (ATRIP). ATRIP binds to the N-terminus of RPA1 and recruits its binding partner, ATR, to chromatin[65-67] Once docked at RPA-coated ssDNA, ATR-ATRIP is activated by the presence of Rad9-Rad1-Hus1 (9-1-1), a clamp that encircles DNA and recruits BRCT-containing proteins, such as TopBP1, to chromatin.[66] TopBP1 contains an

ATR activation domain and strongly stimulates ATR kinase activity to initiate a series of signaling cascades that amplify the DNA damage response.[68, 69]

One important signal transducer downstream of ATR is the S-phase kinase Chk1. ATR-mediated phosphorylation of Chk1 at serines 317 and 345 activates Chk1 via two mechanisms: uncovering of its N-terminal kinase domain[70] and release of inactive chromatin-bound Chk1 to redistribute to centrosomes and block mitotic entry.[71] Like Chk2, activated Chk1 targets Cdc25A for degradation to block cell cycle progression and suppress origin firing via inhibition of the cyclin-dependent kinase Cdk2.[72] Chk1 also blocks cell cycle progression through Cdc25A-independent mechanisms involving via direct phosphorylation of Dbf4.[73] Phosphorylation of Dbf4 is thought to promote removal of Dbf4 from chromatin and inhibit its binding to Cdc7, thus inhibiting the kinase activity of the Dbf4/Cdc7 complex.[73] Inhibition of Cdc7 decreases loading of the MCMs to the pre-replicative complex[74] and also blocks Cdc45 loading[75] and interaction between Cdc45 and MCM7,[76] thereby inhibiting initiation of DNA synthesis at origins of replication.

Although ATM and ATR are frequently described as independent responses to double-strand breaks and stalled replication forks, respectively, extensive cross-talk exists between the two pathways. For example, DSBs can lead to ATR activation in a manner that is dependent on ATM,[77] whereas UV-induced replication stalling can activate ATM via ATR-mediated phosphorylation.[78] Similarly, ionizing radiation can cause ATM- and NBS1-dependent phosphorylation of Chk1 through ATR-

independent mechanisms.[79] Therefore, ATM and ATR function in parallel but also in concert to mediate checkpoint responses to DNA breaks and fork-stalling lesions.

Numerous links between checkpoints and TLS have been demonstrated. First, damage-induced Chk1 activation leads to the phosphorylation-mediated activation of Cdc7 (though Chk1 does not directly phosphorylate Cdc7), which itself phosphorylates Rad18 to drive Rad18-Polη binding and help promote tolerance of UV damage (see section on Rad18 and TLS).[80] Next, Chk1 is reported to facilitate maximal damage-induced PCNA monoubiquitination, albeit via kinase-independent mechanisms.[81] Altogether, DNA damage-induced checkpoints contribute to TLS-mediated DNA damage tolerance by both stalling replication to allow for TLS to occur and through direct stimulation of TLS via Chk1.

5. TLS activation and Rad18 and DNA damage tolerance pathways

Although the precise mechanisms that regulate activation of TLS remain poorly understood, it is generally accepted that TLS activation depends in large part upon physical docking of replicative or TLS polymerases with the homotrimeric replication factor PCNA. Which polymerase interacts with PCNA is determined in large part by the post-translational modification of PCNA by Rad18.

RAD18 is a highly conserved gene that was first identified in yeast in the same epistatis group as *RAD6* on the basis of increased sensitivity of UV and ionizing radiation.[82] Human Rad18 codes for a 495 amino acid protein with several important functional domains. These include an N-terminal RING-finger domain that mediates

catalytic activity and Rad6 binding (amino acids 26-64),[83] a C-terminal Rad6 binding domain (amino acids 340-395),[84] a Zinc finger domain that mediates accumulation at sites of DNA damage (amino acids 201-225),[84] a SAP domain that promotes assembly at stalled replication forks (amino acids 248-284),[85] a C-terminal Polη binding domain (amino acids 402-445),[86] and a C-terminal nuclear localization signal (amino acids 488-494).[85]

Rad18 is an E3 ubiquitin ligase that plays a central role in DNA damage tolerance by catalyzing the damage-induced monoubiquitination of PCNA.[87] In conjunction with its E2 ubiquitin-conjugating enzyme and binding partner, Rad6, Rad18 redistributes to stalled replication forks after DNA damage, where it catalyzes the monoubiquitination of PCNA at lysine 164 (**Figure 1-8**).[88,89] The Rad6-Rad18 complex is highly selective for PCNA monoubiquitination, a signaling event that has important implications for many aspects of the DNA damage response.

Rad18-mediated monoubiquitination of PCNA at lysine 164 serves as a signal to turn on both TLS and template switching. TLS is activated in part due to increased affinity of Y-family polymerases to monoubiquitinated PCNA compared to unmodified PCNA (see below). Template switching is an error-free post-replication repair process that is thus dependent on Rad18 for activation. Template switching is activated by HTLF/SHPRH-mediated (Rad5 in yeast) polyubiquitination of K164 that takes place only after Rad18-mediated K164 monoubiquitination.[90] Rad18 thus stimulates both error-free (template switching) and error-prone (TLS) post-replication repair mechanisms through its catalytic activity at K164 on PCNA.

Rad18 also contributes to DNA damage tolerance through E3 ligase-independent functions. In contrast to post-replication repair of fork-stalling lesions, Rad18 confers tolerance of double-strand breaks by promoting homologous recombination through physical interactions with Rad51C, a key protein in the initial steps of HR.[91] Damage-induced Rad18-Rad51C interactions promote recruitment of Rad51 to DNA DSB, where Rad51 may begin strand invasion to initiate HR.[92] This HR-promoting activity of Rad18 requires the RING and zinc finger domains but not the SAP or Rad6-binding domains. The RING domain of Rad18 facilitates physical interaction with Rad51C, and the zinc finger domain mediates Rad18-binding to ubiquitinated proteins at sites of DNA damage. These Rad18-binding proteins are ubiquitinated at DSBs by RNF8, another damage-inducible E3 ligase. Rad18 recruitment to DSBs thus depends on RNF8-mediated ubiquitination events; Rad18 appears to chaperone Rad51C to such DSB sites. Interestingly RNF8-mediated ubiquitination events seems necessary for recruitment of Rad18 to DSB sites but not stalled replication forks. Rad18 thus uses the same domains for entirely different functions (RING domain for PCNA monoubiquitination in TLS and for Rad51C recruitment in HR), thereby contributing to activation of distinct DNA damage tolerance pathways.

6. TLS activation and Rad18

As illustrated above, Rad18-mediated PCNA monoubiquitination serves as a signal to activate TLS at stalled replication forks. TLS requires replacement of stalled replicative polymerases with TLS polymerases capable of bypassing DNA lesions.

This switching mechanism is attributed to a higher affinity of TLS polymerases to monoubiquitinated PCNA compared to unmodified PCNA. In addition to their highly conserved PIP-boxes (and BRCT domain for Rev1), the Y-family polymerases contain ubiquitin-binding motifs (UBZ domains) that confer high-affinity binding to monoubiquitinated PCNA.[93, 94] Replicative polymerases, conversely, display reduced affinity to monoubiquitinated PCNA. By creating a docking site on PCNA suitable for TLS Pol binding, it has been proposed that PCNA monoubiquitination by Rad18 constitutes the basis for the engagement of TLS Pols with the replication machinery and in turn activation of TLS (**Figure 1-8**).[95,96,97] Such a hypothesis is supported by the findings that TLS Pol mutants in which the UBZ domains have been mutated demonstrate reduced redistribution to stalled replication foci,[94] that XPV cells complemented with UBZ-mutant Polη exhibit UV survival defects,[93, 94] and that UV survival is compromised in knock-in PCNA mutants in which lysine 164 has been mutated to eliminate Rad18-mediated PCNA monoubiquitination.[98] The reliance of TLS on PCNA monoubiquitination is not unanimously accepted, however, as other investigators have reported no UBZ-dependent effect of Polη on UV survival, redistribution to stalled replication forks, or in vitro TLS activity.[99,100]

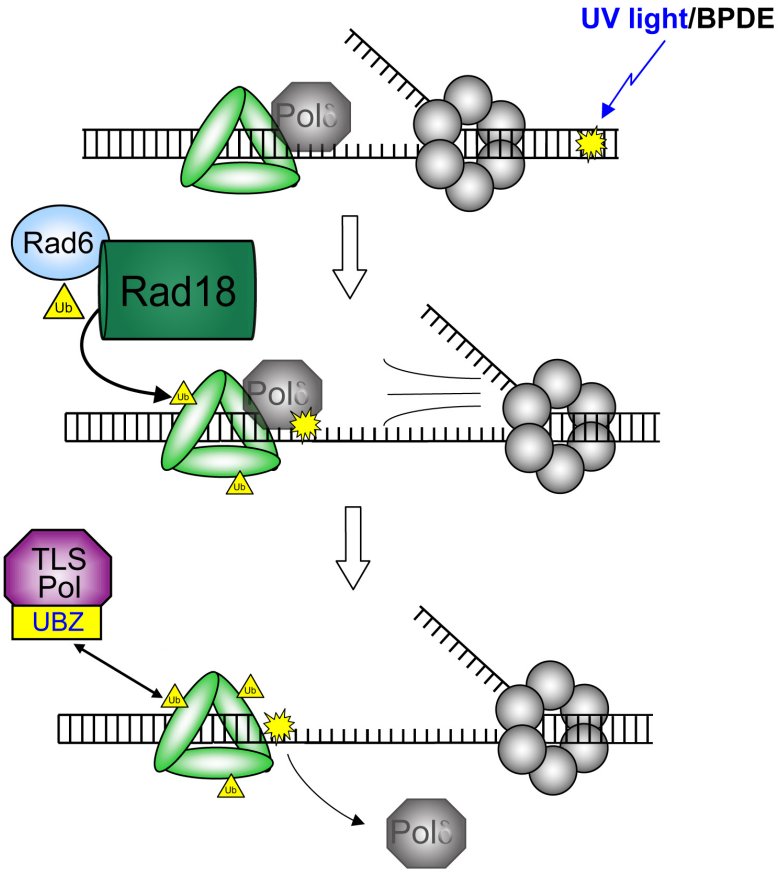


Figure 1-8. Rad18-mediated PCNA monoubiquitination drives binding of TLS polymerases to PCNA.

The mechanisms regulating the monoubiquitination of PCNA by Rad18 are poorly understood, and, specifically, it is not known what regulates the redistribution of Rad18 to stalled replication forks or how it interacts with its monoubiquitination target, PCNA. Rad18 is normally distributed diffusely throughout the nucleus, but it redistributes to discrete nuclear foci and colocalizes with PCNA after DNA damage (**Figure 1-9**).[101] Rad18 redistribution is intimately coupled with Polη redistribution and activity; in addition to its E3 ligase activity, Rad18 physically associates with Polη and contributes to its redistribution to PCNA at stalled replication forks (**Figure**

1-9).[86] Rad18 thus drives TLS by physically chaperoning Polη to stalled RFs in addition to its accepted role of monoubiquitinating PCNA. Indeed, loss of Rad18 eliminates PCNA monoubiquitination and severely compromises redistribution of Polη to replication nuclear foci after UV damage (**Figure 1-10**), though distinguishing between these two functions – E3 ligase activity at PCNA and physical chaperoning – has proven technically challenging (see Chapter 2).

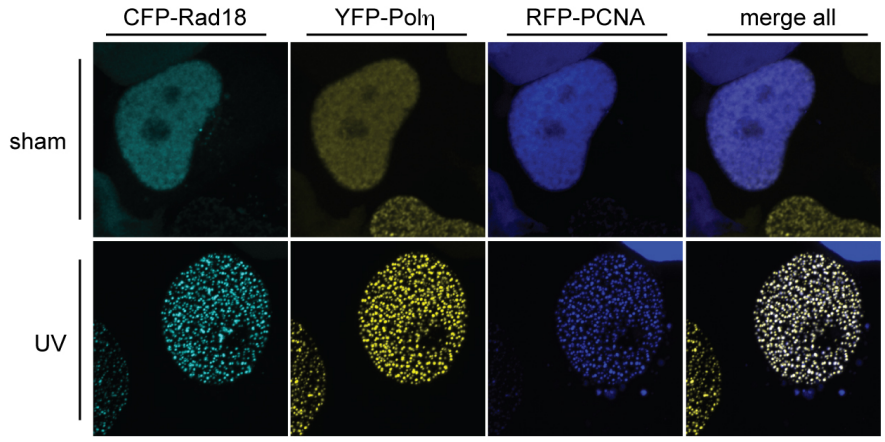


Figure 1-9. Rad18 redistributes to nuclear foci after UV exposure and colocalizes with Polη and PCNA.
(images by MD; RFP-PCNA pseudo-colored in blue for clarity).

Although Rad18 plays an important role in driving TLS polymerase recruitment to sites of DNA damage, the regulatory mechanisms that coordinate this process are only beginning to be delineated. As mentioned above, Rad18-Polη binding is stimulated by Chk1-dependent Cdc7 phosphorylation of Rad18.[80] In addition to the kinase-independent role of Chk1 that potentiates PCNA monoubiquitination, direct interactions between RPA and Rad18 contribute to Rad18 redistribution to stalled replication forks.[102] Checkpoint signaling therefore regulates

Rad18 activity and cellular distribution at several levels, ensuring tight coordination between the acquisition of DNA damage and TLS activation.

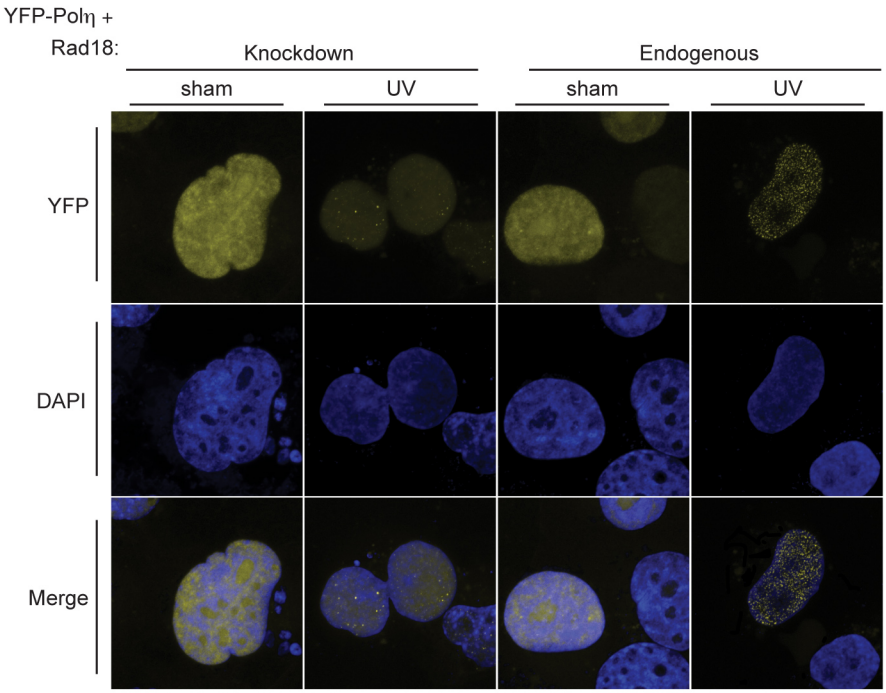


Figure 1-10. Polη redistribution to nuclear foci after UV exposure is compromised after depletion of Rad18.
(images by MD)

7. XPV and TLS

Xeroderma pigmentosum (XP) is a rare cancer propensity syndrome defined by exquisite sensitivity to sunlight and extremely high predisposition to skin cancer. XP affects both males and females equally and follows a pattern of autosomal recessive transmission. XP has been reported in nearly all racial groups with incidences that vary from 1 in 20, 000 in Japan to 1 in 250, 000 in the USA.[103] About 60% of XP individuals first show symptoms of an extreme sunburn reaction to sunlight, often shortly after birth.[103] The remaining 40% do not experience dramatic

sunburn reactions, but rather develop an unusually high number of freckle-like lesions in sun-exposed areas, typically by age 2.

XP tends to follow a pattern of three stages. While the skin appears healthy at birth, the first stage usually begins after about 6 months and is characterized by diffuse erythema, scaling, and areas of increased pigmentation in sunlight-exposed areas.[103] The second stage is characterized by skin atrophy, telangiectasias, and mixed hyperpigmentation and hypopigmentation, a combination of symptoms known as poikiloderma. The third stage involves the progression to malignancy. XP patients are estimated to have a 10,000-fold increased risk of non-melanoma skin cancer and a 2,000-fold increased risk of melanoma before the age of 20.[104] Additionally, ocular abnormalities, including photophobia, conjunctivitis, and ocular neoplasms, occur in approximately 80% of XP patients, and approximately 20% develop neurologic problems, including intellectual deficiency, spasticity or ataxia, and microcephaly.

The cutaneous symptoms of XP, including pigmented skin lesions sunlight-exposed skin, were first documented in 1874 in Vienna by a dermatologist named Moriz Kaposi,[105] and neurologic abnormalities were first described in the 1880's.[106] However, it wasn't until the 1960's that the connection between XP and DNA damage tolerance was appreciated when cultured XP cells were shown to exhibit deficiency of excision repair.[52] In 1971, an excision repair-proficient XP strain was described[107] and subsequently named "variant."[108] Complementation experiments in the 1970's led to identification of 7 XP subtypes, complementation groups A-G, all deficient in nucleotide excision repair, and XP-Variant, which exhibited normal excision repair but defective DNA synthesis after UV exposure.[109] The genes

responsible for each complementation group and their function were subsequently identified (**Table 1-4**).

Table 1-4. Xerderma Pigmentosum complementation groups and their defective gene products.

Group	Defective Gene/Protein	Function	% of XP[110]
XPA	*XPA*/XPA	Binding to UV-damaged DNA[111]	25
XPB	*ERCC3*/XPB	DNA helicase[112]	<1
XPC	*XPC*/XPC	Binding to UV-damaged DNA [113]	25
XPD	*ERCC2*/XPD	DNA helicase[114]	15
XPE	*DDB2*/DDB2	Binding to UV-damaged DNA[115]	<1
XPF	*ERCC4*/ERCC4	Endonuclease[116]	6
XPG	*ERCC5*/XPG	Endonuclease[117]	6
XPV	*POLH*/Polη	TLS	21

XP-Variant (XPV) is unique among the XP complementation groups for several reasons. Unlike groups A-G, which are deficient in proteins that mediate *repair* of UV-damaged DNA via NER, XPV is characterized by deficient TLS-mediated *tolerance* of UV-induced DNA damage while maintaining normal NER. DNA lesions are not actually repaired or removed after TLS, but rather remain in the genome until other mechanisms, like NER, remove them. TLS, rather, allows replication to continue after a replicative polymerase encounters a DNA lesion, thus promoting tolerance of such lesions by preventing replication fork collapse and subsequent DNA breaks. The deleterious effects of XPV are thus due to an entirely different mechanism of coping with UV-damaged DNA (tolerance vs. repair), in addition to a different pathway (TLS vs. NER).

Next, mutations in XPV individuals arise via different mechanisms than the other XP subtypes. Let's first consider NER. NER can be considered as the first-line of defense against DNA damage, through which the vast majority of DNA lesions are

removed via relatively error-free processing. As mentioned earlier, the daily burden of DNA lesions is enormous, estimated in the range of greater than 10,000 DNA lesions per day.[16] In a healthy cell, only a very small percentage of these lesions escape NER to be processed by other DNA damage tolerance mechanisms. Due to the inherent infidelity of TLS enzymes, lesion bypass by TLS will introduce some level of mutation, even when each lesion is processed by the appropriate polymerase (e.g. CPDs by Polη). In other words, TLS of CPDs by Polη carries a greater risk of mutation than normal replication of undamaged DNA by replicative polymerases. Such TLS accounts for at least a large part of the basal mutation rate experienced by all cells over time, even those with intact DNA damage tolerance pathways.

However, in the absence of NER, the burden of DNA lesions that must be processed by secondary damage tolerance mechanisms is enormous. Again in light of the relatively error-prone nature of TLS, lesion bypass via TLS, even when the appropriate TLS polymerase is present, will greatly increase the probability of introducing mutations. In the absence of NER, one can assume that Polη is still performing the vast majority of TLS across UV-induced lesions. Even though Polη-mediated TLS of CPDs is relatively accurate compared to TLS of CPDs by other TLS enzymes, it is still far less accurate than replication of undamaged templates by replicative polymerases. Therefore, tolerance of UV-induced DNA damage carries a dramatically higher risk of mutagenesis in XP A-G, even though Polη is present to perform TLS of CPDs. Indeed, one can assume that the majority of these mutations are in fact introduced by Polη itself.

The situation in XPV is entirely different and conceptually much simpler. The overwhelming majority of UV-induced lesions are removed by NER. Rarely, a CPD that escapes NER will be processed by TLS. However, the preferred polymerase for CPDs is absent (Polη), so polymerases that are far more error-prone than Polη on CPDs (Polι and Polκ) are used instead. Indeed, Polι and Polκ have been implicated in mutagenic TLS in the absence of Polη in XPV cells.[118] Mutagenesis in XPV is thus due to TLS by polymerases other than Polη, whereas Polη plays an important role in mutagenesis in XP A-G.

On a molecular level in XPV cells, the effect of Polη deficiency in the response to CPDs that escape NER can be viewed as having two most likely outcomes. In the first, CPDs are simply not bypassed and persistent stalling of replicative polymerases leads to replication fork collapse and single and double-strand DNA breaks, with obvious deleterious consequences for genomic stability (**Figure 1-11, left**). Alternatively, TLS may occur via the inappropriate TLS polymerase, which will bypass CPDs with a much lower fidelity than Polη and a high probability of introducing mutations (**Figure 1-11, right**). Both potential outcomes severely compromise the genetic integrity of the cell and predispose to malignant transformation.

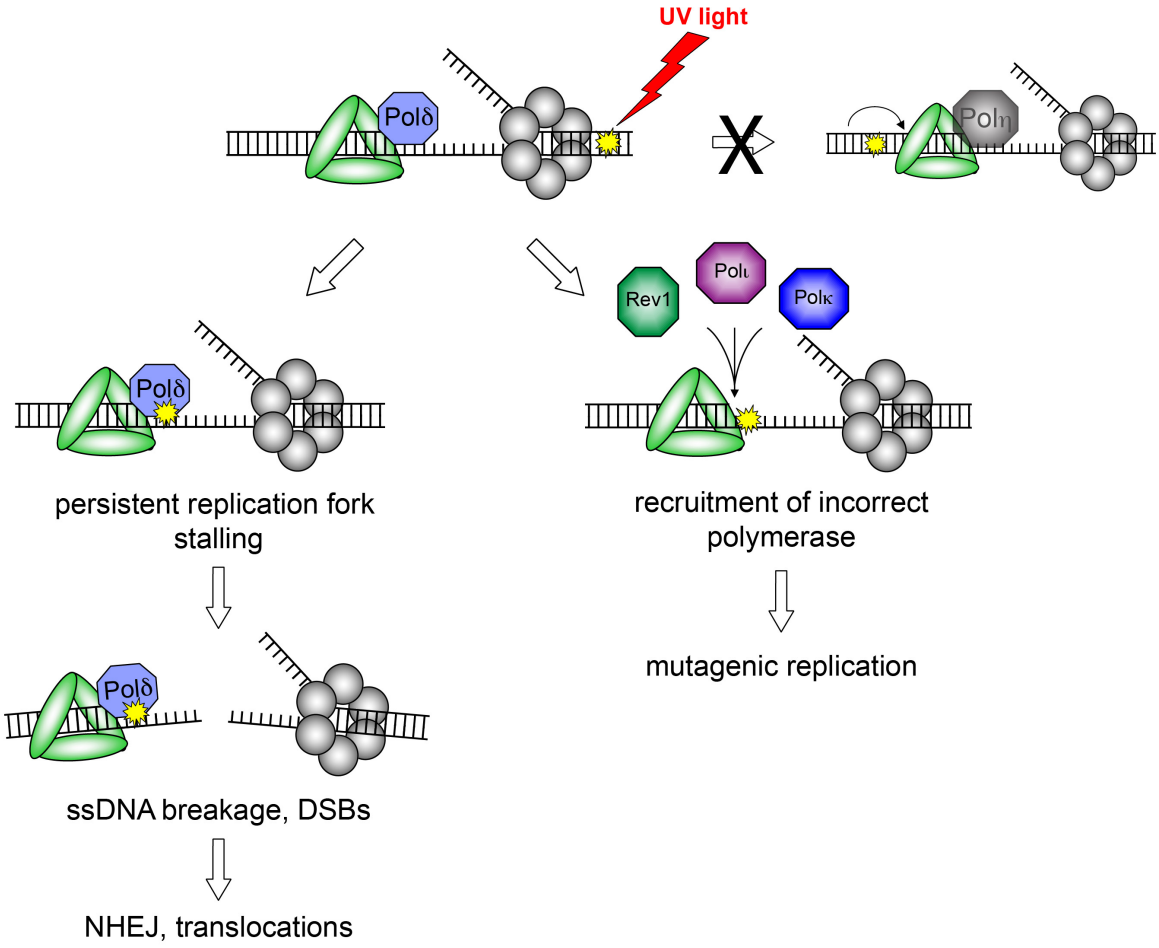


Figure 1-11. Polη deficiency and processing of UV-induced DNA damage
Lack of Polη leads to two main outcomes in XPV cells after UV irradiation. Persistent stalling of replicative polymerases (left) leads to long stretches of ssDNA that are vulnerable to double strand breaks, which can be repaired by non homologous end-joining, predisposing to DNA translocations. Alternatively recruitment of inappropriate TLS polymerases (predominantly Polι and Polκ) predisposes to mutagenic replication (right).

