The Cellular Roles of the Translesion Polymerases Eta, REV1 and Zeta in Bypass and Repair of DNA Damage Induced by Anti-Cancer Agents

by

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List of Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BRCT	BRCA1 C-Terminal domain
DSB	DNA double strand break
FA	Fanconi anemia
FANC	Fanconi anemia complementation group
γ-H2AX	Phosphorylated histone variant 2AX
HR	Homologous recombination
ICL	Interstrand cross-link
IR	Ionizing radiation
MMC	Mitomycin C
NHEJ	Non-homologous end-joining
PAD	Polymerase association domain
PCNA	Proliferating Cell Nuclear Antigen
POLH	Gene encoding polymerase eta
Polη	Polymerase eta
Polζ	Polymerase zeta
REV3	Catalytic subunit of polymerase zeta
REV7/MAD2B/MAD2L2	Accessory subunit of polymerase zeta
RPA	Replicating protein antigen
TLS	Translesion DNA synthesis
UBM	Ubiquitin-binding motif
UBZ	Ubiquitin-binding zinc finger
UV	Ultraviolet radiation
XP-V	Xeroderma pigmentosum varient

Abstract

Translesion DNA synthesis (TLS) is a process whereby specialized DNA polymerases are recruited to bypass DNA lesions that would otherwise stall high-fidelity polymerases. TLS may be a mechanism by which cancer cells resist the cytotoxic actions of chemotherapeutic agents that damage DNA. Here, I addressed if the translesion polymerases eta, REV1 and zeta have a cellular role in protecting against cisplatin-induced cytotoxicity. HeLa cells depleted of polymerase eta, REV1 or polymerase zeta individually were found to display phenotypes suggestive of the inability to bypass cisplatin adducts. In addition, the E3 ubiquitin ligase RAD18 and the Fanconi anemia core complex were found to be crucial for the localization of TLS polymerases to sites of DNA replication stalled by cisplatin adducts. Together, the findings support a model where replicative bypass of cisplatin intrastrand cross-links requires cooperation of multiple translesion polymerases in human cells, and is regulated by both RAD18-dependent PCNA monoubiquitination and the Fanconi anemia core complex.

Recently, translesion DNA polymerases have been implicated in the repair of DNA damage induced by chemotherapeutic agents. I addressed if the translesion polymerases eta, REV1 and zeta were essential for efficient repair of DNA interstrand cross-links induced by mitomycin C and DNA double strand breaks (DSBs) caused by exposure to ionizing radiation (IR). Depletion of REV1 or polymerase zeta in HeLa cells, but not polymerase eta or the RAD18 ubiquitin ligase, resulted in a loss in clonogenic survival following exposure to both mitomycin C and IR. I demonstrated that the loss in survival following mitomycin treatment is likely due to inefficient repair of interstrand cross-links in cells depleted of REV1 or polymerase zeta by measuring the resolution of DSBs, an intermediate of interstrand cross-link repair. Furthermore, I showed that REV1 and polymerase zeta were required for the timely repair of DSBs induced by ionizing radiation. REV1 and polymerase zeta depleted HeLa cells exhibited a relatively mild but significant defect in homologous recombination, an essential DNA

repair pathway important for resolving DSBs. However, the lack of translesion synthesis past damaged DNA bases in REV1 or polymerase zeta deficient cells exposed to ionizing radiation may also explain the loss in survival and apparent deficiency to resolve DSBs. Overall, the results suggest both REV1 and polymerase zeta play an active role in protection against mitomycin C or ionizing radiation-induced cytotoxicity, most likely by directly repairing DNA damage caused by these agents.

CHAPTER 1

Introduction

Efficient and accurate replication of the eukaryotic genome is necessary for exact transmission of genetic information from one generation to the next therefore preventing genomic instability. Replicative polymerases are capable of copying DNA with high fidelity and speed due in part to their stringent active site enforcing correct Watson-Crick base pairing. Any mismatching of nucleotides that does occur by replicative polymerases may be corrected by an intrinsic 3′-5′ exonuclease proofreading function, thus greatly increasing the fidelity of replicative polymerases [1-2]. Due to their low error rates, replicative polymerases are commonly referred to as high fidelity polymerases. The more constrained active site of replicative polymerases is clearly advantageous during DNA replication, but there are certain situations where this property can be deleterious to a cell. One such situation occurs when a replication fork encounters DNA damage. DNA is constantly undergoing endogenous and exogenous damage, which may lead to abasic sites, DNA cross-links, and large bulky adducts covalently attached to DNA bases. Sophisticated DNA repair mechanisms have evolved to correct the damage, however the process can be slow and incomplete [3-4]. If a high fidelity polymerase comes upon damaged DNA, such as bulky adducts or distorted DNA helical structures due to intrastrand cross-links, the stringent active site will not accommodate the damaged template. The end result is a stalled replication fork with long regions of single stranded DNA generated due to the uncoupling of the helicase and polymerase [5-6]. Eventually the stalled replication fork will collapse producing a DNA double strand break, which is one of the most, if not the most, toxic DNA lesions [7-8]. In order to prevent replication stress due to stalled replication forks at sites of damaged DNA, translesion DNA polymerases are employed to synthesize past the sites of DNA damage [9-11].

Translesion DNA polymerases are specialized polymerases that are capable of replicating damaged template DNA, a process referred to as translesion DNA synthesis [12]. Currently eight translesion DNA polymerases are known to exist in humans. Based on homology, the eight human translesion polymerases have been placed into four different families of DNA polymerases. Four of the human translesion polymerases, polymerase eta (pol η), polymerase íota (pol í), polymerase kappa (pol k) and REV1, belong to the Y-Family of DNA polymerases. Polymerase zeta (pol ζ), which consists of the catalytic subunit REV3 and an accessory subunit REV7, is categorized in the B-Family of DNA polymerases, while the translesion polymerase theta (pol θ) belongs to the A-Family of DNA polymerases. The two remaining translesion polymerases, polymerase lamda (pol λ) and polymerase mu (pol μ), are grouped in the X-Family of DNA polymerases of which limited information is known about their cellular functions. There is another polymerase found in the A-Family of DNA polymerases, polymerase nu, which displays similar biological properties to translesion polymerases [13]. However, too little is currently known about polymerase nu to officially declare this polymerase a translesion polymerase. Four homologues of the translesion polymerases known to exist in humans are also found in yeast including Rad30 (pol η), Rev1, Rev3 (pol ζ) and pol4 (pol λ).

Although nine human gene products are recognized to be translesion DNA polymerases, the vast majority of scientific data published thus far on eukaryotic translesion DNA synthesis has focused on the Y-Family polymerases eta and REV1 along with the B-Family polymerase zeta. One reason as to why we have a greater understanding of polymerase eta, REV1 and polymerase zeta is due to the fact homologs exist in *Saccharomyces cerevisiae*, one of the most extensively studied eukaryotes. Polymerase eta is the most widely studied translesion polymerase due to the fact mutations in this polymerase were found to be responsible for the genetic disease Xeroderma pigmentosum variant (XP-V) [6, 14-15]. In addition, many scientific studies have focused upon the translesion polymerases eta, REV1 and zeta because they are currently thought to be the dominate polymerases in terms of translesion synthesis past damaged DNA bases [6].

The other human translesion polymerases discovered to date are thought to have a limited role in translesion synthesis. Polymerases íota and kappa have been demonstrated to participate in translesion DNA synthesis, but their contributions are thought to be restricted and/or redundant [16-17]. Polymerase íota is speculated to have a cellular role in response to oxidative stress, while polymerase kappa may be necessary for bypass of intrastrand cross-links formed between adjacent N^2 linked guanine bases and possibly extension beyond mismatched primer termini [17-22]. A deficiency in either polymerase íota or kappa, though, does not greatly effect spontaneous or damage induce mutagenesis [18, 23]. One explanation for this observation is that both of these polymerases may function as partial replacements for polymerase eta and/or polymerase zeta [24-25]. The X-Family translesion polymerases along with the A-Family translesion polymerase theta are not thought to play a major role in preventing replication stress by synthesizing past damaged DNA bases, but rather may contribute to non-homologous end joining, base excision repair and interstrand cross-link repair [6, 26-28]. It should be emphasized, though, that their impact on non-homologous end joining, base excision repair and interstrand cross-link repair is still poorly understood. As further research is performed in the area of translesion DNA synthesis, it is reasonable to assume more specific biological functions will be assigned to the translesion polymerases íota, kappa, lamda, theta and mu. However, for the purposes of my dissertation I will be focusing on the translesion polymerases currently demonstrated to have the most significant biological roles in eukaryotic translesion synthesis, polymerases eta, REV1 and zeta. Figure 1.1 summarizes the proposed functions of polymerase eta, REV1 and polymerase zeta.

Gene Name	Protein Name	Proposed Functions
POLH	Eta (η)	Insertion of nucleotides directly opposite DNA lesions Extension of D-loops during homologous recombination Somatic hypermutations
REV1L	REV1	Facilitates polymerase switching Somatic hypermutations Bypass of abasic sites Repair of DNA interstrand cross-links Repair of DNA double strand breaks
REV3L	Zeta (ζ)	Catalytic subunit of polymerase zeta Extension beyond DNA damaged bases and mismatched primer termini Repair of DNA interstrand cross-links Repair of DNA double strand breaks *REV3*$^{-/-}$ embryonic lethal in mice
REV7/ MAD2L2/ MAD2B	Zeta (ζ)	Accessory subunit of polymerase zeta Extension beyond DNA damaged bases and mismatched primer termini Repair of DNA interstrand cross-links Repair of DNA double strand breaks Mitotic checkpoint

Figure 1.1 Proposed functions of polymerase eta, REV1 and polymerase zeta

Translesion DNA polymerases are capable of synthesizing damaged template DNA due to possessing a more spacious active site than replicative polymerases [29-30]. All known DNA polymerases contain a catalytic core comprising of subdomains referred to as the thumb, palm and finger [31]. The domains act essentially as a hand allowing the polymerase to grip both the template and the incoming nucleotide. The finger domain of high fidelity polymerases plays a crucial role in ensuring correct Watson-Crick base pairing by tightly gripping the DNA [32-35]. Translesion polymerases, however, have key differences in their structure. First, translesion polymerases have shorter thumb and finger domains resulting in fewer contacts with DNA [23, 36-37]. Second, all translesion polymerases belonging to the Y-Family have an additional subdomain called either the little finger or polymerase-association domain (PAD) [29, 38-40]. In the Y-Family of

translesion polymerases, the little finger and palm domains have a critical role in base pairing during synthesis. Unlike replicative polymerases, the little figure and palm domains in Y-Family translesion polymerases preform a more open and solvent-exposed active site before substrate binding allowing for the accommodation of a damaged template [22, 30]. REV1 appears to be unique among the Y-Family of translesion polymerases in that the protein itself provides specificity for the template and incoming nucleotide and not the DNA [41-42]. The catalytic domain structure of REV3 has yet to be solved, so the exact mechanism of how this polymerase synthesizes damaged template DNA is unknown [43-44]. The general principle that translesion polymerases are able to accommodate damaged template DNA due to having a more open and solvent exposed active site than compared to high fidelity polymerases should still apply to REV3.

There is a trade off, though, for the unique ability of translesion polymerases to accommodate damaged templates. Due to their less stringent active site, translesion polymerases do not enforce correct Watson-Crick base pairing, thus leading to erroneous base pairing and mutagenesis [30, 45]. High fidelity polymerases make very few mistakes during replication, inserting one incorrect nucleotide for every 10^6-10^8 bases replicated, which is due to both their stringent active site and proofreading capability [23]. Translesion polymerases, on the other hand, have a much higher error rate when synthesizing undamaged DNA. Error rates as high as 1 mismatch per 10 bases to 1 mismatch per 10,000 bases replicated have been observed with translesion polymerases [23]. Conversely, translesion polymerases may have higher fidelity rates when replicating a damaged template [6, 46-47]. Any mistakes made by translesion polymerases cannot be corrected during synthesis since they lack an intrinsic 3′-5′ proofreading function [48-49]. Overall, the number of mismatched nucleotides produced by translesion polymerases is thought to be several orders of magnitude greater than high fidelity polymerases [11, 23, 47, 50-51]. The observed increase in mutagenesis, or the lack thereof, is often used as an experimental endpoint to determine if a translesion polymerase may be involved in a particular biological pathway. Since translesion polymerases have such a high error rate when compared to replicative polymerases, they are often referred to as low fidelity polymerases. Even though it may seem unwise to risk an increase of mutagenesis during

translesion synthesis, the alternative, enhanced cell death due to collapsed replication forks, is less desirable.

The ability to synthesize damaged template DNA, thus preventing replication stress, enables a cell to cope with various types of DNA damage. In most circumstances, prevention of collapsed replication forks and ensuing cell death is, evolutionarily speaking, advantageous and allows organisms to survive and reproduce in harsh environmental conditions. Nonetheless, all DNA damage is not inherently bad and in particular situations, such as the treatment of cancer, the damage is purposely induced to cause cell death. The bypassing of chemotherapy associated DNA damage may prevent individual cell death initially, but eventually could result in the demise of the organism. Many of the most successful anti-cancer agents currently used in clinic have a mechanism of action resulting in damaged DNA. Examples of chemotherapeutic agents that induce DNA damage consist of the alkylating drugs such as cyclophosphamide and ifosfamide, the nitrosoureas including carmustine, and methylating anti-cancer compounds such as dacarbazine.

Currently, the extent to which the translesion polymerases promote bypass of DNA damage caused by anti-cancer drugs in intact cancer cells is unknown. To address this issue, I will focus on the platinum compound cisplatin (cis-diammineplatinum(II) dichloride) to determine if the translesion polymerases eta, REV1 and zeta allow for tolerance of platinum adducts. The major DNA lesion induced by cisplatin is an intrastrand cross-link between the N^7 position of two adjacent guanines, accounting for approximately 60-65% of all cross-links produced by cisplatin [52]. Intrastrand cross-links can also be produced between neighboring adenine-guanine bases and guanine bases separated by a cytosine. Overall the dominating lesions are intrastrand cross-links with only about 2% of the DNA damage representing monofunctional guanine adducts and another 2% being interstrand cross-links between guanines on opposite DNA strands [52]. In general, the interstrand cross-link is by far the most physiologically relevant lesion when considering the toxicity induced by anti-cancer agents that produce both intra and interstrand cross-links [53]. However, both DNA intrastrand and interstrand cross-links have been proposed to contribute to the cytotoxicity of cisplatin [54]. The most abundant

intrastrand cross-link induced by cisplatin, 1,2-d(GpG), is thought to be a major contributor to cisplatin cytotoxicity. The toxic nature of 1,2-d(GpG) cross-links may be attributed to the DNA distortions caused by this particular intrastrand cross-link [55].

Translesion polymerases were originally recognized for their ability to replicate past DNA lesions in an error prone manner leading to mutagenesis. A point should be made that in this particular biological process the DNA damage is not corrected, rather the bypass of the lesion allows the cell to cope with the unrepaired lesion until the damage is removed at a later time. Recently, published reports provided evidence to suggest the translesion polymerases eta, REV1 and zeta may also have an active role in repair of DNA damage, notably repair of DNA interstrand cross-links and double strand breaks [56-61]. Specifically, translesion polymerases may be necessary for gap filling during interstrand cross-link repair and extension of D-loops during homologous recombination. Translesion polymerases continue to function by synthesizing damaged or altered templates during repair of the DNA lesion, but the major difference is that their replicating ability is a necessary step during the correction of the DNA damage. As part of my dissertation I will test the hypothesis that the translesion polymerases eta, REV1 and zeta have a role in repair of both DNA interstrand cross-links and double strand breaks in intact human cancer cells, which theoretically could contribute to resistance of chemotherapeutic agents known to induce interstrand cross-links or double strand breaks.

Multiple chemotherapeutic agents used to treat cancer have a mechanism of action that results in the formation of DNA interstrand cross-links. Many of the intrastrand cross-link inducers such as mitomycin C, chlorambucil, carmustine, cisplatin and psoralen also form interstrand cross-links. To address the role of polymerases eta, REV1 and zeta in interstrand cross-link repair I will use mitomycin C as the primary agent for inducing interstrand cross-links. Exposure to Mitomycin C results in a significantly greater percentage of interstrand cross-links when compared to drugs such as cisplatin [52, 62-63]. Interstrand cross-links are formed at the N^2 position of guanines at dCpG sequences in the minor groove following mitomycin C exposure [64-65]. The monoadducts formed by mitomycin C are also located in the minor groove, but they do not perturb the DNA structure and are thought to have little influence upon mitomycin C-

induced cytotoxicity [65]. However, just one mitomycin C interstrand cross-link is capable of killing a bacterial cell if not repaired efficiently [66].

DNA double strand breaks are generated by several anti-cancer agents including the topoisomerase I and II poisons such as camptothecin and etoposide respectively, bleomycin and ionizing radiation. To test the hypothesis that the translesion polymerases eta, REV1 and zeta are necessary for repair of DNA double strand breaks I will use ionizing radiation as the principal inducer of DNA damage. Following exposure to ionizing radiation multiple DNA lesions are formed including DNA adducts such as 7,8-dihydro-8-oxoguanine, abasic sites, DNA single strand breaks and DNA double strand breaks [67]. The proportion of DNA double strand breaks are relativity small when compared to other types of damage induced by ionizing radiation. The ratio of single strand DNA breaks formed by ionizing radiation to double strand breaks formed is approximately 1:0.04 [68]. However, the formation of DNA double strand breaks are by far more toxic to the cell, and the other lesions formed such as DNA adducts and single strand breaks are thought to be repaired relatively quickly and have little if any influence on cell killing due to ionizing radiation [67-69].

In summary, the overall goal of this dissertation is to test the hypothesis that the translesion polymerases eta, REV1 and zeta have a biological role in the bypass of chemotherapeutic associated DNA damage in intact human cancer cells. I will also assess the contribution of the translesion polymerases eta, REV1 and zeta to the repair of interstrand cross-links and DNA double strand breaks induced by anti-cancer agents.

Y-Family translesion DNA polymerases Eta and REV1

In the early years of mutagenesis research there was no clear understanding as to why mutations arose in bacteria and yeast following exposure to DNA damaging agents such as ultraviolet (UV) radiation. In fact, up until the mid to late 1960's scientists were unsure if DNA damage-induced mutations observed in bacteria and yeast occurred naturally or if a gene product played an active role in creating mutations. By the late 1960's a hypothesis was proposed suggesting that mutagenesis was an active process due

to errors in the repair of UV-induced DNA damage, with errors during post-replication repair responsible for the majority of mutagenic events [70-72]. Gene products in both *Escherichia coli* and *Saccharomyces cerevisiae* were found to contribute to the mutagenesis observed following DNA damage. Mutations in either the *Umu* (UV-induced mutability) locus in *Escherichia coli* or the *Rev1* (UV- mutation reversion) locus in *Saccharomyces cerevisiae* resulted in decreased mutation rates following DNA damaging agents [73-76]. The *Rev1* gene product in *Saccharomyces cerevisiae* was later found to have 25% homology with the *UmuC* gene product of *Escherichia coli* suggesting this active process of mutagenesis was conserved through evolution [77]. Additional orthologs were discovered in *Escherichia coli* (DinB), the archeon *Sulfolobus sulfatarirus* (Dbh) and in *Saccharomyces cerevisiae* (Rad30) leading to the identity of a superfamily of proteins responsible for mutations following DNA damage referred to as the UmuC/DinB/Rev1/Rad30 superfamily [78-80]. However, the mechanistic actions of how the UmuC/DinB/Rev1/Rad30 superfamily of proteins induced mutations were still unknown. At the time an hypothesis was formed that the UmuC/DinB/Rev1/Rad30 superfamily of proteins functioned as accessory factors to replicative polymerases in an undefined cellular process allowing for low fidelity synthesis of damaged DNA templates and the emergence of mutagenesis [22].

Two major events occurred in the mid to late 1990's unlocking the mystery as to how the UmuC/DinB/Rev1/Rad30 superfamily of proteins are able to influence mutagenesis following DNA damage. In 1995 it was discovered that purified *Saccharomyces cerevisiae* Rev1 protein had a deoxycytidyl transferase activity, which is the transfer of a dCMP to DNA from a dCTP, and that Rev1 may have a biological role in the bypassing of abasic sites [81-82]. The revelation that Rev1 had a catalytic function was exciting, but due to the observed limited catalytic activity it was still unclear as to exactly how the UmuC/DinB/Rev1/Rad30 superfamily contributed to mutagenesis. Then in 1999 a major discovery was made that forever changed how the scientific community viewed the UmuC/DinB/Rev1/Rad30 superfamily. Johnson *et al.* provided evidence that the yeast Rad30 protein was a DNA polymerase capable of using all four dNTPs and was demonstrated to bypass UV-induced thymine-thymine cyclobutane pyrimidine dimers [46]. The human homolog of Rad30, polymerase eta, was discovered shortly thereafter [83]. The

Escherichia coli UmuD´$_2$C and DinB proteins were demonstrated to be DNA polymerases in 1999 as well and renamed DNA pol IV and pol V respectively [84-85]. By the end of 2000 two other human translesion polymerases were discovered, the DNA translesion polymerases ίota and kappa [86-91]. In fact, translesion polymerases were being described so quickly in the literature in such a short time frame that confusion arose about naming the newly discovered polymerases leading to polymerase kappa being referred to as both kappa and theta [92]. In order to effectively and efficiently communicate about the newly discovered superfamily of mutagenic proteins, the UmuC/DinB/Rev1/Rad30 superfamily of proteins were later grouped together into the Y-Family of translesion polymerases [93]. In retrospect, the time from early 1999 to late 2000 was a major turning point in both identifying human translesion polymerases and understanding the cellular functions of these polymerases.

The translesion DNA polymerase eta (polη), encoded by the *POLH* gene located on chromosome 6p21.1, was the first translesion polymerase identified in humans [83, 94]. The initial biological function described for polymerase eta was its ability to accurately bypass thymine-thymine cyclobutane pyrimidine dimers induced by UV radiation [46, 83, 95]. In other words, polymerase eta always places two adenines across from thymine-thymine dimers caused by UV radiation. Mutations in polymerase eta were found to be responsible for the genetic disease Xeroderma pigmentosum variant (XP-V), which is clinically described as extreme photosensitivity to UV light and the predisposition to develop cancers, especially of the skin, at an early onset [6, 14-15]. Unlike Xeroderma pigmentosum, cells from Xeroderma pigmentosum variant patients have normal nucleotide excision repair activity, but are unable to efficiently bypass UV-induced DNA damage. Currently it is unclear as to why Xeroderma pigmentosum variant patients develop cancer at an early onset. One theory is that another, more error prone translesion polymerase, such as polymerase ίota, replicates past UV-induced DNA damage in the absence of polymerase eta resulting in an increase of mutations [96]. Another hypothesis is that UV-induced DNA damage is not bypassed in the absence of polymerase eta leading to replication fork collapse and subsequent repair of the double strand breaks formed with a noted increase in sister chromatid exchanges [97-99]. The resulting escalation of genomic instability could significantly impact cancer development.

In addition to replicating past UV-induced cyclobutane pyrimidine dimers, polymerase eta is also capable of inserting nucleotides across from other types of damaged DNA bases. Polymerase eta has been demonstrated to replicate past the oxidative lesion 7,8-dihydro-8-oxoguanine in a mostly error free manner via insertion of a cytosine opposite the DNA lesion [48, 100-102]. Similar to the oxidative lesion 7,8-dihydro-8-oxoguanine, Yuan *et al.* found polymerase eta correctly incorporates a cytosine across from an acetylaminofluorene modified guanine [48]. Polymerase eta is capable of bypassing other spontaneous, oxidative, or metabolically induced DNA lesions, though with low fidelity, including O^6-methylguanine, benzo[a]pyrene guanine adducts, 7,8-diol 9,10-epoxide deoxyguanosine, thymine glycol, and the *R* diastereoisomer of cyclodeoxyadenosine [6, 25, 48, 103-105]. Accumulating data suggest polymerase eta is fairly promiscuous in terms of what lesions are bypassed; clearly demonstrating that polymerase eta may have an extremely important biological role in preventing replication stress due to numerous types of endogenous and exogenous DNA damage. Nevertheless, polymerase eta is unable to effectively place nucleotides across from all types of DNA damage, such as abasic sites, suggesting polymerase eta alone is not sufficient for the prevention of all stalled replication forks.

Since polymerase eta can facilitate the bypass of various DNA adducts, there is potential that this polymerase may be capable of synthesizing across chemotherapeutic drug-induced DNA lesions and thus contribute to resistance of anti-cancer drugs. Both Vaisman *et al.* and Alt *et al.* have provided biochemical evidence that polymerase eta is capable of synthesizing past cisplatin induced N^7-guanine-guanine intrastrand cross-links [106-107]. Studies employing cell lines obtained from Xeroderma pigmentosum variant patients found that cells deficient in polymerase eta are sensitive to cisplatin and accumulate more mutations following cisplatin exposure than cells with functional polymerase eta, suggesting that polymerase eta is capable of synthesizing past cisplatin-induced DNA intrastrand cross-links. Furthermore, the observed phenotypes of cisplatin hypersensitivity and enhanced genomic instability in cells derived from Xeroderma pigmentosum variant patients can be rescued by transient expression of polymerase eta [108-109]. Recently, using a quantitative translesion synthesis reporter assay, Schachar *et al.* found polymerase eta bypasses cisplatin guanine-guanine intrastrand cross-links in an

established human cancer cell line [25]. In this investigation, Schachar was able to measure polymerase eta gap filling a plasmid engineered to contain a single-stranded gap region with a cisplatin guanine-guanine intrastrand cross-link placed within the gapped region.

Polymerase eta may have additional biological functions beyond bypassing damaged template DNA such as stabilizing fragile sites during replication, diversification of antibodies and repair of DNA double strand breaks [110]. There is an emerging body of evidence that polymerase eta promotes somatic hypermutations at the Ig locus during immunoglobulin class switch recombination [111-114]. Somatic mutations may arise when activation-induced cytidine deaminase (AID) generates uracils in the DNA creating a uracil-guanine mismatch [112, 115-117]. Although poorly understood, correction of the uracil-guanine mismatch through a process involving polymerase eta and the mismatch repair proteins MSH2 and MSH6 creates mutations at nearby adenine-thymine base pairs [118]. Polymerase eta has also been proposed to function in the homologous recombination repair pathway. Specifically, McIlwraith *et al.* proposed that polymerase eta interacts with the recombinase RAD51, which in turn stimulates polymerase eta-mediated D-loop extension [119]. However, the finding that polymerase eta is necessary for the extension of D-loops during homologous recombination has not been confirmed. I will assess the participation of polymerase eta in homologous recombination.

The cellular functions of polymerase eta are in part regulated by protein-protein interactions. Along with a catalytic site, polymerase eta contains a REV1 interacting region, an ubiquitin-binding zinc finger domain, a PCNA interacting peptide region and a nucleus localization domain (Fig. 1.2) [22-23, 120]. Replication stress initiated by UV exposure results in polymerase eta foci formation presumably at sites of stalled replication forks [121-123]. The ability to form foci in response to DNA damage is critically important since polymerase eta mutants deficient in foci formation cannot complement Xeroderma pigmentosum variant cells [121]. Polymerase eta localizes to sites of replication by interactions with PCNA, proliferating cell nuclear antigen, via its PCNA interacting peptide (PIP) box motif [124-125]. In response to stalled replication forks PCNA is known to be monoubiquitinated by the E3 ubiquitin ligase RAD18. The monoubiquitination of PCNA is thought to increase the affinity of polymerase eta to PCNA through its

ubiquitin-binding zinc finger domain [126]. There is some controversy as to how important the ubiquitin-binding zinc finger domain is for proper foci formation of polymerase eta. Published reports from Acharya *et al.* propose that PCNA monoubiquitination is not necessary for foci formation while reports from both Bienko *et al.* and Sabbioneda *et al.* suggest that the ubiquitin-binding zinc finger domain of polymerase eta is necessary for proper foci formation following DNA damage [125-127]. The REV1 interacting region of polymerase eta may also contribute to foci formation by encouraging localization with REV1, which might act as a scaffold at sites of replication stress [128-129]. This scenario is doubtful, though, since polymerase eta is speculated to localize to sites of replication stress independently of REV1 [130-131]. The more likely role for the REV1 interaction region is to promote polymerase switching between polymerase eta and other translesion polymerases such as polymerase zeta. The p53 and p21 pathway or phosphorylation following DNA damage may also regulate the cellular processes of polymerase eta [132-133].