8. Oncogenic signaling and TLS

Mutations are well-accepted to play an important role in the initiation and progression of many types of cancer.[119] Mutations contribute to malignant transformation when they inactivate tumor suppressor genes or activate oncogenes;

however, the rate of *de novo* mutations is significantly higher in transformed cells compared to normal. Whereas mutation rates in non-transformed cells appear to be in the range of $1X10^{-10}$ mutations/base pair/cell generation,[120,119] mutation rates in neoplastic cells have been shown to be over 20-fold higher,[121] and gene amplification has been shown to be $>10^6$-fold higher in tumor compared to normal cell lines.[122] These data suggest that normal processes of genome maintenance are derailed in malignant cells, leading to inhibited or mis-regulated damage tolerance mechanisms in the context of oncogenic signaling and, in turn, increased mutagenesis.

Central to this idea is the hypothesis that pre-malignant cells acquire somatic mutations that lead to aberrant expression of genes involved in processes important for malignancy, namely cell growth, arrest, invasion, replication, and angiogenesis.[123,124] In particular, aberrant expression and regulation of proteins involved in replication and genome maintenance predisposes to additional subsequent mutations in a feed-forward cycle carrying a dramatic risk of cancer.[125] Because TLS is an error-prone replication process, mis-regulation of TLS in oncogene-expressing cells would run a high risk of introducing early genetic mutations.

One can thus hypothesize that oncogene-expressing cells exhibit a reduced ability to respond properly to environmental DNA damaging agents (*e.g.* UV light and PAHs) or endogenous replication impediments (G4 structures). Similarly, one may hypothesize that TLS alone could be improperly activated or regulated in oncogene-expressing cells, leading to excessive error-prone replication. Malignant cells would thus acquire mutations at an increased rate due to overactive or mis-regulated TLS. Evidence has been reported that directly supports this hypothesis,[126]

and the well-accepted role of environmental DNA damaging agents in carcinogenesis can be further explained by such mechanisms.[127,128] Additionally, oncogene expression is known to induce stalling and collapse of DNA replication forks,[129] as well as replication stress that activates DNA damage responses.[130] For example, oncogenes have been shown to induce re-replication,[131, 132] activate DNA damage response pathways,[132] and induce the formation of reactive oxygen species (**Figure 1-12**).[133]

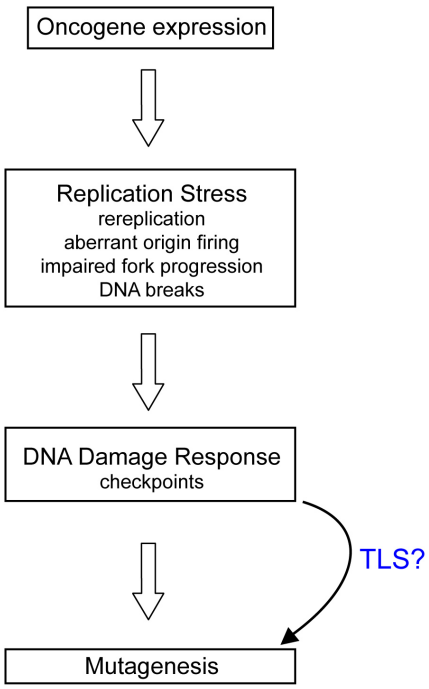


Figure 1-12. Oncogene-induced DNA replication leads to a DNA damage response that may involve TLS.

All of these processes contribute to genetic instability in the context of oncogenic signaling; however, the mechanisms through which oncogenes lead to genetic instability are poorly understood. Expression of oncogenes leads to several

seemingly contradictory cellular responses. Oncogenes such as Ras are known to induce cellular proliferation, but only when expressed in combination with the inactivation of tumor suppressors, such as p53 or Rb.[134] Rather, expression of oncogenes leads to an initial wave of proliferation that is followed by cellular senescence. Such oncogene-induced senescence is characterized by irreversible growth arrest that is associated with accumulation of tumor suppressor genes such as p53, p16, or p21.[135] Therefore, cells respond to oncogenic signaling by expressing proteins that halt cellular proliferation. Importantly, expression of such tumor suppressor genes is a consequence of an oncogene-induced DNA damage response.[131] Specifically, oncogene-expressing cells demonstrate DNA replication stress in the form of stalled replication forks and DSBs, together with a concomitant activation of checkpoint proteins, such as ATM and ATR, and their downstream effectors, Chk2 and Chk1.[136] Importantly, abrogation of these checkpoint responses suppresses oncogene-induced senescence.[131] Therefore, DNA damage response and checkpoint pathways play an integral role in the cellular response to oncogenic signaling; however, the mechanisms of oncogene-induced checkpoint activation, the consequences of such activation, and the DNA damage response pathways affected by such checkpoint activation remains unknown.

As shown blow (see chapter 4) and in numerous reports,[67, 80] there is a clear relationship between checkpoint signaling and TLS, and several lines of evidence suggest that TLS may be involved in the cellular response to oncogene-induced DNA damage. First, because activation of Chk1 stimulates TLS, oncogene-induced checkpoint activation is likely to affect TLS via similar mechanisms as other forms of

DNA damage. Next, TLS is known to contribute to cellular tolerance of abnormal DNA structures, such as G4 DNA, and oncogene-induced replication stress leads to aberrant topological DNA structures, such as reversed replication forks;[130] TLS may also play a role in the processing of such oncogene-induced DNA structures. Considering the predilection for mutagenesis in the context of oncogenic signaling and the connections between TLS and checkpoint signaling, we have hypothesized that TLS is involved in the tolerance of oncogene-induced replication stress and that TLS plays an important role in oncogene-dependent mutagenesis (**Figure 1-12**).

Important unexplored questions include whether TLS is activated in the context of oncogenic signaling, whether checkpoint activation impacts oncogene-dependent TLS activity, how TLS contributes to mutagenesis in the context of oncogene expression, and whether oncogene-expressing cells are sensitized in the absence of TLS-mediated DNA damage tolerance.

9. Remaining major unkowns

The preceding text is intended to provide a background of specific areas relevant to the studies presented in this dissertation. For each area, however, several important processes have remained unknown:

DNA Damage Response Checkpoints and TLS

Although it is clear that checkpoints facilitate activation of TLS via signaling mechanisms (Chk1-dependent binding of Rad18 and Polη) and direct physical

interactions (kinase-independent Chk1-mediated potentiation of PCNA monoubiuqitination), a broader understanding of the relationship between checkpoint signaling and TLS is needed. Specifically, it remains entirely unknown whether checkpoint signaling contributes to binding of other TLS polymerases with Rad18, the mechanism through which Chk1 facilitates recovery from genotoxin-induced S-phase checkpoints, and what additional effector proteins function upstream and downstream of Chk1 to initiate a TLS-stimulatory checkpoint response. Understanding these concepts will help explain the mechanisms through which TLS-mediated damage tolerance and mutagenesis take place. These concepts are addressed in Chapter 4 of this dissertation.

Rad18 and TLS Activation

Although Rad18/Rad6 is known to catalyze damage-responsive monoubiquitination of PCNA, the mechanisms that regulate this process remain entirely unknown. It is known that Rad18 does not physically interact with PCNA, so the processes that drive redistribution of Rad18 to PCNA and subsequent physical binding of the two are unknown. Additionally, whereas UV-induced binding of Rad18 and Polη has been clearly demonstrated, the specific mechanistic aspects of this interaction remain unknown: where does Polη bind to Rad18 and vice versa? Do known effector domains of Polη, such as the UBZ domains, contribute to Rad18 binding? Where does this interaction take place? Is the interaction direct or are other binding partners required? Do the components of the Rad18-Polη complex change as a function of genotoxin stimulation or cell cycle? Further, details of analogous Rad18 interactions with TLS polymerases remain unknown. What are the binding domains

necessary for Rad18-Polκ interactions? Are these interactions promoted only by BPDE? Does Rad18 interact at all with Polι or Rev1? Finally, the mechanisms that dictate selection of the proper polymerase for the appropriate DNA lesion are completely unknown. This point is of crucial importance because use of the wrong polymerase greatly predisposes to mutagenesis. Additionally, because of the role that TLS plays in cellular tolerance anticancer drugs such as cisplatin,[137] understanding the mechanisms that regulate TLS initiation and activation may help guide the development of therapeutic targets.

Oncogenes and TLS

It is widely accepted that oncogenic signaling activates a DNA damage response and checkpoints and simultaneously promotes mutagenesis in a manner that feeds into the mutator phenotype. However, it remains entirely unknown what are the consequences of such oncogene-induced activation of the DDR and what mechanisms are responsible for the oncogene-induced increase in mutagenesis. A putative role for TLS in this process is conceptually feasible but has never been explored. A role for TLS in the tolerance of oncogenic replication stress concepts is addressed in Chapter 5. Understanding the mechanisms that dictate mutagenesis and survival in the context of oncogenic signaling is necessary for the discovery of novel targets for anti-proliferative therapies.

CHAPTER 2

MATERIALS AND METHODS

1. Cell Culture and Transfection

H1299, HDF, XP115LO (GM02359[46, 138]), and HCT-116 WT and Rad18-/- cells[139] cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin. SiRNA and pcDNA, pACCMV, and pCAGGS plasmid transfections were done using Lipofectamine 2000 (Invitrogen) as previously described[80].

2. Materials, siRNA, plasmad and adenovirus construction

siRNA oligonucleotide sequences were as follows: non-targeting Control, 5′–UAGCGACUAAACACAUCAAUU–3′; Polη, 5′–GCAGAAAGGCAGAAAGUUA–3′; Polη-3′ UTR, 5′–CCAUUUAGGUGCUGAGUUA–3′; Polη-5′ UTR, 5′–GAAUAAAUCUCGCUCGAAA–3′; Chk1, 5′–GCGUGCCGUAGACUGUCCA–3′; USP1, 5′–TCGGCAATACTTGCTATCTTA–3′; Polκ, 5′–GUAAAGAGGUUAAGGAAA–3′; Rad18 3′ UTR, 5′–UUAUAAAUGCCCAAGGAAAUU–3′; Spartan 5′–ACCGGACUUGCAGGCACUGUUUGUU–3′. CFP was cloned onto the C-terminus of Rad18 in pACCMV using BamH1 and Xba1 restriction sites. Rad18 and CFP were separated by a linker of the sequence 5′–

ACCTCTTCCGGTTCCAGTCCCTGTTCCGGGTCCTGCTCCTATGCGTATGGC TCC–5′. Rad18-Δ(402-225) and Rad18-C28F were generated as described previously[86] and cloned into pACCMV using EcoRI and BamHI restriction sites. Polη-ΔPIP was cloned into pACCMV EcoRI and BamHI restriction sites and a C-terminal primer containing phenylalanine to alanine mutations at AA 705 and 707. Catalytically-inactive Polη was generated by mutating codons D13, E22, D115, and E116 to alanine in the N-terminal catalytic active site to disrupt coordination of Mg2+ ions between dNTP, primer, and active site moieties and block nucleotide incorporation[140]; this construct was then cloned into pACCMV using EcoRI and BamHI restriction sites. N-terminal Polη truncations were generated with 5′ and 3′ primers containing EcoRI and BamHI restriction sites, respectively, and cloned into pACCMV. The Rad18-Polη fusion was constructed by PCR amplification of Polη with primers containing 3′ BamHI and 5′ XbaI restriction sites, followed by ligation into pACCMV-Rad18. Polη-ΔPLTH and Polκ+PLTH were generated with C-terminal primers omitting or adding, respectively, codons for the PLTH domain, followed by a BamHI restriction site for ligation into pACCMV. pDEST-SFB-Spartan was obtained from Lee Zou (MGH Cancer Center). Adenovirus constructions were performed by recombination of pACCMV constructs with pJM17 as described previously[141]. UV-C irradiation using a UV cross-linker (Stratagene, Santa Clara, CA) and BPDE (NCI Carcinogen Repository) treatments were performed as previously described.[142]

3. Adenovirual expression and titration

Adenoviral infection was performed as described previously by adding to cultured cells CsCl purified adenovirus.[141] Infections in H1299 cells were typically done at 0.1 − 1.0×10^9 pfu/mL and in XPV/HDF cells at 0.1 − 5.0×10^9 pfu/mL. Titration to expression levels approximately equal to endogenous was done by serial infections followed by immunoblotting of extracts with antibodies against the endogenous protein.

4. Fluorescence microscopy

H1299 or XPV cells were grown to ~ 60% confluency on glass-bottom plates (Mat-tek) and then infected with adenovirus (CFP-Rad18-WT, YFP-Polη, GFP-Polκ, and respective mutants) to achieve expression approximately equal to endogenous as determined by Western blot. For co-expression and knockdown experiments, co-infection or transfection were performed 6 hours before adenoviral infection. 20 hours after infection, cells were exposed to genotoxins and then prepared for live or fixed-cell imaging on a Zeiss 710 confocal microscope. For high-magnification representative images, Z-stacks at 0.5 um intervals were collected throughout the entire cell volume using a 63X oil-immersion objective and 2.3X optical zoom. 3D projections of Z-stacks were performed using Grouped Zprojector on ImageJ. For cells expressing multiple chromophores (YFP and CFP-tagged proteins) appropriate excitation lasers, laser intensities, and emission filter bandwidths were selected to eliminate bleedthrough. For live-cell imaging, cells were kept out of the incubator for no more than 10 minutes. For fixed-cell imaging, H1299 cells were washed 3X with

cold PBS, then extracted for 60 s in cold CSK buffer, washed 3X with PBS, then fixed for 10 min in 2% PFA in PBS; XPV cells were washed 3X in cold PBS and then fixed for 15 min in methanol at -20C. Post-fixation, all cells were covered with Vectashield Solution (Vector Laboratories) and imaged within 24 hours. For foci quantification, five representative images containing approximately 60 cells were captured using 0.5 um Z-stacks with a 40X using oil-immersion lens. After 3D-projection, and the number of cells clearly containing more than 100 nuclear foci were counted as a fraction of total chromophore-expressing cells.

5. Triton extraction, immunoprecipitation, and immunoblotting

Extracts containing soluble and chromatin-associated proteins were prepared as previously described[80] by lysing cultured cells into cold cytoskeleton buffer (CSK buffer; 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM ATP, 1 mM Na_3VO_4, 10 mM NaF, and 0.1% Triton X-100) supplemented phosphatase and protease inhibitors (Roche Diagnostics, Indianapolis, IN). For immunoprecipitation of whole cell lysate (WCL) or chromatin-bound proteins, Triton-insoluble proteins were released by sonication on ice for three 10 s intervals followed by centrifugation at 15k x g for 10 min. After normalizing to a protein concentration of 1 ug/ul, immunoprecipitation was conducted at 4°C by rotating overnight with HA-coupled or primary antibody-bound sepharose beads (Roche Diagnostics, Indianapolis, IN). After immunoprecipitation, the beads were washed five times for 15 min in CSK buffer and then resuspended in minimum volume of Laemmli buffer. For Immunoblot experiments, cell extracts or

immunoprecipitates were separated by SDS-PAGE, followed by incubation overnight with the following primary antibodies: PCNA (sc-56), Chk1 (sc-7898), β-Actin (sc-130656), all from Santa Cruz Biotech (Santa Cruz, CA); Polη (A301-231A), Polκ (A301-975A), Polι (A301-304A), and R18 (A301-340A), all from Bethyl Laboratories (Montgomery, TX); and p53 (Ab-6) from Lab Vision, (Fremont, CA).

6. Genotoxin treatments

UV-irradiation and BPDE treatment were performed as previously described [80], and BPDE (National Cancer Institute Carcinogen Repository) was dissolved in anhydrous Me_2SO and added directly to the growth medium as a 1000× stock to give various final concentrations, as indicated in the figure legends. For UVC treatment, the growth medium was removed from the cells, reserved, and replaced with phosphate-buffered saline (PBS). The plates were transferred to a UV cross-linker (Stratagene, Santa Clara, CA) and then irradiated. The UVC dose delivered to the cells was confirmed with a UV radiometer (UVP BioImaging Systems, Upland, CA). The reserved medium from the cells was replaced, and cells were returned to the incubator.

7. *In vitro* Binding and Ubiquitination assays

C-His_6-PCNA-expressing Top10 *E. coli* (acquired from Marila Cordiero-Stone, UNC-CH) were collected and lysed in pH 8 buffer containing 50 mM $NaPO_4$, 300 mM NaCl, 20 mM imidazole, and 0.1% Triton-X. After sonication and clarification,

His_6-PCNA was purified over Ni-NTA beads. For His_6-PCNA pulldown experiments, HA-Rad18 was adenovirally expressed in H1299 cells alone or together with YFP-Polη and lysed in $NaPO_4$/NaCl/imidazole/Triton-X buffer. After sonication, clarification, and normalization to a protein concentration of 1 ug/ul, cell lysates were rotated overnight at 4°C with His_6-PCNA on Ni-NTA beads. The beads were then washed 5 times in the same buffer before addition of Laemmli buffer, boiling, and analysis by SDS-PAGE/Western Blot. For *in vitro* ubiquitination assays, H1299 cells expressing HA-Rad18 alone or together with YFP-Polη were lysed in $NaPO_4$/NaCl/imidazole/Triton-X buffer and immunoprecipitated with HA-sepharose beads. After washing the beads extensively, the beads were resuspended in 50 uL buffer and the following were added: His_6-PCNA (eluted from Ni-NTA beads with the same buffer plus 200 mM imidazole), 500 uM FLAG-ubiquitin, 10 X Energy Regeneration Solution, and 100 nM Ubiquitin Activating Enzyme (UBE1), all from Boston Biochem (Cambridge, MA). After incubation for 16 hours at 4°C, the mixture was mixed with Laemmli buffer, boiled, and analyzed by SDS-PAGE and Western Blot.

8. UV cytotoxicity assays

XPV or HDF cells were split into 24-well plates to a density of approximately 25%. 12 hours later, the cells were infected with empty control adenovirus or adenovirus expressing YFP-Polη. 24 hours after infection, the cells were exposed to UV light in the presence or absence of 1 mM caffeine. After 48 hours, 50 mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich, St. Louis, MO) was added to each well

and let to incubate at 37°C for 2 hours. The cells were then rinsed with PBS and dissolved in 0.5 mL DMSO. The absorbance at 570 nm was then measured for each well and normalized to the sham-treated samples. The minimum dose of YFP-Polη that conferred resistance to UV light in XPV cells (approximately 0.5×10^9 pfu/mL, **Figure 2-1**) was determined by this method and used for survival assays.

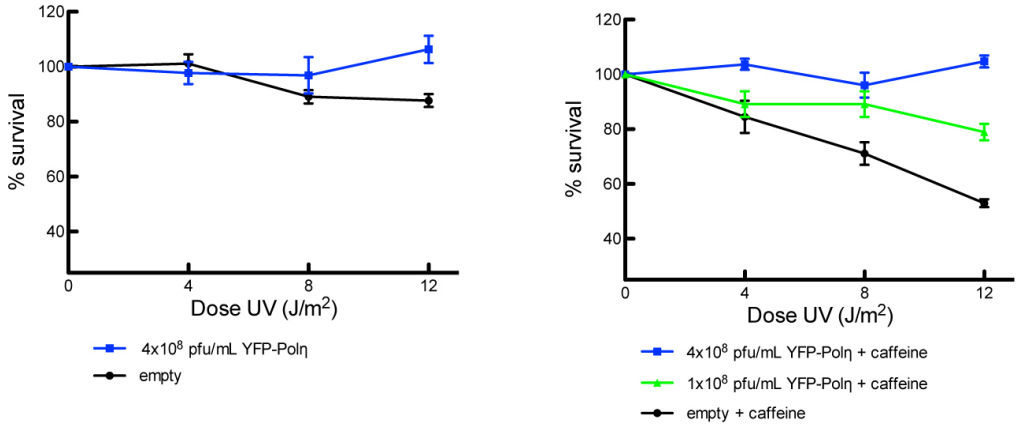


Figure 2-1. Killing curves for XPV cells complemented with Polη.
UV-sensitivity of XP115LO cells infected with adenovirus expressing YFP-Polη and exposed to increased doses of UV 24 hours later in the absence (left) and presence of 1mM caffeine (right). Caffeine was added to cells 2 hrs before UV and maintained for duration of the experiment. Cell viability was assayed by MTT 48 hrs after UV. The minimum dose of YFP-Polη virus (approximately $4x10^8$ pfu/mL, blue circles) that yielded 100% UV survival at 12 J/m^2 UV was used for all ectopic expression experiments in XP115LO and HDF cells.

9. *In vitro* kinase assays

Recombinant Rad18–Rad6 complex from insect cells and GST-Rad18 from bacteria were expressed, purified, and tested as DDK and JNK substrates using in vitro phosphorylation reactions as described previously.[80] Recombinant DDK was purified

from insect cells as described previously,[80] and recombinant JNK1α1 was purchased from Upstate (Millipore, Billerica, MA).

10. Clonogenic survival assays

H1299 cells were grown to ~30–50% confluence in 60-mm plates and transfected with 4 μg of pAC.CMV GFP vector (for controls) or with pAC.CMV expression vectors encoding wild-type or mutant Rad18 using Lipofectamine. Transfected cells were treated with UVC (as described) and then split into replicate 10-cm plates at a density of 1000 cells/plate. After 10–14 d, colonies on the plates were fixed in methanol, stained with crystal violet, and counted as described previously.[80]

11. Statistics

P values for statistical significance were determined by the unpaired Student's t-test with a two-tailed 95% confidence interval.

CHAPTER 3

A NOVEL NON-CATALYTIC FUNCTION OF DNA POLYMERASE η IN PROMOTING PCNA MONOUBIQUITINATION AT STALLED REPLICATION FORKS[1]

1. Introduction

Living organisms are constantly exposed to ubiquitous genotoxins from endogenous and external sources.[143] However, cells have evolved numerous DNA damage response (DDR) pathways that protect genomic DNA and prevent genetic instability.[144] Translesion Synthesis (TLS) is a DDR mechanism involving specialized DNA polymerases that can replicate damaged DNA templates. [145]

TLS relies on inherently error-prone DNA Polymerases of the Y family to replicate damaged DNA.[146] TLS by Y-family polymerases (Polη, Polι, Polκ, and Rev1)[147] maintains replication in cells harboring damaged DNA, albeit at the cost of reduced fidelity. Each TLS polymerase performs relatively error-free replication past a preferred cognate lesion; in the absence of the appropriate TLS polymerase for its preferred lesion, mutagenic replication by error-prone polymerases predisposes to genetic instability.[144]

Polη is unique among Y-Family Polymerases in its ability to perform accurate replication past UV-damaged DNA.[109, 148] Lack of Polη in the inherited cancer-

[1] This chapter is based on the following manuscript, which was accepted for publication in December 2012: Durando M, Tateishi S, Vaziri C. A non-catalytic role of DNA polymerase η in recruiting Rad18 and promoting PCNA monoubiquitination at stalled replication forks. Nucleic Acids Res. 2013 Mar 1;41(5):3079-93.

propensity syndrome xeroderma pigmentosum Variant (XPV)[149] results in error-prone replication by other Y-family Polymerases in sunlight-exposed cells.[118, 150] Thus, UV-induced mutagenesis due to Polη-deficiency compromises genetic integrity to manifest as exquisite sunlight-sensitivity and early skin cancer-propensity.

A prerequisite for error-prone replication in TLS is the Rad6/Rad18-mediated monoubiquitination of PCNA at the highly conserved lysine K164.[93, 151] Y-family polymerases contain ubiquitin-binding (UBZ) domains that confer affinity to monoubiquitinated PCNA.[94, 152] Failure to monoubiquitinate PCNA at K164 phenocopies XPV by compromising TLS and sensitizing cells to UV light and other ubiquitous genotoxins.[98, 153-155] Several other DDR pathways also depend on PCNA monoubiquitination, including SHPRH/HTLF-mediated template switching,[156] ZRANB3-dependent replication fork restart,[157] SNM1A-dependent intrastrand cross-link repair,[158] the Fanconi Anemia pathway activation.[159]

In spite of its pivotal role in the DDR, the molecular mechanisms regulating Rad18-mediated PCNA monoubiquitination are incompletely understood. The Rad18-Rad6 complex is thought to be recruited to the vicinity of damaged DNA via direct interactions with RPA-coated ssDNA.[102, 160] However, Rad18 lacks PCNA-binding motifs and it is unclear how Rad18 is targeted specifically to PCNA at stalled forks (or other sites of post-replication repair). A recent report by Zou and colleagues identified Spartan as a binding partner of both Rad18 and PCNA and proposed that Spartan acts as a scaffold for recruiting Rad18 to PCNA.[161] Consistent with a role for Spartan in targeting Rad18 to PCNA, those workers found DNA damage-induced PCNA monoubiquitination was modestly attenuated in Spartan-depleted cells.

However, several other more recent publications have reported alternative roles for Spartan in DNA damage signaling[162-165] and it is unclear whether Spartan or alternative putative mediators exist to facilitate recruitment of Rad18 to PCNA.