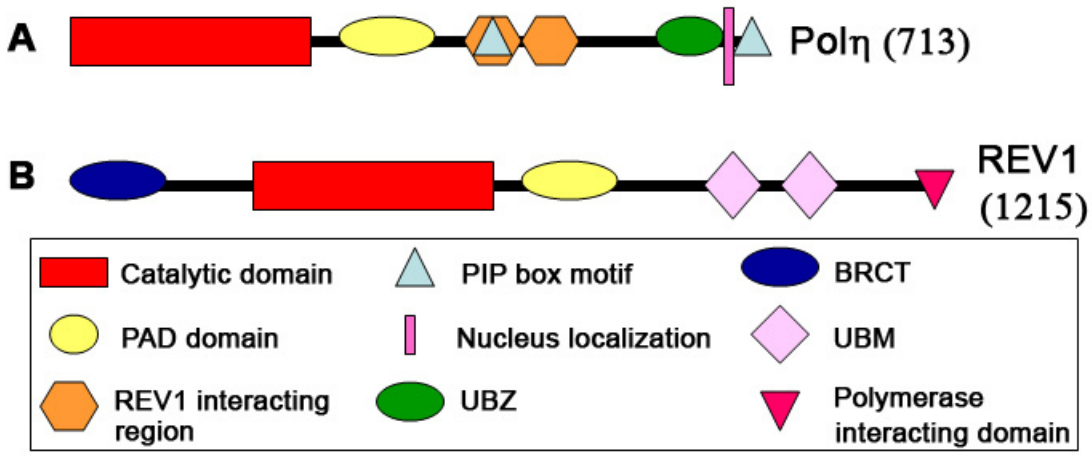

Figure 1.2 Structural domains of polymerase eta and REV1. Sites common to both proteins include the catalytic site and PAD, polymerase association domain. (A) Structural domains of polymerase eta (Polη), a 713 amino acid protein. Polymerase eta contains an ubiquitin-binding zinc finger (UBZ) domain, REV1 interacting region, PCNA interacting domain (PIP) and a nucleus localization domain. (B) Structural domains of REV1, a 1251 amino acid protein. REV1 contains a BRCT domain, an ubiquitin-binding motif (UBM) and a polymerase interacting domain.

Yeast Rev1 was the first translesion polymerase described to have catalytic activity, functioning as a deoxycytidyl transferase [82]. Human REV1 was discovered in 1999 and is encoded by the *REV1L* gene located between chromosome 2q11.1 and 2q11.2 [134]. Similar to the yeast homolog, human REV1 also functions as a deoxycytidyl transferase. REV1 is capable of inserting cytosines across from uracil residues and undamaged guanine, adenine, thymine or cytosine bases, and is thought to have an important role in bypassing abasic sites [81, 134-137]. REV1 may also incorporate a single cytosine opposite various types of base adducts, especially guanines bearing a large adduct, and extend beyond mismatched bases by insertion of a cytosine at the primer terminus [6, 135-136, 138-139].

A mechanistic role for REV1 in translesion DNA synthesis was originally difficult to envision due to its limited catalytic activity. To add to the confusion, Haracska *et al.* found that yeast strains with double amino acid substitutions rendering Rev1 catalytically dead had no differences in observed mutagenesis induced by DNA damage when compared to strains with wild type Rev1, suggesting that Rev1 contributes to translesion DNA synthesis independently of its catalytic function [137]. Furthermore, cells deficient of REV1 were found to have an observable loss of mutagenesis and to be hypersensitive to agents that induce lesions REV1 is incapable of synthesizing across *in vitro* [137, 140-143]. An explanation of how REV1 contributes to translesion DNA synthesis was revealed when several reports were published describing that REV1 interacts with numerous translesion polymerases, including polymerases eta and zeta, through its C-terminal domain [128-129, 144-146]. Based on these observations, REV1 is thought to participate in translesion DNA synthesis via two different mechanisms. First, REV1 might act as a scaffold that promotes localization of multiple translesion polymerases to sites of damaged DNA. Second, REV1 may be essential for polymerase switching at sites of replication stress by facilitating interactions between different translesion polymerases via its C-terminal domain [12, 147].

Along with a C-terminal domain that allows for interactions with other translesion polymerases, REV1 also contains a BRCA1 C-terminal (BRCT) domain at the N-terminus, along with two ubiquitin-binding motifs near the C-terminus (Fig. 1.2) [22-23, 120].

REV1 is unique among the Y-Family of translesion polymerases in that it has a BRCT domain. In general, BRCT domains are found in many cell cycle and DNA repair proteins and are thought to mediate protein-protein interactions. Mutations in the BRCT domain can drastically alter the localization of DNA repair proteins to sites of DNA damage [148]. The BRCT domain of REV1 is thought to be required for participation in the bypass of damaged template DNA, specifically through promoting protein interactions with PCNA [149-151]. There are, though, some discrepancies in the literature as to whether or not the REV1 BRCT domain is necessary for translesion synthesis [152]. The ubiquitin-binding motifs are necessary for localization of REV1 to stalled replication forks as well, presumably by interacting with monoubiquitinated PCNA [153-154].

Due to REV1's apparent involvement in translesion DNA synthesis, an extremely important question is whether or not REV1 has a cellular role in the bypassing of chemotherapy-induced DNA lesions. *REV1* knockout DT40 cells, a cell line derived from a chicken B-lymphocyte, are known to be hypersensitive to chemotherapeutic agents including cisplatin [61, 155]. Okuda *et al.* demonstrated that human ovarian carcinoma cells depleted of REV1 are hypersensitive to cisplatin, have a decrease in measurable mutagenesis and a lower rate of acquired resistance to cisplatin [156]. Furthermore, over-expression of REV1 in human ovarian carcinoma cells results in decreased cisplatin sensitivity and enhanced development of cisplatin resistant variants [157]. It should be noted that in the context of bypassing cisplatin DNA adducts, REV1's catalytic activity may be dispensable. The most likely role of REV1 in terms of translesion synthesis past cisplatin intrastrand cross-links is localization of polymerase zeta to sites of replication stress, and promotion of polymerase switching between polymerase eta and polymerase zeta in order for translesion synthesis to be completed.

In addition to participating in translesion synthesis past damaged template DNA, REV1 may also have a biological role in antibody diversification and DNA repair. REV1 may insert a cytosine across from uracils or abasic sites that are created during somatic hypermutations [158-160]. Along with contributing to somatic hypermutations, REV1 may also have an important cellular role in the repair of DNA interstrand cross-links and double strand breaks [59]. *REV1*$^{-/-}$ DT40 cells are hypersensitive to interstrand cross-

linking agents such as mitomycin C and genetic epistasis exist between REV1 and the interstrand cross-linking repair protein Fanconi anemia complementation group C (FANCC) [60-61]. Employing a luciferase reporter reactivation assay, Shen *et al*. demonstrated that REV1 is required for efficient repair of a mitomycin C interstrand cross-link [161]. Although currently unknown, REV1 may insert a cytosine across from the base containing an unhooked interstrand cross-link. There is also speculation that REV1 has a crucial role in response to ionizing radiation based on observations that *REV1*$^{-/-}$ DT40 cells are hypersensitive to ionizing radiation [155]. In response to ionizing radiation, REV1 may simply bypass abasic sites caused by ionizing radiation, or REV1 could contribute to D-loop extension during homologous recombination to repair DNA double strand breaks. Unlike translesion synthesis across cisplatin cross-links, the catalytic activity of REV1 may be crucial for antibody diversification, and repair of DNA interstrand cross-links and double strand breaks.

Polymerase Zeta (REV3 and REV7)

The *Rev3* gene was originally discovered in *Saccharomyces cerevisiae* using the same screen for reversionless mutants that identified *Rev1*, and shortly thereafter a similar screen was employed to isolate *Rev7* [162-163]. *Saccharomyces cerevisiae* deficient of functional Rev3 or Rev7 are known to exhibit defects in both spontaneous and DNA damaged induced mutagenesis, but it was not initially appreciated that Rev3 and Rev7 cooperate with each other to form a translesion polymerase. Nelson *et al.* discovered Rev3 and Rev7 associate with each other to form the translesion polymerase zeta, with the Rev3 subunit consisting of the catalytic activity and the Rev7 subunit functioning as an accessory protein for catalytic enhancement [164]. In yeast, polymerase zeta is not essential, but this polymerase does contribute to viability following DNA damage noted by the decrease in survival of *Rev3*$^{-/-}$ *Saccharomyces cerevisiae* exposed to UV, hydroxyurea and methyl methanesulfonate [49, 165-166]. Polymerase zeta does have a crucial role in UV-induced mutagenesis in yeast, accounting for approximately 90% of frameshift mutations and base alterations [44]. Mutations are also diminished in *Rev3*$^{-/-}$ yeast following treatment by the chemotherapeutic agents hydroxyurea and methyl

methanesulfonate along with ionizing radiation, signifying that polymerase zeta participates in the translesion synthesis of templates containing DNA damage caused by these agents [43, 167]. Polymerase zeta contributes to spontaneous mutations as well, with a 50% observed decrease of point mutations in *Rev3*$^{-/-}$ *Saccharomyces cerevisiae* [44, 168-169].

Mammalian REV3, located on chromosome 6q21 in humans, is unique among the translesion polymerases because it is absolutely required during embryonic development, as *REV3*$^{-/-}$ mice are not viable [144, 170-176]. Currently, the essential role REV3 plays during embryonic development is unknown, but a logical explanation is that REV3 is necessary for bypass, repair or both of endogenous DNA damage; such damage being most likely due to oxidative stress. *REV3*$^{-/-}$ mouse embryos display a reduced size at 10.5 days into development and are usually aborted by roughly day 12.5 [177]. Except for erythrocytes, no hematopoietic cells are developed and mesenchymal tissues including heart and blood vessels lack integrity. As expected, the time when REV3 is most widely expressed in mouse embryos coincides with the time *REV3*$^{-/-}$ mouse embryos die. Polymerase zeta is thought to participate in the bypass of chemotherapeutic drug-induced DNA damage, thus it is considered a possible drug target to sensitize cancers to anti-cancer agents [57, 161, 178-180]. Therefore, knowing whether or not REV3 is absolutely indispensable beyond embryonic development is of extreme importance. *REV3*$^{-/-}$ mouse embryonic fibroblast are capable of being rescued in a p53 null background and *REV3* can be knocked out or depleted in other vertebrate cell lines including chicken DT40 cells, human fibroblast and human BL2 cells derived from Burkitt's lymphoma, suggesting REV3 is dispensable beyond, but not during, embryonic development.

Human REV7, described approximately two years after the discovery of human REV3, is located on chromosome 1p36 [144]. REV7 has not been as intensely investigated as REV3 primarily due to the fact that REV7 contains no catalytic function, but rather supports the catalytic activity of REV3. Recently published reports strongly suggest human REV7 is truly the homolog of yeast Rev7 [178, 181-183], but there still remains some skepticism in the scientific community if the currently identified human REV7 is the true homolog of yeast Rev7 and if it has a crucial role in translesion synthesis. Contributing to the confusion over REV7 is the fact this protein has 23% identity and 54% similarity to

the human mitotic checkpoint protein MAD2, therefore resulting in REV7 also being referred to as MAD2B or MAD2L2 [184]. REV7/MAD2B is thought to interact with several cell cycle proteins and is proposed to have a role in cell cycle regulation [23, 183]. Nonetheless, the role that REV7 may have in cell cycle control is still poorly understood.

Biochemical studies demonstrated polymerase zeta has limited ability to replicate across from DNA lesions such as 6-4 thymine-thymine photoproducts, thymine glycol and acetylaminofluorene-adducted guanine, while insertion directly opposite cyclobutane pyrimidine dimers or abasic sites is basically non-existent [88, 137, 185-186]. Polymerase zeta, though, is an efficient extender beyond distorted base pairs resulting from an incorrect base insertion opposite a DNA lesion or a base containing a bulky DNA lesion [88, 137, 164, 185]. Therefore, polymerase zeta is unlikely to directly insert a nucleotide opposite a DNA adduct, but rather functions in the translesion DNA synthesis pathway by extending beyond distorted primer termini.

Polymerase zeta is crucial for efficient replication of damaged template DNA, including DNA damage due to chemotherapeutic agents. Multiple published reports, using DT40 cells as a model for translesion synthesis in intact cells, have provided evidence that REV3 deficiency is extremely toxic to cells in the presence of chemotherapeutic agents such as cisplatin, melphalan, mitomycin C, transplatin and methyl methanesulfonate [56, 187]. REV7 depleted nasopharyngeal carcinoma cells are hypersensitive to cisplatin while REV3 protects colorectal cancer cells from cisplatin-induced cytotoxicity [178, 180]. Wu *et al.* determined depletion of REV3 from human fibroblasts had no measurable effect on the removal of cisplatin adducts, but the down-regulation of REV3 did result in greater sensitivity to cisplatin and a decrease in cisplatin resistant variants [179]. Employing a luciferase reactivation reporter plasmid, REV3 was found to be necessary for translesion synthesis across platinum adducts. Taken together, the data suggest that polymerase zeta has no major role in repair of cisplatin adducts, but rather participates in the bypass of cisplatin-induced intrastrand cross-links and may contribute to cisplatin resistance.

Very little is known about REV3 structurally, thus the exact mechanism of how REV3 extends beyond distorted primer termini or how REV3 even localizes to sites of

DNA damage is still a mystery. Human REV3 is approximately twice as large as *Saccharomyces cerevisiae* Rev3, containing a large exon region not found in yeast [44]. Even though human REV3 is a very large protein, it is currently known to have only two domains, a catalytic region and a REV7 interaction domain (Fig. 1.3) [23, 44]. There is speculation that REV3 is able to interact with REV1 as well. REV7 is known to interact with REV1, and also contains a HORMA domain allowing for interactions with chromatin and other proteins (Fig. 1.3) [129, 188]. Hence, not only does REV7 enhance catalytic activity, but may also direct REV3 to sites of DNA damage by interacting with REV1 and other proteins via the HORMA domain [189].

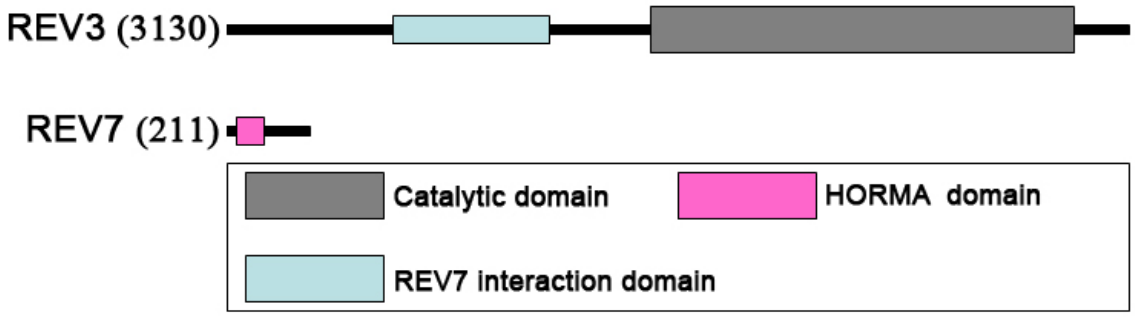

Figure 1.3 Structural domains of REV3 and REV7

In addition to translesion synthesis past DNA adducts, polymerase zeta is hypothesized to participate in interstrand cross-link repair and double strand break repair. *REV3*$^{-/-}$ DT40 cells are hypersensitive to interstrand cross-linking agents such as mitomycin C and genetic epistasis exists between REV3 and the interstrand cross-linking repair protein Fanconi anemia complementation group C (FANCC) [60, 187]. Via construction of a luciferase reaction reporter assay containing both a psoralen and mitomycin C interstrand cross-link, Shen *et al.* proposed that REV3 is necessary for efficient recombination-independent repair of interstrand cross-links [161]. Polymerase zeta is also thought to have a cellular role in recombination-dependent repair of interstrand cross-links as well [57]. Raschle *et al.*, using a Xenopus egg extract model, elegantly demonstrated that polymerase zeta is necessary for extension beyond a cisplatin interstrand cross-link during recombination-dependent repair of the cross-link [59].

Polymerase zeta is also hypothesized to have a biological role in repair of DNA double strand breaks, possibly by participating in homologous recombination. REV7 depleted nasopharyngeal carcinoma cells are hypersensitive to ionizing radiation, while *REV3*$^{-/-}$ DT40 cells are sensitive to ionizing radiation and have an increase of ionizing radiation-induced chromosome aberrations [56, 178]. However, it is unclear if polymerase zeta extends beyond nucleotides placed opposite DNA adducts induced by ionizing radiation or if this polymerase has an active role in repair of ionizing radiation-induced DNA double strand breaks.

Insertion and extension model of translesion DNA synthesis

Translesion DNA synthesis is the process whereby specialized translesion DNA polymerases replicate damaged template DNA that would otherwise stall high fidelity polymerases [12]. Translesion polymerases insert nucleotides opposite the DNA lesion and extend beyond the DNA damage to prevent prolonged replication stress and the eventual formation of DNA double strand breaks. Translesion synthesis is drastically different in prokaryotes and lower eukaryotes when compared to lesion bypass in vertebrates. Both prokaryotes and yeast have two different branches of translesion synthesis, an error free pathway and an error prone post-replication repair pathway [6]. Polymerase eta is thought to operate independently of REV1 or polymerase zeta during replication of DNA damage in the error free pathway, while the REV1/ polymerase zeta functional complex bypasses DNA damage in an error prone manner [190-192]. Based almost entirely on *in vitro* biochemical experiments and plasmid reactivation assays, polymerases eta, REV1 and zeta are all thought to cooperate with each other, referred to as an insertion and extension model, in higher eukaryotes to replicate past DNA lesions 6, [12, 22, 25]. A representative diagram depicting the insertion and extension model of translesion synthesis is shown in Figure 1.4. Since I am addressing the bypass of chemotherapeutic drug-induced DNA damage in human cancer cells, I will focus only on the insertion and extension model of translesion synthesis.

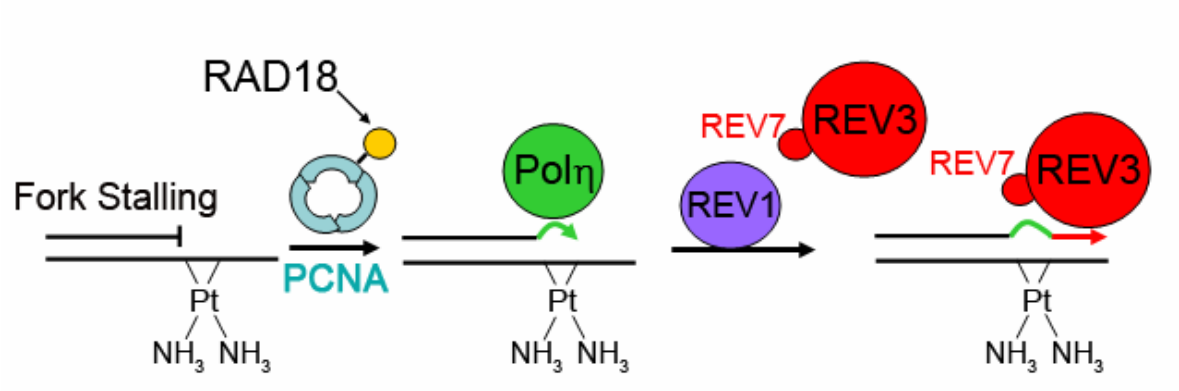

Figure 1.4 Insertion and extension model of translesion synthesis. In response to a cisplatin-induced stalled replication fork, the E3 ubiquitin ligase RAD18 monoubiquitinates PCNA on lysine 164. The monoubiquitination of PCNA is thought to act as a molecular switch from high fidelity replication to translesion synthesis. Polymerase eta (Polη), via interactions with PCNA, localizes to the site of the stalled fork and synthesizes directly across from the cisplatin intrastrand cross-link. REV1 then promotes translesion polymerase switching between polymerase eta and polymerase zeta (REV3 and REV7). Polymerase zeta extends beyond the distorted primer terminus.

Once a replication fork encounters a DNA adduct that a high fidelity polymerase is incapable of replicating past, the fork stalls and the helicase and polymerase uncouple resulting in continued unwinding of the DNA and formation of long stretches of single stranded DNA [193-194]. A lesion located on the lagging strand would immediately result in a single stranded DNA gap. A lesion located on the leading strand may also cause a single stranded DNA gap to form if the leading strand is reprimed to allow for continuation of replication (Fig. 1.5) [194]. RPA, replication protein antigen, coats and stabilizes the single stranded DNA protecting it from nucleases and hairpin loop formation. RPA also acts as an indicator of replication stress and initiates the localization of proteins involved in the replication stress response to stalled replication forks [195]. Chang *et al.* demonstrated, using a Xenopus egg extract model, that the formation of single stranded DNA coated with RPA is crucial for localizing the RAD6 (E2)-RAD18 (E3) ubiquitin ligase to sites of stalled replication forks [196]. The RAD6 (E2)-RAD18 (E3) ubiquitin ligase complex specifically monoubiquitinates proliferating cell nuclear antigen (PCNA) on lysine 164 in response to replication fork stalling. The monoubiquitination of

PCNA by RAD18 is thought to be a molecular switch between high fidelity synthesis and translesion DNA synthesis [121, 197-199]. Cells deficient in RAD18 are known to be hypersensitive to DNA damaging agents that induce replication fork stalling along with polymerase and helicase uncoupling signifying RAD18 is necessary for a proper response to this particular type of replication stress [6, 200-202].

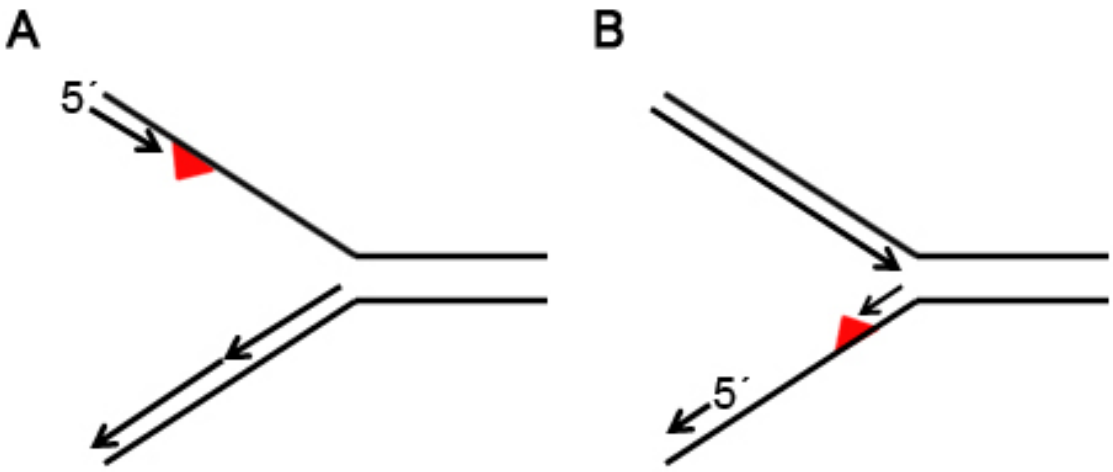

Figure 1.5 Single stranded DNA gap formation in response to stalled replication forks. Depiction of a replication fork encountering a DNA lesion resulting in replication blockage. (A) After blockage of the leading strand, the helicase and polymerase uncouple resulting in the formation of single stranded DNA. The leading strand may eventually be reprimed leading to the formation of a single stranded gap. (B) When a DNA lesion is located on the lagging strand a single stranded DNA gap will be formed due to incomplete replication of the Okazaki fragement [194].

Y-Family translesion polymerases such as eta and REV1 are known to contain ubiquitin binding motifs (Fig. 1.2) Following PCNA monoubiquitination by RAD18, the affinity of polymerase eta and REV1 to PCNA drastically increases [126, 153]. Therefore, RAD18-dependent PCNA monoubiquitination is thought to be the primary initiator of translesion synthesis by promoting localization of translesion polymerases to the site of replication stress. Once localized to the stalled replication fork, polymerase eta is thought to incorporate a nucleotide opposite the adducted base(s). However, polymerase eta is not very efficient extending beyond a terminus containing distorted base pairs resulting from either an incorrect base insertion opposite a DNA lesion or a base pair

containing a bulky DNA lesion [6, 12, 203]. In the insertion and extension model, polymerase eta replicates across from the damaged base(s), but another translesion polymerase is necessary to extend beyond the distorted primer terminus [25]. As discussed earlier, REV1 is thought to promote localization of translesion polymerases to sites of stalled replication forks and facilitate polymerase switching through interactions with its C-terminal domain. After polymerase eta inserts nucleotides opposite the DNA lesion, REV1 is thought to promote localization of polymerase zeta to the site of the stalled fork via interactions with REV7 and facilitate switching between polymerase eta and polymerase zeta [12]. Even though polymerase zeta is not very proficient at inserting bases opposite DNA lesions, this polymerase is efficient at extending beyond distorted primer termini.

Taken together, the insertion and extension model proposes RAD18 initiates translesion synthesis by monoubiquitinating PCNA, and this promotes localization of translesion polymerases to the site of a stalled replication fork. Once at the site of replication stress, polymerase eta inserts a nucleotide opposite the DNA lesion. REV1 promotes localization of polymerase zeta to the stalled replication fork and facilitates polymerase switching between polymerase eta and polymerase zeta. Polymerase zeta extends beyond the inserted base until either the gap is filled or high fidelity polymerases take over replication responsibilities. A point should be made that translesion synthesis in vertebrates is more complicated than previously stated and depends on the type of damage present. In certain situations, such as the bypass of cylcobutane pyrimidine dimers, polymerase eta may be able to replicate past the DNA lesion error free independently of REV1 or polymerase zeta. In other circumstances REV1 and polymerase zeta may be essential for extension beyond the damaged DNA template, while another translesion polymerase besides eta inserts opposite the lesion [25]. However, multiple scientific publications employing both biochemical assays and plasmid reactivation reporter assays suggest polymerases eta, REV1and zeta are all necessary for efficient bypass of DNA damage induced by chemotherapeutic agents such as cisplatin.

Translesion polymerases and interstrand cross-link repair

DNA interstrand cross-links are formed when a bifunctional chemical covalently links both strands of a DNA duplex. The linking of opposite strands of DNA prevents vital biological processes from proceeding, such as the unwinding of DNA by helicases, thereby halting both the replication and transcription machinery. Interstrand cross-links are extremely toxic to cells, and in fact one interstrand cross-link is thought to be capable of killing a yeast cell if not repaired efficiently [204-206]. Nitric oxide, byproducts of metabolism such as malonic dialdehyde along with other naturally occurring environmental chemicals, including crontonaldehyde or acetaldehyde, induce interstrand cross-link formation [205]. Since endogenous and environmental chemicals are capable of interacting with DNA forming cross-linked duplexes, it would be reasonable to assume that organisms have evolved efficient cellular pathways to remove interstrand cross-links. The removal of these toxic lesions is advantageous for cell survival, but the evolution of sophisticated mechanisms for interstrand cross-link repair may result in chemotherapy resistance.

The highly toxic nature of interstrand cross-links have been noted in the clinic setting and several chemotherapeutic agents that promote interstrand cross-link formation, including mitomycin C, are used for cancer treatment. Unfortunately, cancerous cells can become resistant to interstrand cross-linking agents impairing the usefulness of these agents. Furthermore, cross-resistance between interstrand cross-linking agents is known to occur, that is, acquired resistance to one cross-linking agent results in the resistance to multiple interstrand cross-linking agents [207]. The exact mechanism of how resistance arises is unknown, but the occurrence of cross-resistance suggests the involvement of a biological pathway common to all interstrand cross-linking agents. One possible cellular process ubiquitous to all interstrand cross-linking agents that may promote the development of resistance is alterations in repair of interstrand cross-links [207-209]. Currently, interstrand cross-link repair is poorly understood. Due to acquired cross-resistance of chemotherapeutic agents that induce DNA interstrand cross-links, determining how interstrand cross-links are repaired and if alterations in repair promote resistance is of uttermost importance. Comprehending interstrand cross-link

repair may also reveal novel targets that can influence drug design of new chemotherapeutic agents.

DNA interstrand cross-links can be repaired in a recombination-independent manner or a recombination-dependent manner (Fig. 1.6). Recombination-independent repair does not take place at sites of DNA replication and is thought to be independent of homologous recombination. However, recombination-independent repair may be dependent upon monoubiquitinated PCNA [161]. Recombination-dependent repair, on the other hand, is initiated when replication forks encounter interstrand cross-links. The unhooking of the DNA interstrand cross-link results in a DNA double strand break, which in turn is repaired by homologous recombination. The majority of interstrand cross-links are thought be repaired in a recombination-dependent pathway during S phase in human cells [59, 161, 210-211]. Translesion DNA polymerases are thought to play a role during interstrand cross-link repair by either gap filling across from the unhooked cross-link or by extending the D-loop during homologous recombination.