In mammalian cells, Rad18 exists in complex with Polη [80, 86] and association of Rad18 with Polη is necessary for normal DNA damage tolerance.[80, 86, 142] Assembly of the Rad18-Polη complex is stringently controlled by Cdc7 and Chk1 kinases, which serve to integrate TLS with S-phase progression and the S-phase checkpoint respectively.[80, 142] Here we report that the Polη-Rad18 interaction plays a key role in targeting Rad18 to PCNA and facilitating efficient PCNA monoubiquitination. Interestingly, the novel role of Polη in stimulation of PCNA monoubiquitination is fully dissociable from its activity as a DNA polymerase. We show that the Polη-Rad18 interaction provides the basis for coupling PCNA monoubiquitination with DNA damage-inducible checkpoint pathways mediated by p53 and Chk1. Our results also provide a potential explanation for numerous reports that Polη confers tolerance of non-cognate lesions[166, 167] and that catalytically-inactive Polη can partially rescue the DNA damage-sensitivity phenotypes of XPV cells.[168, 169] Moreover, because some XPV cells express a catalytically-inactive Polη that retains the ability to promote PCNA monoubiquitination, our results also indicate a new molecular mechanism for the mutagenesis and cancer-propensity of XPV patients.

2. Results

2.1. Polη promotes Rad18-mediated PCNA monoubiquitination

Current models suggest that Rad18 plays proximal roles in TLS, chaperoning Polη to damaged chromatin and monoubiquitinating PCNA to stably engage Y family polymerases at sites of PRR.[93, 94] Unexpectedly, we observed deficient redistribution of Rad18 to nuclear foci (representing sites of replication stalling) in patient-derived XPV cells (XP115LO) following UV-irradiation (**Figure 3-1 A, B**). In Polη-corrected XP115LO cells, Rad18 redistributed to nuclear foci in a UV-inducible manner and co-localized with Polη, indicating that Rad18 redistribution to repair foci is Polη-dependent in XP115LO cells. Similar to results obtained with XPV cells, redistribution of Rad18 was defective in Polη-depleted H1299 cells (**Figure 3-1 C, E**), which have intact TLS and rely on Polη for UV-tolerance.[80] As shown in **Figure 3-1 D**, basal and UV-induced formation of Rad18 foci were dependent on Polη, indicating a general role for Polη in impacting Rad18 redistribution.

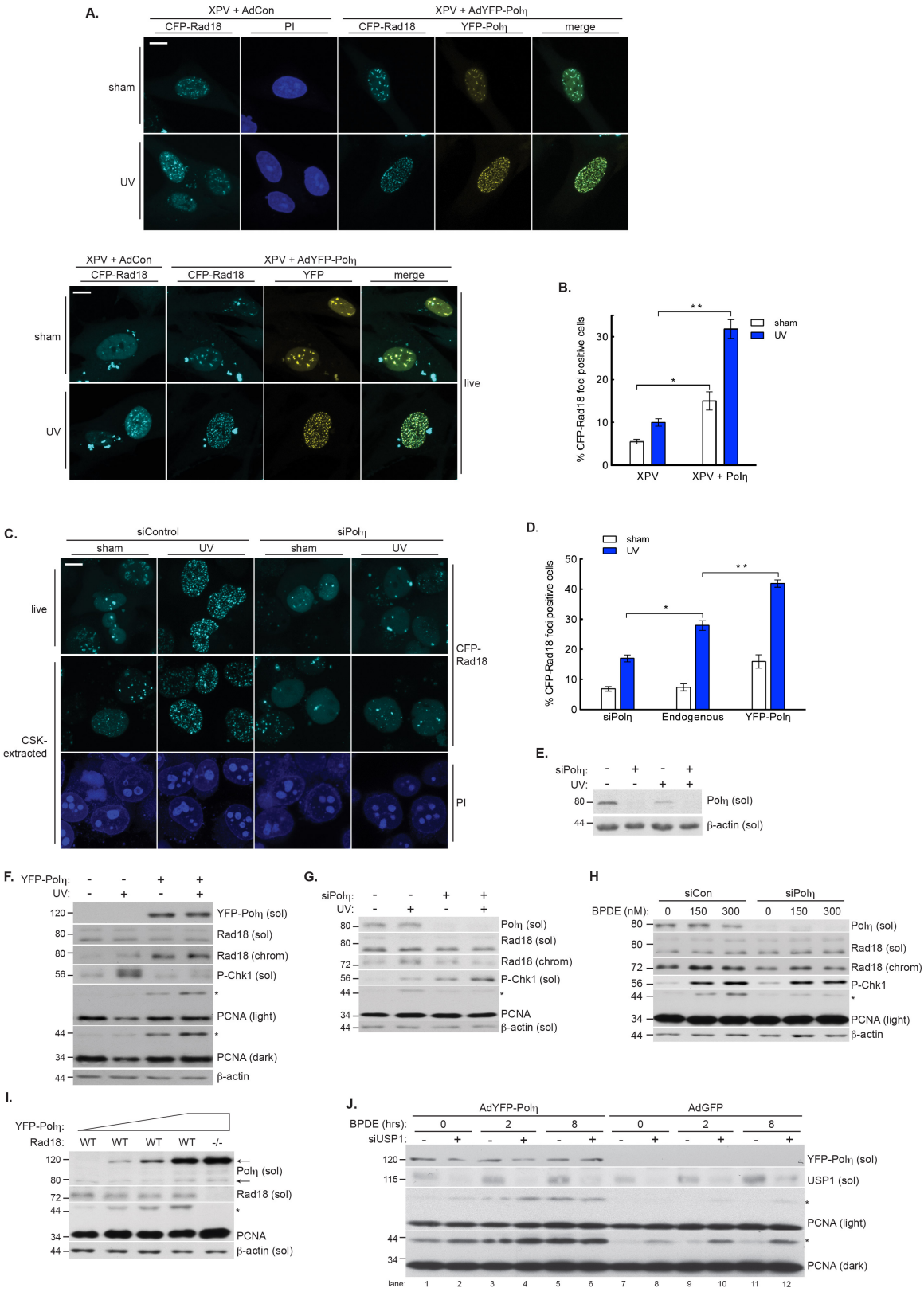
We next asked whether Polη status also affected PCNA-directed Rad18 E3 ligase activity. Polη-complemented XPV cells exhibited higher basal and damage-induced PCNA monoubiquitination compared to parental XPV cells, and Polη expression was associated with increased chromatin-binding of Rad18 (**Figure 3-1 F**). Conversely, UV-induced PCNA monoubiquitination was compromised in Polη-depleted normal Human Diploid Fibroblasts (HDF) relative to Polη-replete controls (**Figure 3-1 G**). Polη depletion thus partially phenocopies the expected effect of depleting RPA, which is thought to initiate TLS by coating ssDNA and triggering

ATR/Chk1 signaling and subsequent PCNA monoubiquitination.[66, 67] Rad18 redistribution and PCNA monoubiquitination were also attenuated in Polη-depleted cells after BPDE-treatment (**Figure 3-1 H**), indicating that the effect of Polη on Rad18 activity is not genotoxin-specific.

Next, we determined whether increased Polη expression affects Rad18 and PCNA monoubiquitination. When expressed in HCT-116 cells at levels ranging from approximately two- to 25-fold higher than endogenous, PCNA monoubiquitination increased in a dose-dependent fashion with Polη (**Figure 3-1 I**). Importantly, PCNA monoubiquitination was not induced by Polη in isogenic Rad18-null HCT-116 cells, indicating that the effect of Polη on PCNA modification is Rad18-mediated (**Figure 3-1 I**, right lane).

Potentially, the stimulatory effect of Polη expression on PCNA monoubiquitination could result (at least in part) from reduced PCNA de-ubiquitylation activity. Ubiquitin-Specific Protease 1 (USP1) is the only known PCNA-directed de-ubiquitylating (DUB) enzyme.[170] To determine whether inhibition of USP1 activity contributes to Polη-dependent PCNA monoubiquitination, we determined the effects of Polη-expression on PCNA modification in USP1-depleted cells. As expected, basal levels of PCNA monoubiquitination were increased by USP1-depletion (**Figure 3-1 J**). However, Polη expression further increased PCNA monoubiquitination in cells lacking USP1 (compare lanes 2 and 8), both basally and 2 and 8 hours after DNA damage. We conclude that that Polη stimulates PCNA monoubiquitination by Rad18 via USP1-independent mechanisms. We cannot exclude the formal possibility that Polη-dependent PCNA monoubiquitination is not

mediated by reduced activity of putative alternative PCNA-directed DUBs. However, the results of Figure 1 and data presented below indicate that Polη facilitates redistribution of Rad18 to sites of DNA damage and promotes efficient PCNA mono-ubiquitination.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XPV + AdYFP-Polη
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sham
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XPV + AdYFP-Polη
CFP-Rad18
CFP-Rad18
YFP
merge
sham
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% CFP-Rad18 foci positive cells
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Rad18 (chrom)
P-Chk1 (sol)
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Figure 3-1. Polη promotes damage-induced Rad18 redistribution and PCNA monoubiquitination..

(A) Representative images of CSK-extracted nuclei (top) or live (bottom) from CFP-Rad18-expressing XP115LO XPV cells co-infected with empty control adenovirus or adenovirus expressing YFP-Polη (at levels that restore UV-tolerance - see Supplementary Fig. S1) and exposed to UV (10 J/m^2) or sham-irradiated. Scalebar = 10 μm.

(B) Quantification of CFP-Rad18 foci-positive H1299 nuclei as a percentage of CFP-Rad18-expressing cells as shown in (A); *, p=0.0001; **, p=0.001. Error bars = SEM.

(C) Representative images of live (top) and CSK-extracted (bottom) nuclei from H1299 cells treated with non-targeting control siRNA (left) or siRNA targeting Polη (right) and imaged 2 hours after sham or UV (10 J/m^2)-irradiation. Scalebar = 10 microns.

(D) Quantification of CFP-Rad18 foci-positive nuclei as a percentage of CFP-Rad18-expressing cells as shown in (D). *, p=0.001; **, p=0.0003.

(E) Immunoblot of fractionated lysates from H1299 cells expressing CFP-tagged Rad18 as shown in (D) and treated with non-targeting control siRNA or siRNA against Polη and then lysed 2 hours after treatment with 10 J/m^2 UV or sham-treated.

(F) Immunoblot of fractionated lysates from XP115LO XPV cells treated with empty control adenovirus or adenovirus expressing YFP-Polη at levels shown in (A) and lysed 2 hours after treatment with 10 J/m^2 UV.

(G) Immunoblot of fractionated lysates from HDF cells treated with non-targeting control siRNA or siRNA against Polη and then lysed 2 hours after treatment with 10 J/m^2 UV or sham-irradiation.

(H) Immunoblot of fractionated lysates from HDF cells treated with non-targeting control siRNA or siRNA against Polη and then lysed 2 hours after treatment with increasing doses of BPDE.

(I) Immunoblot of fractionated lysates from HCT-116 WT cells (lanes 1-4) or RAD18$^{-/-}$ cells (lane 5) treated with increasing titers of YFP-Polη adenovirus and lysed 24 hours post-infection. Upper and lower arrows denote YFP-tagged and endogenous Polη, respectively.

(J) Immunoblot of fractionated lysates from H1299 cells expressing empty control adenovirus or adenovirus expressing YFP-Polη and treated with non-targeting control siRNA or siRNA against USP1 and then lysed at indicated times after treatment with 200 nM BPDE. On all Western Blots, asterisk denotes monoubiquitinated PCNA and bar graphs represent intensity of monoubiquitinated PCNA band relative to the maximum band on each film.

2.2. Rad18-Polη interactions drive PCNA monoubiquitination

Because Rad18 and Polη form a complex after DNA damage,[86] we next asked whether Polη-dependent redistribution of Rad18 and PCNA monoubiquitination required Rad18-Polη interactions. The Polη-binding region of Rad18 has been mapped to amino acid (AA) residues 402-445 (**Figure 3-2 A**).[86] Therefore, we

determined the effect of Polη status on the activity of a Polη-interaction defective Rad18 mutant, Rad18-Δ(402-445), that retains E3 Ub-ligase activity and other DNA repair functions.[86]

Consistent with *in vitro* binding studies,[86] Rad18-Δ(402-445) failed to co-immunoprecipitate Polη from cell lysates (**Figure 3-2 B**). To test how Polη-Rad18 binding affected subcellular Rad18 distribution, we depleted H1299 cultures of endogenous Rad18 and reconstituted with near-physiological levels of siRNA-resistant CFP-Rad18-WT or CFP-Rad18-Δ(402-445). As shown in **Figure 3-2 C** and **Figure 3-3**, co-expression of Polη significantly increased basal and damage-induced redistribution of Rad18-WT to nuclear foci but had no effect on the redistribution of Rad18-Δ(402-445). In replicate cultures of Rad18-complemented cells, Polη-induced PCNA monoubiquitination was severely compromised in cells complemented with Rad18-Δ(402-445) when compared with cells expressing Rad18-WT (**Figure 3-2 D**, compare lanes 1 & 8 to 11 & 12). Therefore, Polη-Rad18 interactions are necessary for Polη-dependent PCNA monoubiquitination.

The stable engagement of TLS polymerases with stalled replication forks depends on their UBZ/UBM-mediated interactions with monoubiquitinated PCNA.[93, 94, 152] As expected, the reduced PCNA monoubiquitination in cells complemented with Rad18-Δ(402-445) was associated with decreased Polη chromatin binding (**Figure 3-2 D**) and reduced formation of Polη nuclear foci (**Figure 3-2 E, F**), when compared with Rad18-WT-expressing cells. Thus, Rad18-Δ(402-445) partially recapitulates phenotypes conferred by the E3 ubiquitin ligase-deficient Rad18-C28F

mutant (**Figure 3-2 A**), including defective PCNA monoubiquitination (**Figure 3-2 G**) and reduced recruitment of Polη to chromatin (**Figure 3-2 E, F**).

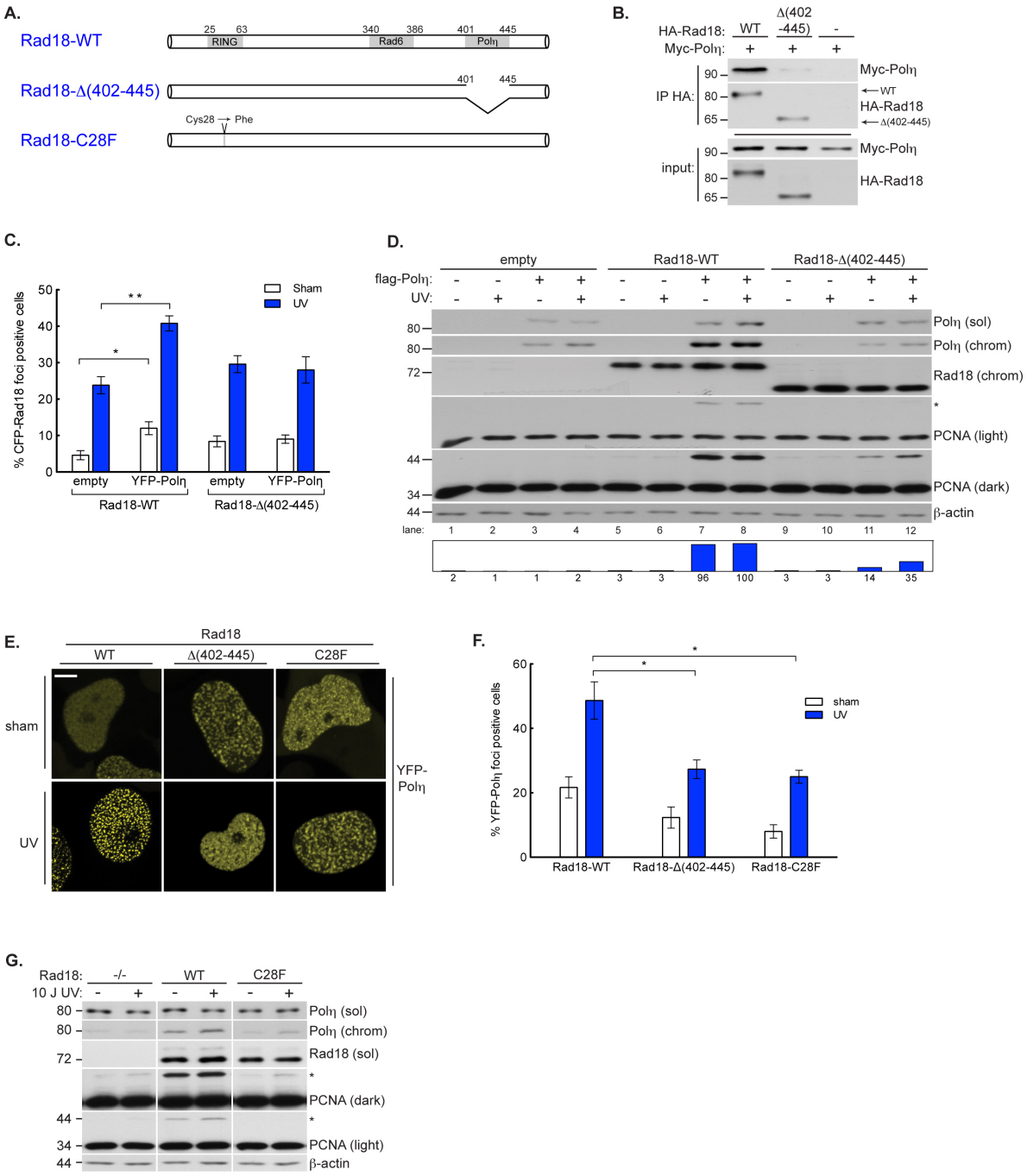


Figure 3-2. Physical interaction between Rad18 and Polη drives efficient damage-induced PCNA ubiquitination.

(A) Schematic of Rad18-WT (top); Rad18-Δ(402-445), a Polη-binding deficient mutant lacking the Polη binding domain (AA 402-445); Rad18-C28F, an E3 ligase-inactive mutant in which the RING-finger cysteine has been substituted with phenylalanine.

(B) Immunoblot analysis of anti-HA immunoprecipitates from H1299 cells co-expressing HA-Rad18-WT or HA-Rad18-Δ(402-445) with Myc-Polη. **(C)** Quantification of CFP-Rad18 foci-positive nuclei as a percentage of H1299 cells expressing CFP-Rad18-WT or CFP-Rad18-

Δ(402-445) and treated with empty adenovirus or Myc-Polη-expressing adenovirus followed by UV (10 J/m^2) treatment or sham irradiation. *, p=0.095; **, p=0.0006. Error bars = SEM.
(D) Immunoblots of fractionated lysates from Rad18-depleted H1299 cells that were reconstituted with siRNA-resistant Rad18-WT, Rad18-Δ(402-445), or empty vector (for control) alone or together with FLAG-Polη, followed by treatment with UV (10 J/m^2) or sham-irradiation.
(E) Representative images of CSK-extracted nuclei from YPF-Polη expressing H1299 cells that were depleted of endogenous Rad18 and then reconstituted with siRNA-resistant Rad18-WT, or Rad18-Δ(402-445), or Rad18-C28F and treated with UV (10 J/m^2) or sham-irradiated. Scalebar = 10 um.
(F) Quantification of YFP-Polη foci-positive nuclei as a percentage of YFP-Polη-expressing cells in cultures complemented with Rad18-WT, Rad18-Δ(402-445), or Rad18-C28F as shown in (E). *, upper p=0.026, lower p=0.0238.
(G) Immunoblots of fractionated lysates from Rad18-depleted H1299 cells that were reconstituted with siRNA-resistant WT-Rad18, C28F-Rad18, or empty vector, and then treated with UV (10 J/ m^2) or sham-irradiated.

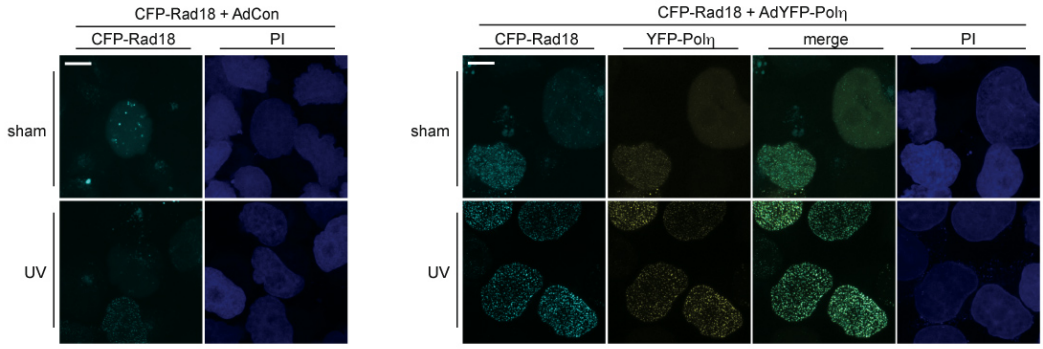


Figure 3-3. Polη overexpression drives Rad18 redistribution to nuclear foci.
Representative images of live CFP-Rad18-expressing H1299 cells co-infected with empty control adenovirus (left) or adenovirus expressing YFP-Polη (right) and treated with UV (10J/m^2) or sham irradiated. Scalebar = 10 μm.

2.3. Polη-PCNA binding drives PCNA monoubiquitination

C-terminal Polη truncations are the most common defect in XPV,[149, 171, 172] in which both the PCNA-Interacting Peptide (PIP) box[173] and the Rad18-binding domains[86] are deleted. To test whether Polη XPV C-terminal truncation mutants

exhibit defects in Rad18 regulation, we complemented XPV cells with WT-Polη or similar levels of a common XPV Polη mutant that retains full catalytic activity[174] but fails to confer UV-resistance (Polη-Δ(1-512), lacking residues 513-713, **Figure 3-4 A**). As expected, complementation of XPV cells with Polη-WT conferred normal Rad18 redistribution (**Figure 3-4 B, C**) and resulted in increased PCNA monoubiquitination (**Figure 3-4 D**). However, complementation of XPV cells with Polη-Δ(1-512) failed to restore Rad18 redistribution or PCNA monoubiquitination to the same extent as those complemented with Polη-WT (**Figure 3-4 B, D**), indicating that the C-terminal domain of Polη is important not only for Polη chromatin binding, but also for Rad18 nuclear redistribution and Rad18-mediated PCNA monoubiquitination.

To test whether loss of PCNA binding contributes to defective PCNA monoubiquitination in Polη-Δ(1-512)-complemented XPV cells, we generated point mutations in the PIP-box that abrogate PCNA binding [173] (**Figure 3-4 A**). In Rad18-depleted cells complemented with physiological levels of Rad18-WT, Polη-WT but not Polη-ΔPIP promoted PCNA monoubiquitination by Rad18 (**Figure 3-4 E**, compare lanes 7 & 8 with 11 & 12). Therefore, Polη-PCNA association via the PIP-box of Polη contributes to maximal Rad18-mediated PCNA monoubiquitination.

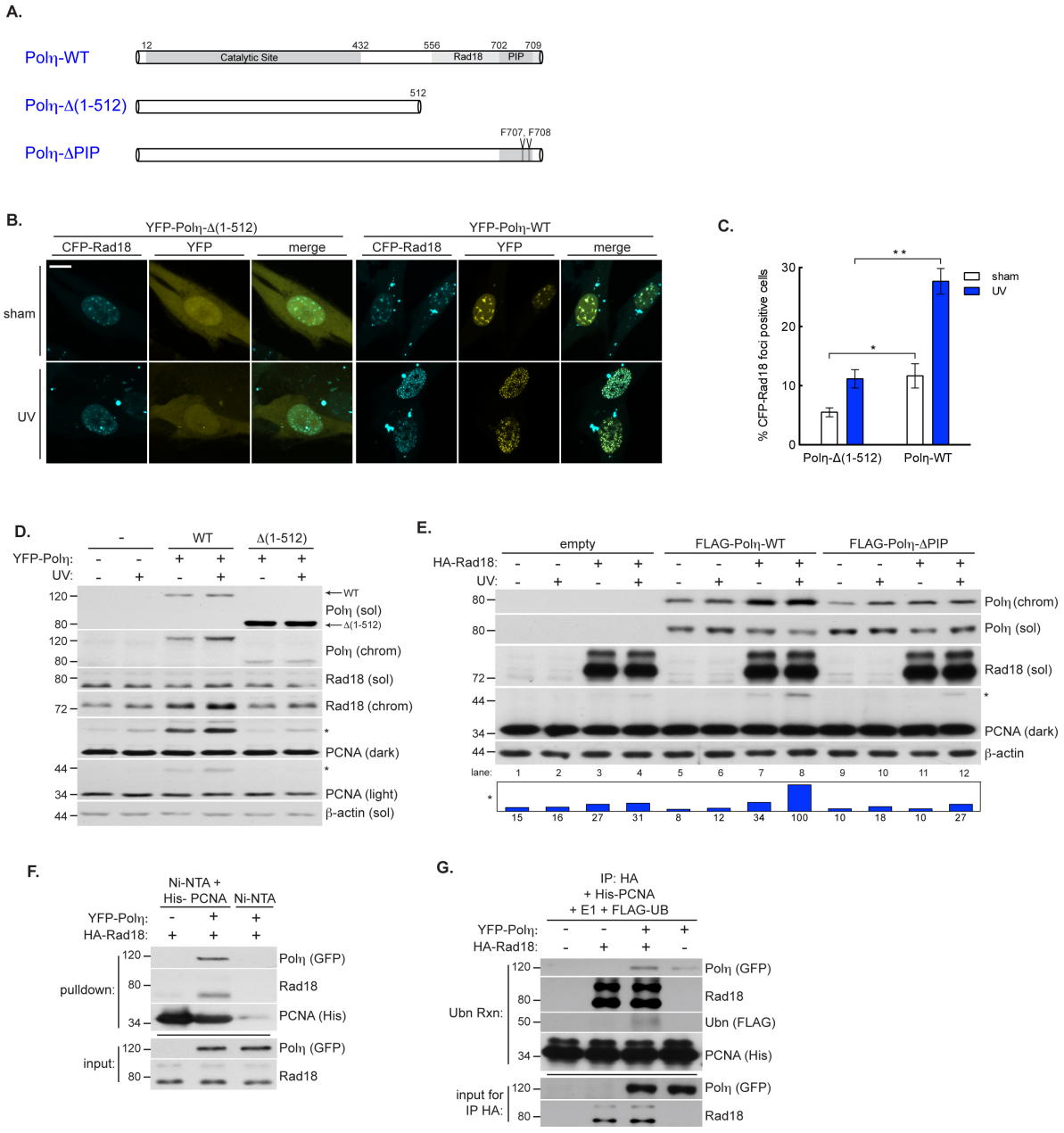


Figure 3-4. Polη physically bridges Rad18 and PCNA to promote efficient PCNA monoubiquitination after DNA damage.