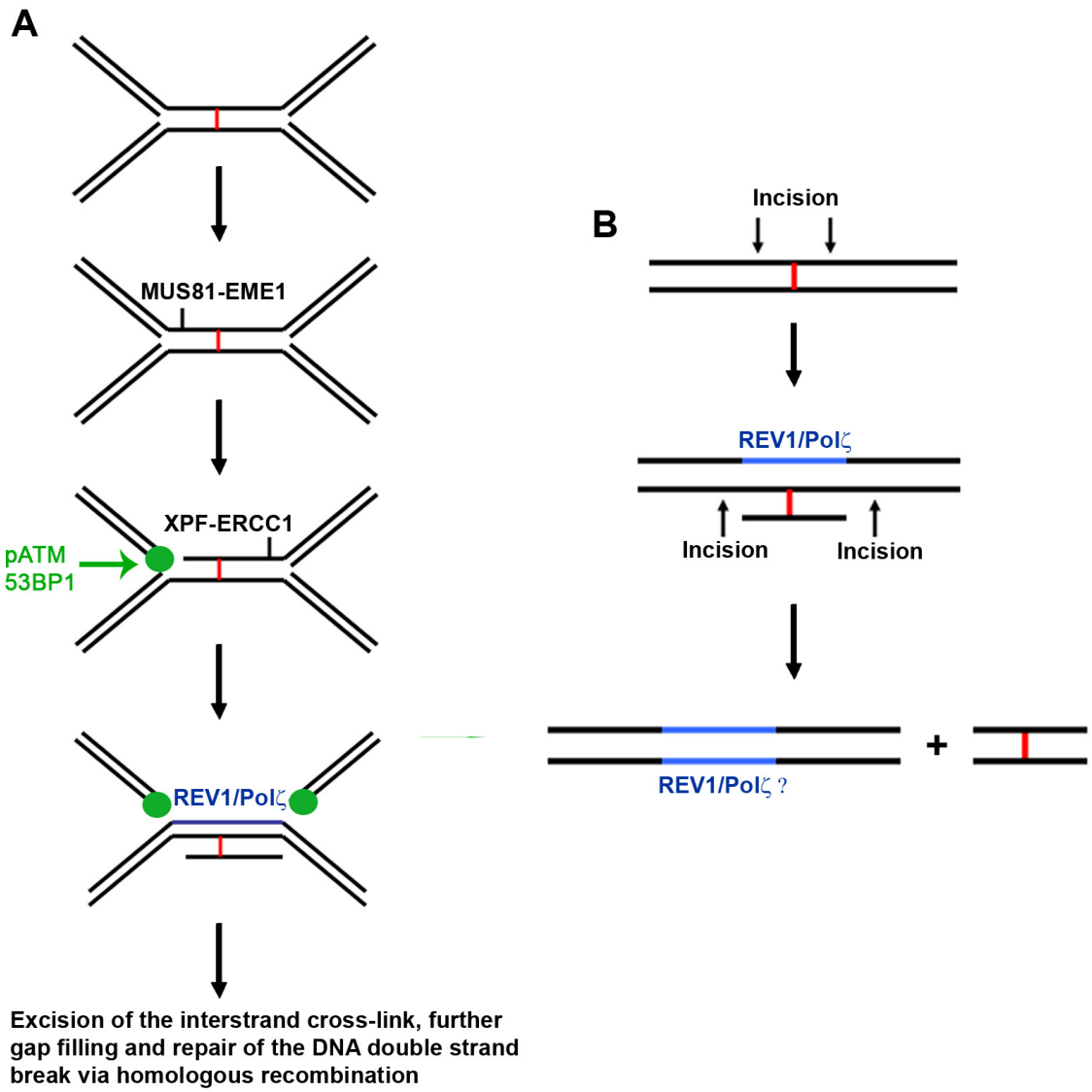

Figure 1.6 A model of recombination-dependent and recombination-independent repair of DNA interstrand cross-links. (A) During recombination-dependent repair, replication forks approach the interstrand cross-link from both sides. MUS81-EME1 makes an incision on side of the cross-link resulting in the formation of a DNA double strand break. XPF-ERCC1 makes an incision on the other side of the interstrand cross-link allowing for unhooking of the cross-link. The REV1/polymerase zeta functional complex may replicate DNA opposite the unhooked cross-link. The interstrand cross-link is excised followed by further gap filling, and repair of the DNA double strand break by homologous recombination. (B) During recombination-independent repair, an incision is made on both sides of the interstrand cross-link on the same strand of DNA allowing for unhooking of the cross-link. The REV1/polymerase zeta functional complex is thought to replicate DNA opposite the unhooked cross-link. The cross-link is excised followed by gap filling of the excised interstrand cross-link.

Based on biochemical and genetic studies several DNA repair pathways appear to be required for repair of interstrand cross-links including nucleotide excision repair, translesion synthesis and homologous recombination [6, 60, 187, 205-206, 212]. The most widely accepted model of interstrand cross-link repair at present time is based upon work from multiple laboratories and is in described in Figure 1.6A [6, 206, 213]. Raschle *et al.* recently proposed a recombination-dependent model of interstrand cross-link repair where replication forks approach the interstrand cross-link from both sides and stall multiple base pairs away [59]. Eventually one of the two stalled replication forks will progress up to the site of the interstrand cross-link where structure-specific endonucleases will recognize the resulting branched DNA structure cleaving on both sides and unhooking the interstrand cross-link. The endonuclease MUS81-EME1 is thought to make the first incision resulting in the formation of a DNA double strand break [214]. The endonuclease XPF-ERCC1 then makes an incision on the other side uncoupling the cross-linked DNA duplex and unhooking the interstrand cross-link [215]. Exactly how an interstrand cross-link is repaired after the unhooking step occurs is unknown, but the currently accepted model proposes gap filling via translesion synthesis opposite from the unhooked cross-link. The unhooked cross-link is then removed by the nucleotide excision repair pathway followed by further gap filling of the excised cross-link, and lastly repair of the DNA double strand break via homologous recombination [206, 212].

The Fanconi anemia pathway, via an undefined cellular process, is thought to recognize interstrand cross-links and initiate repair along with coordinating all of the DNA repair pathways involved in removal of interstrand cross-links [206, 213]. The genetic disease Fanconi anemia arises due to mutations in genes associated with 13 different complementation groups, and is characterized by chromosomal instability, bone marrow failure, congenital defects and early onset of cancer. Fanconi anemia patients are known to be hypersensitive to interstrand cross-linking agents such as mitomycin-C and this observation was one of the first pieces of data linking the Fanconi anemia pathway with interstrand cross-link repair [216-218]. The Fanconi anemia complex of proteins consist of 8 Fanconi anemia complementation group (FANC) proteins including A, B, C, E, F, G, L and M which form a core complex. There are also 5 FANC proteins that operate outside the core complex including D2 and I (known as the I-D complex), D1, J and N. Gene

products of *FAAP24* and *FAAP100* are associated with the Fanconi anemia core complex, but presently no mutations in *FAAP24* or *FAAP100* have been associated with disease [206].

Fanconi anemia proteins are thought to initiate and coordinate interstrand cross-link removal in the recombination-dependent repair pathway. The FANCM -FAAP24 heterodimer recognizes stalled replication forks located at interstrand cross-links, and via DNA translocase activity recruits the Fanconi anemia core complex to the site of DNA damage where the core complex is activated and functions as a large multisubunit ubiquitin ligase [219-220]. Upon localization to stalled replication forks the Fanconi anemia core complex, through the E3 ubiquitin ligase FANCL, monoubiquitinates both FANCD2 and FANCI, which is required for activation of the Fanconi anemia pathway [221-223]. In the absence of FANCM the core complex is stable although binding to chromatin is reduced. Under these conditions only a partial decrease in FANCD2 monoubiquitination is noted signifying FANCM is not absolutely required for activation of the Fanconi anemia pathway [222, 224-225]. FANCM may contribute to interstrand cross-link repair outside activation of the Fanconi anemia pathway. FANCM is a helicase that, *in vitro*, is capable of regressing replication forks, which could be an important remodeling step for initiation of cross-link removal [219, 226]. Along with activating the Fanconi anemia pathway, the core complex also recruits translesion polymerases to sites of stalled replication forks [130].

Ubiquitination of FANCD2 is indispensable for cross-link repair, whereas FANCI ubiquitination is non essential but may enhance repair [206]. FANCI is phosphorylated by ataxia telangiectasia-Rad3 related (ATR) in response to stalled replication forks located at sites of interstrand cross-links, and this phosphorylation is thought to promote localization of the FANCD2-I complex to sites of DNA damage [227]. Inhibition of FANCI phosphorylation ablates FANCD2 ubiquitination, suggesting ATR dependent phosphorylation of FANCI is crucial for localization of the FANCD2-I complex to the site of DNA damage where FANCD2-I is monoubiquitinated by the Fanconi anemia core complex [227]. Ubiquitination of FANCD2 and FANCI by the core complex promotes localization of these proteins into chromatin bound foci presumably at sites of DNA repair since the foci contain other DNA repair proteins such as RAD51 and γ-H2AX [228-

[231]. The precise function of FANCD2 and FANCI at sites of DNA repair is unknown, but it is speculated that monoubiquitinated FANCD2 may promote localization of repair proteins to the site of interstrand cross-links such as the chromatin remodeling protein Tip60 [206, 213]. Since translesion polymerase eta and REV1 have ubiquitin binding domains, ubiquitination of the FANCD2-I complex may promote localization of these translesion polymerases to certain types of DNA damage.

The Fanconi anemia proteins FANCD1, FANCJ and FANCN are not required for FANCD2-I monoubiquitination, thus these proteins are thought to function downstream of both the core complex and FANCD2-I or possibly operate independently in a parallel pathway of interstrand cross-link repair [206]. In general FANCD1, FANCJ and FANCN are thought to be necessary for correction of the DNA double strand breaks formed during recombination-dependent repair of interstrand cross-links via regulation or participation in homologous recombination. FANCJ, also known as BRIP1 or BACH1, is a helicase with unknown function in cross-link removal, but may contribute to both repair of the DNA double strand break and endonucleolytic cross-link unhooking [206, 213]. One hypothesis is that FANCJ, via its 5′ to 3′ helicase activity, may facilitate cross-link repair by remodeling DNA at sites of interstrand cross-link unhooking [213, 232]. FANCJ may also contribute to homologous recombination repair of interstrand cross-links by unwinding D-loop structures and promoting RAD51 nucleofilament removal to allow for completion of homologous recombination [206, 233-234]. However, *FANCJ* knockout cells are not sensitive to ionizing radiation, thus bringing into question its role in homologous recombination [235]. FANCD1, more famously known as BRAC2, is necessary for homologous recombination and functions by promoting RAD51 loading onto DNA [236-237]. FANCN, also known as PALB2, may participate in homologous recombination through interactions with FANCD1 [206, 238].

Translesion synthesis has been implicated in repair of DNA interstrand cross-links based on the fact cells deficient of REV1, REV3 or REV7 are known to be hypersensitive to interstrand cross-linking agents such as mitomycin-C. Also, both REV1 and REV3 are epistatic with the Fanconi anemia core complex protein FANCC in terms of cisplatin cytotoxicity suggesting REV1 and REV3 have an active role in repair of interstrand

cross-links. However, only recently was the contribution of polymerase zeta to repair of DNA interstrand cross-links revealed. Raschle *et al.*, via a Xenopus egg extract model, elegantly demonstrated polymerase zeta is necessary for extension beyond an unhooked cisplatin cross-link during gap filling [59]. The polymerase responsible for inserting directly opposite the base containing the covalently bound cross-link has not yet been identified, but REV1 is a lead candidate. Mitomycin C and cisplatin-induced interstrand cross-links are formed between guanines on opposite strands of DNA. Since REV1 is capable of inserting a cytosine opposite a guanine containing a covalently bound bulky group, REV1 is a logical choice for inserting opposite the unhooked cross-link. Currently it is unknown if translesion synthesis is necessary for gap filling following excision of the unhooked cross-link or if translesion polymerases have a role in homologous recombination during recombination-dependent repair of interstrand cross-links. Nonetheless, translesion synthesis has yet to be directly linked to recombination-dependent interstrand cross-link repair in humans.

One of the most intriguing questions is how the Fanconi anemia pathway regulates translesion synthesis. Fanconi anemia patients lacking a functional core complex and *FANCC*$^{-/-}$ DT40 cells are known to have decreased mutation rates suggesting that the Fanconi anemia core complex somehow affects translesion synthesis [130, 239-241]. Furthermore, *FANCD2* or *FANCI* deficient cells display an increase of mutagenesis further supporting the hypothesis that the Fanconi anemia pathway influences translesion synthesis [130]. Mirchandani *et al.* provided direct evidence that the Fanconi anemia pathway regulates translesion synthesis by showing the Fanconi anemia core complex, independently of FANCD2-I, is necessary for REV1 foci formation after exposure to UV [130]. Therefore, the Fanconi anemia core complex appears to be necessary for localization of REV1 to sites of stalled replication forks due to adducted DNA. Although unproven, the core complex is assumed to promote localization of translesion polymerases to sites of stalled replication forks induced by interstrand cross-links in order for gap filling to occur opposite unhooked cross-links. This assumption is made because PCNA is not thought to be monoubiquitinated to a great extent following mitomycin C exposure. There is speculation that the Fanconi anemia pathway is forked, in which case the core complex and FANCD2-I may regulate translesion synthesis during gap filling

while Fanconi anemia proteins downstream of the core complex would regulate homologous recombination repair of DNA double strand breaks formed during recombination-dependent repair of interstrand cross-links [57, 206, 213]. Translesion polymerases may also be needed for homologous recombination repair of double strand breaks, which if true presents the possibility that translesion polymerases may also be regulated by Fanconi anemia proteins downstream of the core complex [57, 213].

To further complicate the relationship between the Fanconi anemia pathway and translesion synthesis, Shen *et al.* recently proposed a drastically different view of how the Fanconi anemia pathway contributes to interstrand cross-link repair [211]. Shen *et al*. suggest the Fanconi anemia core complex along with FANCD2-I is necessary for recombination-independent cross-link repair while FANCD1/BRAC2, FANCN and FANCJ are necessary for recombination-dependent cross-link repair [211]. REV1 and REV3 are known to participate in recombination-independent repair of interstrand cross-links, which is dependent upon PCNA monoubiquitination [161]. Presumably in the Fanconi anemia pathway proposed by Shen *et al.*, the core complex would regulate translesion synthesis at sites of recombination-independent cross-link repair. However, Raschle *et al.* provided evidence that polymerase zeta and possibly REV1 are essential for recombination-dependent repair of interstrand cross-links [59]. In the Fanconi anemia pathway model presented by Shen *et al.*, the possibility exists for translesion synthesis to regulated by FANCD1/BRAC2, FANCN and/or FANCJ during recombination-dependent repair of interstrand cross-links.

Nevertheless, much work is needed to understand the cellular process of interstrand cross-link repair in human cells and how translesion synthesis contributes to removal of cross-links. I will address if the translesion DNA polymerases eta, REV1 and zeta participate in repair of DNA interstrand cross-links.

Translesion polymerases and DNA double strand break repair

DNA double strand breaks are thought to be the most toxic form of DNA damage [242]. If unrepaired in a timely manner, double strand breaks will lead to cell cycle arrest, apoptosis, and possible loss of genetic material which can lead to mitotic cell death [243]. Several anti-cancer treatments have a mechanism of action that results in DNA double stand break formation including ionizing radiation, bleomycin, the topoisomerase I poisons such as camptothecin, and the topoisomerase II poisons including etoposide and doxorubicin. Even though DNA double strand breaks are exceptionally lethal, cancer cells can become refractory to the damage [244]. The ability to repair DNA double strand breaks, thus lessening the cytotoxic effects, may contribute to resistance of double strand break-inducing agents [245-247]. Recently, the translesion polymerases eta, REV1 and zeta have been proposed to play a role in the repair of DNA double strand breaks [56, 119, 155, 178, 248]. However, several questions remain unanswered as to how translesion polymerases protect against cytotoxicity caused by DNA double strand break-inducing agents, particularly if translesion polymerases are simply bypassing damaged DNA bases induced by these agents or if translesion polymerases have an active role in double strand break repair.

Translesion polymerases have been linked to DNA double stand break repair mostly based on the observation that in the absence of REV1 or polymerase zeta cells are hypersensitive to double strand break-inducing agents. Chen *et al.* demonstrated that *Rev1*, *Rev3* or *Rev7* knockout *Saccharomyces cerevisiae* are sensitive to ionizing radiation, a known inducer of DNA double strand breaks [249]. Also, *Rev1*$^{-/-}$, *Rev3*$^{-/-}$ or *Rev7*$^{-/-}$ *Saccharomyces cerevisiae* display a decrease in ionizing radiation-associated mutagenesis implying these translesion polymerases have a cellular role in response to ionizing radiation [249]. REV7-depleted nasopharyngeal carcinoma cells are hypersensitive to ionizing radiation, while *REV1*$^{-/-}$ or *REV3*$^{-/-}$ DT40 cells are sensitive to ionizing radiation and exhibit an increase of ionizing radiation-induced chromosomal aberrations [56, 178]. In addition, polymerase eta has been proposed to be necessary for D-loop extension during homologous recombination [119].

Nonetheless, many types of DNA lesions are formed in addition to DNA double strand breaks by the aforementioned agents rendering results difficult to interpret. In the case of ionizing radiation, DNA adducts such as 7,8-dihydro-8-oxoguanine, abasic sites, DNA single strand breaks and DNA double strand breaks are all formed following exposure [67]. The translesion polymerases eta, REV1 and zeta may be necessary to bypass DNA abasic sites or adducts formed by ionizing radiation [48, 81, 100, 102, 137]. On the other hand, translesion polymerases may also be necessary for repair of DNA double strand breaks by participating in either non-homologous end-joining or homologous recombination [6, 26-27, 119, 250]. A depiction of the different cellular pathways the translesion polymerases eta, REV1 and zeta may participate in following ionizing radiation is contained in Figure 1.7.

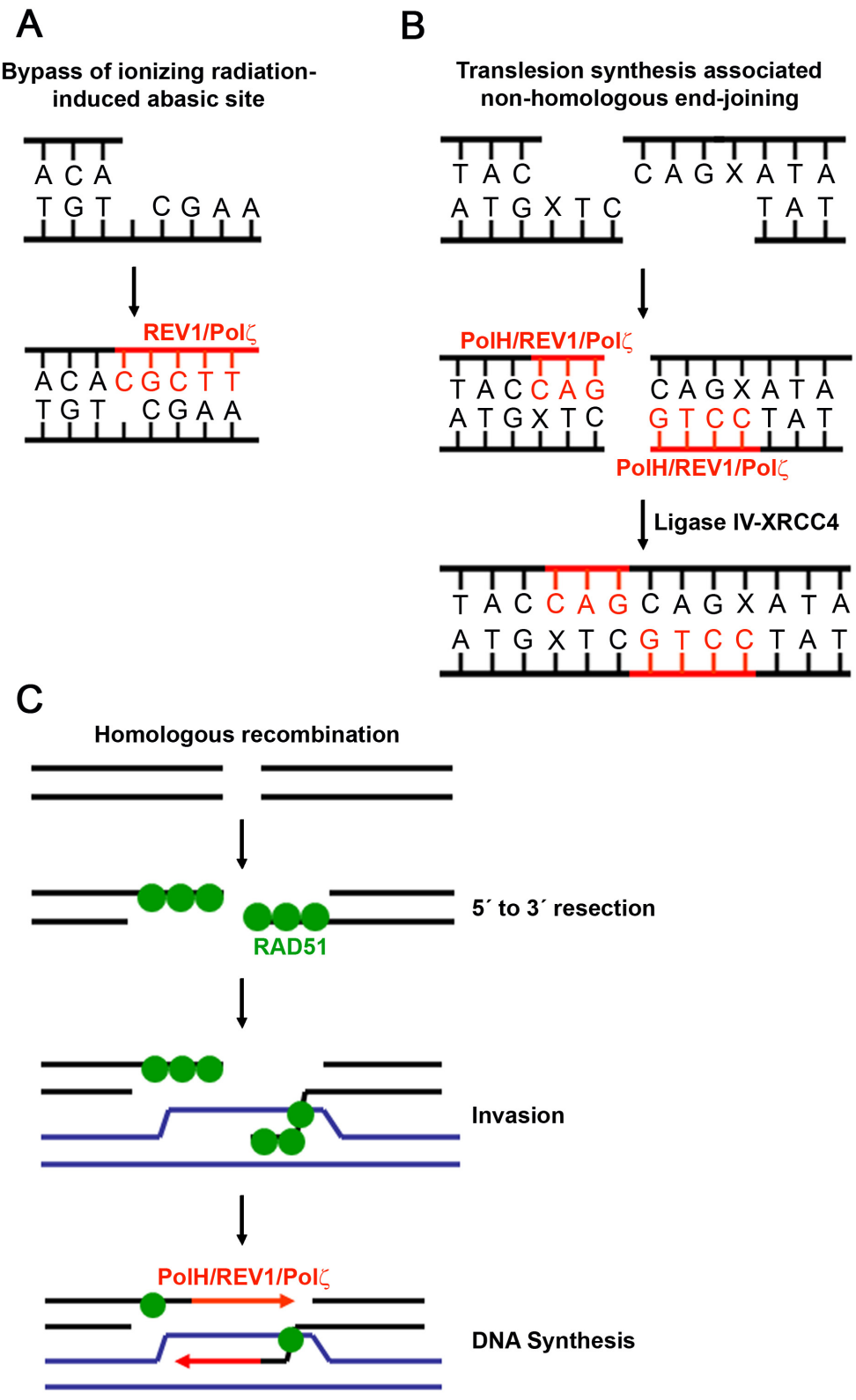

Figure 1.7 Different cellular pathways the translesion polymerases eta, REV1 and zeta may participate in following ionizing radiation. (A) In response to ionizing

radiation-induced abasic sites, the REV1/polymerase zeta function complex may be necessary for inserting a cytosine opposite the abasic site and extending beyond the damage. Replicative bypass of abasic sites would be expected to dependent upon PCNA monoubiquitination since helicase and polymerase uncoupling should occur. (B) It is hypothesized that in certain situations translesion polymerases may be needed for completion of non-homologous end-joining [250]. Ionizing radiation may produce DNA double strand breaks with non-complementary overhangs containing DNA damage denoted by X. In order to conserve DNA bases within the overhangs, the translesion polymerases eta, REV1 and zeta may be necessary for gap filling. The blunt ends created by gap filling are ligated together by ligase IV-XRCC4. (C) During homologous recombination, the termini of DNA double strand breaks are resected in a 5′ to 3′ manner in order to reveal 3′ overhangs. The single stranded overhangs are first coated by RPA, then FANCD1 along with RAD51 paralogues displaces RPA and loads RAD51 onto the single stranded DNA. RAD51 searches for a homologous repair template, and once found promotes strand invasion forming a D-loop structure. The translesion polymerases eta, REV1 and zeta may be essential for D-loop extension, especially if the repair template contains DNA damage.

Non-homologous end-joining can occur during any phase of the cell cycle, and is the only operational DNA double strand break repair mechanism during the cell cycle phases G0, G1 and early S since a homologous repair template is not present in these phases of the cell cycle [251-252]. Non-homologous end-joining is thought to be a fast process, capable of being completed within 30 minutes, and is the dominate repair pathway of double strand breaks in eukaryotic cells [253-254]. Upon the formation of a DNA double strand break, the Ku heterodimer complex, which consists of the Ku70 and Ku80 subunits, binds to the DNA termini [255-256]. The Ku heterodimer complex allows for alignment of the broken ends and promotes localization of other proteins involved in non-homologous end-joining to the site of the DNA break including the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) and the XRCC4-DNA ligase IV complex [256-258]. The Ku heterodimer complex translocates into the DNA leaving DNA-PK_{cs} at the DNA terminus on each of the aligned broken ends [259]. Upon binding the DNA ends, DNA-PK_{cs} is activated and functions as a synapse between the two DNA termini [257, 260]. The broken DNA ends are rejoined by the actions of DNA ligase IV stimulated by XRCC4 and XLF [261-263]. It should be pointed out this is a simplistic over view of non-homologous end-joining, and this repair pathway is not as sequentially ordered as depicted here.

When DNA double strand breaks are formed due to exposure of ionizing radiation, the ends of the DNA can be damaged or contain single strand overhangs that are non-complimentary resulting in DNA termini that cannot be ligated. In order to join the broken ends together, end-processing enzymes such as Artemis transform the termini into 5′-phosphorylated ends capable of being ligated together [264]. If non-complimentary single strand DNA overhangs are present, gap filling may occur before ligation in order to conserve the overhang sequence [265]. The presence of modified bases in the vicinity of the processed ends, though, may interfere with gap filling and repair of the DNA double strand break. Covo *et al.* developed a plasmid-based assay containing an abasic site on a short 5′overhang located at the terminus of a DNA strand break to determine what effect altered bases have upon non-homologous end-joining [250]. The presence of the abasic site reduced the overall repair of the DNA double strand break, while translesion synthesis across from the abasic site allowed non-homologous end-joining to proceed with out loss of DNA bases [250]. Therefore, in certain situations translesion synthesis may be necessary for non-homologous end-joining to proceed, such as gap filling of DNA overhangs where the overhang contains an altered base. Translesion polymerases contained within the X-Family of polymerases may contribute to gap filling during non-homologous end-joining, therefore it is unclear if the translesion polymerases eta, REV1 and zeta are also necessary for non-homologous end-joining. Furthermore, McElhinny *et al.* pointed out that in the absence of polymerases, non-homologous end-joining should still occur via the actions of a nuclease [265]. In this situation a nuclease would cleave off any non-complimentary overhangs allowing for non-homologous end-joining to proceed. Consequently, translesion synthesis across altered bases contained within DNA overhangs may allow for conservation of DNA bases, but should not influence the efficiency of non-homologous end-joining.

Even though non-homologous end-joining is the major repair pathway for DNA double strand breaks in mammalian cells, homologous recombination repair may occur if a homologous repair template is present. Homologous recombination is a slow repair process taking seven hours or longer to complete and utilizes a homologous sister chromatid as a template for DNA repair [253]. In the homologous recombination repair pathway DNA is resected at the site of the DNA double strand break, perhaps by the

MRN complex, revealing a single stranded 3′ overhang [266-268]. RPA binds the single stranded DNA overhang in order to stabilize it and prevent formation of secondary structures [268-271]. For the homology search to take place, RPA must be displaced to allow for the recombinase RAD51 to form a nucleofilament on the single stranded 3′ overhang. RAD51 mediators including FANCD1 and the RAD51 paralogues, such as RAD51B and RAD51C, are responsible for RPA dissociation, and RAD51 loading onto the single stranded DNA 3′ overhang [272-278]. Once formed, the RAD51 nucleofilament searches for a homologous repair template and promotes strand invasion at the site of the homologous template resulting in the formation of a D-loop structure [279-280]. DNA synthesis occurs at the site of the D-loop structure utilizing the homologous repair template, and once synthesis is complete the invading structure is resolved resulting in a repaired double strand break [268].

Translesion polymerases may contribute to homologous recombination by participating in DNA synthesis at the D-loop structure. Polymerase eta is proposed to associate with RAD51 and extend the 3′ overhang repair substrate [119]. Sonoda *et al.* suggested that ionizing radiation-induced DNA double strand breaks may contain unsuitable 3′ ends for initiation of D-loop extension and the translesion polymerase zeta may be critical for commencement of synthesis during homologous recombination repair [56]. This scenario is unlikely due to the end-processing that takes place to ready the DNA termini for repair. However, if altered bases are contained within the repair template, such as ionizing radiation-induced abasic sites, then translesion polymerases may be required for extension of the D-loop.

Hypothesis

The overall goals of my dissertation are as follows:

1. To determine if the translesion DNA polymerases eta, REV1, and zeta are all necessary to bypass cisplatin-induced DNA adducts in a human cancer cell line, and gain an understanding of how these translesion polymerases are regulated during replicative bypass.

Several lines of evidence suggest that in vertebrates multiple translesion polymerases are necessary for translesion synthesis past damaged DNA bases. However, few studies have addressed if multiple translesion polymerases are essential for replicative bypass in intact human cells. Furthermore, biochemical assays have suggested that translesion polymerases are capable of replicating past chemotherapeutic-induced DNA damage, including cisplatin intrastrand cross-links. Based upon these studies, I hypothesize that the translesion polymerases eta, REV1 and zeta are crucial for translesion synthesis past cisplatin-induced intrastrand cross-links in human cancer cells, thus reducing the cytotoxicity of this anti-cancer agent. Monoubiquitination of PCNA by RAD18 at sites of stalled replication forks is thought to be an important event in terms of initiating translesion synthesis. Recently, the Fanconi anemia core complex has been suggested to regulate translesion synthesis by promoting localization of REV1 to sites of ultraviolet radiation-induced replication stress. I hypothesize that both RAD18 and the Fanconi anemia complex have important regulatory roles during translesion synthesis past cisplatin adducts.

2. To determine if the translesion DNA polymerases eta, REV1 and zeta participate in the repair of chemotherapeutic drug-induced DNA interstrand cross-links in a human cancer cell line.

Published studies have suggested the translesion polymerases REV1 and zeta have a cellular role in repairing interstrand cross-links in Xenopus egg extracts, DT40 cells and *Saccharomyces cerevisiae*. Experiments utilizing plasmid reaction assays, where the plasmid contains a cisplatin or mitomycin C interstrand cross-link, have further implied that REV1 and polymerase zeta are essential for interstrand cross-link repair. Multiple DNA repair pathways are thought be involved in the repair of interstrand cross-links including homologous recombination. Polymerase eta has been proposed to extend D-loops during homologous recombination. Founded upon the data published in the aforementioned studies, I hypothesize that the translesion DNA polymerases eta, REV1, and zeta are required for efficient repair of DNA interstrand cross-links induced by the chemotherapeutic agents cisplatin and mitomycin C in a human cancer cell line.

3. To address if the translesion DNA polymerases eta, REV1 and zeta have a cellular role in bypass or repair of DNA damage induced by ionizing radiation.

DT40 cells and *Saccharomyces cerevisiae* deficient in REV1, REV3 or REV7 are known to be sensitive to ionizing radiation, suggesting REV1 and polymerase zeta have a biological role in response to ionizing radiation. In addition, *REV3*$^{-/-}$ DT40 cells display enhanced chromosomal aberrations following ionizing radiation exposure. Polymerase eta may be necessary for repair of ionizing radiation-induced DNA double strand breaks by extending D-loops during homologous recombination. I hypothesize that the translesion polymerases eta, REV1 and zeta are necessary for protection against ionizing-radiation induced cytotoxicity by either replicative bypass of damaged DNA bases or repair of DNA double strand breaks.