(A) Schematic of Polη-WT (top); Polh-Δ(1-512), a C-terminal truncation lacking AA 513-713 (middle); and Polη-ΔPIP, full length Polη with two PIP-box phenylalanines mutated to alanine (bottom).

(B) Representative images of CSK-extracted nuclei from XPV cells that were co-infected with CFP-Rad18 and YFP-Polη-Δ(1-512) adenovirus (left) or YFP-Polη-WT (right) and treated with UV (10 J/m^2) or sham-irradiated. Scalebar = 10 um.

(C) Quantification of CFP-Rad18 foci-positive nuclei as a percentage of CFP-Rad18-expressing XPV cells expressing YFP-Polη-Δ(1-512) or YFP- Polη-WT adenovirus. *, upper p=0.018; **, p=0.0001; Error bars = SEM.
(D) Immunoblots of fractionated lysates from XPV cells complemented with Polη-WT or Polη-Δ(1-512) and treated with 10 J/m^2 UV.
(E) Immunoblots of fractionated lysates from Rad18-depleted H1299 cells that were reconstituted with siRNA-resistant Rad18-WT together with FLAG-tagged Polη-WT or Polη-ΔPIP and treated with sham or 10 J/m^2 UV.
(F) *In vitro* pulldown assay. His_6-PCNA-loaded Nickel beads (or unloaded beads) were incubated with lysates from UV-irradiated H1299 cells expressing HA-Rad18 or both HA-Rad18 and YFP-Polη.
(G) *In vitro* ubiquitination assay. HA-Rad18 complexes immunoprecipitated from UV-irradiated H1299 cells expressing HA-Rad18 alone or in combination with YFP-Polη were mixed with recombinant His_6-PCNA, E1, FLAG-ubiquitin, and an ATP-regenerating system and conjugated FLAG-Ub was detected by immunoblotting with anti-FLAG antibodies.

2.4. Polη scaffolding mediates Rad18-PCNA association

While Polη possesses a PIP-box[173, 175] and interacts directly with PCNA,[173] no PCNA-interacting domain has been identified for Rad18, and the mechanism for association of Rad18 with PCNA is unknown. The Polη-dependence of Rad18-mediated PCNA monoubiquitination (**Figures 3-1 - 3-3**) suggested that Polη may serve as a 'molecular bridge' or scaffold to facilitate Rad18-PCNA interactions. To test this hypothesis, we developed a cell-free system to determine the Polη-dependence of PCNA-Rad18 interactions (if any). Recombinant PCNA was immobilized on Ni-NTA beads (or unloaded beads for controls) and incubated with extracts from cells expressing Rad18 alone or in combination with Polη. When extracts from Rad18-expressing cells were incubated with PCNA-Ni beads, we were unable to detect association between Rad18 and PCNA (**Figure 3-4 F**, lane 1). However, we readily detected Rad18 association with immobilized PCNA incubated with lysates from Rad18 and Polη co-expressing cultures (**Figure 3-4 F**, lane 2).

Therefore, we conclude that Polη promotes Rad18-PCNA interactions or stabilizes Rad18-PCNA complexes.

We modified this cell-free assay to test whether the presence of Polη influenced PCNA monoubiquitination by Rad18. HA-Rad18 was expressed in UV-irradiated H1299 cells individually or in combination with Polη and then immunoprecipitated using anti-HA antibodies. The resulting immune complexes were then mixed with recombinant PCNA, E1, FLAG-ubiquitin and an ATP-regenerating system. As shown in **Figure 3-4 G**, Rad18 immune complexes conjugated FLAG-ubiquitin to PCNA in a manner that was stimulated by Polη (compare lanes 2 and 3).

Taken together, the results of Figure 2-3 suggest that Polη promotes efficient PCNA monoubiquitination via a bridging mechanism that facilitates physical interaction between Rad18 and PCNA.

2.5. Polη-induced PCNA-Ub is dissociable from catalytic activity

To test whether DNA polymerase activity was required for Polη to promote PCNA monoubiquitination, we generated a Polη mutant harboring 4 inactivating point substitutions in conserved residues necessary for catalytic activity[140] (**Figure 3-5 A**). Catalytically-inactive mutant Polη (Polη-C.I) and wild-type Polη both stimulated PCNA monoubiquitination to similar levels (**Figure 3-5 B**). Additionally, Polη-C.I. caused Rad18 redistribution to nuclear foci in a manner nearly identical to Polη-WT (**Figure 3-5 C, D**). Thus, the function of Polη in promoting Rad18-

mediated PCNA monoubiquitination is dissociable from its catalytic role as a DNA polymerase.

To probe the molecular determinants of PCNA monoubiquitination induced by Polη, we performed structure-function studies using Polη truncation mutants that progressively eliminated AAs 1-400 spanning the catalytic domain (**Figure 3-5 A**). Interestingly, when expressed at equal levels, the Polη-truncation mutants mobilized Rad18 to nuclear foci in a manner similar to Polη-WT (**Figure 3-5 E**). A Polη truncation constituting only 300 C-terminal amino acids induced a level of PCNA monoubiquitination comparable to WT-Polη (**Figure 3-5 F**). Therefore, the Rad18- and PCNA-binding C-terminus of Polη represents the minimal domain that is necessary and sufficient to regulate Rad18 activity and promote PCNA monoubiquitination.

Recent work identified a novel PIP box and UBZ-containing protein termed 'Spartan' that promotes PCNA monoubiquitination via a bridging mechanism between PCNA and Rad18, similar to that which we have defined for Polη.[161] To compare the relative contribution of Spartan and Polη to Rad18-mediated PCNA monoubiquitination, we expressed FLAG-Polη or FLAG-Spartan in H1299 cells. When expressed at comparable levels, Polη induced an increase in PCNA monoubiquitination that was nearly 10-fold higher than that conferred by Spartan (**Figure 3-5 G**) and siRNA-mediated knockdown of Polη decreased UV-induced PCNA monoubiquitination significantly more than Spartan knockdown (**Figure 3-5 H** compare lanes 4 and 6). Together, these data indicate that scaffolding of PCNA and

Rad18 by Polη plays an important role in the regulation of PCNA monoubiquitination.

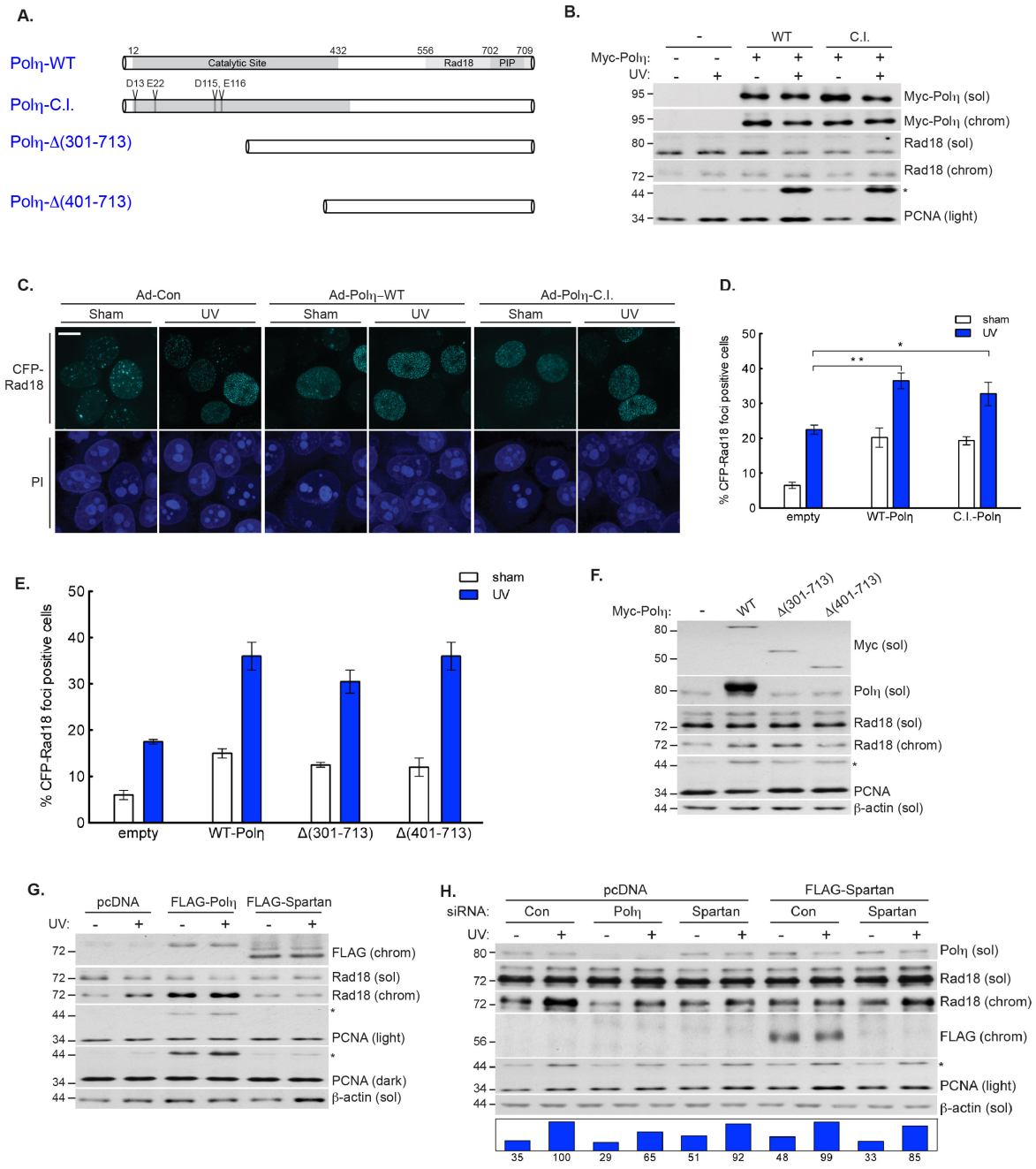


Figure 3-5. Physical bridging of Rad18 and PCNA by Polη is dissociable from its DNA Polymerase activity.

(A) Schematic of Polη-WT (top); full-length catalytically-inactive Polη, Polη-C.I, in which amino acids D13, E22, D115, and E116 are mutated to alanine; and N-terminal Polη truncation mutants, Polη-Δ(301-713) and Polη-Δ(401-713).

(B) Immunoblots of fractionated lysates from Myc-Polη-WT or Myc-Polη-C.I.-expressing H1299 cells that were treated with UV (10 J/m^2) or sham-irradiated.

(C) Representative images of CSK-extracted nuclei from H1299 cells that were co-infected with CFP-Rad18 and empty control adenovirus (left), Myc-Polη-WT (middle), or Myc-Polη-C.I. (right) and treated with UV (10 J/m^2) or sham-irradiated. Scalebar = 10 um.

(D) Quantification of CFP-Rad18 foci-positive nuclei as a percentage of CFP-Rad18-expressing H1299 cells expressing empty control adenovirus, Myc-Polη-WT, or Myc-Polη-C.I. **, p=0.0016; *, p=0.0287; Error bars = SEM.

(E) Quantification of CFP-Rad18 foci-positive nuclei as a percentage of CFP-Rad18-expressing H1299 cells expressing empty control adenovirus, Myc-Polη-WT, Myc-Polη-Δ(301-713), or Myc-Polη-Δ(401-713). Error bars = SEM.

(F) Immunoblot of fractionated lysates from H1299 cells expressing empty control adenovirus, Myc-Polη-WT, Myc-Polη-Δ(301-713), or Myc-Polη-Δ(401-713).

(G) Immunoblot of fractionated lysates from H1299 cells expressing empty vector control, FLAG-Polη, or FLAG-Spartan and lysed two hours after treatment with 10 J/m^2 UV or sham-treatment.

(H) Immunoblot of fractionated lysates from H1299 cells treated with scramble control siRNA or siRNA against Polη or Spartan and lysed two hours after UV treatment or sham. Spartan knockdown was validated by depletion of co-expressed FLAG-Spartan (lanes 6-10).

2.6. Specificity of Y-family Pol–dependent PCNA-Ub

We next asked whether the stimulatory effect of Polη on Rad18 activity was shared by other Y-family TLS Polymerases. Similar to Polη, Polκ associates with Rad18,[141] redistributes to form nuclear foci in response to DNA damage, and associates with PCNA via C-terminal PIP box. Therefore, for the purpose of comparison with Polη, we determined the effects of manipulating Polκ expression levels on PCNA monoubiquitination. In contrast with Polη knockdown, Polκ depletion did not attenuate PCNA monoubiquitination basally or after genotoxin treatment (**Figure 3-6 A**). Even when expressed at levels approximately 15-fold higher than Polη, Polκ did not induce the robust PCNA monoubiquitination response

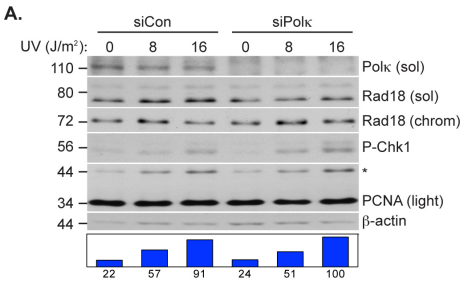
elicited by Polη (**Figure 3-6 B**). Polκ also exhibited induced far less Rad18 redistribution to chromatin. Hence, the role of Polη in promoting genotoxin-induced PCNA monoubiquitination is not shared by all Y-family TLS polymerases.

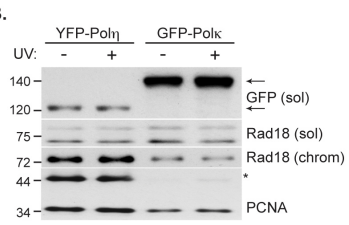
Because Polη and Polκ both associate with Rad18 after DNA damage,[80, 141] differences in Rad18 binding do not explain the inability of Polκ to promote PCNA monoubiquitination. We therefore hypothesized that differences in TLS Polymerase-PCNA binding account for the differential contributions of Polη and Polκ to PCNA monoubiquitination. Domains flanking the PIP boxes in the various Y family members confer dramatically different PCNA-binding affinities;[175] specifically, the high PCNA-binding affinity of Polη relative to other TLS Polymerases is attributed in large part to the 'PLTH' sequence immediately C-terminal to its PIP-box (**Figure 3-6 C**).

To test whether PCNA-binding affinity influences relative PCNA monoubiquitination activity, we performed domain-swap experiments in which we removed the PLTH motif from Polη (generating Polη-ΔPLTH) or added it to Polκ (generating Polκ+PLTH) (**Figure 3-6 C**). We then compared the subcellular distribution of wild-type and mutant forms of Polη and Polκ. As expected, Polη-ΔPLTH showed reduced nuclear focus formation and was also compromised for PCNA monoubiquitination activity relative to Polη-WT (**Figure 3-6 D-F**). Conversely, whereas Polκ-WT was localized diffusely throughout the nucleus, Polκ+PLTH showed a focal distribution pattern more similar to that of Polη-WT. Interestingly, Polκ+PLTH induced more robust PCNA monoubiquitination than Polκ-WT (**Figure 3-6 F**), demonstrating that addition of the PLTH (from the Polη PIP) to

the Polκ PIP increases its ability to induce PCNA monoubiquitination. Therefore, high affinity binding of Polη to PCNA confers the unique ability among Y-family polymerases to promote PCNA monoubiquitination.

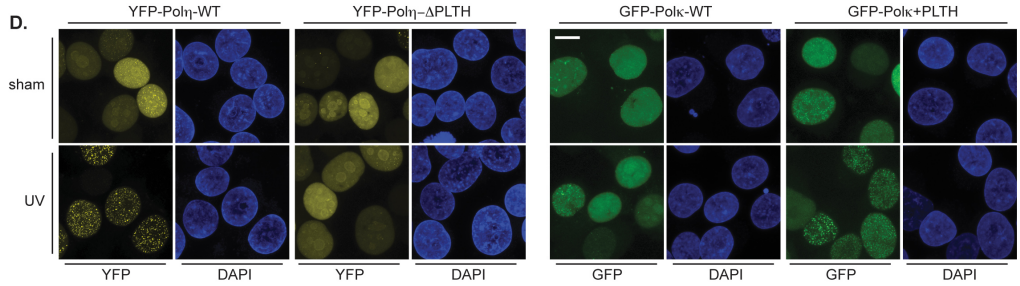
DNA damage-induced PCNA monoubiquitination contributes to the PCNA-binding of all Y-family TLS polymerase.[93, 94, 151] We hypothesized that Polη would influence other Y-family TLS polymerases by facilitating PCNA monoubiquitination, independently of its catalytic activity. Therefore, we compared the UV-inducible redistribution of Polι and Polκ in parental XPV cells or XPV cells reconstituted with Polη-WT or Polη-C.I. Consistent with prior studies,[97, 176] we found that basal and UV-induced Polι and Polκ redistribution to nuclear foci was higher in Polη-WT-reconstituted XPV cells compared to the Polη-defective parental cell line (**Figure 3-6 G, H**). Importantly, we found that Polη-C.I. dramatically increased both basal and UV-induced redistribution of Polι and Polκ to nuclear foci. We conclude that cells expressing full-length, catalytically inactive Polη retain Rad18-stimulatory activity, which in turn promotes recruitment of alternative error-prone polymerases to stalled replication forks.

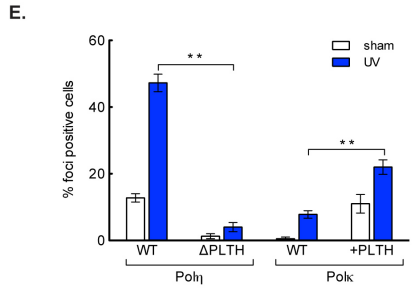


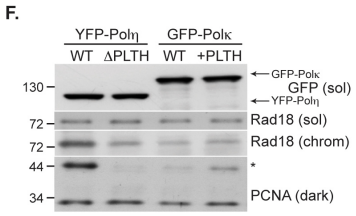


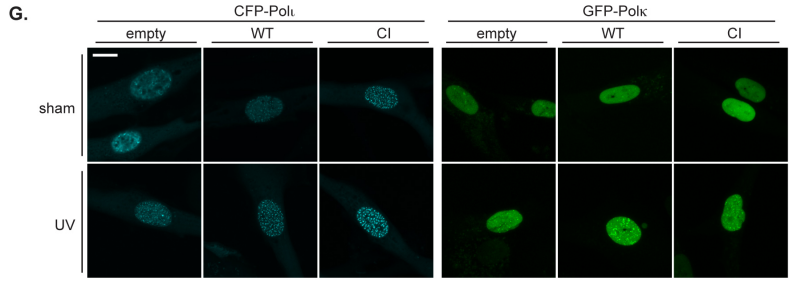
C.

	1 2 3 4 5 6 7 8
PIP Box Consensus:	Q - - ψ - - θ θ
Polη-WT (699-713):	E G M Q T L E S F F K P L T H
Polη-ΔPLTH (699-709):	E G M Q T L E S F F K - - - -
Polκ-WT (860-870):	N P K H T L D I F F K
Polκ+PLTH (860-874):	N P K H T L D I F F K P L T H









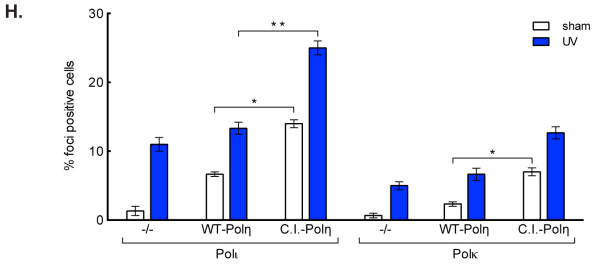


Figure 3-6. High-affinity interaction with PCNA drives Polη-specific induction of PCNA monoubiquitination.

(A) Immunoblot of fractionated lysates from control or Polκ-depleted H1299 cells that were lysed 2 hours after treatment with UV (10 J/m2) or sham irradiation.

(B) Immunoblot of fractionated lysates from H1299 cells expressing YFP-Polη or GFP-Polκ and lysed 2 hours after treatment with UV (10 J/m^2) or sham-irradiated.

(C) Sequence of the C-terminus of Polη and Polκ and the mutants used in domain-swap experiments: Polη-ΔPLTH and Polκ+PLTH. PIP-box consensus amino acids are in bold, where ψ = I/L/M; θ = Y/F.

(D) Representative images of CSK-extracted nuclei from H1299 cells that were infected with GFP-Polκ-WT, GFP-Polκ+PLTH, YFP-Polη-WT, or YFP-Polη-ΔPLTH and treated with 10 J/m^2 UV or sham-irradiated. Scalebar = 10 μm.

(E) Quantification of foci-positive nuclei as a percentage of H1299 cells expressing in YFP-Polη-WT, YFP-Polη-ΔPLTH, GFP-Polκ-WT, or GFP-Polκ+PLTH. *, left p=0.0001; **, p=0.0004; Error bars = SEM.

(F) Immunoblot of fractionated lysates from H1299 expressing YFP-Polη-WT, YFP-Polη-ΔPLTH, GFP-Polκ-WT, or GFP-Polκ+PLTH.

(G) Representative images of CSK-extracted nuclei from XPV cells that were co-infected with CFP-Polι or GFP-Polκ and empty control adenovirus (left), Myc-Polη-WT (middle), or Myc-Polη-C.I. and treated with UV (10 J/m^2) or sham-irradiated. Scalebar = 10 um.

(H) Quantification of CFP-Polι foci-positive nuclei as a percentage of CFP-Polι-expressing XPV cells (left) and GFP-Polκ foci-positive nuclei as a percentage of GFP-Polκ-expressing XPV cells (right), after co-infection with empty control adenovirus, Myc-Polη-WT, or Myc-Polη-C.I. and treatment with UV (10 J/m^2) or sham-irradiation *, left p=0.0009; **, p=0.0004, *, right p=0.0022; Error bars = SEM.

f Polη-WT (top); full-length catalytically-inactive Polη, Polη-C.I, in which amino acids D13, E22, D115, and E116 are mutated to alanine; and N-terminal Polη truncation mutants, Polη-Δ(301-713) and Polη-Δ(401-713).

2.7. p53 promotes PCNA monoubiquitination through Polη

Because Rad18-mediated PCNA monoubiquitination was sensitive to Polη expression, it was of interest to determine relative levels of Rad18 and Polη within cells. Therefore, we expressed an in-frame fusion of full-length Polη and full-length Rad18 in cultured cells, which allowed us to perform quantitative comparison of each endogenous protein relative to the Rad18-Polη fusion (**Figure 3-7 A**) using appropriate antibodies.

At expression levels comparable to endogenous Polη, the Rad18-Polη fusion protein was nearly undetectable in immunoblots with anti-Rad18 antibody (Rad18

light), and prolonged exposures revealed that expression of the fusion at these levels was substantially lower than endogenous Rad18 (Rad18 dark). This surprising result demonstrated that cellular Rad18 protein expression is several orders of magnitude higher than Polη in human cells; we estimate that Rad18 expression exceeds Polη by approximately 75-fold. Importantly, expression of the Rad18-Polη fusion protein to a level double that of endogenous Polη (and negligible compared to endogenous Rad18) induced a six-fold increase in PCNA monoubiquitination (right lane), showing that Rad18-mediated PCNA monoubiquitination is highly sensitive to Polη levels. These findings prompted us to determine whether physiologically-relevant changes in Polη expression influence PCNA monoubiquitination.

POLH is a transcriptional target of activated p53 and DNA damage stimulates p53-dependent increases in Polη protein expression.[177] Because endogenous Polη levels are limiting for Rad18 activity, we hypothesized that p53-induced Polη expression contributes to PCNA monoubiquitination. Therefore, we compared UV-induced PCNA monoubiquitination in WT HCT-116 cells and an isogenic p53-null HCT-116 line (**Figure 3-7 B**). As expected, Polη protein levels were lower in p53-null cells (compared to WT) after UV. Interestingly, PCNA was monoubiquitinated after UV-treatment in a manner that was temporally co-incident with Polη expression in p53-expressing HCT-116 cells, but not in $p53^{-/-}$ cells.

To test whether the p53-induced PCNA monoubiquitination was Polη-dependent, we depleted Polη in p53-null H1299 cells that were transfected with empty-vector or pcDNAp53. As shown in **Figure 3-7 C**, transient expression of p53 led to concomitant increases in Polη expression and UV-induced PCNA

monoubiquitination (compare lanes 1 & 2 with 5 & 6). Importantly, Polη depletion severely impaired PCNA monoubiquitination in p53-expressing cells compared to controls. Therefore, the p53-dependent component of PCNA monoubiquitination is Polη-mediated.

Because loss of p53 sensitizes normal fibroblasts, but not XPV cells, to UV,[178] [179] we hypothesized that the UV protection conferred by Polη is mediated by p53. To test this hypothesis, we compared UV survival in p53-depleted HDF cells infected with 'empty' control adenovirus or adenovirus expressing Polη at levels that confer UV-survival in XPV fibroblasts (see **Figure 2-1**). We found that Polη-expression modestly but significantly increased UV survival in $p53^{-/-}$ cells (**Figure 3-7 D**). Therefore, loss of p53-mediated Polη regulation indeed contributed to the UV sensitivity of p53 null fibroblasts. Together, these results suggest that Polη facilitates PCNA monoubiquitination in a p53-dependent manner, thereby revealing a novel link between the p53 pathway and TLS (**Figure 3-8**).

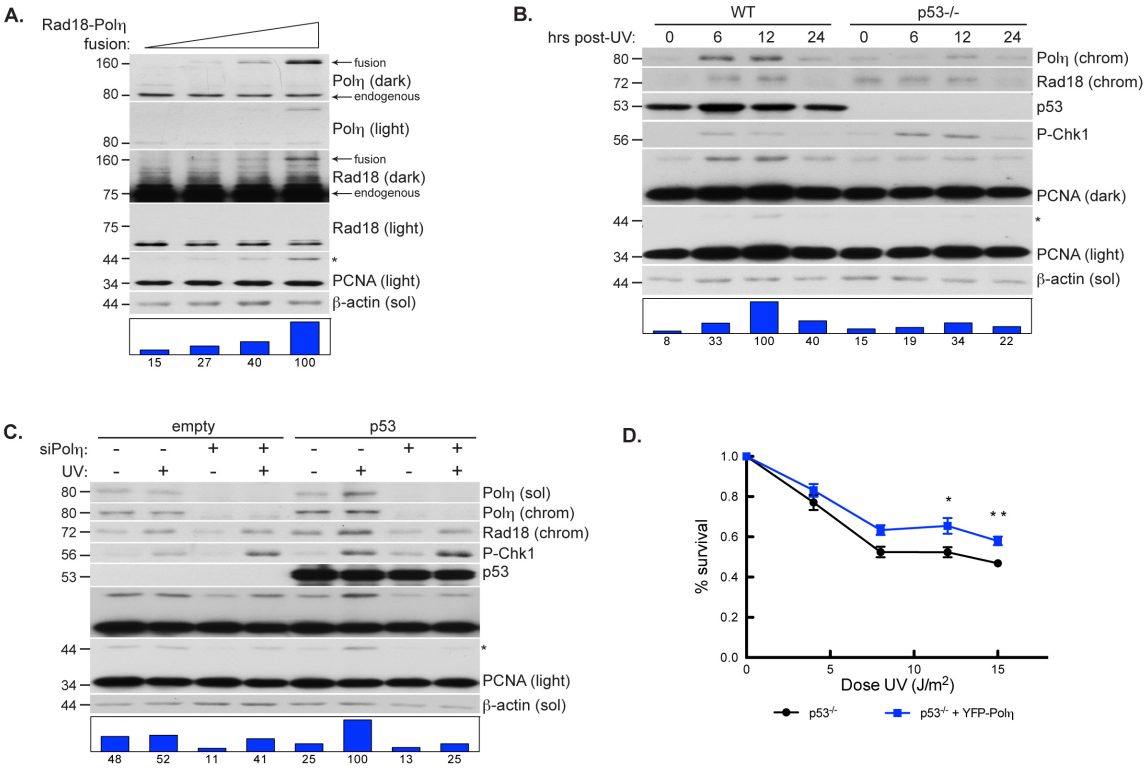


Figure 3-7. Rad18-Polη interaction is checkpoint sensitive and p53-regulated in response to DNA damage.

(A) Immunoblots of fractionated lysates from H1299 cells transfected with increasing quantities of pACCMV-Rad18-Polη fusion construct.

(B) Immunoblot of fractionated lysates from HCT-116 WT or HCT-116 $p53^{-/-}$ cells that were UV-treated (30 J/m^2) and lysed at indicated times after irradiation.

(C) Immunoblots of fractionated lysates of H1299 cells that were transfected with empty pcDNA as control or pcDNA-p53, followed by non-targeting control siRNA or siRNA against Polη. Cells were lysed 6 hours after 10 J/m^2 UV.

(D) UV-sensitivity of WT or $p53^{-/-}$ HDF incubated in 1mM caffeine and exposed to increasing doses of UV. Cells were infected with YFP-Polη adenovirus at a dose that confers UV-survival in XP115LO cells (see Supplementary Fig. S6). **, p=0.0305 at 12 J/m^2.**, p=0.0036 at 15 J/m^2.

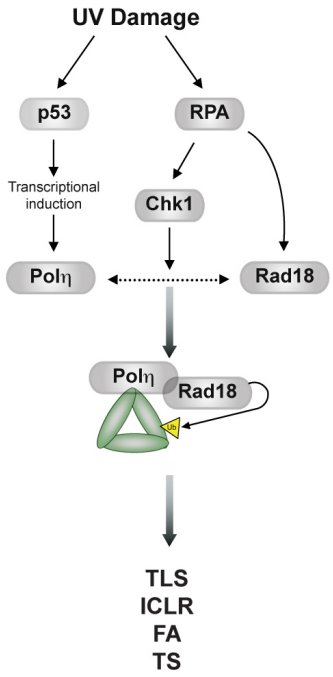


Figure 3-8. Contributions of p53 and Chk1 signaling to Polη-facilitated PCNA monoubiquitination.

UV-induced p53 activity leads to transcriptional induction of Polη expression (left). RPA-coated ssDNA generated through helicase-polymerase uncoupling directly recruits Rad18 and promotes Polη-Rad18 association via Chk1 signaling (right), thereby stimulating PCNA monoubiquitination and dependent DDR pathways.

3. Discussion

The results described here are consistent with existing models of TLS pathway activation involving an initial redistribution of the Rad18-Polη complex to the vicinity of damaged DNA (most likely via association of Rad18 with RPA-coated ssDNA).[102, 180] However, our results extend current models in that we propose Rad18 is in turn targeted to PCNA, its relevant substrate at the stalled replication fork, by Polη (**Figure 3-8**). Specifically, the extreme C-terminus of Polη physically bridges

Rad18 and PCNA to stimulate PCNA monoubiquitination (**Figure 3-4**), a function unique to Polη among TLS polymerases and fully dissociable from its TLS polymerase activity. The results of this study challenge the notion that TLS constitutes a simple linear pathway in which Rad18 acts upstream of Polη to promote TLS. Instead, we propose that Rad18 and Polη play mutually-dependent roles in TLS pathway activation.

Non-catalytic effector functions have been identified for other participants of the DDR, including Rev1,[181] Rad18,[86] NBS1,[182] and Chk1,[81] but this is the first demonstration of a DNA polymerase-independent activity for Polη. A non-catalytic role for Polη in stimulating PCNA monoubiquitination helps explain results of recent studies by other labs. For example, XPV cells are hypersensitive to BPDE and other genotoxins whose DNA lesions are not bypassed by Polη;[166, 167] clearly, a polymerase-independent function of Polη that promotes PCNA monoubiquitination and activation of Polκ (the TLS polymerase that mediates bypass of BPDE adducts) explains the BPDE-sensitivity of XPV cells. In other studies, catalytically-dead Polη mutants conferred DNA damage tolerance,[169, 183] and mutagenesis.[168, 169] Because PCNA monoubiquitination at K164 is necessary for tolerance of UV and other genotoxins,[98, 153, 154] restoration of UV-survival by catalytically-dead Polη[169] is explained by its scaffold function that promotes PCNA monoubiquitination, thus recruiting other TLS polymerases that facilitate tolerance, albeit at a cost of increased mutagenesis.

The Polη scaffolding function identified here has important implications for the molecular basis of genetic instability in XPV patients. Mutagenesis in XPV cells

is widely believed to result solely from deficient Polη polymerase activity,[184] leading to error-prone TLS of UV-damaged DNA by alternative and inappropriate TLS polymerases.[118] Many XPV mutations encode C-terminally-truncated forms of Polη that lack Rad18- and PCNA-binding domains.[171] However, in XPV cells in which Polη catalytic activity is perturbed while Rad18-PCNA bridging activity remains intact, high rates of UV-induced mutation frequencies may be conferred not only by loss of thymine dimer bypass activity by Polη, but also by stimulation of Rad18-mediated PCNA monoubiquitination and recruitment of alternative error-prone DNA polymerases.

Our finding that cellular Rad18 expression vastly exceeds Polη was unexpected, yet fully explains why PCNA monoubiquitination is exquisitely sensitive to slight alterations in Polη levels (**Figure 3-7**). Potentially, any process that affects Polη expression,[177] stability,[185-187] or nuclear localization[188] or its association with Rad18 is likely to affect PCNA monoubiquitination and in turn influence TLS. Indeed, we show here that transcriptional induction of *POLH* by p53 contributes to PCNA monoubiquitination. The Polη-Rad18 interaction is dependent on checkpoint signaling via Chk1.[80] Therefore, the results of this study may explain the long-standing observation that Chk1 signaling is required for efficient PCNA monoubiquitination.[81, 141] In fact, the Rad18-Δ(402-445) mutant in this study that failed to monoubiquitinate PCNA inducibly in response to Polη expression lacks the Chk1-dependent phosphorylation sites required for Polη binding.[80] Therefore, the Polη-dependent mechanism for PCNA monoubiquitination described here may

provide the basis for crosstalk between TLS and multiple processes including p53 signaling and the S-phase checkpoint.

Several DNA damage tolerance pathways depend on PCNA monoubiquitination, including replication fork restart[157] template switching,[156] intrastrand cross-link repair,[158] and the Fanconi Anemia pathway.[159] Hence, Polη contributes to cross-talk between multiple DDR pathways via PCNA monoubiquitination; loss of this element of the DDR in XPV underscores the importance of their orchestrated convergence to preserve genetic stability.

CHAPTER 4

PHOSPHORYLATION-MEDIATED REGULATION OF POLη-DEPENDENT PCNA MONOUBIQUITINATION AND RAD18

1. Checkpoint signaling and Polη-dependent PCNA monoubiquitination

1.1. Introduction

The results reported in Chapter 2 demonstrate a novel role for Polη in promoting DNA damage tolerance through mechanisms that are entirely dissociable from its well-known polymerase function. By promoting physical interaction of Rad18 with PCNA, Polη helps facilitate damage-induced monoubiquitination of PCNA and in turn initiate TLS. The exquisite sensitivity of PCNA monoubiquitination to changes in Polη levels (**Figure 3-2**) and the extent to which Rad18 expression levels exceed Polη (**Figure 3-6**) suggest that regulation of Polη expression contributes to regulation of PCNA monoubiquitination. Polη-dependent PCNA monoubiquitination is regulated at least in part by p53 (**Figure 3-6**), which transcriptionally up-regulates Polη expression after UV. However, several additional questions regarding the regulation of this process remain unanswered.

Previous studies have implicated checkpoint signaling in various steps of the mechanism of Polη-dependent PCNA monoubiquitination described above. Importantly, Polη-Rad18 association has been shown to be Chk1-dependent, as a function of the S-phase kinase Cdc7.

Cdc7 is required for initiation of DNA replication by phosphorylating MCM2-7 in the pre-replication complex, thus stimulating origin firing.[189] It has been shown

that Cdc7 is a target of S-phase checkpoint signaling pathways that block firing of origins.[190,191] Recent evidence also shows that Cdc7 participates in recovery from checkpoint-mediated replication arrest, although few downstream targets of Cdc7 have been identified.[192] Rad18 is necessary for TLS of genotoxin-induced DNA damage and for recovery from damage-induced S-phase checkpoints.[141,193] Mechanisms that regulate Rad18 in these TLS processes are not understood.

Phosphorylation of Rad18 takes place in parallel with Rad18 redistribution to stalled replication forks,[101] and our lab previously found that Cdc7 phosphorylates Rad18 in a UV-inducible manner.[80] This phosphorylation takes place at a specific serine (S434) in the C-terminus of Rad18, and it stabilizes Rad18-Polη associations and contributes to Polη translocation to sites of DNA damage.[80, 142] Mutation of S434 eliminates the UV-inducible Rad18-Polη interaction and sensitizes cells to UV irradiation. These data show that Rad18 is a Cdc7 target in checkpoint recovery pathways and led us to hypothesize that Chk1 regulates the UV-responsive recruitment of Polη to stalled RFs, presumably via Cdc7-mediated phosphorylation Rad18 **(Figure 4-1)**.

The results shown demonstrate a novel role for checkpoint signaling in the regulation of Rad18-mediated PCNA monoubiquitination. Specifically, these results show that Chk1 promotes UV-inducible PCNA monoubiquitination by facilitating physical interaction between Rad18 and Polη. Interestingly, we found that Rad18-Polη interactions are Chk1-dependent only in the chromatin fraction of cell lysates. Together, these results demonstrate a novel role for checkpoint signaling in the activation and regulation of TLS.

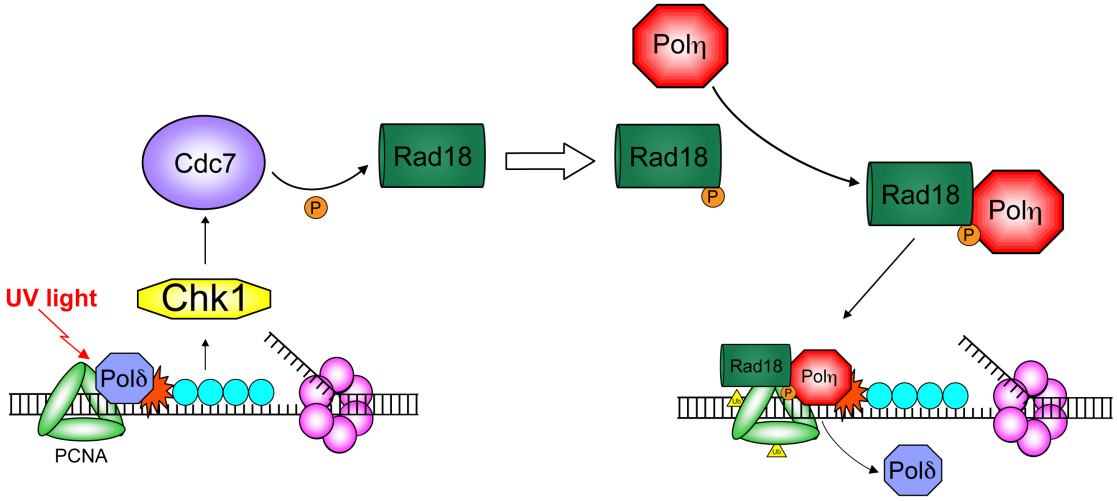


Figure 4-1. Hypothesized regulation of Polη recruitment to stalled RFs due to Cdc7-mediated phosphorylation of Rad18.

1.2. Results

Based on the importance of Rad18-Polη interactions in stimulating PCNA monoubiquitination, we hypothesized that loss of checkpoint signaling would compromise PCNA monoubiquitination. Indeed, when we depleted H1299 cells of Chk1 using siRNA, we found that PCNA monoubiquitination was severely compromised, as was the chromatin-bound fraction of Rad18 (**Figure 4-2**). This finding was consistent with other reports that Chk1 mediates maximum PCNA monoubiquitination, albeit through kinase-independent mechanisms.[81]

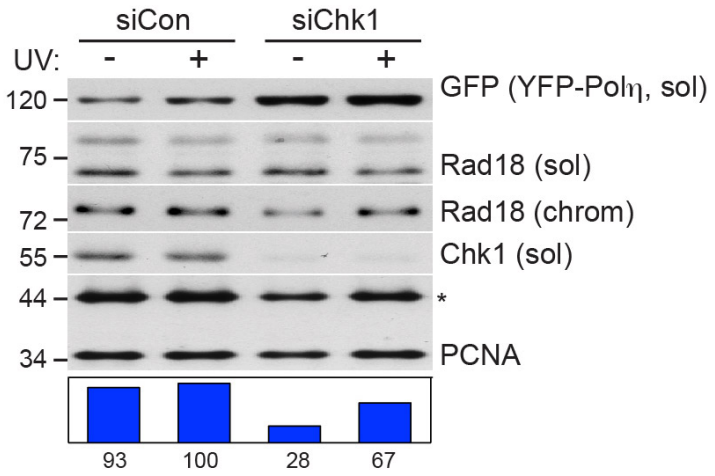


Figure 4-2. Immunoblot of lysates from H1299 cells that were treated with non-targeting control siRNA or siRNA against Chk1.

To determine how Chk1 depletion compromised PCNA monoubiquitination, we performed co-immunoprecipitation experiments in cells expressing HA-tagged Rad18 that were treated with UV, fractionated after lysis, and then incubated with HA-coupled sepharose beads. Consistent with the finding that Chk1 drives Rad18-Polη interactions, Chk1-depletion led to reduced co-immunoprecipitation of chromatin-bound Rad18 with Polη, together with attenuation of PCNA monoubiquitination (**Figure 4-3**). Interestingly, Chk1 status did not affect binding of co-immunoprecipitation of Rad18 and Polη from the soluble fraction, indicating that checkpoint signaling only regulates chromatin bound, and presumably active, states of Rad18 and Polη.

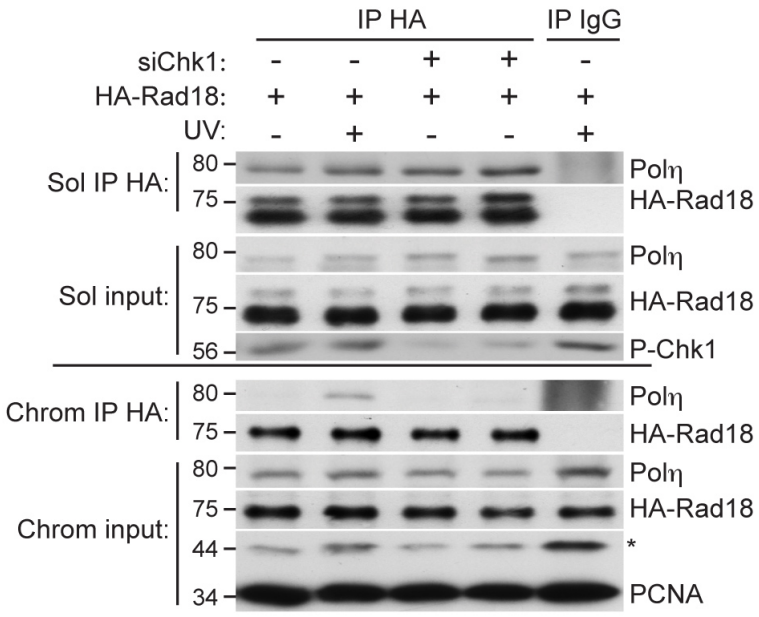


Figure 4-3. Immunoblot analysis of anti-HA precipitates from HA-Rad18-expressing H1299 cells transfected with non-targeting control siRNA or siRNA against Chk1 followed by treatment with UV (10 J/m^2) or sham irradiation.

The results presented above show that the Polη-Rad18 interaction is checkpoint dependent, as expected, and that this binding is important for efficient PCNA monoubiquitination. However, the unexpected finding that Chk1 status only impacts Rad18- Polη binding in the chromatin fraction indicates that Chk1 signaling is involved in targeting the complex to sites of DNA damage. Most likely, Chk1-signaling either drives translocation of the Rad18-Polη to chromatin, which would be consistent with the Chk1-Cdc7 kinase-dependent mechanism,[80] or stabilizes the complex at replication forks, which would be consistent with the kinase-independent mechanism.[81]

To distinguish between these two possibilities, we measured the redistribution of Rad18 to nuclear foci in the presence or absence of Chk1 signaling. When we

expressed CFP-Rad18 in cells treated with non-targeting control siRNA or siRNA against Chk1, Chk1-depletion severely compromised the UV-induced redistribution of Rad18 to nuclear foci (**Figure 4-4 and 4-5)**, consistent with the reduced chromatin-binding of Rad18 shown in **Figure 4-2**. We observed similar results after depletion of Cdc7, a distal target of Chk1 that promotes association between Rad18 and Polη.[80] These results indicate that the kinase activity of Chk1 is involved in driving Rad18 redistribution to chromatin, presumably via stimulation of Rad18-Polη physical interaction, which recruits Rad18 to PCNA via the Polη-mediated bridging mechanism. The kinase-independent mechanism of stimulating PCNA monoubiquitination involves a platform-like mechanism, and its impact is thus limited to stalled forks that only affects chromatin-bound Rad18. However, the results of **Figure 4-4 and 4-5** show that Chk1 influences translocation of Rad18 from soluble to chromatin, thus involving steps distal to chromatin, such as soluble Chk1 kinase signaling. These results thus implicate the kinase activity of Chk1 in promoting PCNA monoubiquitination, though kinase-independent mechanism is not contradicted.

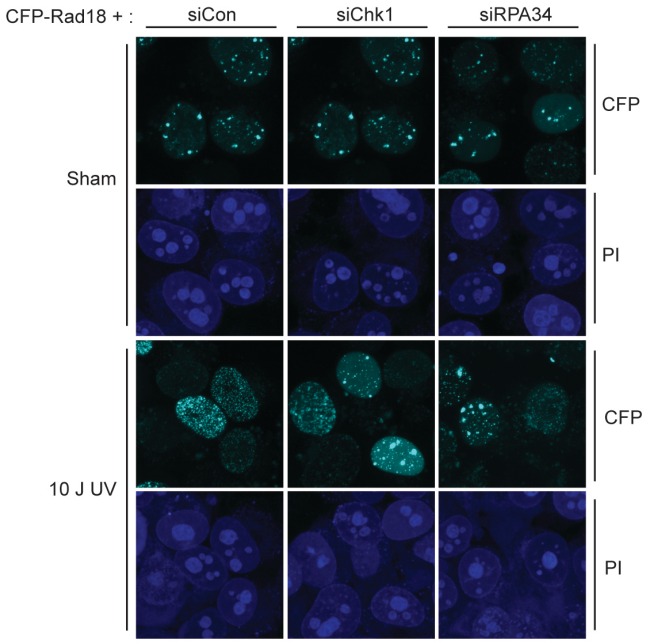


Figure 4-4. Representative images of H1299 cells expressing CFP-Rad18 and UV- or sham-treated.
Cell were treated with siRNA against Chk1, RPA, or non-targeting controls, exposed to sham or UV, and then CSK-extracted after two hours.

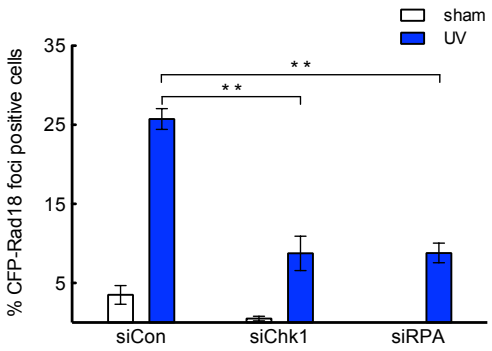


Figure 4-5. Quantification of CFP-Rad18 expressing H1299 cells as a function of RPA and Chk1.

To further distinguish between kinase dependent or independent contributions of Chk1 to Rad18 E3 ligase activity and PCNA monoubiquitination, we looked

upstream of Chk1 activation. Current models explain that Chk1 is activated after RPA coats ssDNA to trigger ATR/ATRIP interactions, which stimulate Chk1 kinase activity.[64] Additionally, RPA has been reported to recruit Rad18 directly to chromatin via physical interaction with Rad18.[102] [80]When we depleted CFP-Rad18 expressing cells of RPA with siRNA, we found that RPA-depleted cells exhibited diminished Rad18 redistribution after UV-irradiation, similar to Chk1-depleted cells (**Figures 4-4 and 4-5**). Our demonstration that depletion of Chk1 (a distal effector of RPA in the S-phase checkpoint) also compromises Rad18 activity in a manner similar to RPA indicates that RPA regulates initiation of TLS via 2 mechanisms: (1) a Chk1-independent pathway recruiting Rad18 complexes directly to ssDNA and (2) a Chk1-dependent mechanism that facilitates Polη-Rad18 complex formation, which promotes productive interactions between Rad18 and its substrate, PCNA.

2. JNK-mediated Rad18 phosphorylation and Y family Polymerase activation[1]

2.1. Introduction

Improper coordination of TLS poses great risks of mutagenesis by favoring error-prone DNA replication, as best exemplified by XPV.[194] However, the processes that regulate activation of TLS and physical interactions between TLS enzymes are

[1] Results presented in this section were published in in May 2012 in the following manuscript: Barkley LR, Palle K, Durando M, Day TA, Gurkar A, Kakusho N, Li J, Masai H, Vaziri C. c-Jun N-terminal kinase-mediated Rad18 phosphorylation facilitates Polη recruitment to stalled replication forks. Mol Biol Cell. 2012 May;23(10):1943-54.

poorly inderstood. Direct interplay between checkpoint signaling and TLS activation has been reported[80], and the results shown above demonstrate coordination between checkpoint signaling and TLS via Chk1-dependent phosphorylation of Rad18 and through physical interactions with RPA. Due to the detection of numerous UV-inducible phosphorylation sites on Rad18, we hypothesized that phosphorylation of Rad18 contributes to the activation of TLS and facilitates interaction with TLS Polymerases. Indeed, we found that Cdc7-mediated phosphorylation of Rad18 at serine 434 drives binding between Rad18 and Polη and contributes to tolerance of UV light. However, Cdc7-mediated phosphorylation at serine 434 is only one of many phosphorylation sites on Rad18, and we thus hypothesized that other phosphorylation sites may contributes to TLS activation via similar mechanisms.