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CHAPTER 2

Translesion DNA Polymerases Eta, REV1 and Zeta in lesion bypass of cisplatin DNA intrastrand cross-links

Summary

Translesion DNA synthesis (TLS) is a process whereby specialized DNA polymerases are recruited to bypass DNA lesions that would otherwise stall high-fidelity polymerases. Currently, the process by which translesion polymerases bypass damaged template DNA in intact human cells is poorly understood. In this chapter I provide evidence that multiple translesion polymerases are necessary to synthesize across cisplatin intrastrand cross-links in human cancer cells, supporting the insertion and extension model of translesion DNA synthesis. Furthermore, I demonstrated that PCNA monoubiquitination by RAD18 is necessary for efficient bypass of cisplatin adducts by the translesion synthesis polymerases eta (Polη), REV1, and zeta (Polζ) based on the observations that depletion of these proteins individually leads to decreased cell survival, cell cycle arrest in S phase, and activation of the DNA damage response. In addition to PCNA monoubiquitination by RAD18, the Fanconi anemia core complex is also crucial for recruitment of REV1 to stalled replication forks in cisplatin treated cells. Together the findings support a model where replicative bypass of cisplatin intrastrand cross-links requires cooperation of multiple TLS polymerases in mammalian cells and is regulated by both PCNA monoubiquitination via RAD18 and the Fanconi anemia core complex.

Introduction

Efficient and accurate replication of the eukaryotic genome is necessary for exact transmission of genetic information from generation to generation therefore preventing genomic instability. In the presence of DNA damage, maintenance of genomic integrity involves the activation of cell cycle checkpoints coupled with DNA repair to remove any damaged bases before synthesis occurs. Despite these sophisticated mechanisms to remove DNA lesions prior to DNA replication or cell division, DNA replication forks inevitably encounter lesions that block high fidelity polymerases potentially leading to replication fork instability, gaps in replicated DNA, and generation of DNA double strand breaks (DSBs). In order to preserve replication fork stability by allowing completion of DNA replication, template DNA containing a damaged base or abasic site can be bypassed through the actions of specialized translesion DNA synthesis (TLS) polymerases [1]. A key event in the regulation of TLS is the monoubiquitination of PCNA, a homotrimeric protein that functions as an auxiliary factor for DNA polymerases [2-5]. The RAD6 (E2)-RAD18 (E3) complex specifically monoubiquitinates PCNA (proliferating cell nuclear antigen) on Lysine-164 in response to replication fork stalling. This event is thought to operate as a molecular switch from normal DNA replication to the TLS pathway based on the observations that association of Y-Family TLS polymerases with monoubiquitinated PCNA is strengthened through the cooperative binding of one or more ubiquitin binding domains (UBM or UBZ) plus a PCNA-interacting domain [6-7].

In yeast and higher eukaryotes, the Y-Family polymerase eta (Polη) plays a key role in the efficient bypass of cyclobutane pyrimidine (TT) dimers, one of the major lesions resulting from exposure to ultraviolet (UV) radiation [8]. Inactivation of polymerase eta in humans results in the variant form of xeroderma pigmentosum (XP-V), an inherited disease associated with an increased incidence of skin cancers and sensitivity to sunlight, thus highlighting the importance of polymerase eta in performing error-free bypass of UV-induced DNA damage [9-10]. In yeast, most mutagenesis induced by various DNA damaging agents is thought to arise from the activity of a non-Y-Family TLS polymerase, polymerase zeta (Polζ) [11]. Polymerase zeta is a B-Family polymerase

composed of the Rev3 catalytic subunit and its accessory subunit, Rev7. The *Rev* (for "defective mutation reversion") genes were originally identified from genetic screens searching for mutants that are defective in UV-induced mutagenesis and include the *Rev1* gene encoding the Y-Family TLS polymerase rev1. Biochemically, Rev1 is characterized as having limited polymerase activity restricted to inserting dCMP opposite guanine, adducted guanine, or abasic sites in DNA [8]. Rev1 is essential for polymerase zeta-dependent mutagenesis in yeast and in this role, rev1 appears to perform a regulatory function independent of catalytic activity [12]. With regards to DNA damage-induced mutagenesis, the genetic epistasis observed in yeast between *Rev1*, *Rev3*, and *Rev7* contributed to the prevailing model that Rev1 and polymerase zeta function together during lesion bypass [11]. Recent evidence suggests that Rev1 is necessary to target polymerase zeta to stalled replication forks [13].

Although the genetic studies performed in yeast suggest that polymerase eta and the Rev1/polymerase zeta functional complex operate independently of one another in an error-free and error-prone mode of lesion bypass, recent studies *in vitro* implicate multiple translesion synthesis polymerases working in concert to bypass a single DNA adduct. For example, polymerase eta efficiently replicates templates containing cyclobutane pyrimidine dimers but not other lesions (*e.g.* 6-4 pyrimidine-pyrimidone photoproducts induced by UV radiation). In the latter case, polymerase eta can only insert a nucleotide directly opposite the adducted base and requires an additional translesion polymerase, such as polymerase zeta, to extend beyond the insertion [8]. Polymerase zeta is unique among translesion polymerases in that its primary role appears to be extending mispaired primer termini and nucleotides already inserted opposite a variety of DNA lesions [8]. These observations brought forth a two polymerase insertion and extension model for translesion synthesis in higher eukaryotes and implicates polymerase zeta as being essential for completing lesion bypass across multiple DNA lesions and adducts, a concept recently supported through mutagenesis reporter studies conducted in REV3-depleted mammalian cells [14-15].

Rather than directly participating in nucleotide insertion opposite DNA lesions, vertebrate REV1 is thought to serve as a molecular scaffold during translesion synthesis

polymerase switching events. In addition to a UBM domain that directly interacts with monoubiquitinated PCNA, REV1 is unique among the Y-Family polymerases in that it possesses an N-terminal BRCT motif that directly interacts with PCNA [7, 16]. In addition, REV1 possesses a unique protein interaction domain in its carboxy-terminus which makes mutually exclusive contact with the polymerase zeta accessory subunit, REV7, and other translesion polymerases including polymerase eta and the catalytic subunit of polymerase zeta, REV3 [13, 17-20].

Agents which introduce intrastrand cross-links are widely used in cancer chemotherapy and thus understanding the means by which cells cope with these lesions will be instrumental in identifying novel mechanisms leading to drug resistance and designing new agents refractory to DNA damage tolerance mechanisms. Polymerase eta, REV1, and polymerase zeta have all been implicated in mediating translesion synthesis past cisplatin intrastrand cross-links based on the findings that inhibiting their expression increases sensitivity and reduces cisplatin-induced mutagenesis in human cancer cells [21-25]. Furthermore, biochemical and structural analyses of polymerase eta demonstrate that this translesion polymerase is capable of efficiently inserting dCTP opposite the 3'dG of a 1,2-d(GpG) cisplatin intrastrand cross-link [26]. Here, RAD18, polymerase eta, and REV1 are all demonstrated to localize to sites of replication stress marked by PCNA and γ-H2AX foci following treatment of cells with cisplatin; however, REV1 focus formation is specifically dependent upon a functional Fanconi anemia (FA) core complex suggesting FA core proteins play an important role in directing REV1 to cisplatin-induced stalled replication forks. In addition, depletion of RAD18, polymerase eta, REV1, or polymerase zeta proteins lead to the induction of cellular responses indicative of inefficient lesion bypass of cisplatin adducts.

Together the findings support a model where replicative bypass of cisplatin intrastrand cross-links requires cooperation of multiple translesion polymerases in mammalian cells. This is one of the first reports directly supporting an insertion and extension model in a human cell line. Evidence is also provided that translesion synthesis is triggered by PCNA monoubiquitination and REV1 dependent bypass of cisplatin adducts is specifically regulated by the Fanconi anemia core complex.

Materials and Methods

Cell lines and culture conditions. HeLa, U2OS, and 293T/17 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The BL2 human Burkitt's lymphoma cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Construction of *POLI*, *POLH*, and *REV3L* $^{-/-}$ BL2 lines have been described [27-28]. Cisplatin (cis-diammineplatinum(II)dichloride) was purchased from Sigma-Aldrich and dissolved in 0.9% NaCl as a 3.3 mM stock solution.

RNA interference. HeLa cells were seeded at a density of $1x10^5$ per well of a 6 well plate. Introduction of siRNA into HeLa cells was carried out with X-tremeGENE (Roche). Each well was exposed to 50 nM siRNA plus 5µl X-tremeGENE overnight in the presence of serum followed by a change in medium the next morning. For FANCA and FANCD2 knockdown, U2OS cells were plated at a density of $2x10^5$ per well of a 6 well plate and transfected with 50 nM siRNA plus 5µl X-tremeGENE for 8 hours on sequential days. When delivering siRNA into cells cultured in 12 well plates, the conditions for transfection was reduced by one half. For most experiments, cells were treated with cisplatin or mitomycin C approximately 40 hours after the addition of siRNA. siRNAs directed against RAD18, Polη, REV1, REV3, REV7, FANCA, or FANCD2 were obtained from Qiagen. The gene-specific target sequences are as follows: RAD18-3 (GAG CAT GGA TTA TCT ATT CAA), Polη-1 (CTG GTT GTG AGC ATT CGT GTA), REV1-4 (ATC GGT GGA ATC GGT TTG GAA), REV3-2 (CCC ACT GGA ATT AAT GCA CAA), REV7-1 (GTG GAA GAG CGC GCT CAT AAA), FANCA-6 (AGG CCT ATG CTA ATC ATT CTA), FANCA-7 (CAG GGC CAT GCT TTC TGA TTT), FANCD2-2 (CAG AGT TTG CTT CAC TCT CTA), and FANCD2-5 (AAG CAG CTC TCT AGC ACC GTA). The negative control Non-si sequence (AAT TCT CCG AAC GTG TCA CGT) was purchased from Qiagen. To create stable RAD18 knockdowns, U2OS cells were infected with pLKO.1 lentivirus (Sigma-Aldrich) that encodes no shRNA (control) or shRNA targeting the following sequences: RAD18-2 (TGC TTC GAG TAT TTC AAC ATT) or RAD18-6 (ATG GTT GTT GCC CGA GGT TAA). Cells were selected in puromycin and used within one week after drug selection.

Assessment of cell viability. For clonogenic survival assays, HeLa cells were transfected overnight with siRNA as described above. When assessing survival following cisplatin treatment, cells were seeded at known densities the next day, allowed to attach to culture plates overnight, and then treated with cisplatin for two hours. Cells were washed and allowed to form colonies for approximately 12 days. Twelve days later, colonies were simultaneously stained and fixed in a solution containing 3:1 methanol and glacial acetic acid plus 1% trypan blue (Sigma). Colonies of 50 cells or greater were counted and the surviving fractions for each siRNA treatment group represent the plating efficiency for each treatment divided by the plating efficiency of the corresponding untreated control. The BL2 lymphoma lines were treated with cisplatin for 2 hrs and then harvested 48 hours later. Cells were then washed with PBS and assessed as to their ability to exclude the trypan blue as a measure of viability.

Antibodies. Rabbit polyclonal anti-Polη (H-300), anti-GAPDH (FL-335), and anti-REV1 (H-300) antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-CHK1 (ser345) and anti-phospho-Ser139 Histone H2AX were purchased from Cell Signaling and Active Motif respectively. The following mouse monoclonal antibodies were used in these studies: anti-PCNA PC10 (Ab-1, Oncogene Research Products), anti-RAD18 (3H7, Abnova), anti-phospho-histone H2A.X (Ser139, clone JBW301, Millipore), and anti-MAD2B/REV7 (BD Biosciences). The anti-β-tubulin (TUB 2.1) and anti-Flag M2 monoclonal antibodies were purchased from Sigma-Aldrich Inc.

Immunoblotting and rtPCR. Cells were lysed in a sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris pH 8.0, 2% SDS, 1 × protease inhibitor cocktail (Roche) and 1 × phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich)), sonicated, heated at 95°C, and equal amounts of protein were separated on SDS-PAGE gels. Proteins were transferred onto a nitrocellulose membrane and probed with the appropriate primary antibodies described above followed by secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse antibody (Thermo Scientific). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). To detect REV3L mRNA, total cellular RNA was extracted with TRIzol

reagent (Invitrogen), and 1μg of RNA was reverse transcribed with the High Capacity Reverse Transcription Kit (Applied Biosystems) according to manufacturer's protocol. REV3L cDNA were PCR amplified using primers flanking either side of the *REV3L* siRNA targeting sequence. GAPDH cDNA primers were included in each rtPCR reaction as an internal control. The following PCR primers were used in these studies: REV3L forward 5′-CTT TCT CAG ATG GCA TTC AG -3′, REV3L reverse 5′- TTT CGG AAC TTG ACA GCA GC-3′; GAPDH forward 5′- AAG GTC GGA GTC AAC GGA TTT GGT -3′, GAPDH reverse 5′- AGT GAT GGC ATG GAC TGT GGT CAT -3′. The PCR protocol was as follows: 94°C for 5 min, 25 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 2 min. The RT-PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining.

Immunofluorescence. For γ-H2AX, cells cultured on glass coverslips were washed in phosphate buffered saline (PBS) and then fixed in ice cold 100% methanol for 10 minutes. Samples were blocked with 5% fetal bovine serum, 0.05% Triton X-100, and 1% goat serum, and then incubated with primary antibodies for 45 minutes. Coverslips were washed three times with PBS and then incubated with the appropriate secondary goat anti-rabbit or goat anti-mouse Alexa Fluor dye conjugated secondary antibody (Molecular Probes) for 45 minutes, washed with PBS, counterstained with DAPI to visualize nuclear DNA, and then mounted onto slides with ProLong Gold antifade reagent (Invitrogen). For experiments assessing PCNA colocalization with EGFP-tagged proteins, soluble proteins were first extracted with a Triton X-100 containing buffer (0.5% triton X-100, 20 mM HEPES pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl and 300 mM sucrose) for 5 minutes at 4°C in order to reveal detergent extraction-resistant proteins in the nucleus. Cells were then fixed with a 3.7% para-formaldehyde solution for 20 minutes at 4°C, washed once with PBS, and then fixed again in ice cold 100% methanol to expose the PCNA (PC-10) epitope. Immunofluorescence staining for PCNA was the same as described above. To visualize EGFP-tagged proteins and FANCD2 in siRNA transfected U2OS cells, the cells were washed in PBS and then fixed in a 3.7% para-formaldehyde solution containing 0.5% Triton-X-100 for 20 minutes, stained for FANCD2 along with counterstaining with DAPI, and then mounted onto slides with Prolong Gold antifade reagent. Staining for FANCD2 protein was as described above.

Imaging was performed on either an Olympus BX-51 fluorescence microscope or Olympus FV-500 confocal microscope. Images were further processed and merged using Adobe Photoshop CS.

Flow cytometry. For single parameter flow cytometry, cells were fixed with ice cold 70% ethanol and then resuspended in PBS containing RNAse A and propidium iodide to determine DNA content. For two parameter flow cytometry, cells were fixed with 70% ice cold ethanol, blocked with 5% fetal bovine serum, 1% goat serum plus 0.05% Tween 20, and then stained with anti-γ-H2AX monoclonal antibody. Cells were washed, incubated with goat anti-mouse FITC-conjugated secondary antibody, and then counterstained with propidium iodide in PBS containing RNAse A. Cells were acquired on a BD FACSCaliber system using CellQuest software.

Lentiviral vectors and virus production. The cDNA for human *POLH* (Polη) and *REV1L* were purchased from Enzymax. Human *RAD18* cDNA was PCR amplified from I.M.A.G.E. clone No. 3451960 (American Tissue Culture Collection). *POLH*, *REV1*, and *RAD18* cDNAs were subloned into pEGFP-C1 (Clontech) such that EGFP was fused in frame at the amino terminal end of each protein. The entire cDNA encoding EGFP fused with the open reading frame of Polη, REV1, or RAD18 was excised and then subcloned into pLenti EV (University of Michigan Vector core facility) to generate pLLEV-EGFP-Polη, pLLEV-EGFP-REV1, and pLLEV-EGFP-RAD18. To generate lentivirus, 293T/17 cells were cotransfected with individual lentiviral vectors plus the following packaging plasmids at a 1:1:1:1 ratio (pLP1, pLP2, and pLP/VSVG, Invitrogen) using the calcium phosphate method to deliver plasmid DNA into cells. U2OS cells were seeded onto coverslips in twelve well plates and then infected with 293T/17 supernatent in the presence of 4 μg/ml polybrene overnight. The following morning, the medium was changed and treatments were initiated 24 hours later.

Results

Replicative bypass of cisplatin intrastrand cross-links requires PCNA monoubiquitination, Polη, and the REV1/ Polζ functional complex.

Upon entering a cell, the major adduct cisplatin forms is covalent bonds with the N^7 position of adjacent guanines within the same DNA strand [29]. Intrastrand cross-links are also formed between neighboring adenine-guanine bases and guanine-guanine bases separated by a cytosine. Approximately 2% of the DNA damage represents interstrand cross-links between guanines on opposite DNA strands. Both DNA intrastrand and interstrand cross-links have been proposed to contribute to the cytotoxicity of cisplatin [29-30]. To assess the importance of each component of the translesion synthesis pathway hypothesized as being necessary for replicative bypass of cisplatin DNA adducts (a simplistic model is shown in Fig. 2.1A), HeLa cells were transiently depleted of RAD18, polymerase eta (Polη), REV1, or polymerase zeta (REV3, and REV7) individually via siRNA and then analyzed for their ability to progress through the cell cycle following cisplatin-treatment along with their cellular response to DNA damage. The ability to specifically deplete endogenous proteins with siRNA in HeLa cells was determined by immunoblot analysis (RAD18, Polη, REV1, or REV7) (Fig. 2.1C and supplemental data Fig. S2.2). Since antibodies are not available to detect endogenous REV3, rtPCR was used to confirm reduction of REV3 mRNA following transfection with REV3-specific siRNA (Fig. 2.1B). Further validation of the specificity and potency of the siRNAs used in this study were examined by demonstrating the ability of different siRNA sequences targeting REV1 or REV3 to deplete ectopically expressed proteins in 293T cells (supplemental data Fig. S2.1). In addition, depletion of RAD18 via siRNA prevented efficient monoubiquitination of PCNA in response to UV-C or cisplatin demonstrating effective knockdown of the principle regulator of the translesion synthesis pathway (Fig. 2.1D).

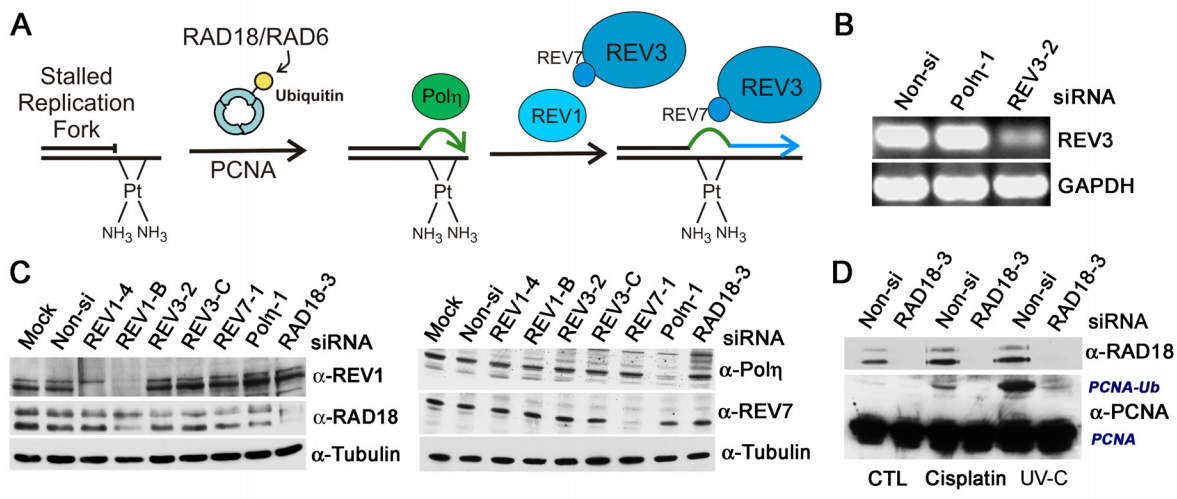


Figure 2.1 Model for lesion bypass of cisplatin intrastrand cross-links and validation of siRNAs used to deplete individual components of the TLS pathway. (A) RAD18 responds to stalled replication forks by monoubiquitinating PCNA at K164. This event serves as a molecular switch by increasing the affinity of Y-Family TLS polymerases for PCNA via their ubiquitin binding domains. Evidence suggests that Polη performs the initial nucleotide insertion step opposite the cisplatin intrastrand cross-link while Polζ (composed of the catalytic REV3 domain and the REV7 accessory subunit) performs the extension step beyond the initial insertion. Polζ activity requires REV1, a Y-Family polymerase thought to promote TLS polymerase switching events. See introduction for details**.** (B and C) HeLa cells were transfected with the indicated siRNAs as described in Materials and Methods. Forty-eight hours later, total cellular RNA was subjected to rtPCR using primers specific for either REV3 or GAPDH (B) or whole cell lysates were separated by SDS-PAGE and subjected to immunoblot analysis using the indicated primary antibodies (C). Non-si indicates a nonspecific control siRNA. (D) Depletion of RAD18 prevents monoubiquitination of PCNA. Control or RAD18-depleted HeLa cells were exposed to 40 J/m^2 UV-C or 150 μM cisplatin for 2 hours and then harvested 8 hrs later to measure the ubiquitination of PCNA. Whole cell lysates were subjected to immunoblot analysis with anti-PCNA antibody.

Phosphorylation of the histone variant H2AX at serine 139 occurs in response to DNA damage such as double strand breaks or replication fork stalling, which are primarily mediated by ATM and ATR, respectively [31], and is an extremely sensitive marker for the presence of DNA damage or perturbation of DNA replication. To determine if depletion of RAD18, Polη, REV1, or REV3 in HeLa cells leads to enhanced DNA damage following cisplatin treatment, these proteins were down-regulated in HeLa cells, exposed to cisplatin, and stained for γ-H2AX (Fig. 2.2A). Rather than seeing individual foci in cisplatin-treated cells, the nuclei of RAD18, Polη, REV1 or REV3

siRNA-transfected cells exhibited indistinguishable, intense, and almost diffuse staining patterns 24 hours after drug exposure. In comparison, non-specific (Non-si) siRNA-transfected cells displayed little staining suggesting these cells were capable of replicating DNA containing cisplatin intrastrand cross-links in the presence of an intact translesion synthesis pathway. Similar results were found when HeLa cells were transfected with several different siRNAs targeting RAD18, Polη, REV1, REV3 or REV7 thus demonstrating that the enhanced γ-H2AX response induced by cisplatin treatment is not due to siRNA off-target effects (supplemental data Fig. S2.3 and S2.4). In response to ionizing radiation, γ-H2AX staining patterns present as individual foci directly correlating with the number of DNA double strand breaks expected for a given dose [32]. Since it is unlikely that the intense γ-H2AX staining observed in RAD18, Polη, REV1, or REV3 knock-down cells represents mass accumulations of double strand breaks, this staining pattern is interpreted as an indication of extensive DNA replication stalling due to the inability to perform lesion bypass, and this in turn results in the induction of a sustained checkpoint response mediated by ATR [33-34].

To determine whether the increased γ-H2AX staining detected by immunofluorescence was specifically associated with cells residing in S phase, HeLa cells were co-stained with propidium iodide for DNA content along with γ-H2AX and analyzed individually by flow cytometry. REV7 knockdown was also examined to determine whether depletion gives rise to the same phenotype as observed with knockdown of RAD18, Polη, REV1, or REV3. Transfection of siRNA specific for each component of the translesion synthesis pathway, including REV7, was associated with essentially identical γ-H2AX responses in that the majority of cells arrested primarily in S and G2 phases of the cell cycle and stained positive for γ-H2AX after cisplatin treatment (Fig. 2.2B and C). This result also demonstrates that the gene identified as human *REV7* (*MAD2L2* or *MAD2B*) is likely to be the true homologue of yeast *Rev7* based on the observation that essentially an identical phenotype was exhibited in REV7-depleted cells as compared to REV3-depleted cells challenged with cisplatin. Depletion of translesion synthesis proteins using several different siRNA sequences targeting RAD18, Polη, REV1, REV3 or REV7 lead to similar cell cycle arrest profiles following cisplatin treatment demonstrating that the effects observed here are gene-specific

(supplemental data Fig. S2.5). Time course studies analyzing γ-H2AX formation by immunoblot analysis indicate that the γ-H2AX response developed slowly over time and became prominent by 16 hours after cisplatin treatment as cells accumulated in S phase (supplemental data Fig. S2.6). Consistent with enhanced ATR-dependent checkpoint activation in response to replication fork stalling, depletion of RAD18, Polη, REV1, REV3, or REV7 lead to increased phosphorylation of the CHK1 cell cycle checkpoint kinase, a direct and specific target of the ATR kinase (Fig. 2.2D) [13].

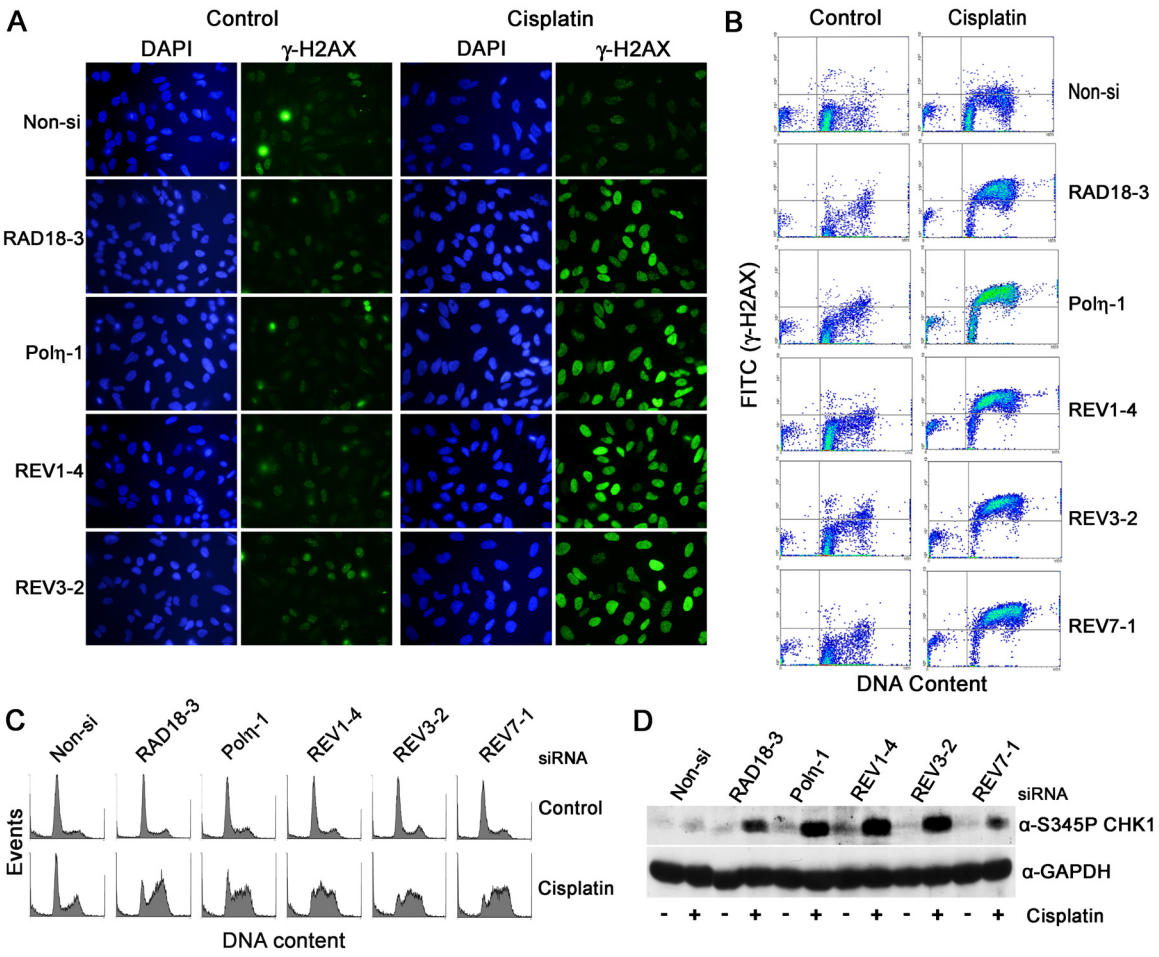


Figure 2.2 Lesion bypass of cisplatin adducts in Hela cells requires PCNA monoubiquitination by RAD18, Polη, and the REV1/Polζ functional complex. HeLa cells transfected with the indicated siRNAs were treated with 10 μM cisplatin for 1 hr. Twenty-four hours later the cells were fixed, stained for γ-H2AX (green), and then imaged by fluorescent microscopy (A) or analyzed by flow cytometry (B and C). Dot plots showing the level of γ-H2AX staining versus DNA content are shown in (B) and corresponding histograms from the same experiment showing DNA content per event are shown in (C). (D) RAD18, Polη, REV1, REV3 or REV7-depleted HeLa cells were

treated with 10 μM cisplatin as above. Whole cell lysates were subjected to immunoblot analysis for CHK1 specifically phosphorylated at Ser 345 and GAPDH as a loading control. Depletion of RAD18, Polη, REV1, REV3, or REV7 leads to an enhanced DNA damage response indicative of incomplete DNA replication in cisplatin treated cells.