2.2. Rad18 is a JNK phosphorylation target

Whereas serine 434 of Rad18 was shown to be a direct phosphorylation target of Cdc7, numerous other phosphorylation sites of unknown consequence were also identified in this same region.[80] Additionally, a recent mass spectrometry-based screen of phosphorylation sites in proteins purified from human mitotic spindles identified another phosphorylation site of Rad18 at serine 409 (**Figure 4-6**).[195] Because this site is highly conserved among species and in the vicinity of the Cdc7 target S434, our lab tested whether this phosphorylation event may regulate Rad18 activity in a manner analogous to S434.

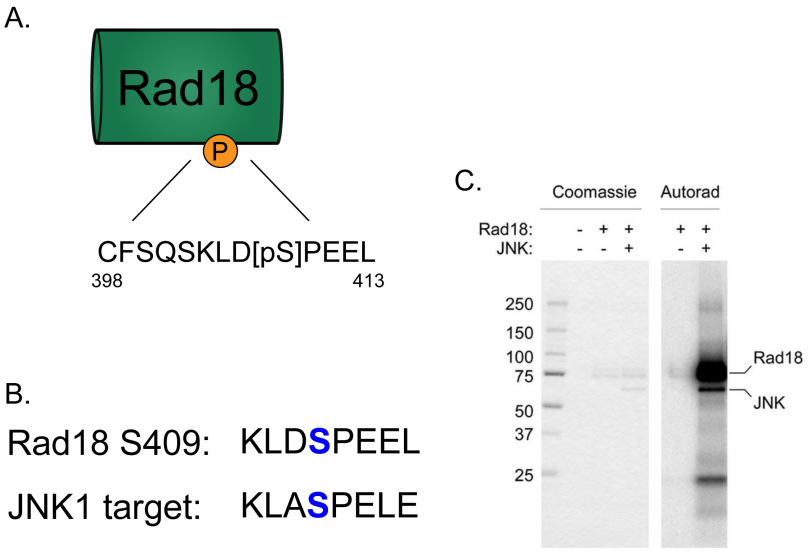


Figure 4-6. Rad18 is a phosphorylation target of JNK.
A. Serine 409 phosphorylation site identified in large-scale screen of phosphoproteins.
B. Comparison of sequences flanking Rad18 S409 with JNK1 consensus phosphorylation sites.
C. *In vitro* phosphorylation of Rad18 by JNK (expt by L. Barkley).

After generating phosphospecific antibodies to the phosphopeptide corresponding to residues 398–413 of Rad18 (**Figure 4-6A**), we found that S409 was phosphorylated in a UV-inducible manner. Similar to S434, S409 phosphorylation was found to be Chk1-dependent, consistent with the hypothesis that Chk1-dependent phosphorylation of TLS proteins links S-phase checkpoint signaling with TLS. However, the conserved sequences flanking S409 do not correspond to the preferred substrate motifs for Chk1[196] and *in vitro* kinase assays with recombinant Chk1 and Rad18 elicited negligible phosphorylation at S409, together indicating that phosphorylation of Rad18 S409, like S434, is not mediated by Chk1. Instead, it was noted that the sequences flanking S409 greatly resemble the consensus sequence for preferred c-Jun N-terminal kinase (JNK) substrates (**Figure 4-6B**). *In vitro* kinase

assays with recombinant Rad18 indeed showed strong phosphorylation by JNK (**Figure 4-6C**), indicating that Rad18 is indeed a phosphorylation target of JNK.

2.3. JNK-mediated Rad18 phosphorylation promotes Rad18-Polη binding

To determine whether Rad18 is a phosphorylation target of JNK in live cells, HA-tagged Rad18 was immunoprecipitated from cells treated with UV or sham irradiation in the presence or absence of the pharmacological inhibitor of JNK, SP600125. Probing with the S409 phosphospecific antibody revealed a marked reduction in Rad18 phosphorylation in the presence of the JNK inhibitor, indicating that JNK activity indeed targets Rad18 in live cells in a UV-inducible manner (**Figure 4-7A**).

The UV-inducible nature of S409 phosphorylation drew further parallels to Cdc7-mediated S434 phosphorylation, which facilitates UV-dependent Rad18-Polη binding. To determine whether phosphorylation at S409 contributes to interaction with Polη, we created a mutant Rad18 in which S409 is mutated to Alanine, Rad18S409A (**Figure 4-7B**). Rad18S409A was not reactive with the p-S409 phosphospecific antibody, as expected. When we immunoprecipitated HA-tagged WT or S409A Rad18 from Rad18$^{-/-}$ HCT-116 cells treated with sham irradiation or UV, we detected association only between WT-Rad18 and Polη, indicating that phosphorylation at S409 is important for Rad18-Polη binding (**Figure 4-7C**). To further determine whether phosphorylation at S409 drives Rad18-Polη association, we created a phosphomimetic substitution at S409, replacing serine with the negatively charged glutamic acid to yield Rad18-S409E (**Figure 4-7-B**). Indeed,

when we expressed WT, S409A, or S409E at equal levels and immunoprecipitated Rad18 after sham irradiation or UV, we found that basal and UV-induced association between Rad18-S409E was slightly greater than WT-Rad18, indicating that the phosphomimetic substitution at S409, though an imperfect recreating of a phosphate moiety, increases binding between Rad18 and Polη. Together, these results demonstrate that JNK-kinase mediated phosphorylation at S409 on Rad18 contributes to UV-inducible binding between Rad18 and Polη.

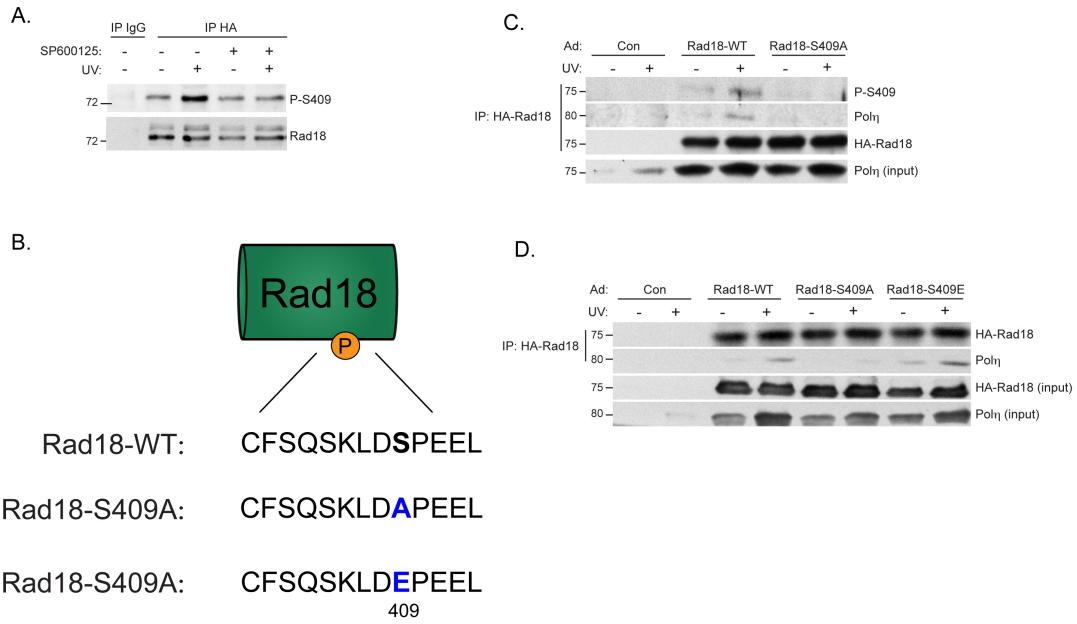


Figure 4-7. Serine 409 phosphorylation drives UV-inducible interaction of Rad18 with Polη.
A. Immunoblots of lysates from H1299 cells expressing HA-Rad18 that were treated for 1 h with 1 μM SP600125 or left untreated for controls, followed by UV or sham irradiation. After 2 h, Rad18 was immunoprecipitated from cell extracts using HA-coupled beads, and immune complexes were resolved by SDS–PAGE and probed sequentially with antibodies against Rad18 pS409 and HA. (expt by T. Da)
B. Sequences Rad18 Wt and the Rad18 mutants harboring alanine and glutamic acid substitutions at S409.
C. Immunoblots of immunoprecipitates from *RAD18*$^{-/-}$ cells expressing HA-Rad18 WT or HA-Rad18 S409A were treated with UV or sham irradiation and lysed after 6 h. Chromatin fractions from the cells were solubilized and immunoprecipitated with anti-HA antibodies. The resulting immune complexes

were resolved by SDS–PAGE and analyzed by immunoblotting with antibodies against Rad18 S409, HA, and Polη.
D. Immunoblots of immunoprecipitates from *RAD18*$^{-/-}$ cells expressing HA-Rad18 WT, HA-Rad18 S409A, or HA-Rad18 S409E that were treated with UV or sham irradiation and lysed after 6 h. (expt by L. Barkley)

2.4. JNK-mediated Rad18 phosphorylation drives Polη to nuclear foci

As demonstrated extensively in Chapter 2 of this thesis and in many previous reports,[80, 86, 197] the cellular redistribution of Rad18 and Polη is tightly coordinated in cells, especially after exposure to DNA damaging agents. To determine whether JNK-mediated phosphorylation of Rad18 contributes to the UV-inducible interplay between Rad18 and Polη, we measured the redistribution of Polη in cells expressing WT Rad18 or Rad18 S409A. Consistent with a role for Rad18 in directing UV-inducible Polη redistribution, the percentage of cells harboring nuclear Polη foci were greatly increased after co-expression with WT-Rad18 (**Figure 4-8A, B**). However, Polη foci were greatly diminished in the presence of Rad18-S409A, indicating that JNK-mediated phosphorylation of Rad18 contributes to the Rad18-dependent redistribution of Polη after exposure to UV.

Rad18 has also been shown to bind to Polκ and to contribute to its damage-dependent redistribution to nuclear foci[141], although the binding sites on the two proteins are completely unknown. To determine whether JNK-mediated phosphorylation of Rad18 contributes to mobilization of Polκ as well, we tested the effect of the S409A on Polκ redistribution after UV. As expected, the UV-inducible redistribution of Polκ was dramatically less than for Polη (compare ordinates in **Figure 4-8 B and D**) but was also diminished in the context of Rad18-S409A

compared to Rad18-WT (**Figure 4-8D**). This indicates that JNK-mediated phosphorylation of Rad18 does not specifically affect Rad18-Polη binding, but rather contributes to the Rad18-mediated mobilization of TLS Polymerases in a general fashion. It is of interest to test how this JNK-mediated phosphorylation is affected by different genotoxins (e.g. UV vs BPDE vs MMC) and how this impacts association with and mobilization of the different TLS polymerases.

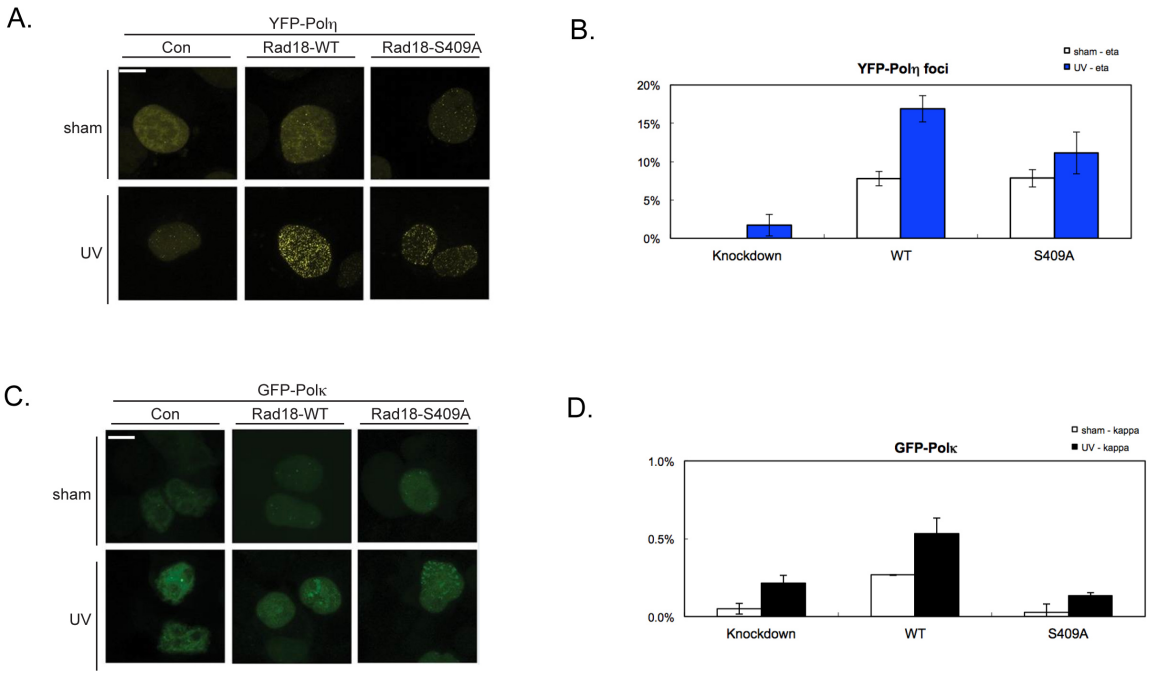


Figure 4-8. JNK-mediated Rad18 phosphorylation contributes to UV-inducible redistribution of Polη.

A. Representative images of CSK-extracted nuclei from H1299 cells that were infected with YFP-Polη and co-infected with empty control adenovirus, Rad18-WT, or Rad18-S409A and exposed to UV or sham irradiation.

B. Quantification of YFP-Polη foci-positive nuclei as a percentage of YFP-Polη-expressing H1299 cells as a function of Rad18 status.

C. Representative images of CSK-extracted nuclei from H1299 cells that were infected with GFP-Polκ and co-infected with empty control adenovirus, Rad18-WT, or Rad18-S409A and exposed to UV or sham irradiation.

D. Quantification of GFP-Polκ foci-positive nuclei as a percentage of GFP-Polκ - expressing H1299 cells as a function of Rad18 status.

The results of **Figures 4-5** through **4-8** show that JNK-mediated phosphorylation drives the UV-inducible binding of Rad18 and Polη, a process that is known to promote tolerance of UV-damaged DNA. To test whether phosphorylation at S409 contributes to tolerance of UV damage, we compared the UV survival of cells expressing WT-Rad18 or Rad18-S409A. When we depleted H1299 cells of endogenous Rad18 (using siRNA targeting the Rad18 3'UTR) and then complemented with WT-Rad18 or Rad18-S409A, we found that Rad18-S409A-expressing cells were sensitized to UV light, compared to Rad18-WT (Figure 3-9). Phosphorylation of Rad18 at serine 409 by JNK thus contributes to the UV tolerance of cells mediated by Polη, most likely via promotion of Rad18-Polη physical interactions (**Figure 4-7**) and subsequent mobilization of Polη to replication forks (**Figure 4-8**),

2.5. Discussion

The interaction of Rad18 with Polη represents one of several mechanisms that facilitate recruitment of Polη to the replication machinery. It is generally believed that Rad18 guides Polη to sites of stalled replication[86] and that Rad18-mediated Polη chaperone activity promotes stable engagement of Polη with PCNA via PCNA-interacting protein (PIP) box and UBZ-mediated interactions.[94] These results demonstrate a novel UV damage–inducible Rad18 phosphorylation site at the

conserved residue Serine-409 that resides in the Polη-binding domain of Rad18. S409 phosphorylation is important for Rad18–Polη association and confers more efficient chaperoning of Polη to nuclear foci at sites of replication stalling, all of which promotes tolerance of UV-induced DNA damage (**Figure 4-9**). The impact and phenotype conferred by JNK-mediated phosphorylation at S409 greatly parallels the Cdc7-mediated phosphorylation at S434. Our results show that Rad18-mediated tolerance of UV damage requires both JNK- and Cdc7-mediated phosphorylation of Rad18.

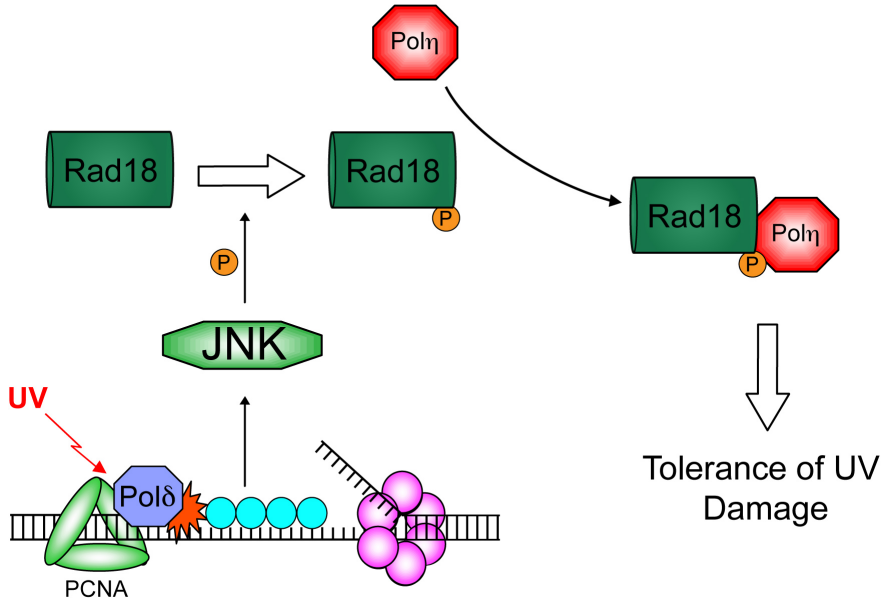


Figure 4-9. Schematic of JNK-mediated regulation of Rad18-Polη binding in response to UV light.

These results represent the first demonstration of a relationship between JNK signaling and TLS. The activation of JNK in response to replication stress–inducing agents such as UV radiation[198] and BPDE[199] has been studied extensively, but

relationships between JNK and checkpoint signaling in the TLS response have not been explored. Our finding that JNK regulates TLS in a manner that is Chk1 dependent further demonstrates the intricate coordination of the various effector branches of the DNA damage response, including checkpoint signaling and TLS.

Phosphorylation of Rad18 by JNK has interesting implications. JNK is generally considered a mediator of apoptosis, not processes that mediate tolerance of DNA damage, such as TLS. It is therefore surprising that JNK promotes Rad18–Polη recruitment to stalled replication forks to protect against DNA damage and presumably apoptosis. However, JNK activity is not exclusively associated with apoptosis. Many stimuli that activate JNK do not lead to apoptosis, perhaps because of active survival signaling pathways that prevent the apoptotic response.[200] In addition, the kinetics of JNK activation may determine whether or not there is an apoptotic outcome. For example, transient JNK activation (e.g., in response to cytokines) may mediate survival, whereas prolonged activation can mediate apoptosis.[201] Although SAPK signaling has not previously been implicated in TLS or postreplication repair, recent studies by other labs have implicated JNK in control of DNA replication. For example, Miotto and Struhl reported that JNK phosphorylates Cdt1 (a replication licensing factor) on T29, leading to dissociation of HBO1 from replication origins and blocking DNA replication licensing.[202] Similarly, Cook and colleagues identified multiple JNK and p38 target sites on Cdt1 the phosphorylation of which serves to rapidly inactivate Cdt1 and inhibit origin licensing.[203] JNK thus helps to ensure appropriate DNA replication and genome maintenance in genotoxin-treated cells via at least two mechanisms: inhibition of origin licensing, and the

results presented here demonstrate a role for stimulation of TLS at sites of ongoing DNA replication. It is of interest to determine whether additional proteins involved in other stages of DNA synthesis (e.g., initiation) or other modes of DNA repair are similarly subject to regulation by SAPKs.

Potentially consistent with additional roles for SAPKs in DNA replication and repair, Lannigan and colleagues demonstrated that extracellular signal-regulated kinase 8 (ERK8; a SAPK family member) contains a conserved PIP box that mediates PCNA binding and subsequent PCNA turnover via Mdm2.[204] As demonstrated in Chapter 2 (**Figure 2-5**), affinity to PCNA is crucial for activation of TLS Polymerases, and in general, PCNA represents a central hub for numerous DNA repair and replication processes. Therefore recruitment of ERK8 and perhaps additional PIP box–containing mitogen-activated protein kinase (MAPK) family members to PCNA may provide the basis for cross-talk between MAPK signaling and DNA replication and repair events. By analogy, JNK2 has been detected at RPA–coated ssDNA, which both initiates checkpoint signaling that regulates TLS and directly recruits Rad18 to stalled forks[205] (see previous section). Therefore, similar to ERK8, JNK2 may be able to regulate DNA replication and repair events in the vicinity of stalled replication forks. Further work is needed to investigate potential regulators and targets of the SAPKs at sites of DNA replication and repair.

It has become increasingly evident that TLS is coordinated with other elements of the DNA damage response, in particular checkpoints. JNK- and DDK-dependent Rad18 phosphorylation is clearly Chk1 dependent (**Figure 4-2 through 4-5**), demonstrating further links between S-phase checkpoint signaling and TLS. The

finding that mutation of S409 to alanine influences UV-induced Polκ redistribution, in the context of a dramatically UV response compared to Polη, indicates that the role of JNK in regulating TLS may be general. Specifically, it is of interest to determine whether BPDE-induced Polη redistribution (which is orders of magnitude greater than that induced by UV) and whether sensitivity to BPDE is also influenced by JNK. The interaction domains for Rad18 and Polκ are yet to be determined. These results suggest that S409 may reside in a putative Rad18 binding domain on Polκ.

Regarding the generality of JNK-mediated Rad18 phosphorylation, there is a strong precedent for phosphorylation-dependent regulation of E3 ubiquitin ligases. For example, sustained (but not transient) JNK signaling mediates tumor necrosis factor α–induced cell death via the E3 ligase Itch, which degrades the caspase inhibitor cFLIPL.[206] Another E3 ligase, Siah2, is regulated by p38-dependent phosphorylation.[207] Because Rad18 plays a central role in TLS, and perhaps additional DNA repair pathways, Rad18 phosphorylation has the potential to regulate multiple effector pathways, perhaps by influencing associations with its substrates (e.g., PCNA, RFC2, 53BP) or regulatory binding partners (e.g., Polη, Rad6, Rad5). Rad18 phosphorylation may serve as a molecular switch for determining various physiological outcomes including but not limited to TLS.

Post-translational modification of TLS enzymes has been reported to impact TLS activation in several different ways. Aside from the obvious monoubiquitination of PCNA that coordinates stabilization of TLS polymerases in place of replicative polymerases, ubiquitination of Polη inhibits interaction of Polη with PCNA, thus regulating its ability to perform TLS.[208] Polη has also been shown to be modified by

phosphorylation in a manner that regulates its recruitment to stalled replication forks.[209] Additionally, the ubiquitin conjugating enzyme Rad6 is phosphorylated by the cyclin-dependent kinase CDK2, suggesting that Rad6 activity and potentially its affinity to known binding partners (e.g. Rad18) may be coordinated with cell cycle progression.[210] Taken together, the results reports here and those of previous studies indicate that post-translational modification of TLS enzymes, in particular via phosphorylation, serves as a molecular signal to coordinate the activation of TLS with DNA damage sensing (RPA, Chk1), cell cycle progression (Cdc7 and CDK2), and potentially apoptosis (JNK).

CHAPTER 5

ONCOGENIC SIGNALING DRIVES MISREGULATION OF TLS

1. Introduction

The interplay between oncogenes, DNA repair pathways, genetic stability, and tumorigenesis is poorly understood. Activation of the DNA damage response appears to function as a protective barrier to the progression of malignant transformation.[129, 211] Expression of oncogenes induces abnormal replication patterns, including re-replication and aberrant origin firing, which causes replication stress that activates various arms of the DNA damage response. Replication stresses that lead to DNA breaks pose a great threat to genome integrity; activation of checkpoints in response to oncogene-induced replication stress can be considered a mechanism of protecting against proliferation of oncogene-expressing cells.[212, 213] This notion is supported by the observation that DSBs in precancerous lesions activate p53 and other checkpoint proteins, such as Chk2 and ATM, whereas cancerous lesions harbor inactivated or compromised DNA damage checkpoints, often due to mutation in p53 and loss of expression of other checkpoint proteins.[129, 214]

Various arms of the DNA damage response are implicated as protective barriers against oncogene-induced replication stress, but the role of TLS has never been considered in this context. Many lines of evidence suggest that TLS may play both protective and harmful roles in the response to oncogene-induced replication stress. Putative protective roles for TLS involve permitting normal replication to continue in the wake of replication stress, thus avoiding persistence of stalled

replication forks and subsequent progression to DNA breakage. Deleterious roles involve over-activation of error-prone TLS that leads to mutagenesis, a concept that is consistent with the mutator phenotype of malignant cells.

Various lines of evidence link error-prone replication to a mutator phenotype in cancer.[215] For example, activating mutations in the low-fidelity base excision repair enzyme Polβ have been associated with mutagenesis in various cancers.[216, 217] Even a mild over-production (4-7 fold) of Polβ causes up to an 8-fold increase in mutation rate.[126] Upregulation of the Y-family polymerase Polκ confers a similar mutator phenotype.[218] Considering that both Polη and Polι are substantially more mutagenic than Polβ and Polκ, it is reasonable to hypothesize that their overexpression may also confer a similar, if not more dramatic, mutator phenotype (**Table 5-1**). Importantly, studies in cultured human fibroblasts have found that the mutation rate is not elevated by Polη overexpression; however, these cells contain intact checkpoints that help protect against the mutagenic potential of overexpressed Polη.[219] Because pre-malignant cells and especially oncogene-expressing cells lack intact DNA damage and cell cycle checkpoints, misregulation and/or overexpression of TLS polymerases is likely to induce a mutator phenotype in the context of oncogene expression.

Table 5-1. Properties of select eukaryotic polymerases.[126]

Polymerase	Substitution error rate(X10-5)	Normal function
α	9.6	Replication
δ	<1.3	Replication
ε	<0.2	Replication
β	67	BER
ζ	110	TLS
η	3500	TLS
ι	7200	TLS
κ	580	TLS

The studies described in this section are intended to address the hypothesis that TLS plays a role in the tolerance of replication stress induced by oncogenic signaling. Specifically, the studies are intended to test how the expression and activation of TLS enzymes are affected by oncogenic signaling, how TLS contributes to survival and genome integrity in the presence of oncogenic signaling, and how loss of TLS impacts models of tumorigenesis *in vivo*.

2. Results

2.1. TLS is activated differently by different oncogenes

To test the hypothesis that TLS is activated in the context of oncogenic signaling, we studied the effect of expressing a panel of oncogenes in a non-transformed human diploid fibroblast cell line (**Table 5-2 and Figure 5-1**). Each of these oncogenes is reported to induce replication stress or activate a DNA damage response.[220-222] Additionally, we overexpressed the replication licensing factor Cdt1 and depleted cells of geminin. During the G1 phase of the cell cycle, DNA synthesis begins after the MCM complex is loaded to origins of replication by Cdt1;

overexpression of Cdt1 causes aberrant MCM loading and subsequent re-licensing of origins and re-replication,[203] which lead to replication stress that promotes genome instability and tumorigenesis in a manner similar to oncogenes.[223, 224] Geminin physically binds to Cdt1 and blocks its interaction with MCMs,[225] thus counteracting the activity of Cdt1 to inhibit MCM loading and origin licensing. Gemenin depletion thus phenocopies Cdt1 overexpression to result in replication stress, such as re-replication.[223]

Table 5-2. Properties of oncogenes and replication proteins considered in this study.

Gene	Mechanism of action	Cellular Functions	Effect of overexpression
Myc	Transcription factor	Metabolism, protein biosynthesis, cell cycle regulation, cell adhesion	Replication stalling, re-replication, ROS[226]
Ras	GTPase, signal transduction via MAPK	Cell polarity, proliferation, Differentiation, adhesion, migration, and apoptosis	Replication stress, re-replication[227]
CycE	Cdk2 binding and activation, S-phase entry	Cell cycle progression	Chromosome instability[228]
Cdt1	MCM loading	Replication licensing, cell cycle	Re-replication
Geminin	Inhibition of Cdt1	Replication licensing, cell cycle	Re-replication (due to depletion)

Unexpectedly, markers of TLS activation were vastly different after expression of these oncogenes or alteration of Cdt1 and geminin expression. As shown in **Figure 5-1**, Myc and Cdt1, which are reported to activate checkpoints via several mechanisms, including re-replication and replication stalling,[229, 230] induced negligible Chk1 phosphorylation and PCNA monoubiquitination compared to UV-treated cells. Conversely, Cyclin E induced a massive PCNA monoubiquitination and moderate Chk1 phosphorylation, whereas Ras induced massive Chk1

phosphorylation, consistent with published reports,[220] but only moderate PCNA monoubiquitination. Interestingly, depletion of the replication licensing protein geminin, caused Chk1 activation but failed to cause PCNA monoubiquitination.

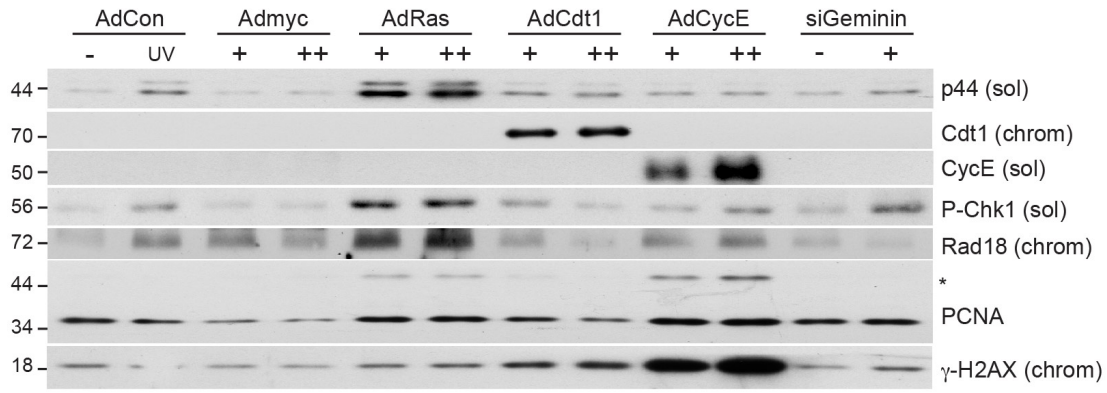


Figure 5-1. Expression of different oncogenes affects TLS differently.
HDF cells were infected with empty-control adenovirus or low (+, 5.0EE9 vir/ml) and high (++, 1.0EE10 vir/ml) doses of different oncogenes and lysed 48 hours later.

These results indicate that activation of checkpoints and TLS are not always coordinated in the context of oncogenic signaling and that expression of oncogenes or manipulation of licensing proteins activate checkpoints and TLS very differently.

2.2. Cyclin E and Ras activate TLS

We next asked how effector proteins in the checkpoint and TLS pathways impact the cellular response to replication stress induced by oncogenic signaling. The results in **Figure 5-1** indicated that Cyclin E and Ras cause the greatest activation of TLS as demonstrated by PCNA monoubiquitination, whereas Myc had a relatively negligible effect on PCNA monoubiquitination. To test how TLS and checkpoints coordinate a response to these oncogenes, we depleted HDF cells of Rad18 or Chk1 using siRNA (or treated cells with non-targettng siRNA for control) and then

expressed Myc, Ras, or Cycln E using adenovirus. Consistent with **Figure 5-1**, expression of CycE and Ras induced more monubiquitination of PCNA than Myc, and this effect was entirely eliminiated after depletion of Rad18, showing that oncogene-induced PCNA monoubiquitination was mediated by Rad18 (**Figure 5-2**). Depletion of Rad18 greatly potentiated the oncogene-induced phosphorylation of Chk1, especially that induced by Ras. Conversely, depletion of Chk1 had virtually no effect on PCNA monoubiquitination induced by Myc and Ras but greatly attenuated Cyclin E-induced PCNA monoubiquitination. Interestingly, expression of Myc induced an approximately ten-fold inrcease in binding of NBS1/p95 to chromatin, in a manner that was largely independent of Rad18 or Chk1, indicating that Myc-induced replication stress activates NBS1-dependent stress tolerance pathways, such as those induced by double-strand breaks.

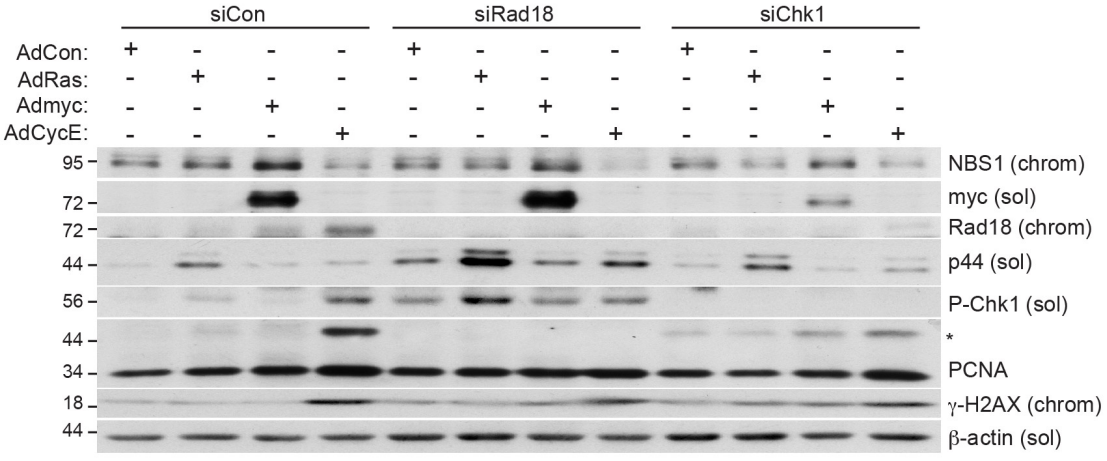


Figure 5-2. Impact of TLS and checkpoints in the response to different oncogenes.
HDF cells were treated with non-targeting control siRNA, or siRNA against Rad18 or Chk1 and then infected with adenovirus expressing the indicated oncogenes followed by lysing and fractionation 48 hours after infection with the oncogene viruses.

These results show that expression of Cyclin E and Ras, but not Myc, potentially activate TLS via PCNA monoubiquitination, a process dependent upon the recruitment of Rad18 to stalled replication. We thus tested whether expression of these oncogenes would result in Rad18 redistribution to nuclear foci. Consistent with the effect on PCNA monoubiquitination shown in **Figure 5-1** and **5-2**, Ras- and Cyclin E-expressing cells exhibited increased CFP-Rad18 redistribution to nuclear foci, whereas Myc had a slightly inhibitory effect on Rad18 redistribution (**Figure 5-3, A & B**). Recruitment of Polη to stalled replication forks is stimulated by Rad18-mediated PCNA monoubiquitination and represents activation of TLS. As shown in **Figure 5-3**, Ras and Cyclin E induced a three- and seven-fold increase in Polη nuclear foci formation, indicating that these oncogenes indeed lead to activation of TLS.

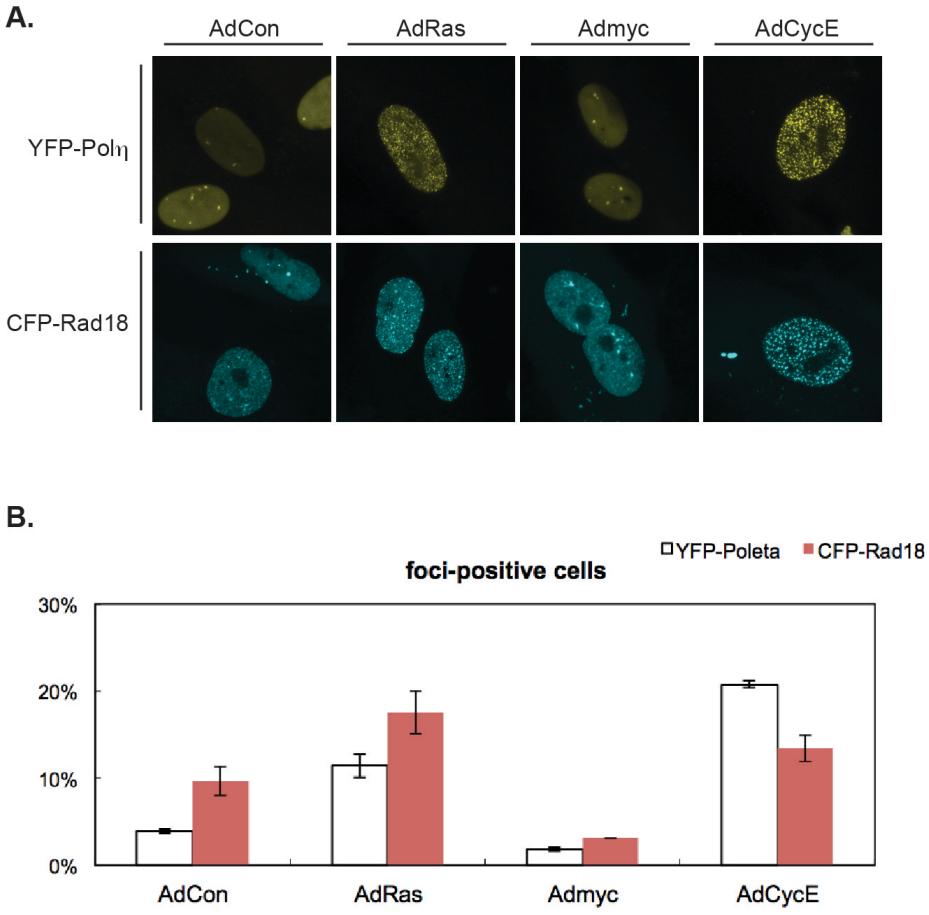


Figure 5-3. Oncogene-induced redistribution of Ra18 and Polη to nuclear foci.
A. HDF cells were treated adenovirus expressing CFP-Rad18 or YFP-Polη followed by infection with empty-vector control, Ras, myc, or Cyclin E adenovirus and imaged live 48 hours later. B. Quantification of foci-positive cells as a fraction of total chromophore-expressing cells.

2.3. TLS and Checkpoints drive tolerance of oncogenic stress

To test whether the oncogene-induced activation of Rad18 and Polη shown in **Figures 5-1 - 5-3** contributes to tolerance of oncogene-induced replication stress, we measured the survival of oncogene-expressing HDF cells after depletion of Rad18 and Polη. As shown in **Figure 5-4**, depletion of Rad18 and Polη decreased the

survival of Cyclin E and Ras-expressing cells by approximately 20 and 15%, respectively, but had no effect on the survival of Myc-expressing cells. Depletion of Chk1 only impacted the survival of Ras-expressing cells. These results suggest that the markers of TLS activation demonstrated via PCNA monoubiquitination and Rad18/Polη redistribution to nuclear foci are indeed functionally significant to help facilitate survival in oncogene-expressing cells.

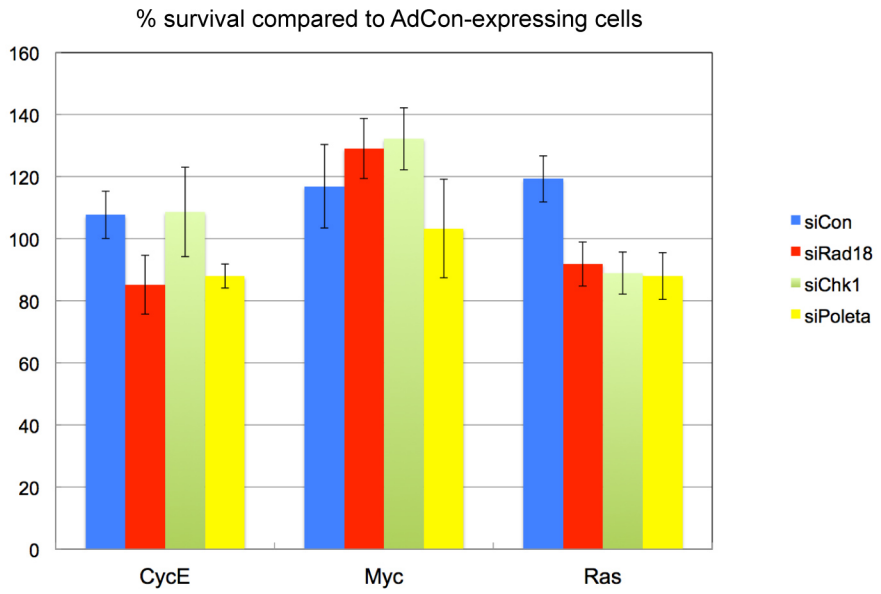


Figure 5-4. Percent survival of oncogene-expressing cells.
A. HDF cells were treated with non-targeting control siRNA or siRNA against Rad18, Chk1, or Poleta and then treated with empty vector control adenovirus or adenovirus expressing Cyclin E, Myc, or Ras. Percent survival compared to AdCon-expressing cells was measured 48 hours later by MTT.

2.4. RNR-induced tumors and Rad18 expression *in vivo*

The results shown in **Figure 5-1 – 5-4** show that Ras and Cyclin E expression leads to activation of checkpoints and TLS enzymes and that this activation is important for survival in ongogene-expressing cells. We next asked whether

malignant cells demonstrate activation of TLS *in vivo* using a mouse model in which lung tumors are induced by expression of ribonucleotide reductase (RNR).[231] RNR expression selectively causes tumor growth in lung tissue and induces reactive oxygen species that causes replication stress. We found that Rad18 protein expression was markedly increased in all RNR-induced tumors compared to normal lung, indicating that malignant cells *in vivo* up-regulate Rad18 expression, perhaps to use the TLS pathway to facilitate tolerance of replication stress (**Figure 5-5**).

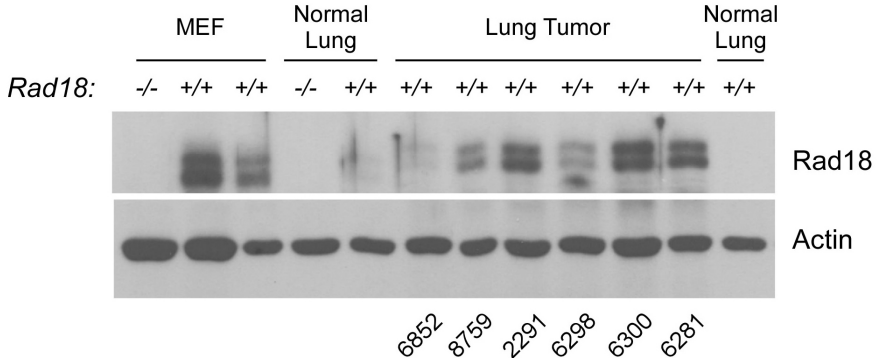


Figure 5-5. Rad18 expression is increased in RNR-induced lung tumors.

2.5. Rad18 modulates Ras-driven tumorigenesis

Figure 5-1 through **5-5** show that expression of oncogenes such as Ras affect expression and activity of key mediators of TLS, namely Rad18, both in cultured cells and *in vivo*. We next asked whether TLS impacts Ras-dependent tumorigenesis *in vivo* using wildtype or Rad18$^{-/-}$ mice. After lung-specific expression of K-Ras, all mice developed a large number of small lung tumors (<1mm diameter), but Rad18$^{-/-}$ mice also developed massive lung tumors (>3mm diameter) that were not present in

wildtype mice (**Figure 5-6A, B)**. Surprisingly, in spite of the stark contrast in lung tumor histopathology, the wildtype and Rad18$^{-/-}$ mice demonstrated no difference in overall survival (**Figure 5-6C**). These results indicate that Rad18 status dramatically impacts the development and growth of Ras-induced lung tumors *in vivo*, through the mechanism behind this phenotype and its implication remains unclear.

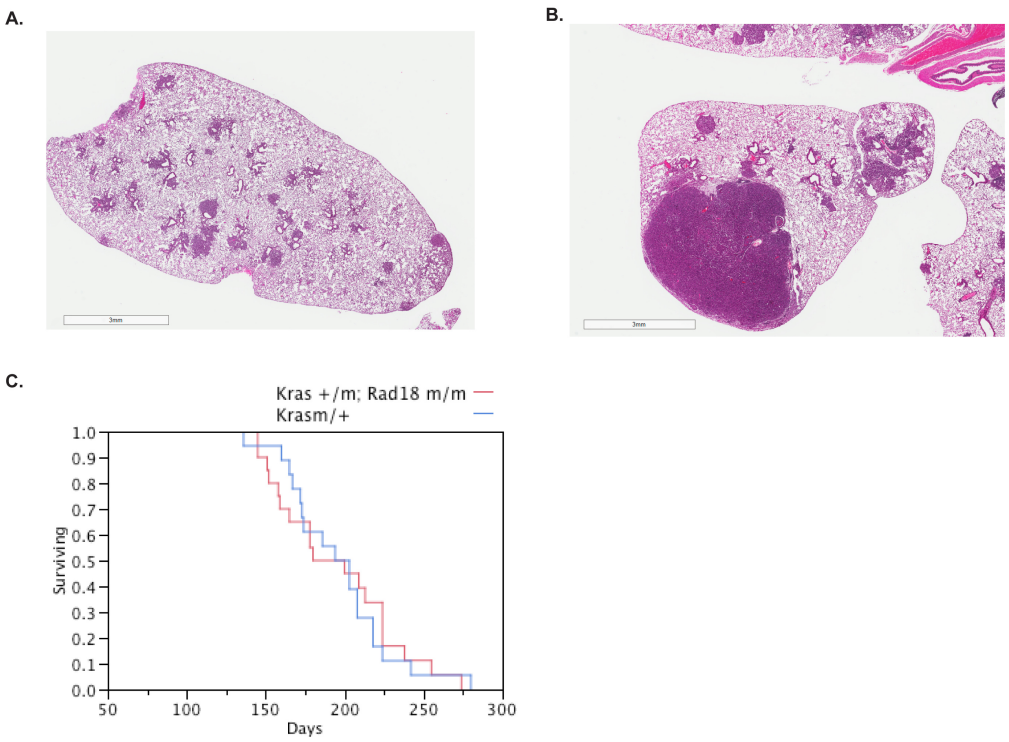


Figure 5-6. Rad18 status impacts Ras-induced tumorigenesis but not survival. Representative histopathological image lung tissue from Ras-expressing A. wildtype mouse and B. Rad18$^{-/-}$ mouse. C. Survival curves of wildtype and Rad18-/- mice after development of Ras-induced lung tumors.

3. Discussion

Previous reports have demonstrated that expression of oncogenes induces replication stress that activates a DNA damage response and checkpoints.[212, 229]

However, the relationship between oncogenic signaling and TLS has never been explored. Several lines of evidence led us to hypothesize that TLS is activated under conditions of oncogenic signaling. First, oncogene-transformed cells accumulate mutations at a higher rate than normal cells[121] and point mutations are introduced in large part by error-prone TLS polymerases. Next, checkpoint signaling has a stimulatory effect on TLS,[80] and checkpoints are activated by many oncogenes.[131] Finally, TLS is utilized by cells to tolerate abnormal DNA structures and replication stress,[232] and expression of certain oncogenes, such as Cyclin E, induces aberrant replication structures that results in DNA damage response.[130]

The results presented above demonstrate a novel link between oncogenic signaling and TLS. We show that expression of certain oncogenes robustly activates TLS, and that checkpoint signaling may contribute to this activation. Our finding that different oncogenes activate checkpoints and TLS differently demonstrates that the mechanisms by which cells tolerate oncogene-induced replication stress differs between oncogenes (**Figure 5-1**). The affect of Ras, Myc, and Cyclin E expression on markers of DNA damage and TLS activation are listed in **Table 5-3**. Whereas Myc- and Ras-induced PCNA monoubiquitination appears to be checkpoint-independent, Cyclin E-induced PCNA monoubiquitination is clearly Chk1-dependent (compare PCNA-Ub in **Figure 5-2**, lanes 4 and 12). Conversely, Rad18 depletion has no effect on Cyc E-induced Chk1 phosphorylation (compare P-Chk1 in **Figure 5-2**, lanes 4 and 8), but Cyc E-induced PCNA monoubiquitination is greatly attenuated after Chk1 depletion. These results suggest that Cyc E-induced TLS activation takes place downstream of Chk1. Because Chk1 is most active during S-phase, a strong Chk1-

dependence of Cyc C-induced PCNA monoubiquitination is consistent with the role of Cyclin E in promoting S-phase entry and cell cycle progression.

Table 5-3. Oncogene expression and markers of checkpoint and TLS pathways.

Oncogene	Chk1 phosphorylation	g-H2AX	PCNA monoubiquitination
Cyclin E	↑↑	↑	↑↑↑
Ras	↑↑	–	↑
Myc	–	–	–

The dramatic redistribution of NBS1 to chromatin after Myc expression, which is attenuated after Rad18 depletion, suggests that Myc induces double strand breaks of which Rad18 contributes to tolerance (**Figure 5-2**). Similarly, the massive increase in Ras-induced Chk1-phosphorylation after depletion of Rad18 indicates that Rad18 contributes to tolerance of Ras-mediated replications stress. The Chk1-independence of Ras-induced PCNA monoubiquitination is consistent with cell cycle-independent mechanisms of replication stress that are induced by Ras.

The finding that Rad18 and Polη are necessary for cellular tolerance of expression of oncogenes demonstrates a physiological significance of oncogene-induced TLS activation. The finding that loss of Rad18 and Polη compromises survival of Ras and Cyclin E-expressing cells but not Myc cells is consistent with the PCNA monoubiquitination and Rad18/Polη redistribution that are induced by Ras and Cyclin E but not Myc (**Figures 5-2** and **5-3**). Taken together, these data show that the TLS pathway is activated in response to different transforming oncogenes, that the

mechanism of TLS activation differs among oncogenes, and that TLS contributes to tolerance of oncogenic expression, namely Ras and Cyclin E.

Our *in vivo* data provide further physiological significance to the relationship between TLS and oncogenic signaling. Our finding that Rad18 expression is increased in RNR-induced lung tumors suggests that Rad18 plays a role in the progression of RNR-induced malignant transformation or the tolerance of RNR-induced replication stress (**Figure 5-3**). A dramatic increase in the size of Ras-induced tumors in $Rad18^{-/-}$ mice compared to wildtype mice suggests that Rad18 protects against overproliferation of Ras-transformed cells, though the mechanism for this phenomenon remains unknown (**Figure 5-4**).

Together, these results demonstrate a novel role for TLS in the tolerance of oncogene-induced replication stress. Our findings describe a model in which expression of certain oncogenes (Ras and Cyclin E) leads to checkpoint activation and subsequent stimulation of Rad18-mediated PCNA monoubiquitination, which recruits TLS polymerases to mediate tolerance of oncogene-induced replication stress (**Figure 5-7**). This TLS activation promotes survival of oncogene-expressing cells, which carries therapeutic implications for potentially inhibiting the TLS pathway to decrease survival of oncogene-expressing cells. Rad18 expression clearly impacts the development and proliferation of malignantly transformed cells, such as RNR-induced and Ras-induced lung tumors, but the mechanisms through which Rad18 impacts tolerance of replication stress in these malignantly transformed cells requires further study.