The γ-H2AX staining patterns compared to cisplatin treatment were essentially identical both in intensity and pattern when RAD18, Polη, REV1, REV3, or REV7 siRNA-transfected HeLa cells were exposed to a relatively low dose of UV-C light (4 J/m^2) (Fig. 2.3). Depletion of translesion synthesis proteins using several different siRNA sequences targeting RAD18, Polη, REV1, REV3 or REV7 lead to similar γ-H2AX staining patterns via immunofluorescence following UV-C exposure demonstrating that the effects observed are gene-specific (supplemental data Fig. S2.7 and S2.8). The staining pattern observed in Figures 2.2A and 2.3 is very similar to other published reports where replication fork stalling activates ATR leading to pan-nuclear like γ-H2AX staining patterns [35]. Taken together, the data supports an insertion and extension model of translesion synthesis in a human cancer cell line where RAD18 along with translesion polymerases eta, REV1 and zeta are all necessary to bypass cisplatin and UV-C induced DNA intrastrand cross-links.

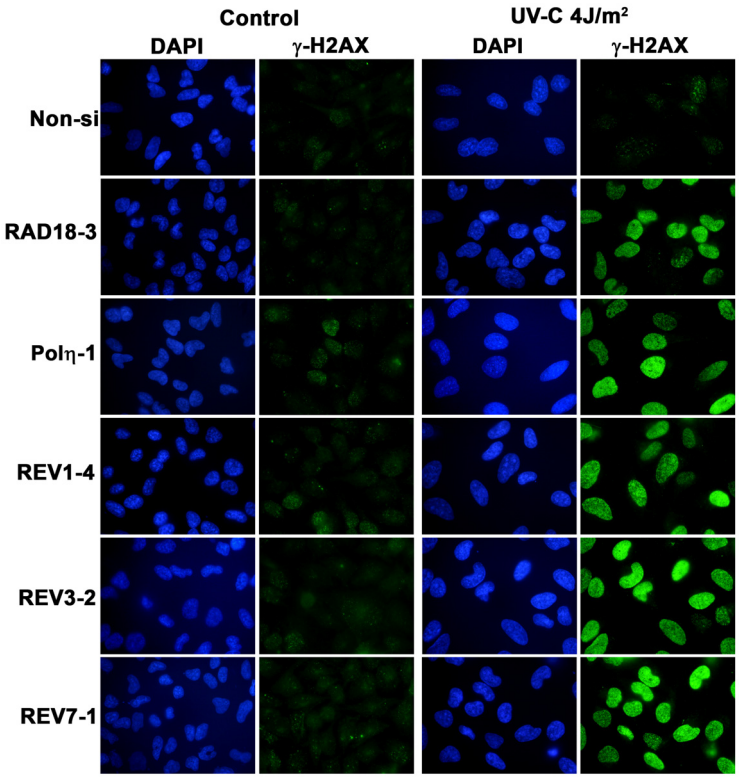


Figure 2.3 RAD18, Polη, and the REV1/Polζ functional complex are necessary for lesion bypass of UV-C adducts. HeLa cells depleted of RAD18, Polη, REV1, REV3 or REV7 individually were exposed to 4 J/m^2 UV-C radiation. Twenty-four hours later the cells were fixed, stained for γ-H2AX (green), and then imaged by fluorescent microscopy.

RAD18, polymerase eta, and REV1 colocalize with PCNA at sites of cisplatin-induced replication stress

To gain additional evidence that RAD18, polymerase eta, and REV1 are involved in replicative bypass of cisplatin intrastrand cross-links, the proteins were assessed as to whether they colocalize with PCNA foci in a cisplatin-dependent manner. To determine sites of cisplatin-induced stalled replication forks, PCNA foci were utilized as a surrogate marker of replication stress. To distinguish whether cisplatin-induced PCNA foci represent sites of replication stress (*e.g.* stalled replication forks) as opposed to unperturbed DNA replication, a determination was made as to whether cisplatin-induced PCNA foci co-localize with γ-H2AX. As expected, untreated U2OS cells exhibited both PCNA and γ-H2AX foci; however, these foci rarely colocalized (Fig. 2.4A). In response to agents known to cause replicative fork stalling, helicase-polymerase uncoupling and monoubiquitination of PCNA, such as UV-C, hydroxyurea, aphidicolin or cisplatin, PCNA focus formation greatly increased and exhibited significant colocalization with γ-H2AX (Fig. 2.4A and B). Even though replication forks may stall due to DNA double strand breaks or interstrand cross-links, these lesions are unlikely to promote or cause helicase-polymerase uncoupling thus prohibiting formation of long stretches of single stranded DNA. Chang *et al.* demonstrated that the single stranded DNA formed due to helicase-polymerase uncoupling at sites of stalled replication forks is coated with RPA and is crucial for localizing the RAD6 (E2)-RAD18 (E3) ubiquitin ligase to sites of replication stress [36]. As expected, monoubiquitination of PCNA was not detected after exposure to ionizing radiation (DNA double strand break inducer), mitomycin C (interstrand cross-linker), or camptothecin (topoisomerase I poison that induces formation of replication-associated DNA double strand breaks) and extensive colocalization was not observed between PCNA and γ-H2AX (Fig. 2.4A and B). Taken together, PCNA foci mark sites of replication stress induced by lesions that promote induction of RAD18-dependent PCNA monoubiquitination.

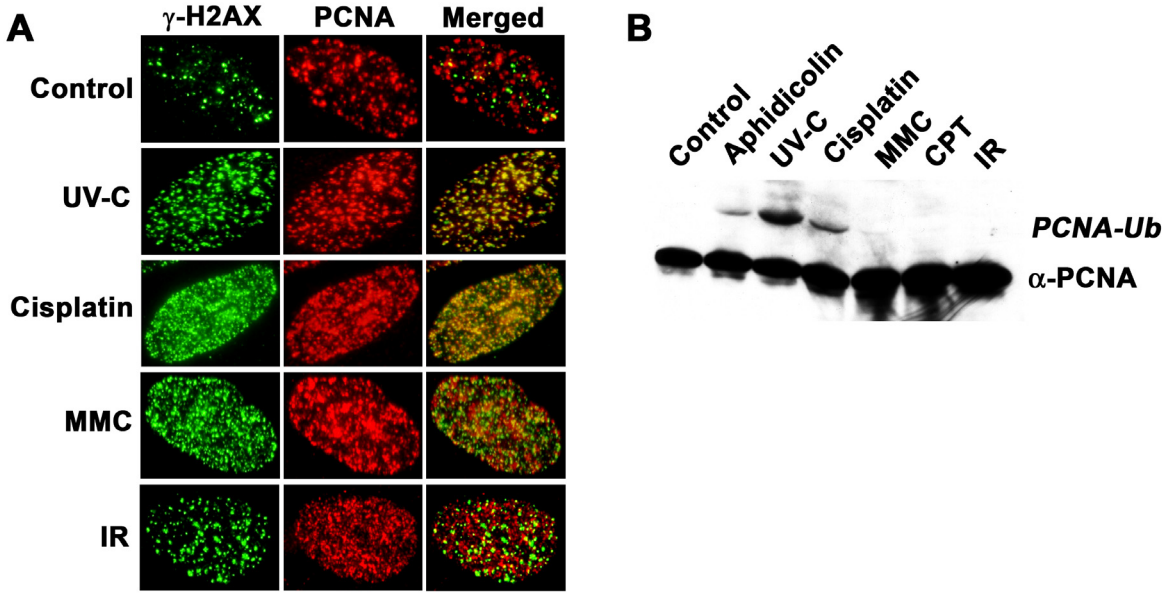


Figure 2.4 Agents that induce replication fork stalling and helicase/replicase uncoupling promote extensive colocalization between PCNA and γ-H2AX foci along with PCNA monoubiquitination. (A) U2OS cells grown on coverslips or 6 well dishes were left untreated or treated with 8 J/m^2 UV-C, 33 μM cisplatin for 2 hours, 100 ng/ml mitomycin C (MMC) for 2 hours, or 10 Gy ionizing radiation (IR). Six hours later, Triton-X-100 detergent soluble proteins were extracted from the nuclei prior to fixation with para-formaldehyde followed by additional fixation with 100% methanol to reveal the PCNA epitope. Cells were stained with anti-γH2AX (green) and anti-PCNA (red) and then imaged on an Olympus BX-51 fluorescence microscope. (B) HeLa cells were exposed to 5 μM aphidicolin, 40 J/m^2 UV-C, 150 μM cisplatin for 2 hours, 300 ng/ml mitomycin C (MMC) for 2 hours, 100nM camptothecin (CPT), or 10 Gy ionizing radiation (IR). Cells were harvested 24 hours after the initiation of treatment, lysed, and subjected to SDS-PAGE. Shown is a representative immmunoblot probing for PCNA. MMC, CPT and IR did not induce detectable PCNA monoubiquitination even when cells were treated with or exposed to relatively high doses of drug or radiation.

To determine if RAD18, polymerase eta, and REV1 localize to sites of cisplatin-induced replication stress, replication-deficient lentivirus was engineered to deliver EGFP-tagged *RAD18*, *POLH*, or *REV1* genes into mammalian cells in a highly efficient manner such that nearly 100% of cells express EGFP-tagged protein after lentiviral transduction. Since HeLa cells expressed extremely low levels of EGFP-REV1 protein, U2OS osteosarcoma cells were used for these studies. In agreement with previous studies, untreated or cisplatin exposed cells exhibited EGFP-Polη foci that colocalize

nearly 1 to 1 with PCNA foci (Fig. 2.5A and D) [21, 37]. Following cisplatin treatment, the number of EGFP-Polη foci within a given cell increased dramatically (Fig. 2.5A and supplement data Fig. S2.9). In contrast, a significant number of untreated cells exhibited EGFP-REV1 or RAD18 foci that failed to show co-localization with PCNA suggesting these two proteins do not strictly localize to replication factories. In response to cisplatin treatment, the majority of cisplatin-induced EGFP-REV1 or RAD18 foci colocalized with PCNA, similar to EGFP-Polη (Fig. 2.5A and D). While the majority of EGFP-RAD18 expressing cells contained 10 or greater foci in the presence or absence of cisplatin treatment, EGFP-REV1 expressing cells exhibited cisplatin-induced foci formation (Fig. 2.5A and supplemental data Fig. S2.9). Cisplatin-induced EGFP-Polη, REV1 and RAD18 foci also displayed extensive colocalization with γ-H2AX (Fig. 2.5B). Consistent with the model that Polη and REV1 cooperate to perform lesion bypass of cisplatin-DNA adducts, cisplatin induced the formation of foci consisting of EGFP-Polη and Flag-REV1 colocalized together (Fig. 2.5C).

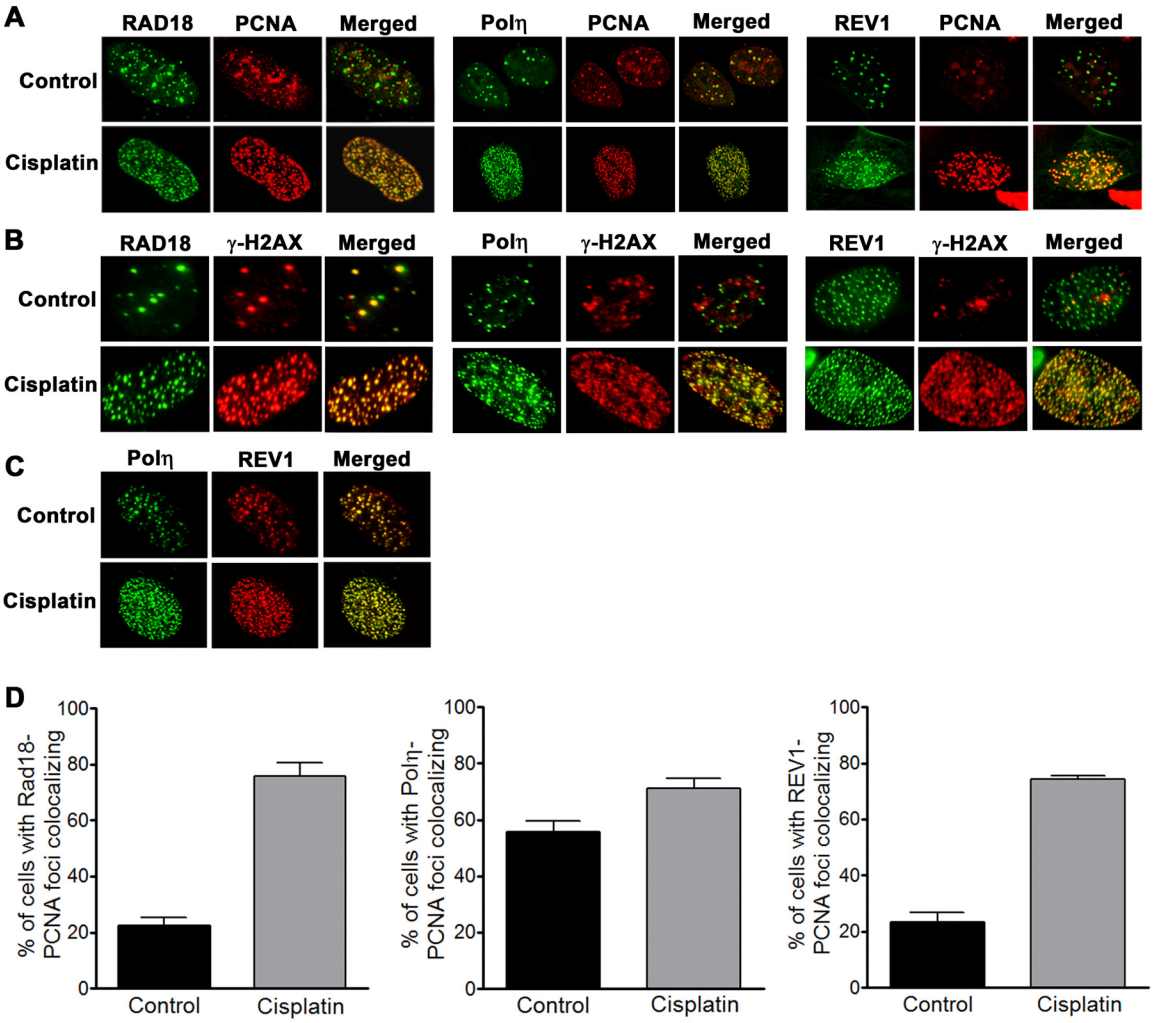


Figure 2.5 RAD18, Polη and REV1 localize to sites of cisplatin-induced replication stress marked by PCNA and γ-H2AX. (A and B) Cisplatin induces RAD18, Polη, and REV1 foci that extensively colocalize with PCNA and γ-H2AX. U2OS cells grown on cover slips were infected with lentivirus designed to express EGFP-tagged RAD18, Polη, or REV1 as described in Materials and Methods. Cells were treated with 33 μM cisplatin for 2 hrs, allowed to recover for 8 hours and then fixed. Prior to fixation, Triton-X-100 soluble proteins were extracted from nuclei in order to enhance visualization of focus formation. Samples were stained with anti-PCNA antibody (red) and imaged using confocal microscopy (A) or stained with anti-γ-H2AX antibody (red) and imaged using an Olympus BX-51 fluorescence microscope (B). (C) Cisplatin induces the formation of Polη and REV1 foci that colocalize. U2OS cells were first infected with lentivirus encoding EGFP-tagged Polη. Thirty hours later, the same cells were infected with lentivirus encoding Flag-tagged REV1. Cells were treated with cisplatin, fixed as described above, and then stained with anti-Flag antibody (red). Cells were imaged using confocal microscopy. (D) Graphs represent the average percentage of cells exhibiting greater than 10 foci per cell of either EGFP-tagged RAD18, Polη or REV1 foci

colocalizing with PCNA foci. Data represent the mean ± S.E.M of three independent experiments where >100 cells were counted in each experiment.

RAD18 is essential for localization of polymerase eta and REV1 to cisplatin-induced stalled replication forks

As described in Figure 2.5 the translesion polymerases eta and REV1 form cisplatin-induced foci at sites of stalled replication forks. To access how foci formation is regulated in response to replication stress, RAD18 depleted U2OS were examined to determine whether polymerase eta and REV1 foci form in response to cisplatin treatment. The RAD6 (E2)-RAD18 (E3) ubiquitin ligase complex specifically monoubiquitinates PCNA in response to replication fork stalling and is thought to be a molecular switch between high fidelity synthesis and translesion DNA synthesis [38-41]. Cells deficient of RAD18 are known to be hypersensitive to DNA damaging agents that induce replication fork stalling signifying RAD18 is necessary for a proper response to replication stress [42-45]. Furthermore, the affinity of translesion polymerases such as eta and REV1 to PCNA drastically increases following monoubiquitination due to interactions via UBZ and UBM domains respectively [7, 46]. Therefore, RAD18 is thought to be the primary initiator of translesion synthesis promoting localization of translesion polymerases to the site of replication stress. However, discrepancies in the literature exist over whether the monoubiquitination of PCNA is truly a regulator of translesion synthesis [47].

U2OS cells with stable knockdown of RAD18 were developed as described in Materials in Methods. RAD18 was depleted from U2OS cells via two different shRNA sequences independently of each other. Exposure of the RAD18-depleted cells to cisplatin did not result in monoubiquitination of PCNA demonstrating efficient stable knockdown of RAD18 (Fig. 2.6A). After stable knockdown of RAD18 was established, both EGFP-Polη and EGFP-REV1 were introduced into U2OS cells as in Figure 2.5. In the cells deficient of RAD18, EGFP-Polη or EGFP-REV1 foci formation was abrogated in response to cisplatin treatment (Fig. 2.6B, C, and D). The reduction in both EGFP-Polη or EGFP-REV1 foci formation in the RAD18-depleted cells suggests RAD18 is an important initiator of translesion synthesis. Taking into consideration the data presented here and in Figure 2.5C, both polymerase eta and REV1, via RAD18 regulation, appear

to operate together in the same translesion synthesis pathway to bypass cisplatin adducts consistent with previously published reports [15].

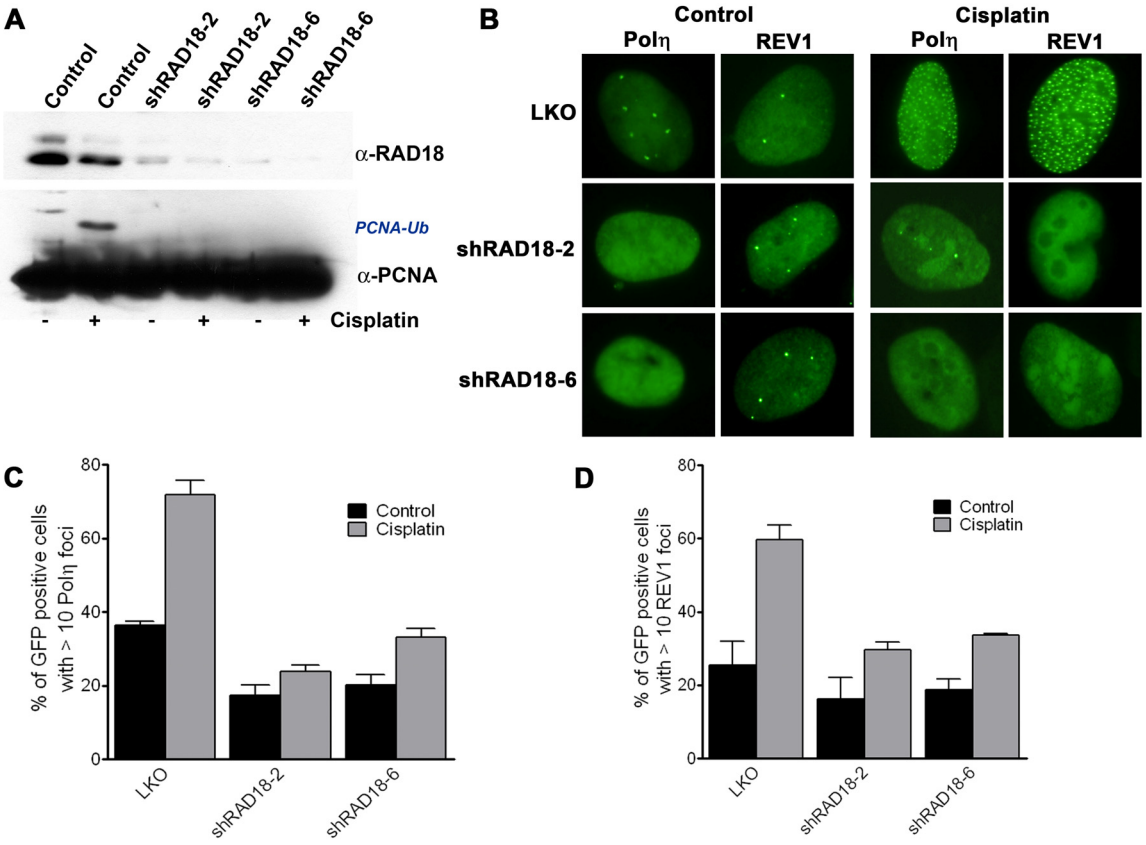


Figure 2.6 RAD18 is necessary for efficient Polη and REV1 focus formation in U2OS cells following cisplatin treatment. U2OS cells were infected with lentivirus designed to deliver shRNA targeting RAD18 as detailed in Material and Methods. (A) Control or RAD18-depleted HeLa cells were exposed to 150 μM cisplatin for 2 hours and then harvested 24 hrs later to measure the ubiquitination of PCNA. Whole cell lysates were subjected to immunoblot analysis with anti-PCNA antibody. (B) U2OS cells stably depleted of RAD18 via two different shRAD18 sequences independently of each other were grown on cover slips and infected with lentivirus designed to express EGFP-tagged Polη or REV1. Cells were treated with 33 μM cisplatin for 2 hrs, allowed to recover for 6 hours and then fixed with para-formaldehyde and imaged using an Olympus BX-51 fluorescence microscope. (C and D) Graphs represent the average percentage of cells exhibiting greater than 10 foci of either EGFP-tagged Polη or REV1. Data represent the mean ± S.E.M of three independent experiments where >100 cells were counted in each experiment.

The Fanconi anemia (FA) core complex recruits REV1, but not Polη, to sites of cisplatin-induced replication stress

Given that RAD18 appears to initiate translesion synthesis at sites of cisplatin-induced replication stress, it was of interest to determine if any other proteins may contribute to the regulation of translesion synthesis. Several lines of evidence implicate the Fanconi anemia pathway in regulating REV1 and polymerase zeta-dependent translesion synthesis. First, cells deficient in components of the Fanconi anemia pathway have been characterized as being hypomutagenic at the *HPRT* locus [48-50]. In addition to the genetic epistasis observed between FANCC and REV1 or REV3 for cisplatin sensitivity in DT40 cells, FANCC-deleted cells are also deficient for somatic hypermutation of abasic sites at the IgM locus, a process dependent upon the TLS pathway [51]. Mirchandani *et al.* have recently shown that the Fanconi anemia core complex is necessary for both spontaneous and UV-C induced mutagenesis, as well as proper recruitment of ectopically expressed REV1 protein to nuclear foci upon exposure to UV-C irradiation [52]. Interestingly, regulation of REV1 localization into UV-C induced foci does not require the Fanconi anemia complementation group I (FANCI) or Fanconi anemia complementation group D2 (FANCD2) proteins, which are believed to act downstream of the FA core complex to direct DNA repair. Regulation of REV1 focus formation by the FA core complex also appears to be a parallel pathway to RAD18-dependent PCNA monoubiquitination since localization of RAD18 and its activity are unperturbed in UV-C irradiated cells deficient in FANCA or FANCG protein, components of the Fanconi anemia core complex [52]. Based on these observations, the influence of the FA-pathway on recruitment of REV1 or Polη into foci after cisplatin treatment was investigated.

U2OS cells were transfected twice with two different siRNAs specific for either the FANCA or FANCD2 mRNA, and then infected with lentivirus encoding EGFP-RAD18, EGFP-Polη, or EGFP-REV1. Cells were exposed to cisplatin, fixed, and then analyzed for foci formation. Eliminating FANCA or FANCD2 expression had little impact on cisplatin-induced RAD18 or Polη foci formation (Fig. 2.7A and B). However, cells depleted of FANCA protein failed to exhibit cisplatin-induced EGFP-REV1 foci

demonstrating that the Fanconi anemia core complex specifically regulates REV1 localization at stalled replication forks. The efficiency of FANCA knockdown was confirmed by assessing disruption of cisplatin-induced FANCD2 foci formation (Fig. 2.7C) and also by demonstrating that FANCD2 monoubiquitination was abrogated in FANCA siRNA transfected cells (Fig. 2.7D). Both FANCD2 foci formation and monoubiquitination are known to require a functional Fanconi anemia core complex [53].

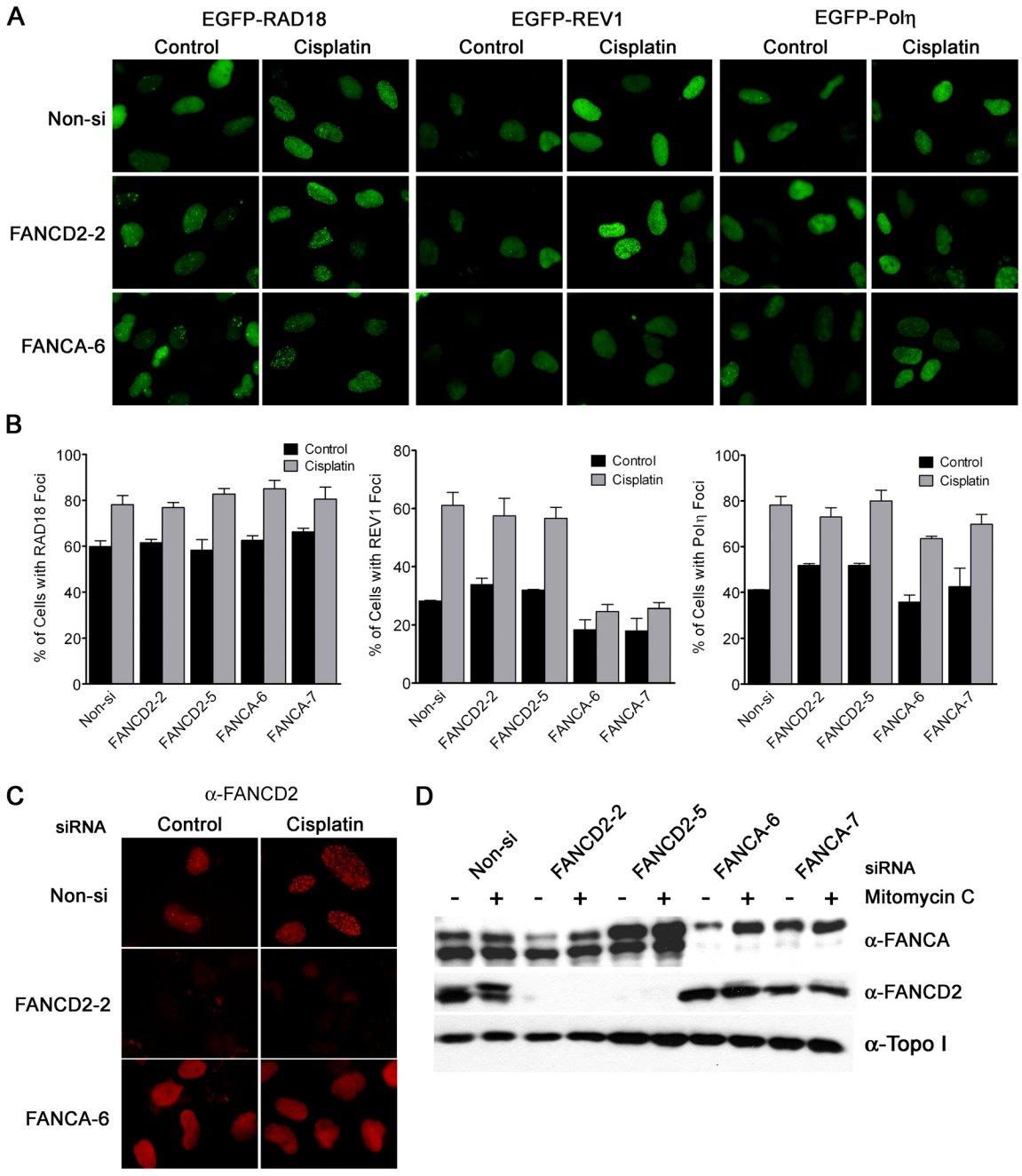


Figure 2.7 The Fanconi anemia core complex is necessary for cisplatin-induced

REV1 focus formation. U2OS cells were transfected twice with Non-si siRNA or siRNA specific for FANCA or FANCD2 mRNA. After the second transfection, cells were infected with lentivirus encoding EGFP-RAD18, Polη, or REV1 as described in Materials and Methods. Forty hours after infection, cells were treated with 33 μM cisplatin for 2 hrs and 6 hrs later samples were fixed in para-formaldehyde plus 0.5% Triton-X-100, and then stained for FANCD2 protein. Cells were imaged using an Olympus BX-51 fluorescence microscope. (A) FANCA-deficient cells are defective in REV1 focus formation in response to cisplatin treatment. (B) The percentage of EGFP-RAD18, Polη or REV1 positive cells exhibiting 10 or more foci per cell was determined. Results represent the mean ± S.E.M. of two independent experiments. Effective knockdown of FANCA or FANCD2 protein was assessed by analyzing FANCD2 foci formation by immunofluorescence (C) and FANCD2 monoubiquitination by immunoblotting (D). Depletion of FANCA protein abrogates both cisplatin-induced FANCD2 foci formation and mitomycin C-induced FANCD2 monoubiquitination.

REV1, REV3 and REV7 protect against cisplatin-induced cytotoxicity

Since depletion of RAD18, Polη, REV1, REV3, or REV7 lead to indistinguishable responses with regards to phosphorylation of H2AX and cell cycle redistribution (Fig. 2.2), the expectation was that knock-down of these proteins in HeLa cells would result in equivalent hypersensitivity to cisplatin as measured by a standard colony formation assay. RAD18 or the translesion synthesis polymerases were noted to promote tolerance of cisplatin. However, REV1, REV3, or REV7 siRNA-transfected HeLa cells were found to exhibit greater sensitivity to cisplatin-induced loss in clonogenicity at lower drug concentrations (*e.g.* 1 and 3 μM) suggesting that REV1 and polymerase zeta may perform additional roles in promoting tolerance to cisplatin (Fig. 2.8A). To confirm this observation, human Burkitt's lymphoma BL2 cells deleted of *POLH* (eta)*, POLI* (ίota) or *REV3L* (332 and 504) were treated with various concentrations of cisplatin for 2 hours and viability measured 48 hours later by trypan blue dye exclusion (Fig. 2.8B). Consistent with the results observed in HeLa cells transfected with REV3 siRNA, REV3 knockout BL2 cells were significantly more sensitive to cisplatin cytotoxicity as compared to Polη or Polι knockout cells.

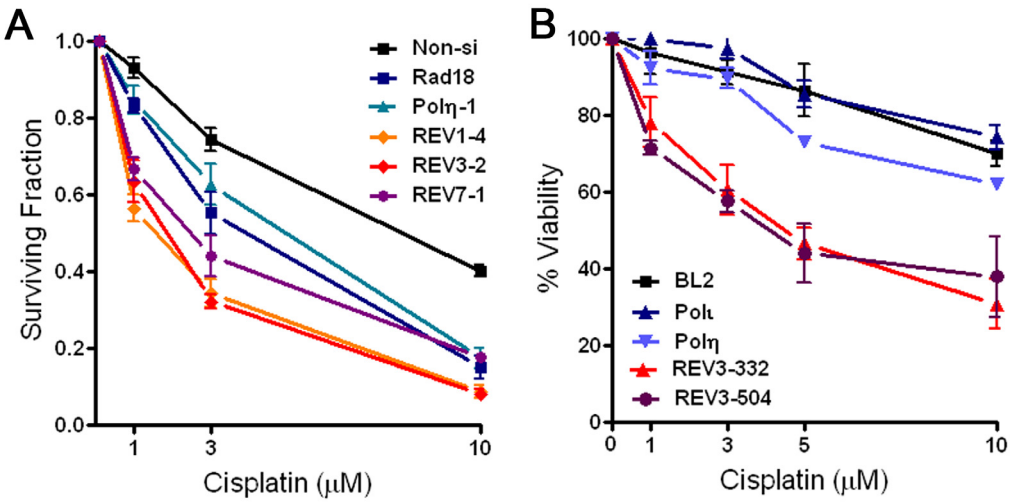


Figure 2.8 Depletion of REV1, REV3 or REV7 renders HeLa or BL2 cells hypersensitive to cisplatin-induced cytotoxicity. (A) HeLa cells were transfected with control (Non-si) siRNA or siRNA targeting RAD18, Polη, REV1, REV3 or REV7. Cells were seeded at known densities, treated with cisplatin for 2 hrs the following day, and then allowed to form colonies for approximately 12 days. Data is expressed as the average surviving fraction ± S.E.M. for each dose of cisplatin. (B) Wild type BL2 cells or BL2 cells lacking expression of polymerase ίota (Polι), Polη, or REV3 (clones 332 and 504) were treated with various doses of cisplatin for 2 hrs. Two days later, the cells were harvested and subjected to the trypan blue exclusion assay to assess viability. Data is expressed as the average percentage of cells stained negative for trypan blue, normalized to untreated control. At least three independent experiments were performed. Error bars represent S.E.M.

Discussion

Here the demonstration is made that elimination of translesion synthesis activity can significantly alter the ability of cells to progress through the cell cycle when treated with the anti-cancer drug cisplatin. In order to fully comprehend the complete molecular apparatus that performs translesion synthesis across cisplatin DNA adducts in intact cells, RAD18, polymerase eta (Polη), REV1, and polymerase zeta (REV3 and REV7) protein were individually depleted via siRNA in HeLa cells and their individual responses to cisplatin were measured. The data demonstrate that PCNA monoubiquitination by RAD18 is necessary to promote translesion synthesis across these lesions. Eliminating this essential first step in the translesion synthesis pathway resulted in the accumulation

of cells in S and G2 phases of the cell cycle and this block of cell cycle progression was associated with a markedly enhanced cell cycle checkpoint response as measured by the phosphorylation of H2AX and the CHK1 kinase. The results indicate that RAD18-depleted cells experienced profound replication stalling due to the inability to bypass cisplatin adducts during DNA replication.

Furthermore, depletion of RAD18 inhibited cisplatin-induced polymerase eta and REV1 focus formation. The fact that Polη, REV1, REV3, or REV7-depleted cells exhibited essentially identical responses to cisplatin as RAD18-depleted cells, demonstrates that polymerase eta, REV1, and polymerase zeta cooperate in order to achieve efficient bypass of cisplatin intrastrand cross-links, the major cisplatin adduct formed on DNA. Consistent with the model that polymerase eta and REV1 cooperate to perform lesion bypass of cisplatin-DNA adducts, cisplatin induced the formation of foci consisting of EGFP-Polη and Flag-REV1 colocalized together (Fig. 5C).

The Y-Family polymerase kappa has also been implicated in performing the insertion step during error prone replicative bypass of cisplatin adducts [15]. The data presented here indicates that polymerase eta is the primary translesion synthesis polymerase that performs the insertion step opposite these lesions during replication of genomic DNA, at least in HeLa cells. This is based on the observation that eliminating polymerase eta expression is just as effective as eliminating REV1, REV3 or REV7 with regards to the ability of cisplatin-treated cells to progress through S phase. Also, phenotypes associated with the phosphorylation of H2AX (Fig. 2.2) is basically identical in RAD18, polymerase eta, REV1 or polymerase zeta depleted HeLa cells exposed to cisplatin. Overall, the findings agree with those of Shachar *et al.* [15], and provide evidence supporting the multiple polymerase insertion and extension model for translesion synthesis across cisplatin intrastrand cross-links in mammalian cells. Together, these observations are consistent with a model where polymerase eta performs the insertion step across a 1,2-d(GpG) cisplatin adduct and polymerase zeta completes translesion synthesis by extending beyond the initial insertion, with the latter polymerase zeta step facilitated through REV1 interactions (Fig. 2.1A).

Consistent with this model, polymerase eta and REV1 form distinct foci in response to cisplatin treatment which colocalize with PCNA. Whether or not translesion polymerase focus formation truly represents an increase in actual translesion synthesis activity at stalled replication forks is not known; however, these visible foci likely represent increased residency times of Y-Family translesion polymerases associated with monoubiquitinated PCNA [54]. The fact that cisplatin-induced PCNA foci also colocalized with γ-H2AX strongly suggests that these sites coincide with a replication stress response that effectively results in the localized phosphorylation of H2AX. It is interesting to note that translesion polymerase focus formation appears to occur only in response to DNA damaging agents that are thought to cause uncoupling of replicative helicase and polymerase activities as an indirect consequence of replication fork stalling [34]. The generation of RPA-primed single stranded DNA, an end result of extensive DNA unwinding at a stalled replication fork, is essential for triggering RAD18-dependent monoubiquitination of PCNA [55-56]. Consistent with this model, DNA damage-induced polymerase eta and REV1 focus formation occurs in response to agents that strongly induce PCNA monoubiquitination, such as UV-C irradiation, hydroxyurea, aphidicolin, and cisplatin [57-60]. Treatments which predominantly introduce interstrand cross-links (*e.g.* mitomycin C) or alternatively, DSBs (*e.g.* ionizing radiation and camptothecin, a topoisomerase I poison), do not effectively induce PCNA monoubiquitination or translesion polymerase focus formation (Fig. 2.4) [37, 57, 61].

In addition to RAD18, the Fanconi anemia core complex was found to have a regulatory role in translesion synthesis. In the absence of the Fanconi anemia core complex protein FANCA, REV1 focus formation following cisplatin treatment was defective. Interestingly, RAD18 and polymerase eta foci formation in response to cisplatin exposure was unaffected in FANCA deficient cells, suggesting the regulatory function of the Fanconi anemia core complex during translesion synthesis is specific to REV1. Furthermore, the depletion of FANCD2 appeared to have no effect upon RAD18, polymerase eta or REV1 focus formation. This result was somewhat surprising since FANCD2 is monoubiquitinated by the core complex and the Y-Family translesion polymerases have a high affinity for monoubiquitinated proteins. The observation that localization of REV1 into foci following cisplatin treatment is deficient in cells lacking

FANCA, but not FANCD2 protein, is consistent with previously published models that Fanconi anemia core proteins facilitate REV1-dependent translesion synthesis [52].

Taken together, a model can be proposed highlighting the stepwise approach to translesion synthesis past cisplatin adducts (Fig. 2.9). In response to cisplatin intrastrand cross-links, the helicase and polymerase uncouple at the site of stalled forks leading to regions of single stranded DNA. RPA coats the single stranded DNA and promotes localization of RAD18 in an ATR-independent manner to sites of replication stress [55, 62]. As discussed in Figure 2.4, agents which do not allow helicase and polymerase uncoupling also do not promote monoubiquitination of PCNA, implying that single strand DNA formation is essential for recruitment of RAD18 to sites of stalled replication forks. Once localized to the stalled forks, RAD18 monoubiquitinates PCNA on lysine 164. The monoubiquitination of PCNA is thought to act as a molecular switch between high fidelity synthesis and translesion synthesis by promoting the localization of translesion polymerases to the site of stalled forks. In agreement with this model, depletion of RAD18 suppresses polymerase eta and REV1 foci formation suggesting PCNA monoubiquitination is indeed vital for localization of the Y-Family polymerases to sites of replication stress (Fig. 2.6). Polymerase eta inserts a cytosine opposite the 3'dG of a 1,2-d(GpG) cisplatin adduct, and is thought to perform this function independently of the REV1/polymerase zeta functional complex [26]. Data presented in Figure 2.7 demonstrated that the Fanconi anemia core complex has a regulatory role in REV1 but not polymerase eta focus formation following cisplatin exposure, thus polymerase eta can localize to the sites of replication stress independently of both REV1 and the Fanconi anemia core complex. In this stepwise approach to translesion synthesis, PCNA monoubiquitination, the Fanconi anemia core complex and possibly polymerase eta [63] are all necessary for REV1 focus formation at sites of stalled forks. REV1 is thought to promote localization of polymerase zeta to sites of replication stress and facilitate a translesion polymerase switch between polymerase eta and polymerase zeta. Polymerase zeta extends beyond the damaged DNA completing translesion synthesis past a cisplatin adduct.

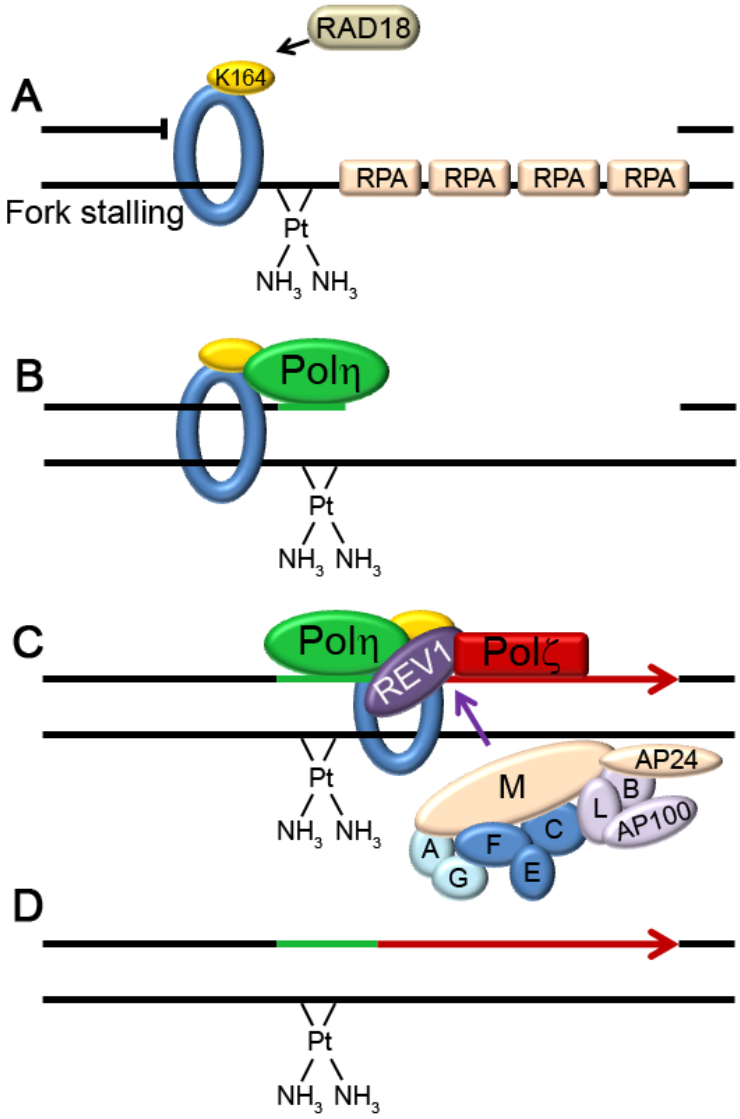
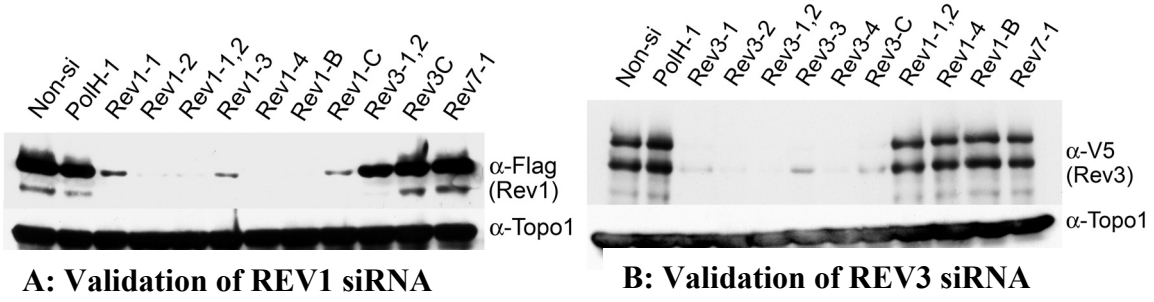


Figure 2.9 A stepwise approach to translesion synthesis past cisplatin adducts. (A) A replication fork encounters a cisplatin intrastrand cross-link and stalls, thus leading to helicase and polymerase uncoupling and the formation of single stranded DNA (ssDNA). Replication may be reinitiated downstream of the stalled fork creating a ssDNA gap. RPA coats the single stranded DNA, and in an ATR-independent manner RAD18 localizes to the site of the stalled fork and monoubiquitinates PCNA on lysine 164. (B) Polymerase eta (Polη) localizes to the site of the stalled replication fork and synthesizes directly across from the cisplatin adduct. (C) REV1/Polζ complex localizes to the stalled replication fork in a monoubiquitinated PCNA**,** Polη, Fanconi anemia core complex dependent manner. The Fanconi anemia core complex likely translocates to the site of replication stress via FANCM-FAAP24 interactions. Once located at the stalled fork polymerase zeta (Polζ) synthesizes beyond the cisplatin intrastrand cross-link filling the ssDNA gap. (D) The completed bypass of a cisplatin intrastrand-cross link. The cisplatin adduct is not removed, but translesion synthesis across the lesion allows the cell more time to cope with the DNA damage, thus promoting resistance to cisplatin intrastrand cross-links.

Lastly, RAD18 along with the translesion polymerases eta, REV1 and zeta were all found to promote cisplatin tolerance noted by the loss of survival in cisplatin treated HeLa cells depleted of these proteins. Unexpectedly, depletion of REV1, REV3 or REV7 resulted in a significantly greater sensitivity to cisplatin when compared to RAD18 or polymerase eta knockdown. The hypersensitivity of REV3-depleted HeLa cells to cisplatin was confirmed in *REV3L*$^{-/-}$ BL2 cells. The results suggest that REV1 and polymerase zeta may have additional biological roles beyond lesion bypass in HeLa cells exposed to cisplatin. Chapters 3 and 4 will address if the REV1/polymerase zeta functional complex participates in other biological pathways besides lesion bypass of DNA adducts.

Here, evidence was provided to support an insertion and extension model in human cells where RAD18 along with the translesion polymerases eta, REV1 and zeta are all required for bypass of cisplatin intrastrand cross-links. Furthermore, RAD18 along with the Fanconi anemia core complex had an important regulatory role over translesion synthesis past cisplatin adducts.

Supplemental Data

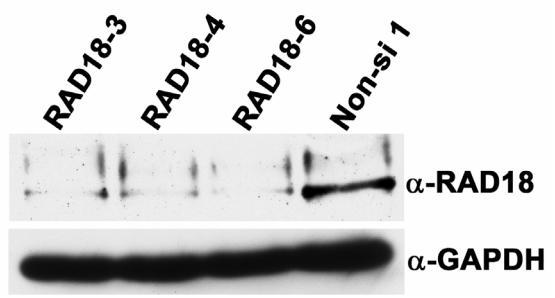


A: Validation of REV1 siRNA **B: Validation of REV3 siRNA**

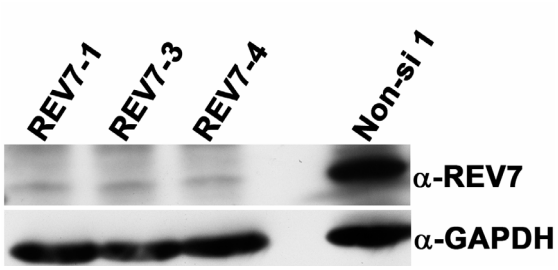
Figure S2.1 Validation of REV1 and REV3 siRNA specificity. Multiple siRNAs were individually tested as to their ability to downregulate ectopically expressed epitope-tagged REV1 (A) or REV3 (B) proteins. cDNA encoding REV1 (Enzymax) was subcloned into the pSG5 vector (Stratagene) such that the Flag epitope was placed at the amino terminus of the REV1 open reading frame. cDNA encoding human REV3L was amplified through rtPCR from HeLa total RNA using Pfu DNA polymerase (Stratagene). Primers for cDNA amplification were designed such that the entire coding frame for REV3L was subcloned in frame with the carboxy-terminal V5-His epitope tag of pEF6/V5-HisA (Invitrogen) and the insert was verified by DNA sequence analysis. For the data shown in A and B, 293T/17 cells were cotransfected with either Flag-REV1 or V5-REV3 expression plasmids plus the indicated siRNAs using X-tremeGENE (Roche) according to manufacturer's suggestions. REV1-1,2 and REV3-1,2 indicate that a combination of REV1-1 and REV1-2 or REV3-1 and REV3-2 siRNAs were used together at a 1:1 ratio. Total cell lysates were separated by SDSPAGE, transferred to nitrocellulose membrane and probed with either anti-Flag (M2) antibody (Sigma) or anti-V5 monoclonal antibody (Invitrogen). Membranes were immunoblotted for Topoisomerase 1 as a loading control. All siRNAs were purchased from Qiagen with the exception of REV1-B, REV1-C, and REV3-C which were obtained from Ambion. This work was performed by Christine Canman.

The gene specific target sequences for each individual siRNA used here and throughout the supplemental data section are as follows:

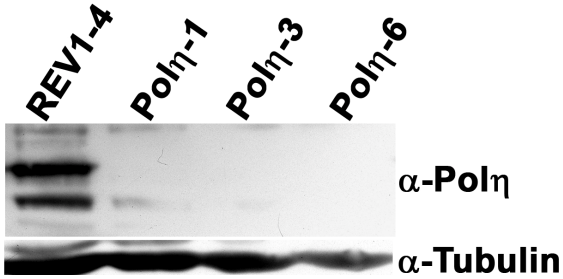
Non-si 1	AATTCTCCGAACGTGTCACGT	REV3-4	ATGAGTATGGATCATATACAA
Non-si 2	Qiagen AllStars Neg. Control (proprietary)	REV3-C	AAGCAATTTTGAACCTTATGG
REV1-1	AGGAGATATGTCAGTATTGAA	REV7-1	GTGGAAGAGCGCGCTCATAAA
REV1-2	CAGCGCATCTGTGCCAAAGAA	REV7-3	AAGATGCAGCTTTACGTGGAA
REV1-3	CTGCCAGGTCCAAGCAATATA	REV7-4	CACCCGGAGCTGAATCAGTAT
REV1-4	ATCGGTGGAATCGGTTTGGAA	Polη-1	CTGGTTGTGAGCATTCGTGTA
REV1-B	AAGCATCAAAGCTGGACGACT	Polη-3	CAGCCAAATGCCCATTCGCAA
REV1-C	AACCAGTAAATGGCTGTAATA	Polη-6	CCCGCTATGATGCTCACAAGA
REV3-1	CGGGATGTAGTCAAACTGCAA	RAD18-3	GAGCATGGATTATCTATTCAA
REV3-2	CCCACTGGAATTAATGCACAA	RAD18-4	TGCGATGCTTTGCATCCTAAA
REV3-3	CCCATTATCAACAGAACCAAA	RAD18-6	ATGGTTGTTGCCCGAGGTTAA



A: Validation of RAD18 siRNA



B: Validation of REV7 siRNA



C: Validation of Polη siRNA

Figure S2.2 Validation of RAD18, Polη and REV7 siRNA specificity. HeLa cells were transfected with Non-si or three different siRNA sequences targeting either RAD18 (A), REV7 (B) or Polη (C). Whole cell lysates were collected forty-eight hours after transfection and separated via SDS-PAGE as described in Materials in Methods. Immunoblot analysis was performed using the indicated primary antibodies to demonstrate depletion of RAD18, REV7 or Polη by each individual siRNA. Membranes were immunoblotted for GAPDH or tubulin as a loading control.

Figure S2.3 Depletion of RAD18 or Polη with siRNA targeting different mRNA sequences results in enhanced H2AX phosphorylation (γ-H2AX) following cisplatin treatment. HeLa cells were transfected with one of three different siRNAs targeting either RAD18 or Polη. Mock transfected or cells transfected with non-specific siRNA (Non-si 1) were included as controls. Forty hours after transfection, cells were treated with 10 μM cisplatin for 1 hour. Twenty-four hours later the cells were fixed, stained for γ-H2AX (green), and then imaged by fluorescent microscopy as detailed in Materials in Methods. Depletion of RAD18 or Polη by each individual siRNA sequence lead to identical γ-H2AX staining patterns and intensities following cisplatin treatment confirming gene specificity of siRNA-mediated protein knock down. The same exposure was used for each image.

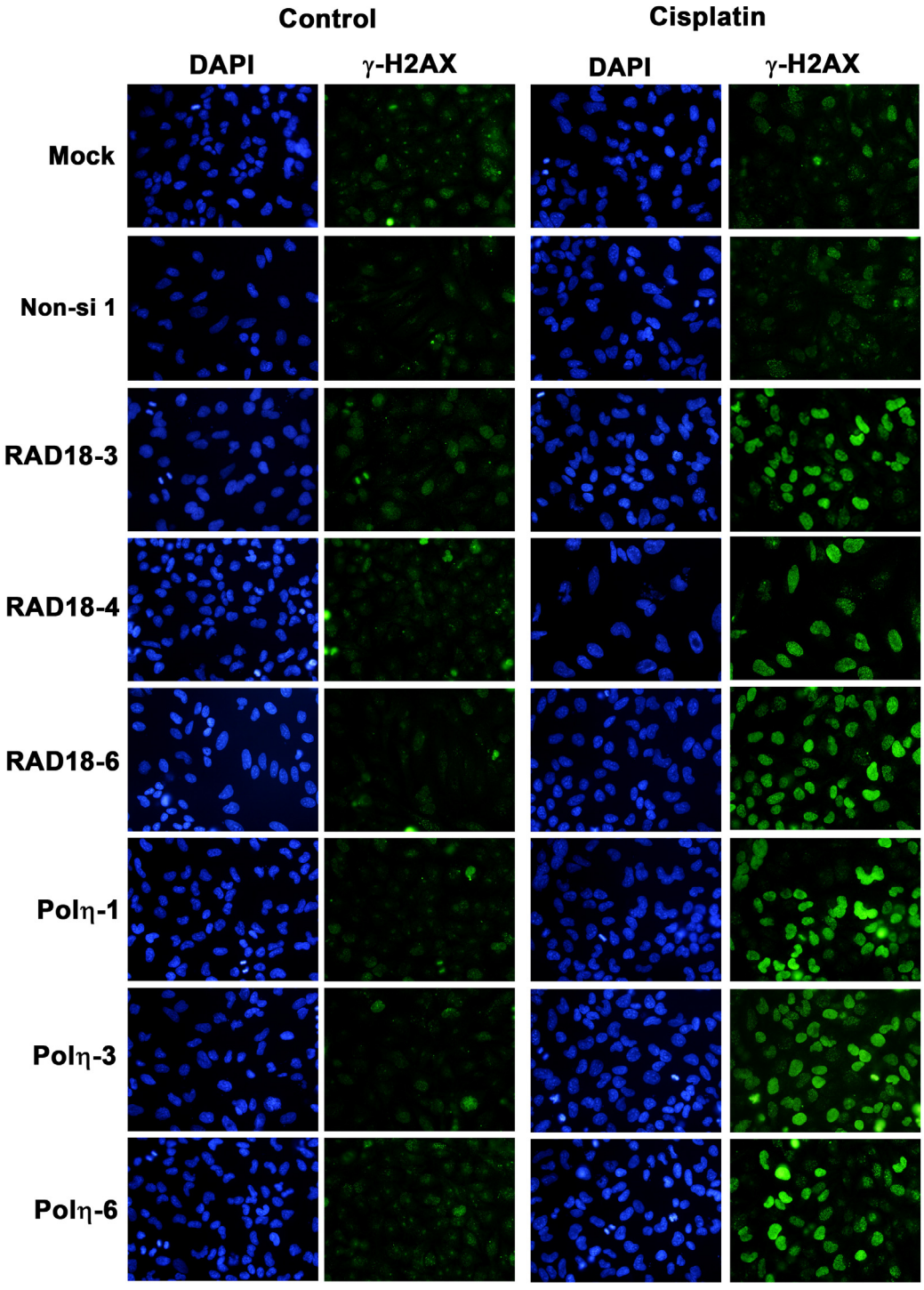
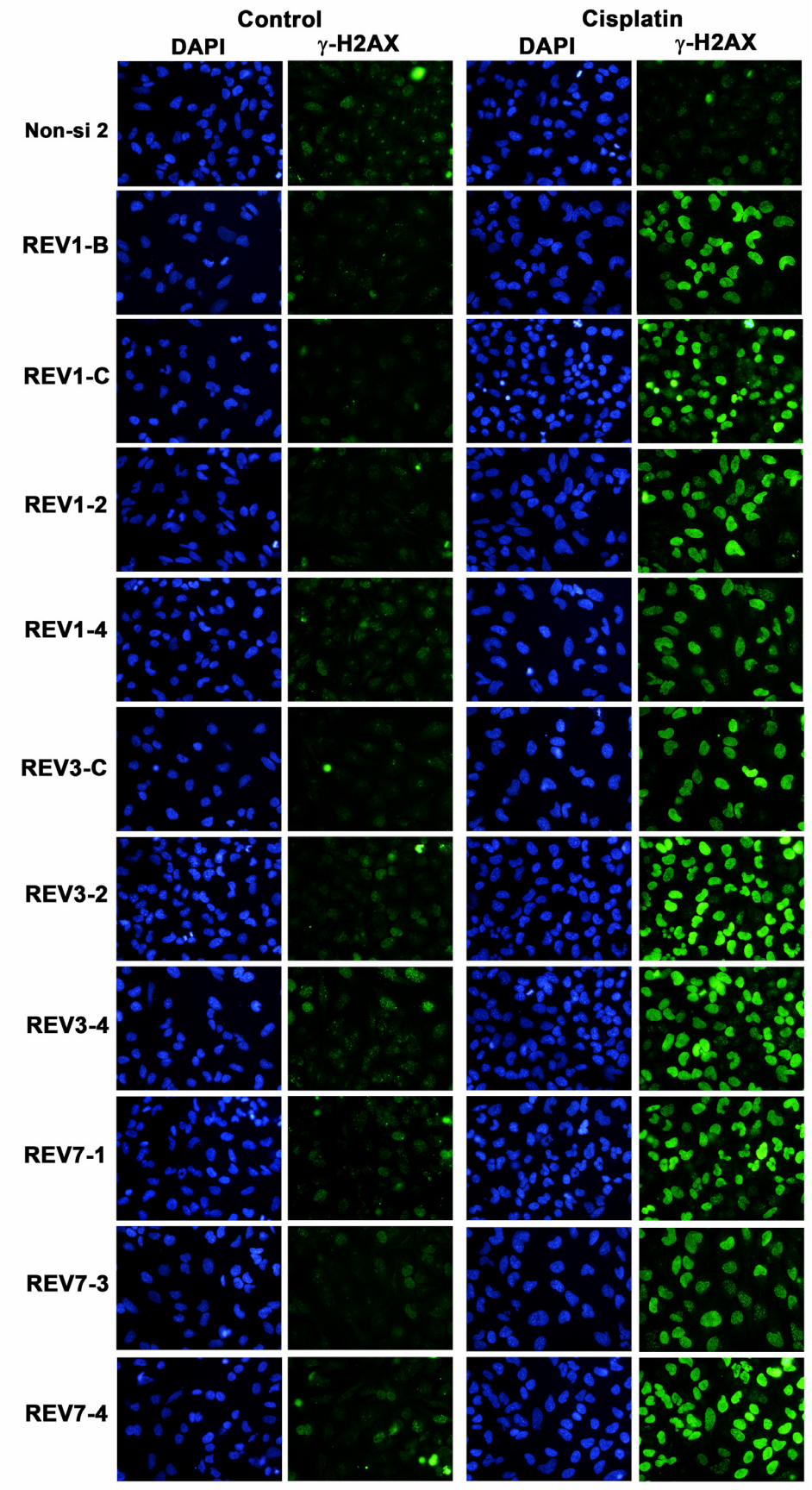

Control
Cisplatin
DAPI
γ-H2AX
DAPI
γ-H2AX
Mock
Non-si 1
RAD18-3
RAD18-4
RAD18-6
Polη-1
Polη-3
Polη-6

Figure S2.4 Depletion of REV1, REV3 or REV7 with siRNA targeting different mRNA sequences leads to enhanced H2AX phosphorylation (γ-H2AX) following cisplatin treatment. HeLa cells were independently transfected with one of at least three different siRNA sequences targeting REV1, REV3 or REV7. Qiagen AllStar Negative Control siRNA (Non-si 2) was included as a control. Forty hours after transfection, cells were treated with 10 μM cisplatin for 1 hour. Twenty-four hours later the cells were fixed, stained for γ-H2AX (green), and then imaged by fluorescent microscopy as detailed in Materials in Methods. Depletion of REV1, REV3 or REV7 by each individual siRNA sequence lead to identical γ-H2AX staining patterns and intensities following cisplatin treatment confirming gene specificity of siRNA-mediated protein knockdown. The same exposure was used for each image shown here.