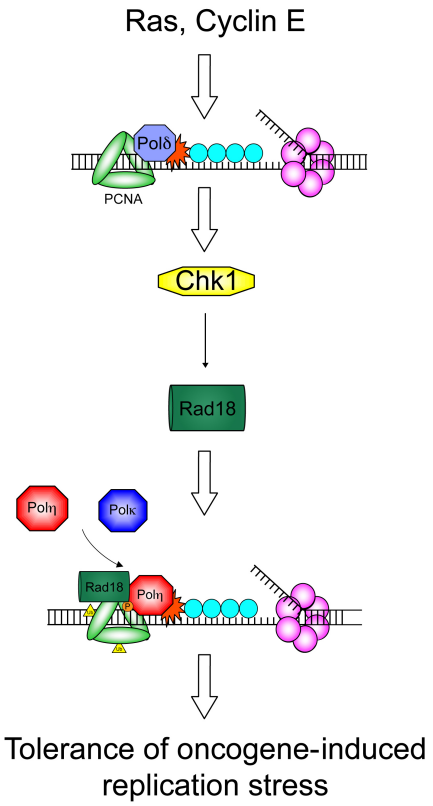


Figure 5-7. TLS promotes tolerance of oncogene-induced replication stress. **Expression oncogenes, such as Ras and Cyclin E, activates checkpoints and stimulates Rad18-mediated PCNA monoubiquitination, both of which stimulate TLS at sites of replication stress and promote cellular survival.**

CHAPTER 6

FUTURE DIRECTIONS AND CONCLUSIONS

1. Future Directions

1.1. Mutagenesis in the context of catalytically inactive Polη

The results of Chapter 3 show that Polη drives Rad18-medited PCNA monoubiquitination via physical bridging of Rad18 and PCNA (**Figure 3-3**), a process that is entirely independent of its catalytic activity (**Figure 3-4**). Importantly, we found that expression of a catalytically-inactive mutant of Polη in XPV cells drives the recruitment of inappropriate polymerases, Polι and Polκ to stalled replication forks both basally and in response to UV damage (**Figure 3-5**). This important finding suggests that inappropriate, error-prone TLS of CPD or of undamaged DNA by Polι and Polκ will caused increased mutagenesis in cells expressing catalytically inactive Polη (**Figure 6-1**), consistent with the finding that Polι accounts for a large percentage of error-prone TLS in Polη-deficient XPV cells.

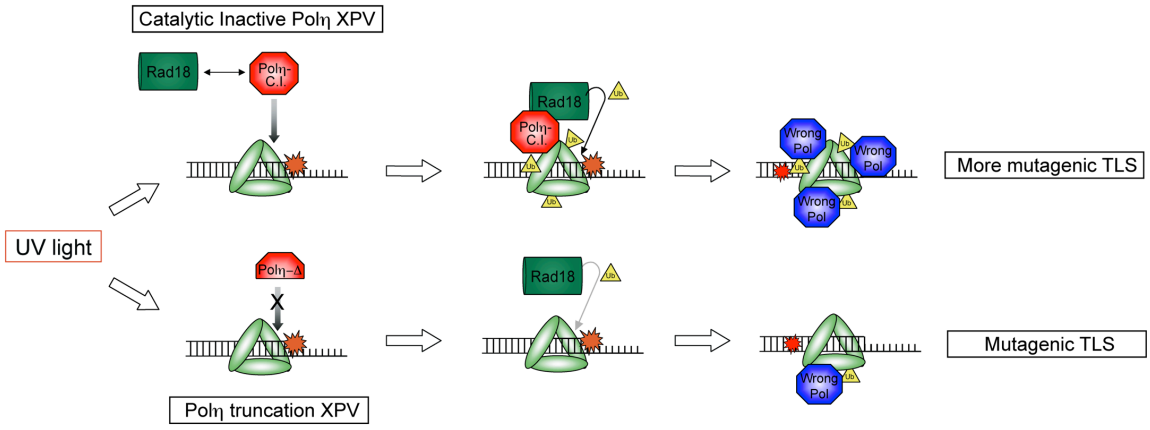


Figure 6-1. Hypothesized increased mutagenesis in the context of catalytically inactive Polη. In XPV cells harboring a truncated Polη, residual but limited monoubiquitination of PCNA leads to limited recruitment of error-prone polymerases (bottom). But in XPV cells expressing catalytically inactive, full-length Polη, stimulation of Rad18-mediated PCNA monoubiquitination in the absence of Polη-mediated lesion bypass leads to excessive recruitment of error-prone polymerases (top).

It has been shown in MEFs[169] and in yeast[168] that complementation of Polη-defeicient cells with catalytically inactive Polη leads to a higher mutation rate than in uncomplemented cells, but this experiement has not been demonstrated in human cells. Importantly, many XPV patients express full-length, catalytically inactive Polη;[171] one may thus hypothesize that mutagenesis in their cells would be worse than in complete Polη null cells.

These experiments should be performed in two ways. In the first, one may use gapped plasmid SupF shuttle vector assays that are based on SV40 T antigen-dependent replication of the SupF plasmid in human cells.[233, 234] Briefly, XPV cells that are transfected with emptry vector control, wildtype Polη, or catalytically inactive Polη are co-transfected with pSP189 plasmid that has been UV irradiated. The pSP189 is then recovered from the cells 48 hours later, digested with Dpn1 to remove unreplicated plasmid, and transformed into an indicator bacterial strain (MBM7070) containing an amber mutation in the β-galactosidase gene, and the transformed bacteria plated on agar containing isopropyl-1-thio-b-D-galactopyranoside (IPTG) and X-gal. The mutation frequency is determined as the ratio of white (mutant) to total (blue & white) colonies that form.

Next, determination of mutation rate may be performed using whole exome sequencing on the Illumina platform. Briefly, this approach involves UV-irradiating

XPV cells that have been transfected with empty vector control plasmid, wildtype Polη, or catalytically inactive Polη and then recovering DNA from these cells using standard phenol/chloroform extraction techniques. The DNA is then sonicated, labeled via PCRwith barcode-tagged primers, enriched for exome DNA by PCR, and sequenced on the Illumina platform. This method is preferable to the SupF assay as it measures mutational events on nuclear DNA, rather than cytosolic plasmids, and is thus a better representation of actual processes at chromatin within cells.

We hypothesize that catalytically inactive Polη-expressing XPV cells will show a significantly higher mutation frequency than control-transfected XPV cells. These experiments will determine whether the mutagenic model shown in Figure 5-3 is valid and will demonstrate the phenotypic significance of the polymerase-independent function of Polη illustrated in Chapter 2.

1.2. Contribution of TLS and Oncogene-induced Mutagenesis

As outlined in the introduction and in Chapter 5, mutations play a fundamental role in the development and progression of cancer. Activating mutations in oncogenes or inactivating mutations in tumor suppressor genes can lead to initiation of carcinogenesis, and malignant cells exhibit subsantially higher rates of mutations[121] and gene amplification[122] than normal cells. This evidence suggests that malignant cells exhibit defective genome maintenance progresses, and, importantly, that cellular pathways that contribute to mutagenesis may be upregulated or mis-regulated in cancer cells.

Considering the role that TLS plays in the introduction of point mutations, it is therefore plausible to hypothesize that TLS may be activated or mis-regulated in the context of oncogenic signaling (**Figure 6-2**). This was the rationale for the experiments described in Chapter 5, and the results shown therein are consistent with such a hypothesis. However, those results show that certain oncogenes contribute to TLS activation and not that such oncogene-induced activation of TLS actually drives mutagenesis. This future aim is based on the hypothesis that mutagenesis in the context of oncogenic signaling is due to the activity of TLS. It will use the same techniques described in the previous section, except that mutagenesis will be measured in the context of oncogene expression in the presence or absence of key TLS enzymes.

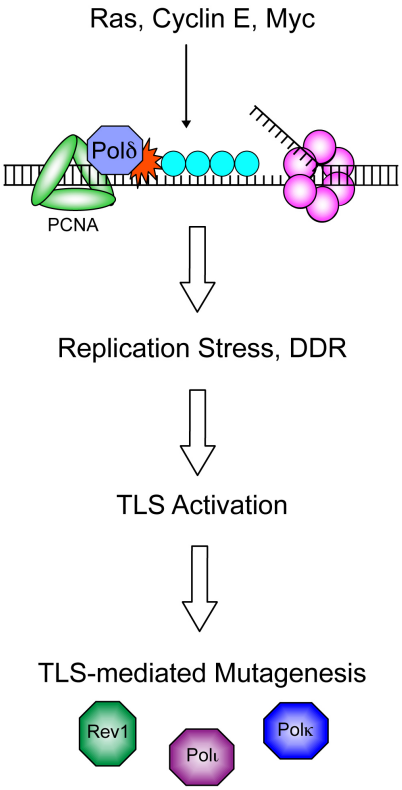


Figure 6-2. Hypothetical models of oncogene-induced activation of TLS that results in mutagenesis.

Two main experimental approaches should be explored. The first involves using HDF cells that have been depleted of Rad18 or Polη, which are then infected with oncogene-expressing adenovirus, as outlined in Chapter 5. 48 hours post-infection, the cells are harvested for DNA and subjected to Illumina sequencing. Mutation rates in cells with and without TLS enzymes are compared. The second approach involved repeating the same experiment in XPV cells that have been reconstittued with Polη or empty vector control before infection with oncogenes. Should either of these two approaches demonstrate a role for TLS in oncogene-induced mutagensis, additional manipuilations of specific TLS polymerases, including knockdown of other TLS polymerases, may help elucidate the roles played by each polymerase in the introduction of mutations. That is, these experiments will determine which polymerase is primarily responsible for introducing point mutations, and if the selection of polymerase differs for different oncogenes.

Together, these experiments will not only reveal whether the activation of TLS reported in Chapter 5 indeed contributes to error-prone replicaiton in the context of oncogenic signaling, but they will also shed light on the mechanisms of mutagenesis induced by oncogenic signaling and how this contributes to initiation of malignancy.

1.3. Damage-specificity of TLS Pol recruitment to DNA damage sites

Replication by error-prone TLS Polymerases carries a high risk for introducing mutations and is thus a crucial step in carcinogenesis after exposure to environmental genotoxins. Because each TLS Pol performs accurate bypass for a specific lesion (ie Polη for UV CPDs and Polκ for BPDE adducts), utilization of the appropriate polymerase is an important means of preventing mutagenic TLS. However, it is not known what mechanisms are responsible for TLS Pol selection, nuclear translocation, and engagement with the replication machinery.

While Rad18 has a dual role in Polη recruitment (chaperone and PCNA-Ub ligase), mechanisms of recruitment for the other TLS Pols (Polι, Polκ and Rev1) are poorly understood. For example, it is unknown whether Rad18 has chaperone activity for TLS Pol members other than Polη. We have devised two hypothetical models describing our hypotheses of TLS Pol selection for TLS **(Figure 6-3)**: (A) Rad18-mediated chaperoning promotes recruitment of all TLS polymerases; (B) Rad18-mediated chaperoning (induced by Cdc7 phosphorylation of Rad18) specifically affects Rad18-Polη recruitment. Future studies may distinguish between these putative mechanisms and determine the effect of the eliciting genotoxin on TLS Pol selection.

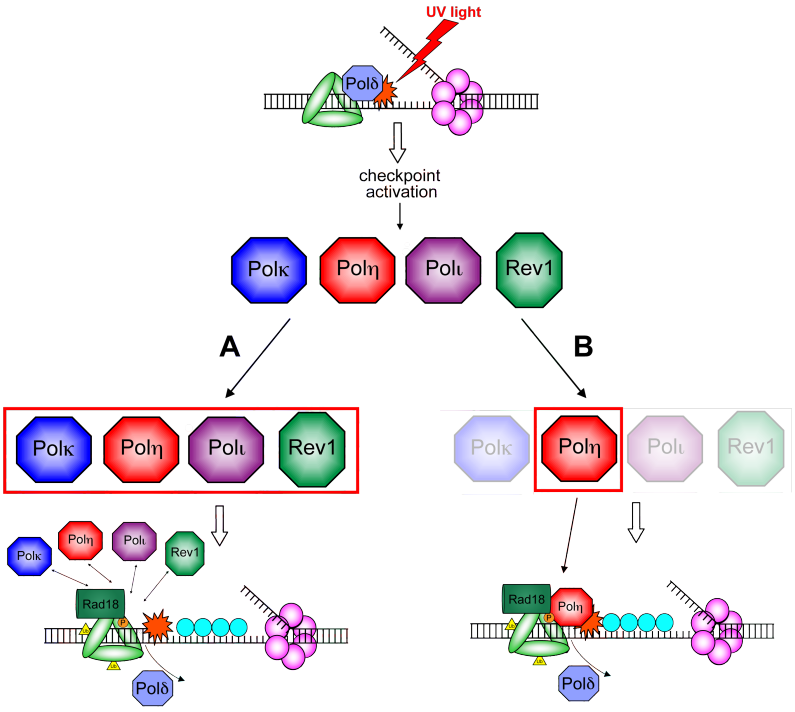


Figure 6-3. Hypothetical models of damage-responsive Y Polymerase selection. Cdc7-mediated Rad18 phosphorylation promotes Rad18 interactions with all TLS polymerases equally (A) or specifically with Polh (B).

Specific experiments to address the question of TLS Pol selection should first focus on the kinetics of TLS Pol recruitment to sites of DNA damage for different genotoxins. Based on the lesion-specific accuracy of Polη and Polκ for bypassing UV- and BPDE-induced DNA lesions, respectively, it is logical to hypothesize that cells utilize regulated rather than random mechanisms of polymerase selection. Specifically, we hypothesize that Polη is recruited to replication forks and engages with PCNA preferentially in response to UV-induced damage and that Polκ is recruited and engaged preferentially in response to BPDE-induced damage. This hypothesis is best tested by a combination of immunoprecipitation of each respective TLS Polymerase and PCNA at increasing timepoints after DNA damage and

immunofluorescent microscopy of chromophore-tagged polymerases at similar timepoints. These experiments will help determine how the TLS Pols are recruited to stalled replication forks and what mechanisms regulate this process.

Next one should determine whether Cdc7 regulates recruitment of Polκ to stalled replication forks and its subsequent bypass of BPDE-induced lesions. UV damage-induced phosphorylation of Rad18 by Cdc7 facilitates binding of Rad18 and Polη, an interaction we hypothesize is necessary for bypass of CPDs (the lesion caused by UV). Rad18 also binds to Polκ in a similar damage-responsive manner, due to BPDE rather than UV, and perturbation of Rad18-Polκ binding (by Rad18 or Polκ depletion) impairs recovery from the BPDE-induced S-phase checkpoint.[141,235] But the impact of Cdc7 signaling on Rad18 and Polκ interactions has never been investigated. Based on the role of Cdc7 in facilitating tolerance of UV-induced CPDs by Polη, it is logical to hypothesize that Cdc7 mediates BPDE-responsive association between Rad18 and Polκ and loss of Cdc7 will impair bypass of BPDE DNA adducts. Such a hypothesis may be tested by first determining whether BPDE-inducible Polκ and Rad18 binding is Cdc7-dependent via co-immunoprecipitation of Rad18 and Polκ after depletion of Cdc7. Measurement of Polκ recruitment to stalled replication forks after Cdc7 depletion by immunofluorescence and analysis of recovery from the BPDE-induced S-phase checkpoint in the presence or absence of Cdc7 will reveal the phenotypic impact of putative Cdc7-dependent Rad18-Polκ interactions. The latter should be performed via measurement of the BPDE sensitivity of Cdc7-depleted cells treated with increasing doses of BPDE and via ^{3}H-thymidine incorporation assays. Together, these experiments will determine whether Cdc7 regulates Rad18-Polκ

binding and Polκ-mediated bypass of BPDE-induced DNA damage. Combined with the kinetic assays above, these studies will help determine the mechanisms that dictate selection of the proper TLS Polymerase for the appropriate DNA lesion.

1.4. Endogenous Impediments to Normal Replication

In addition to the DNA adducts caused by common environmental genotoxins, many naturally occurring aberrant DNA structures also interfere with normal DNA replication.[236] One such replication-blocking structure is G4 (or G-quadruplex) DNA, which naturally develops in guanine-rich stretches of DNA. In G4 DNA, four guanines are arranged in a hydrogen bond-stabilized ring around a mono-valent cation (**Figure 6-4**).[237] These guanine rings are planar and stack on top of each other to form extremely stable structures involving one to four strands of DNA; most endogenous G4 structures in cells are single-stranded.[238] Due in part to the high propensity of many natural sequences in the human genome to form G-quadruplex DNA (estimated at over 300,000 sequences),[239] G4 DNA has been implicated in many biological functions, such as transcriptional regulation and telomere maintenance and protection.[237, 240] Notably, G4 structures have been identified in several important oncogene promoters, such as c-Myc,[241] KRAS[242], and c-KIT,[243] and the presence of G4 structures in telomeres has been known for over two decades.[244,245]

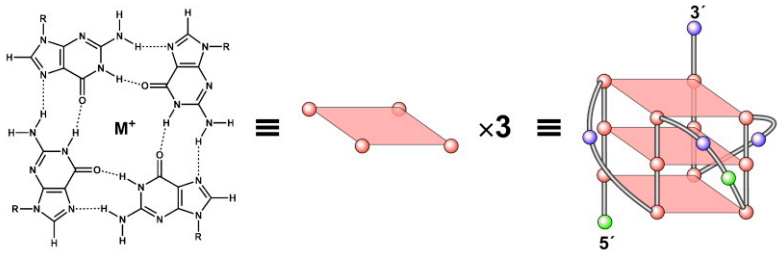


Figure 6-4. Schematic of G4 DNA.
A planar quartet of guanines surrounding a cation (usually potassium) and the stacking of these quartets to form a 3-dimensional G-quadruplex structure. (image modified from Qin, 2008).

Importantly, because normal and G4 DNA differ so dramatically in both structure and stability, most DNA metabolizing enzymes are incapable of properly processing G4 DNA.[237] Few specialized enzymes that can process G4 DNA have been identified, and defective G4-processing by specialized helicases capable of unwinding G4 DNA has been targeted as potential contributing factors in chromosomal instability syndromes, such as Werner Syndrome (WRN protein),[246] Bloom's Syndrome (BLM protein),[247] and Fanconi Anemia (FancJ protein).[248] The consistent presence of G4 DNA in telomeres and the observation that loss of the G4-unwinding helicase activity of WRN protein leads to elevated homologous recombination specifically at telomeres[249] has lead to the hypothesis that improper processing of G4 DNA may contribute genetic instability.[250,251]

It is thus of great importance to understand the mechanisms utilized by cells to tolerate G4 DNA, yet it is largely unknown how G4 structures are processed during important functions such as DNA replication and transcription. It has been shown that TLS polymerase-depleted human cells are sensitized to agents that stabilize G4 DNA and that G4 DNA is a natural substrate of Polη and Polκ.[232,252] However, the impact

of important players in the TLS pathway, such as Rad18, PCNA, and Chk1, has not been studied in this context. Does Polη and Polκ activity in processing G4 structures depend on Rad18? Does PCNA ubiquitination help facilitate tolerance of G4 DNA? Does loss of TLS enzymes, such as Rad18, Polη, and Polκ, sensitize cells to killing by G4-stabilizing compounds?

Preliminary studies have shown that cells treated with the G4 stabilizing substances, such as telomestatin, exhibit prolonged Chk1 phosphorylation and PCNA monoubiquitination, suggesting a role for checkpoints and TLS in tolerance of G4 DNA. Future studies should test the hypothesis that mis-regulated TLS contributes to genomic instability due to improper bypass/processing of G4 DNA, in particular at telomeres. These experiments may compare how the same damage tolerance mechanism (TLS) is activated differently in response to environmental (UV, BPDE) or endogenous (G4) obstacles to replication. For instance, as shown in Chapter 4, TLS activation depends on checkpoint signaling; is this the same for G4-targeted activation of TLS? Experimentally, does depletion of Chk1 reduce telomestatin-induced redistribution of TLS polymerases? Such studies will shed light on the mechanisms used by cells to direct damage tolerance by TLS toward endogenous or environmentally induced obstacles to replication.

Specifically, it is of interest to test the hypothesis that Rad18 confers bypass of G-quadrupled DNA. Thousands of stretches of genomic DNA are capable of adopting G4 conformation, including promoters in clinically relevant oncogenes and in telomeres.[250] Although helicases capable of unwinding G4 DNA have been

identified, it is unknown how cells maintain normal replication after encountering G4 structures. Recent work implicates Polη and Polκ in processing G4 DNA.[232] Because of the known functions of Rad18 in facilitating the activity of Polη and Polκ, we hypothesize that Rad18 is also involved (**Figure 6-5**). Telomestatin is a natural product first isolated in 2001[253] that has been shown to intercalate between the planar guanine quartets to stabilize G-quadruplex DNA with an extremely high specificity and potency.[254] We thus hypothesize that cells depleted of Rad18 will be sensitized to killing by G4-stabilizing agents such as telomestatin.

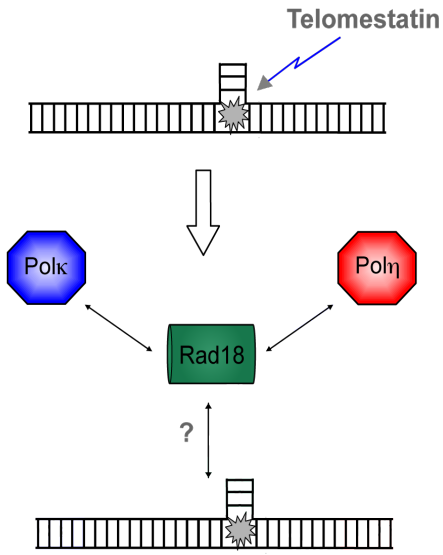


Figure 6-5. Potential function of Rad18 in facilitating Polη and Polκ-mediated processing of G4 DNA.

Next, based on the novel non-catalytic function of Polη demonstrated in Chapter 2 that demonstrates a role for Polη in stimulating Rad18-mediated PCNA monoubiquitination, we would hypothesize that Polη impacts the activity of Rad18 at G4 DNA. Experimentally, this hypothesis is tested by measuring whether

telomestatin-induced chromatin association of Rad18 is compromised in Polh depleted cells.

Together, these studies will determine whether Rad18 mediates tolerance of G4 DNA, thus defining a novel mechanism through which TLS mediates tolerance of aberrant DNA structures.

2. Conclusions

The results presented in this dissertation define new regulatory aspects of TLS that were previously unknown. First, Chapter 3 demonstrates that Polη, previously recognized as a Translesion DNA polymerase with one sole effector function, that of a polymerase, in fact performs an additional function that is entirely independent of is catalytic activity. This function serves to facilitate physical interacts between Rad18 and its ubiquitination target, PCNA, and thus stimulate TLS via PCNA monoubiquitination. Identification of this catalytic-independent function of Polη has important implications for the molecular basis on which mutagenesis is thought to develop in XPV cells, as well as any cell in which Polη function or expression may be altered. Additionally, because so many other DNA damage tolerance pathways depend on PCNA monoubiquitination (eg template switching, instrastrand cross-link repair, Fanconi Anemia), Polη expression is likely to impact numerous other aspects of the cellular DNA damage response.

Next, Chapter 4 demonstrates a novel role for checkpoint signaling that directly contributes to the regulation of TLS via promoting interaction between

Rad18 and Polη. This finding builds on the Polη-induced monoubiquitination of PCNA demonstrated in Chapter 3, such that loss of Chk1 signaling abrogates Rad18-Polη binding and in turn compromises PCNA monoubiquitination. Additionally, the results shown in Chapter 4 reveal a novel link between stress kinase signaling and TLS, such that JNK-mediated phosphorylation of Rad18 contributes to UV-induced PCNA monoubiquitination and TLS activation. Together, the results shown in this chapter demonstrate cross-talk between different DNA damage response pathways and illustrate how cells coordinate various mechanisms to promote genetic stability.

Finally, Chapter 5 presents data that reveal a novel relationship between TLS and oncogenic signaling. Oncogenes have been known to cause replication stress, activate a DNA damage response, and eventually induce cellular senescence. However, the mechanisms by which cells tolerate such oncogenic stresses have remained poorly understood. Our findings reveal that TLS contributes to cellular tolerance of oncogene-induced replication stress. Not only does expression of certain oncogenes lead to a robust activation of TLS, as indicated by PCNA monoubiquitination and mobilization of Rad18 and Polη, but loss of TLS effector proteins Rad18 and Polη compromises the survival of cells expressing oncogenes. We therefore conclude that TLS proves a cellular mechanism of tolerating oncogene-induced replication stress. This conclusion is further supported by the findings that lung neoplasms express abnormally high levels of Rad18 and that loss of Rad18 dramatically changes the growth and progression of Ras-induced lung tumors in vivo.

Altogether, it is hoped that the findings presented in this dissertation will help address some of the remaining unknowns in the field of genome maintenance and tumorigenesis. Specifically, this dissertation shows that Polη contributes to genome stability via polymerase-independent functions and that TLS contributes to tolerance of oncogene-induced replication stress. These novel findings help explain how TLS contributes to genome maintenance and may help guide the development of antiproliferative therapies. For example, consistent with the survival results shown in **Figure 5-4**, can inhibition of TLS reduce the survival of malignant cells *in vivo*? Or, consistent with the role for catalytically-dead Polη in promoting mutagenesis (Chapter 3), would XPV patients harboring full-length, catalytically inactive Polη have a better prognosis if the Rad18-stimulatory function of their mutant Polη were blocked? The author hopes that these and other questions stemming from this research will advance our understanding of molecular carcinogenesis and eventually translate to better treatment for and survival of people afflicted with cancer.

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