Control
Cisplatin
DAPI
γ-H2AX
DAPI
γ-H2AX
Non-si 2
REV1-B
REV1-C
REV1-2
REV1-4
REV3-C
REV3-2
REV3-4
REV7-1
REV7-3
REV7-4

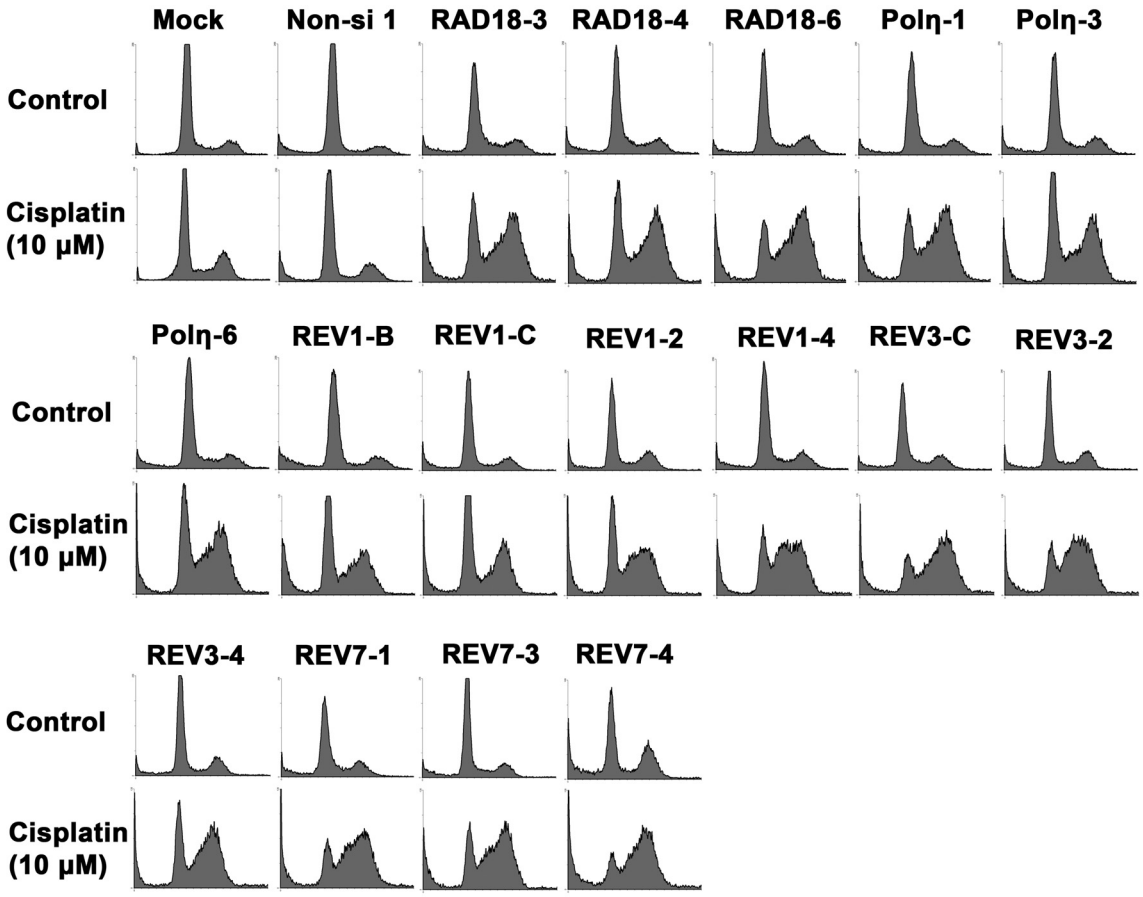


Figure S2.5 Depletion of RAD18, Polη, REV1, REV3 or REV7 leads to cell cycle arrest in S and G2 phases of the cell cycle following cisplatin treatment. HeLa cells were independently transfected with one of at least three different siRNA sequences targeting RAD18, Polη, REV1, REV3 or REV7. Forty hours after transfection the cells were exposed to 10 μM cisplatin for 1 hour. Twenty-four hours later cells were fixed, resuspended in PBS containing RNAse A and propidium iodide, and then acquired on a BD FACSCaliber system using CellQuest software. The y-axis represents number of events. The x-axis reflects DNA content. Depletion of RAD18, Polη, REV1, REV3 or REV7 by each individual siRNA sequence resulted in similar cell cycle profiles following cisplatin treatment. Mock transfected or Non-si 1 siRNA-transfected cells were relatively non-perturbed by this treatment.

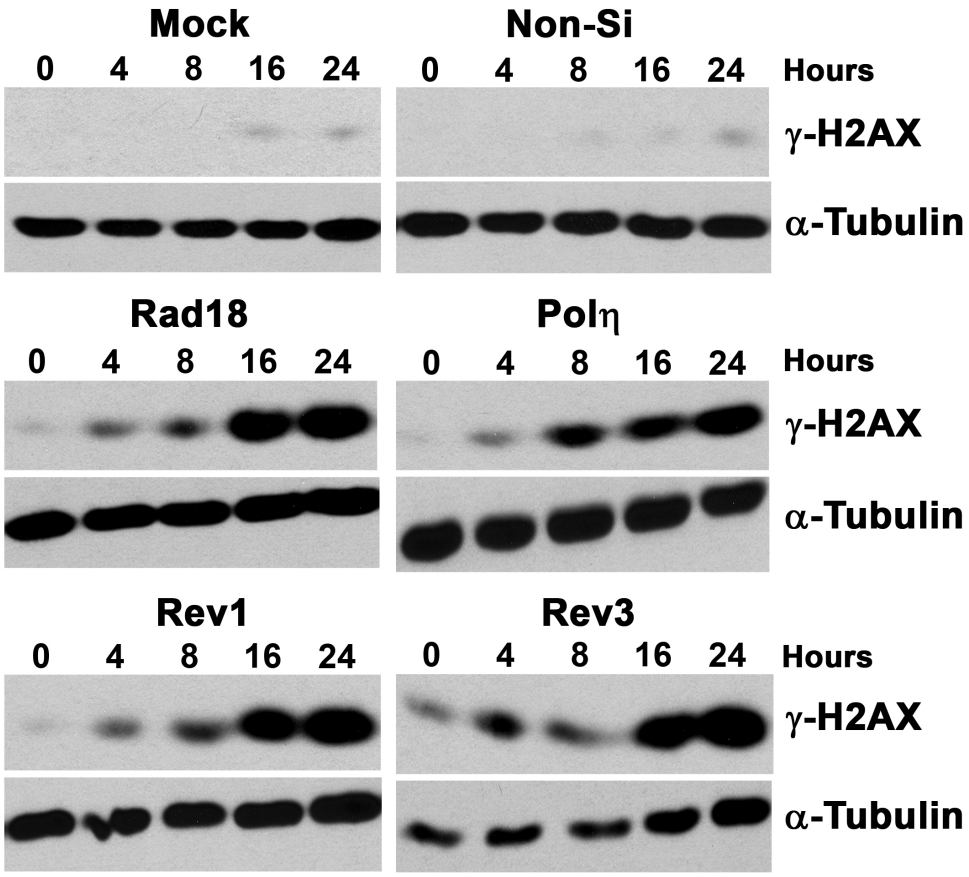


Figure S2.6 Time course analysis of γ-H2AX induction by cisplatin in siRNA-transfected HeLa cells. HeLa cells were transfected with RAD18-3, Polη-1, REV1-4, REV3-2, REV7-1 or Non-si 1 siRNA overnight. Forty hours later, cells were treated with 10 μM cisplatin for 1 hour and then lysed in SDS-sample buffer at 0, 4, 8, 16, and 24 hours after cisplatin treatment. Total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and then probed with anti-S139P-H2AX monoclonal antibody or anti-Tubulin monoclonal antibody as a loading control.

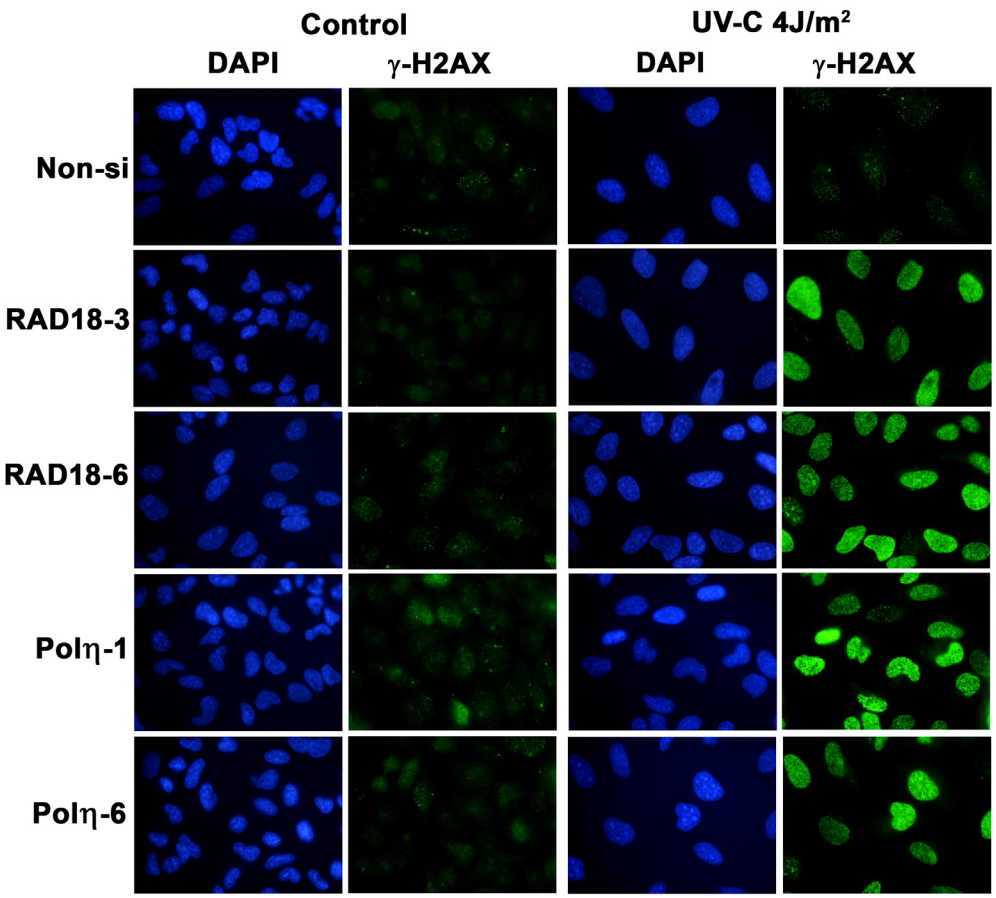


Figure S2.7 Depletion of RAD18 or polymerase eta (Polη) with siRNA targeting different mRNA sequences results in enhanced H2AX phosphorylation (γ-H2AX) following UV-C exposure. HeLa cells were transfected with one of two different siRNAs targeting RAD18 or Polη. Forty hours after transfection, cells were exposed to 4 J/m^2 UV-C radiation. Twenty-four hours later the cells were fixed, stained for γ-H2AX (green), and then imaged by fluorescent microscopy. Depletion of RAD18 or Polη by each individual siRNA sequence lead to identical γ-H2AX staining patterns and intensities following cisplatin treatment confirming gene specificity of siRNA-mediated protein knockdown. The same exposure time was used for each image.

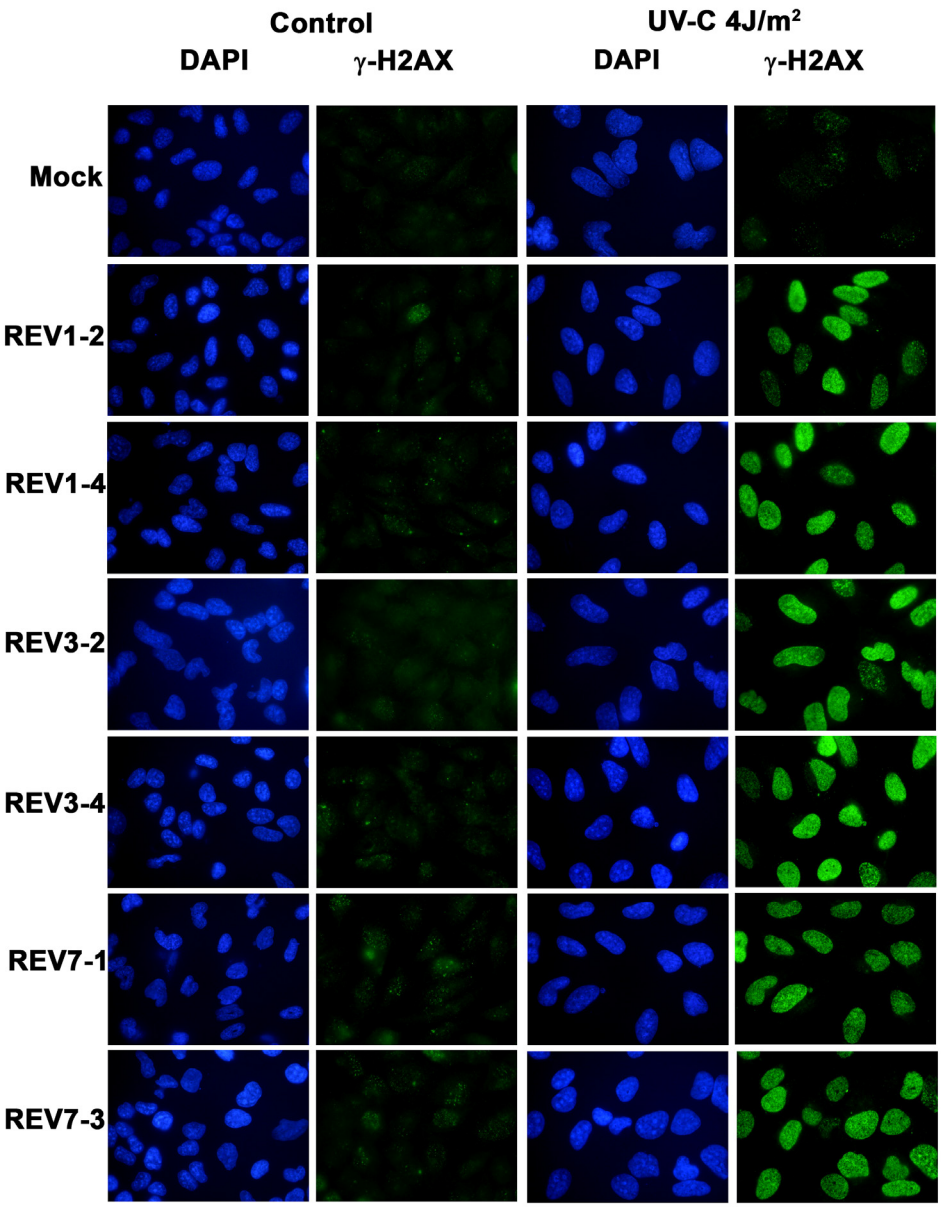


Figure S2.8 Depletion of REV1 or polymerase zeta (REV3 and REV7) with siRNA targeting different mRNA sequences results in enhanced H2AX phosphorylation (γ-H2AX) following UV-C exposure. HeLa cells were transfected with one of two different siRNAs targeting REV1, REV3 or REV7. Mock transfected cells were included as a control. Forty hours after transfection, cells were exposed to 4 J/m^2 UV-C radiation. Twenty-four hours later the cells were fixed, stained for γ-H2AX (green), and then imaged by fluorescent microscopy. Depletion of REV1, REV3 or REV7 by each individual siRNA sequence lead to identical γ-H2AX staining patterns and intensities following cisplatin treatment confirming gene specificity of siRNA-mediated protein knockdown. The same exposure time was used for each image.

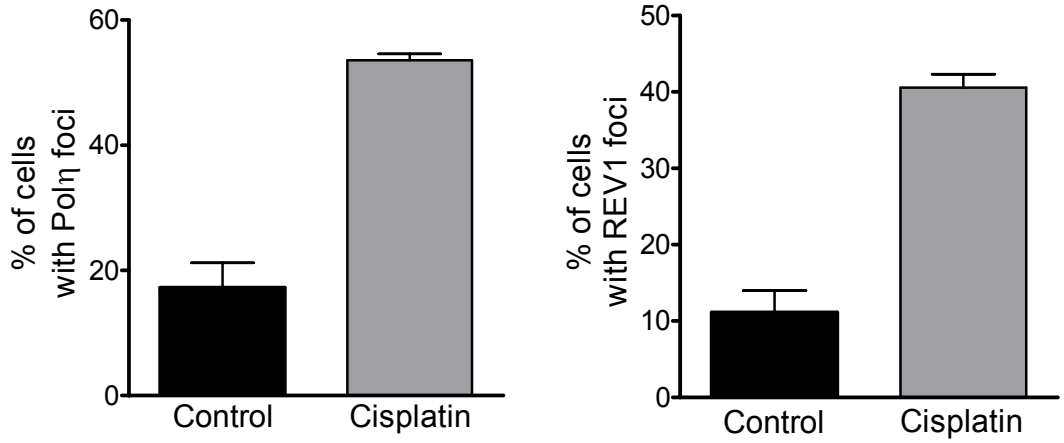


Figure S2.9 Cisplatin-induced polymerase eta (Polη) and REV1 focus formation. U2OS cells transduced with EGFP-Polη or EGFP-REV1 were untreated or exposed to 33μM cisplatin for 2 hours then fixed 8 hours later. Soluble proteins were first extracted with a Triton X-100 containing buffer (0.5% triton X-100, 20 mM HEPES pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl and 300 mM sucrose) for 5 minutes at 4°C in order to reveal detergent extraction-resistant proteins in the nucleus. Cells were then fixed with a 3.7% para-formaldehyde solution for 20 minutes at 4°C. Graphs represent the average percentage of cells exhibiting greater than 10 foci of either EGFP-tagged Polη or REV1 foci. Data represent the mean ± S.E.M of three independent experiments where >100 cells were counted in each experiment.

References

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CHAPTER 3

The Translesion Polymerases REV1 and Zeta, but not Eta, are Required for Interstrand cross-link Repair in a Human Cancer Cell Line

Summary

DNA interstrand cross-links are extremely cytotoxic lesions that halt both the replication and transcription machinery. Multiple chemotherapeutic agents that promote interstrand cross-link formation, including mitomycin C, are successfully used for the treatment of cancer. Unfortunately, cancer cells can become resistant to interstrand cross-linking agents impairing the usefulness of these agents. Removal of interstrand cross-links has been implicated in resistance development, thus determining how interstrand cross-links are repaired in human cells is of uttermost importance. Multiple DNA repair pathways including nucleotide excision repair, homologous recombination and translesion synthesis are thought to participate in interstrand cross-link removal. Here, evidence is presented strongly suggesting translesion synthesis is required for interstrand cross-link repair in human cells. Depletion of the translesion polymerases REV1 and zeta are uniquely associated with protection against cisplatin and mitomycin C-induced chromosomal aberrations when compared to polymerase eta depleted HeLa cells. Furthermore, knockdown of REV1 and polymerase zeta, but not polymerase eta, leads to extraordinary mitomycin C hypersensitive along with cell cycle arrest in late S and G2 phases of the cell cycle, a phenotype characteristically displayed by cells deficient in interstrand cross-link repair. REV1 and polymerase zeta were also found to be necessary for the timely resolution of DNA double strand breaks associated with repair of DNA interstrand cross-links. Together, the findings indicate that REV1 and polymerase zeta facilitate repair of DNA interstrand cross-links independently of PCNA monoubiquitination and polymerase eta.

Introduction

DNA interstrand cross-links are formed when a bifunctional chemical covalently links both strands of a DNA duplex. The linking of opposite strands of DNA prevents vital biological processes from proceeding, such as the unwinding of DNA by helicases, thereby halting both the replication and transcription machinery. DNA interstrand cross-links are extremely toxic, with one interstrand cross-link capable of killing a yeast cell if not repaired efficiently [1-3]. The highly toxic nature of interstrand cross-links have been noted in the clinic setting, and several chemotherapeutic agents that promote interstrand cross-link formation, including mitomycin C, are successfully used for cancer treatment. Unfortunately, cancer cells can become resistant to interstrand cross-linking agents impairing the usefulness of these agents. Furthermore, cross-resistance between interstrand cross-linking agents is known to occur, that is developed resistance to one cross-linking agent results in the resistance to multiple interstrand cross-linking agents [4]. The observance of cross-resistance suggests a biological pathway common to all interstrand cross-linking agents is involved in development of resistance. Although acquired resistance is not completely understood, one possible cellular process that is ubiquitous to all cross-linking agents that may promote resistance is alterations in repair of interstrand cross-links [4-6]. Currently, interstrand cross-link repair is poorly understood, thus determining how interstrand cross-links are repaired and if alterations in repair promote resistance is of uttermost importance. Comprehending interstrand cross-link repair may also reveal novel targets influencing drug design of chemotherapeutic agents.

Based on biochemical and genetic studies, multiple DNA repair pathways appear to be required for removal of interstrand cross-links including nucleotide excision repair, homologous recombination and translesion synthesis [2-3, 7-10]. As discussed in Chapter 2, translesion polymerases are capable of synthesizing past DNA adducts induced by both ultraviolet radiation and the chemotherapeutic agent cisplatin. In addition to facilitating direct lesion bypass and filling in postreplicative gaps in DNA, REV1 and polymerase zeta may also play an important role in the repair of interstrand cross-links [11-12]. Deletion of *REV1*, *REV3* or *REV7* in chicken DT40 cells leads to remarkable hypersensitivity to a wide variety of genotoxic stresses, most notably cisplatin and other DNA cross-linking

agents such as mitomycin C [9, 13-15]. Both Shen *et al.* and Zhang *et al.*, via a plasmid reactivation assay, demonstrated that the translesion polymerases REV1 and zeta are essential for both recombination-dependent and recombination-independent repair of interstrand cross-links [16-17]. Polymerase zeta was recently demonstrated to be required for extension beyond an unhooked cisplatin interstrand cross-link during gap filling via a Xenopus egg extract model, while REV1 may be capable of inserting directly opposite the base containing the unhooked cross-link [7, 11, 18].

The Fanconi anemia pathway is known to be essential for repair of DNA interstrand cross-links, possibly by coordinating the different DNA repair mechanisms involved in cross-link removal [3, 19]. Therefore, DNA repair pathways regulated by the Fanconi anemia complex of proteins have a high probability of participating in interstrand cross-link repair. Fanconi anemia patients lacking a functional Fanconi anemia core complex and *FANCC*$^{-/-}$ DT40 cells are known to have decreased mutation rates suggesting that the Fanconi anemia core complex somehow regulates translesion synthesis [20-23]. The genetic epistasis observed between *REV1*, *REV3* and the Fanconi anemia complementation group C (*FANCC*) gene for cisplatin sensitivity further implicates translesion synthesis in the interstrand cross-link repair pathway [9]. Mirchandani *et al.* demonstrated the Fanconi anemia core complex is crucial for REV1 foci formation following exposure to UV [23]. Data provided in Figure 2.7 confirms that the Fanconi anemia core complex regulates translesion synthesis by promoting the formation of REV1 foci at sites of cisplatin-induced replication stress. Taken together, the data strongly suggest the translesion polymerases REV1 and zeta have an essential biological role in repair of interstrand cross-links. However, direct evidence of translesion polymerases participating in repair of DNA cross-links in human cells is lacking.

Based on evidence that interstrand cross-links are repaired primarily during S-phase of the cell cycle [11, 17, 24-25], the most widely accepted models of interstrand cross-link repair at present time suggest that two replication forks converge upon an interstrand cross-link where the MUS81-EME1 endonuclease recognizes and cleaves the resulting branched DNA structure. The incision by MUS81-EME1 occurs on one side of the

cross-link creating a replication-associated DNA double strand break [26]. The XPF-ERCC1 endonuclease uncouples the cross-linked complementary DNA strands by making an incision on the other side of the interstrand cross-link [27]. Recent biochemical evidence suggests that polymerase zeta performs DNA synthesis opposite the DNA strand containing the residual cross-link (*e.g.* an uncoupled cisplatin interstrand cross-link) and this process may be necessary to prepare the daughter strand for subsequent homologous recombination repair of the replication-associated DNA double strand break [11]. Although unknown, translesion synthesis may also be necessary for gap filling following excision of the unhooked cross-link. Currently there is some confusion in the literature as to whether or not polymerase eta contributes to recombination-dependent interstrand cross-link repair by directly participating in homologous recombination [19, 28-29].

In Chapter 2, I provided substantial evidence to support the hypothesis that the E3 ubiquitin ligase RAD18 along with the translesion polymerases eta, REV1 and zeta are all necessary for bypass of DNA adducts induced by the chemotherapeutic agent cisplatin. Depletion of RAD18 or the translesion polymerases eta, REV1 or zeta individually caused increased sensitivity to cisplatin implying these proteins may protect cells from the cytotoxic effects of anti-cancer agents whose main mechanism of action is adduct formation. Unexpectedly, depletion of REV1, REV3 or REV7 resulted in a significantly greater sensitivity to cisplatin when compared to RAD18 or polymerase eta knockdown, suggesting that REV1 and polymerase zeta may have additional biological roles beyond lesion bypass. In this chapter, I will determine if the increased sensitivity to cisplatin is due to lack of interstrand cross-link repair in HeLa cells depleted of the translesion polymerases REV1 and zeta. I will also investigate if RAD18 and polymerase eta contribute to DNA interstrand cross-link removal in human cells. Several publications have proved strong evidence that REV1 and polymerase zeta are crucial for repair of interstrand cross-links induced by chemotherapeutic agents. However, little data is currently available directly connecting REV1 and polymerase zeta to interstrand cross-link repair in human cells. Furthermore it is unclear if RAD18 regulates translesion synthesis during interstrand cross-link repair and if polymerase eta is vital for cross-link removal.

Chemotherapeutic agents which induce DNA interstrand cross-links are widely used in cancer treatment, thus understanding the means by which cells repair or cope with these lesions will be instrumental in identifying novel mechanisms leading to drug resistance. Determining how interstrand cross-links are repaired may lead to the designing of new agents refractory to DNA damage tolerance mechanisms. Here evidence is provided to demonstrate REV1 or polymerase zeta knockdown cells accumulated chromosome aberrations, and failed to resolve DNA double strand breaks in a timely manner after cisplatin treatment as compared to RAD18 or polymerase eta-depleted cells. These results suggest that REV1 and polymerase zeta may be essential for the repair of cisplatin interstrand cross-links in addition to performing lesion bypass of cisplatin intrastrand cross-links. In agreement with this concept, REV1 and polymerase zeta-depleted cells were uniquely hypersensitive to mitomycin C, accumulated greater numbers of chromosome aberrations, and failed to resolve replication-associated double strand breaks induced by mitomycin C treatment.

Data presented in this chapter contributes to the understanding of how interstrand cross-links are repaired and directly implicates translesion synthesis in cross-link removal in human cells. Together the findings support a model where REV1 and polymerase zeta facilitate repair of interstrand cross-links in intact cells and this process is likely independent of PCNA monoubiquitination and polymerase eta.

Materials and Methods

Cell lines and culture conditions. HeLa and H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. BL2 human Burkitt's lymphoma cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Construction of *POLI*, *POLH*, and *REV3L* -/- BL2 lines have been described [20,22]. Cisplatin (cis-diammineplatinum(II)dichloride) was purchased from Sigma-Aldrich and dissolved in 0.9% NaCl as a 3.3 mM stock solution. Mitomycin C (MMC) was purchased from Roche, dissolved in 95% ethanol at a concentration of 0.2

mg/ml, and stored at -20°C. PD0332991, a gift from Pfizer, was dissolved in DMSO at a concentration of 10 mM and stored at -20°C.

RNA interference. HeLa cells were seeded at a density of $1x10^5$ per well of a 6 well plate. Introduction of siRNA into HeLa cells was carried out with X-tremeGENE (Roche). Each well was exposed to 50 nM siRNA plus 5µl X-tremeGENE overnight in the presence of serum followed by a change in medium the next morning. For most experiments, cells were treated with cisplatin or mitomycin C approximately 40 hours after the addition of siRNA. When delivering siRNA into cells cultured in 12 well plates, the conditions for transfection was reduced by one half. siRNAs directed against RAD18-3, Polη-1, REV1-4, REV3-2, REV7-1, and RAD51 were obtained from Qiagen. The gene-specific target sequences are as follows: RAD18-3 (GAG CAT GGA TTA TCT ATT CAA), Polη-1 (CTG GTT GTG AGC ATT CGT GTA), REV1-4 (ATC GGT GGA ATC GGT TTG GAA), REV3-2 (CCC ACT GGA ATT AAT GCA CAA), REV7-1 (GTG GAA GAG CGC GCT CAT AAA) and RAD51 (AAG CTG AAG CTA TGT TCG CCA). The negative control Non-si sequence (AAT TCT CCG AAC GTG TCA CGT) was purchased from Qiagen. Refer to supplemental data Figure S2.1 for additional siRNA sequences used in this chapter.

Assessment of cell viability. For clonogenic survival assays, HeLa cells were transfected overnight with siRNA as described above. The following afternoon cells were treated with mitomycin C for 24 hours and then seeded at known cell densities for colony formation. Twelve days later, colonies were simultaneously stained and fixed in a solution containing 3:1 methanol and glacial acetic acid plus 1% trypan blue (Sigma). Colonies of 50 cells or greater were counted and the surviving fractions for each siRNA treatment group represent the plating efficiency for each treatment divided by the plating efficiency of the corresponding untreated control. The BL2 lymphoma lines were treated with mitomycin C continuously for 48 hours. Cells were then washed with PBS and assessed as to their ability to exclude the trypan blue as a measure of viability.

Antibodies. The rabbit polyclonal antibodies anti-53BP1 (H-300) and anti-RAD51 (H92) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-

phospho-ATM (Ser 1981) antibody was obtained from Rockland. The anti-β-tubulin (TUB 2.1) antibody was purchased from Sigma-Aldrich Inc.

Immunoblotting. Cells were lysed in a sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris pH 8.0, 2% SDS, 1 × protease inhibitor cocktail (Roche) and 1 × phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich)), sonicated, heated at 95°C, and equal amounts of protein were separated on SDS-PAGE gels. Proteins were transferred onto a nitrocellulose membrane and probed with the appropriate primary antibodies described above followed by secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse antibody (Thermo Scientific). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Immunofluorescence. For S1981P-ATM and 53BP1 immunofluorescence, cells cultured on glass coverslips were washed in phosphate buffered saline (PBS) and then fixed in ice cold 100% methanol for 10 minutes. Samples were blocked with 5% fetal bovine serum, 0.05% Triton X-100, and 1% goat serum, and then incubated with primary antibodies for 45 minutes. Coverslips were washed three times with PBS and then incubated with the appropriate secondary goat anti-rabbit or goat anti-mouse Alexa Fluor dye conjugated secondary antibody (Molecular Probes) for 45 minutes, washed with PBS, counterstained with DAPI to visualize nuclear DNA, and then mounted onto slides with ProLong Gold antifade reagent (Invitrogen).

Cyclin-dependent kinase inhibition. H1299 cells were exposed to either 200 or 500 nM PD0332991 24 hours prior to cell cycle analysis or addition of mitomycin C. The inhibitor was present during and after treatment with mitomycin C.

Analysis of chromosomal aberrations. HeLa or BL2 cells were treated with either cisplatin for 1 hr or mitomycin C continuously. Twenty-four hours later, mitotic cells were enriched through the addition of 50 ng/mL colcemid (Gibco) for 45 minutes prior to cell harvesting. Cells were treated for 18 minute at 37°C with a hypotonic solution consisting of 0.075 M KCl and then fixed in Carnoy's fixative (3:1 methanol:glacial acetic acid). Cells were dropped onto slides, allowed to dry for a day, and then chromosomes were stained with Giemsa prior to analysis as described [30]. 50 mitotic

spreads were analyzed for each treatment. For comparisons of the average number of gaps and breaks per metaphase between control and drug-treated treatment groups, the student's T-test was employed.

Flow cytometry. For single parameter flow cytometry, cells were washed once with PBS then fixed with ice cold 70% ethanol. The cells were resuspended in PBS containing RNAse A and propidium iodide to determine DNA content. Cells were acquired on a BD FACSCaliber system using CellQuest software.

Results

REV1, REV3 and REV7 protect against cisplatin-induced chromosomal aberrations.

As discussed in Chapter 2 (Fig. 2.8A), depletion of REV1 or polymerase zeta (REV3 and REV7) enhanced cisplatin-induced cytotoxicity as measured by clonogenic survival when compared to RAD18 or polymerase eta (Polη) depleted cells. The results were confirmed by treating human Burkitt's lymphoma BL2 cells deleted of *POLH* (eta), *POLI* (ίota) or *REV3L* with various concentrations of cisplatin for 2 hours and measuring viability 48 hours later by trypan blue dye exclusion. Consistent with the results obtained from the clonogenic survival assay, *REV3L* knockout BL2 cells were significantly more sensitive to cisplatin cytotoxicity as compared with polymerase eta or polymerase ίota knockout cells (Fig. 2.8B). Since knockdown of RAD18, polymerase eta, REV1 or polymerase zeta lead to indistinguishable phenotypes in response to cisplatin treatment with regards to phosphorylation of H2AX and cell cycle redistribution (Fig. 2.2), the survival and viability results were unexpected and suggested REV1 and polymerase zeta may perform additional roles in promoting tolerance to cisplatin.

Cisplatin is known to create a small percentage of interstrand DNA cross-links (1-3%) in addition to intrastrand cross-links [31]. Given the proposed role of REV1 and polymerase zeta in interstrand DNA cross-link repair and the genetic epistasis observed between the *FANCC* gene and *REV1* and *REV3* in cisplatin resistance in DT40 cells,

REV1 or polymerase zeta-deficient cells were investigated to determine if they are more prone to developing cisplatin-induced chromosome aberrations, a hallmark of interstrand DNA cross-link repair deficiency [32]. Depletion of RAD18 or polymerase eta in HeLa cells did not result in a significant increase in cisplatin-induced chromatid gaps and breaks per metaphase compared to controls, suggesting disruption of translesion synthesis alone does not cause an increase in chromosomal aberrations (Fig. 3.1A). In contrast, depletion of REV1, REV3, or REV7 resulted in a 3 to 4 fold increase in chromatid gaps and breaks per metaphase in cisplatin-treated cells, consistent with the hypothesis that REV1 and polymerase zeta play an important role in interstrand DNA cross-link repair. Similar results were observed in BL2 cells where *REV3L* knockout cells accumulated twice as many chromosomal aberrations as compared to *POLH* knockout cells treated with cisplatin (Fig. 3.1B). Representative images of chromosomal aberrations observed in RAD18, Polη, REV1, REV3 or REV7 depleted HeLa cells are displayed in Figure 3.1C and supplemental data Figure S3.1. For knockdown of RAD18, polymerase eta, REV1, REV3 or REV7 in HeLa cells refer to Figures 2.1B and C along with supplemental data Figures S2.1 and S2.2.

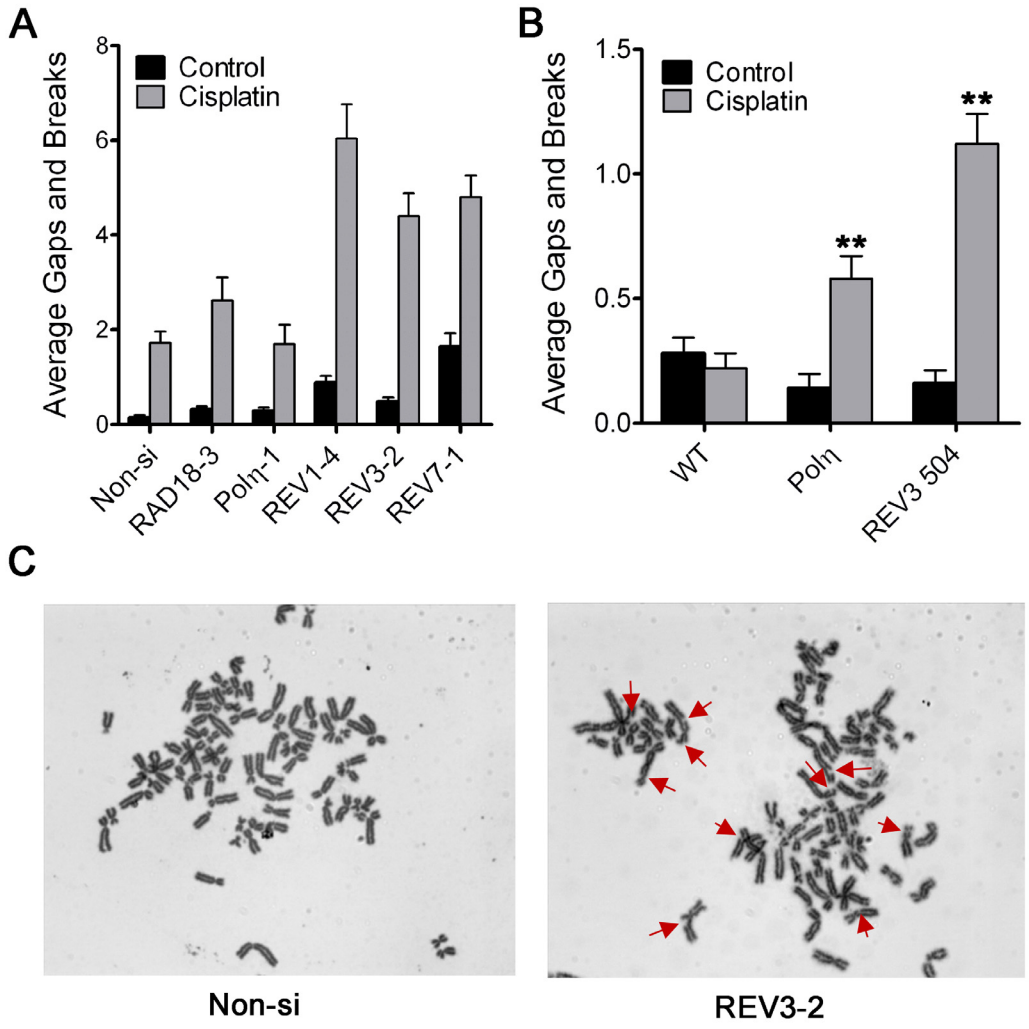


Figure 3.1 Depletion of REV1, REV3 or REV7 leads to genomic instability in HeLa or BL2 cells. (A) siRNA transfected HeLa cells were treated with 10 μM cisplatin for 1 hour and metaphase cells were assessed for chromosomal gaps and breaks 24 hours later as described in Materials and Methods. Data is expressed as the average gaps and breaks per metaphase ± S.E.M. (n=50). (B) Wild type BL2 or BL2 knockout cells were treated with 30 μM cisplatin for 1 hour and then analyzed for chromosomal aberrations 24 hours later. Data is expressed as the average gaps and breaks per metaphase ± S.E.M. (n=50). *p* values were calculated by the Students *t* test, where ** represents p values <0.001. (C) Representative images of chromosomal aberrations observed in siRNA-transfected HeLa cells treated with 10 μM cisplatin. Red arrows indicate chromatid gaps and breaks.

REV1, REV3 and REV7 are necessary for resolution of DNA double strand breaks induced by repair of cisplatin interstrand cross-links

Although interstrand cross-links can be repaired in a recombination-independent manner, the majority of interstrand cross-links are thought to be repaired in a recombination-dependent pathway during DNA replication resulting in the formation of DNA double strand breaks [11, 17, 24-25]. During recombination-dependent interstrand cross-link repair a DNA double strand break is formed when structure specific endonucleases unhook the interstrand cross-link by cleaving on both sides of the cross-link. As a way to measure repair of interstrand cross-links, the formation and resolution of protein foci marking sites of DNA double strand breaks were observed and quantitated. Since γ-H2AX is not a reliable surrogate marker for the presence of DNA double strand breaks under conditions of pronounced replication stalling (Fig. 2.2), foci formation was marked by activated ATM co-localized with 53BP1. ATM is a central cell cycle checkpoint kinase that is specifically activated in response to DNA doubles strand breaks. Activation of ATM is associated with rapid transphosphorylation on S1981 and localization to sites of double strand breaks [33-34]. 53BP1 is a cell cycle checkpoint mediator protein involved in DNA damage signaling and repair and is well characterized for its ability to localize to DNA double strand breaks in cells exposed to DNA damaging agents [35-36]. By examining colocalization of S1981P-ATM and 53BP1, this should be a more specific surrogate marker of double strand breaks under conditions of DNA replication arrest.

To provide evidence that DNA double strand break formation is due to recombination-dependent repair, H1299 cells were arrested in the G1 phase of the cell cycle using a cyclin-dependent kinase (CDK) inhibitor, then treated with mitomycin C to determine if double strand break formation is dependent upon cells entering S phase. PD0332991, a CDK4 and CDK6 specific inhibitor, was utilized to arrest H1299 cells in G1 phase of the cell cycle [37-39]. Transition from G1 into the synthesis phase of the cell cycle requires the phosphorylation of retinoblastoma, a repressor of gene transcription. Both CDK4 and CDK6 phosphorylates retinoblastoma, which in turn prevents

retinoblastoma from inhibiting the E2 family of transcription factors thus allowing for progression into S phase [40-44].

H1299 cells exposed to either 200 or 500 nM PD0332991 for 24 hours resulted in cell cycle arrest in the G1 phase (Fig. 3.2A). To determine if cells must progress through S phase for mitomycin C induced DNA double strand breaks to form, H1299 cells were treated with 500 nM PD0332991 for 24 hours then pulsed with either 30 or 60 ng/ml mitomycin C for 2 hours in the presence of the CDK inhibitor. Eight hours later the cells were collected and stained for S1981P ATM and 53BP1. H1299 cells exposed to PD0332991 displayed a decrease of S1981P-ATM and 53BP1 containing foci, even when treated with mitomycin C, implying the induction of DNA double strand breaks can be attributed to recombination-dependent repair of interstrand cross-links (Fig. 3.2B). Approximately 75% of the cells pulsed with 30 ng/ml mitomycin C exhibited greater than ten S1981P-ATM and 53BP1 containing foci per cell while only 46% of the cells exposed to both the CDK inhibitor and mitomycin C displayed greater than ten S1981P-ATM and 53BP1 containing foci per cell, a reduction of roughly 40% (Fig. 3.2C). Similarly, exposure to both the CDK4/CDK6 inhibitor and 60 ng/ml mitomycin C resulted in a 55% reduction in the number of cells demonstrating greater than ten S1981P-ATM and 53BP1 containing foci per cell when compared to H1299 cells only treated with 60 ng/ml mitomycin C (Fig. 3.2C).

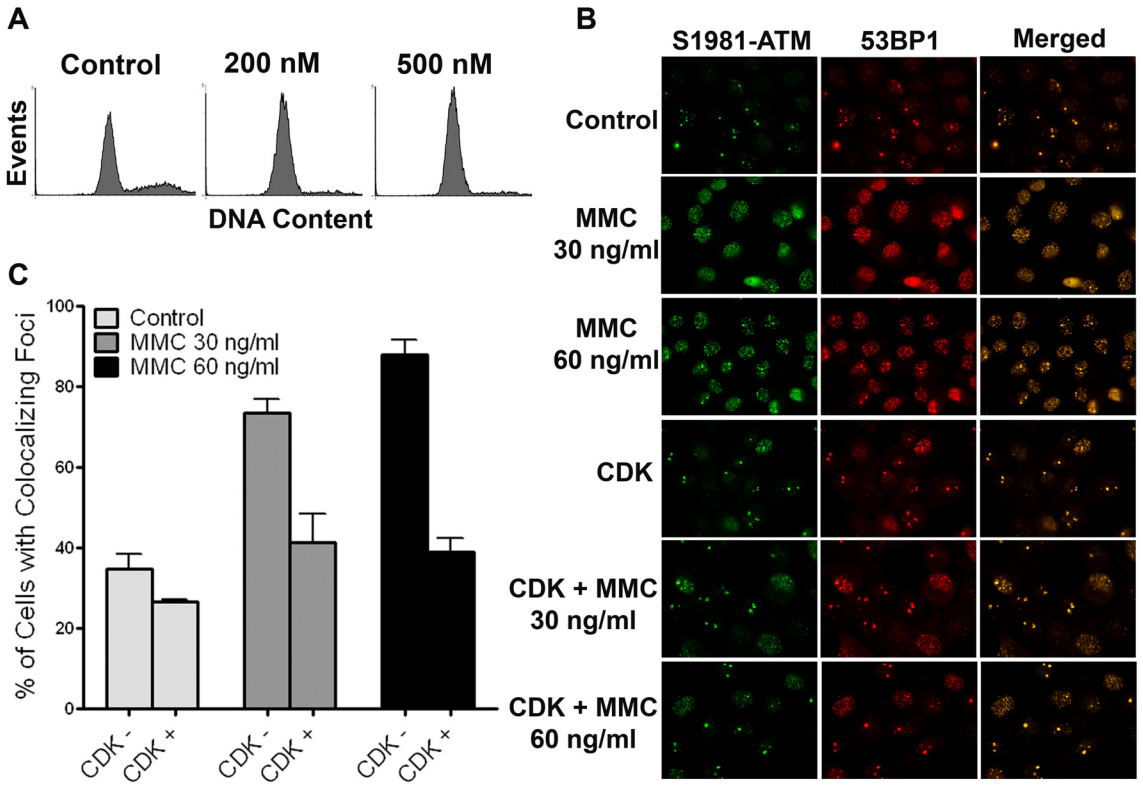


Figure 3.2 DNA double strand break formation following mitomycin treatment may be contributed to recombination-dependent interstrand cross-link repair. (A) H1299 cells were treated with either 200 or 500 nM PD0332991 for 24 hrs, then fixed, stained for DNA content with propidium iodide, and analyzed by flow cytometry. (B) H1299 cells were either untreated or exposed to 500 nM PD0332991 for 24 hrs then pulsed with either 30 or 60 ng/ml mitomycin C for 2 hrs. Eight hours later the cells were fixed in 100% ice cold methanol then stained for S1981P-ATM (green), and 53BP1 (red) as surrogate markers of DNA double stranded DNA breaks. (C) The graph represents the average percentage of cells exhibiting greater than 10 colocalized foci containing both phospho-ATM and 53BP1 per cell. Data represent the mean ± S.E.M of two independent experiments where >100 cells were counted in each experiment. Treatment with the CDK4/CDK6 inhibitor PD0332991 diminishes DNA double strand break formation following mitomycin C treatment.

To further test the hypothesis that REV1, REV3, and REV7 contribute to interstrand DNA cross-link repair in human cells, the formation and resolution of DNA double strand breaks in cisplatin-treated cells were examined as an indirect measurement of interstrand DNA cross-link repair [26-27, 45-46]. HeLa cells were transfected with individual siRNAs, treated with 3 μM cisplatin for 1 hour, and then allowed to recover for 24 or 48 hours before fixation and imaging of S1981P-ATM and 53BP1 by indirect

immunofluorescence. The 3 μM dose was chosen since it produces the greatest difference in loss in clonogenic survival when cells are depleted of Rad18 or polymerase eta versus REV1 or REV3. At 24 hours, all cisplatin-treated cells appeared to accumulate similar numbers of foci containing S1981P-ATM and 53BP1 regardless of which gene the siRNA was targeting (including the non-specific Non-si siRNA) (supplementary date Fig. S2.2). However, at 48 hours, approximately 60% of HeLa cells depleted of REV1, REV3, or REV7 still exhibited greater than ten foci per cell (Fig. 3A and B). In contrast, the majority of Non-si, RAD18 or polymerase eta siRNA-transfected cells contained 5 or fewer foci per cell 48 hours after cisplatin treatment suggesting that interstrand cross-link repair was not impacted to a significant degree. These results were confirmed using two additional siRNA sequences targeting Rad18, Polη, REV1, REV3 or REV7 (supplemental data Fig. S3.3 and S3.4).

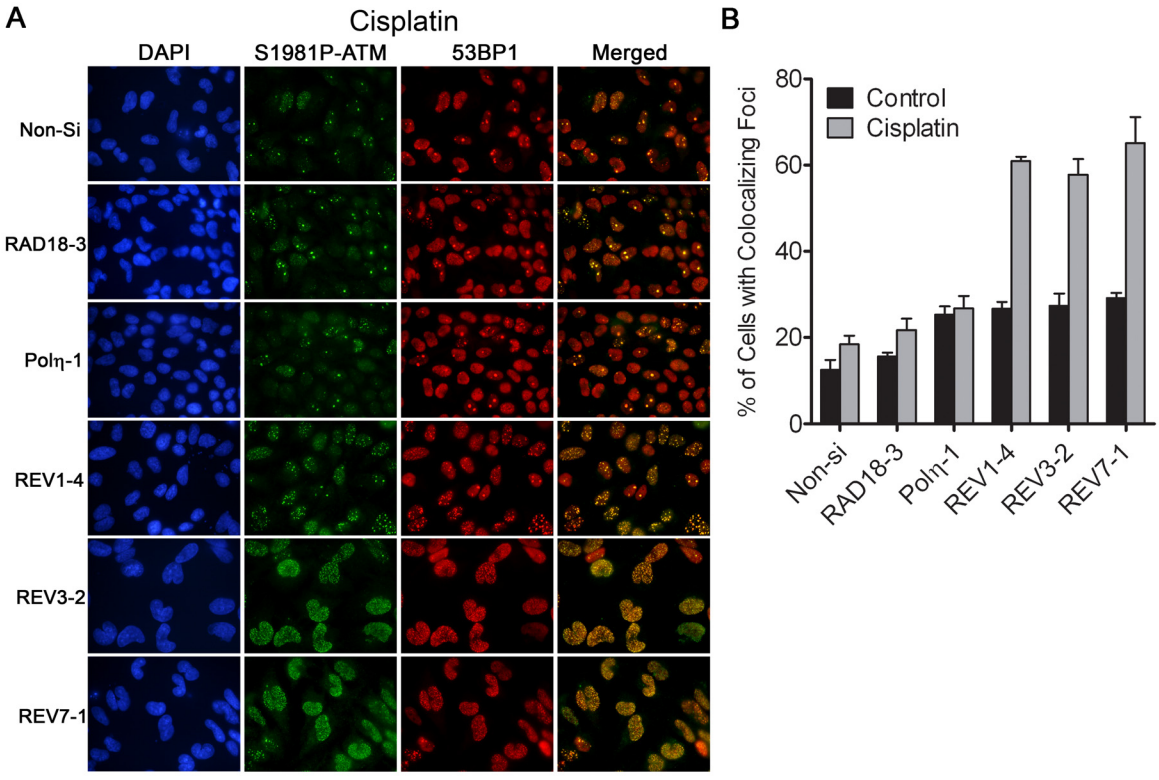


Figure 3.3 REV1 and polymerase zeta (REV3 and REV7) are necessary for repair of cisplatin-induced interstrand cross-links. (A) RAD18, Polη, REV1, REV3 or REV7-depleted HeLa cells were treated with 3 μM cisplatin for 1 hr and fixed 48 hours later. Cells were then stained for S1981P-ATM (green), and 53BP1 (red) as surrogate markers of DNA double strand breaks (DSBs). Nuclear DNA was stained with DAPI (blue). (B) The graph represents the average percentage of cells exhibiting greater than

10 colocalized foci containing both phospho-ATM and 53BP1 per cell. Data represent the mean ± S.E.M of three independent experiments where >300 cells were counted in each experiment. REV1, REV3, or REV7 depleted cells fail to resolve cisplatin-induced DSBs in a timely manner.

To confirm the validity of the foci retention assay, I also analyzed the effect of depletion of RAD51 on foci resolution after cisplatin exposure. Similar to REV1, REV3, or REV7 depleted cells, RAD51-deficient cells (*i.e.* homologous recombination repair deficient cells) failed to resolve S1981P-ATM and 53BP1 foci in a timely manner (Fig. 3.4A). As in supplemental data Figure S3.2, the non-specific siRNA transfected HeLa cells displayed pATM-53BP1 foci formation 24 hours after cisplatin treatment, but by 48 hours the majority of cisplatin-induced foci had resolved implicating repair of the DNA double strand breaks (Fig. 3.4A and B). However, greater than 65% of the RAD51 depleted HeLa cells still exhibited greater than ten pATM-53BP1 foci per cell 48 hours following cisplatin treatment suggesting the replication-associated DNA double strand breaks were not repaired efficiently (Fig. 3.4B). The ability to deplete endogenous RAD51 was demonstrated by immunoblot analysis (Fig. 3.4C). Together, these results are in agreement with the hypothesis that REV1, REV3, and REV7 play an important role in repair of cisplatin interstrand DNA cross-links.

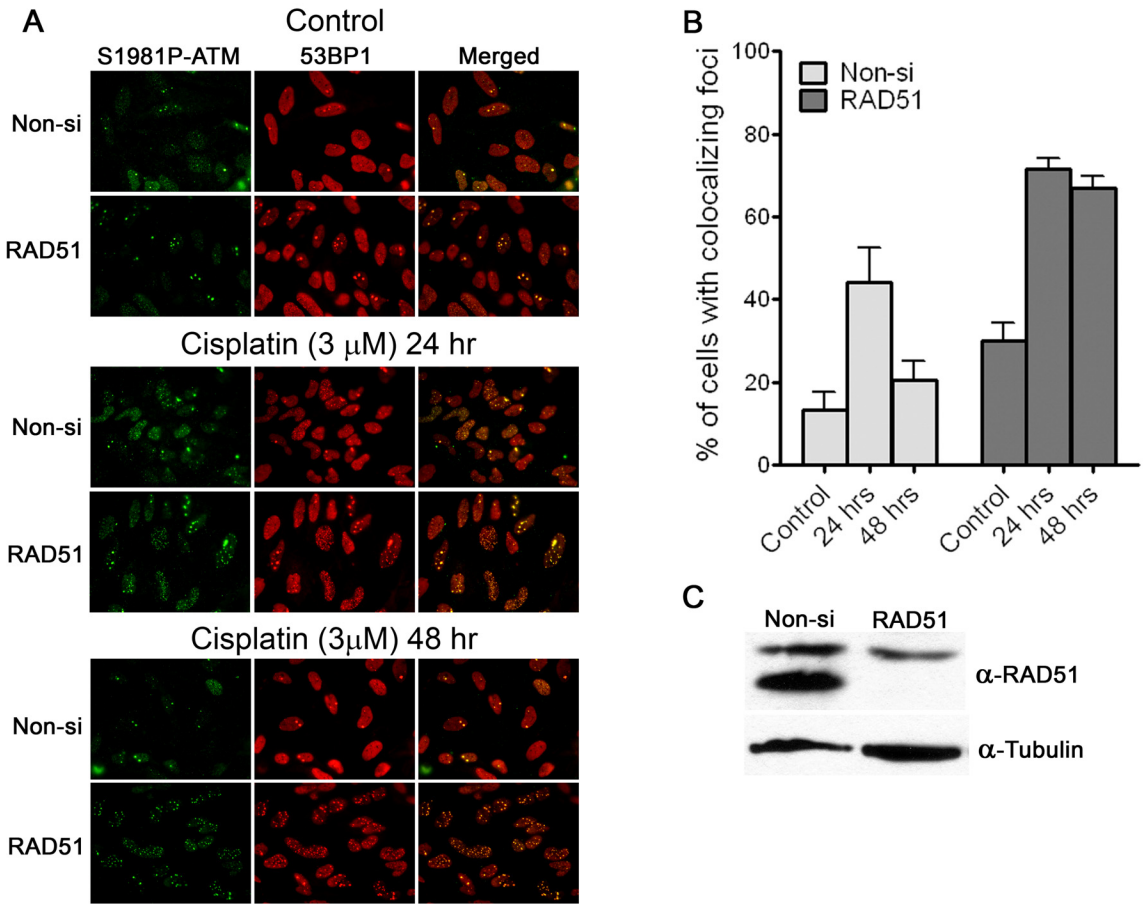


Figure 3.4 RAD51-deficient HeLa cells fail to resolve cisplatin-induced DNA double strand breaks in a timely manner. (A) RAD51 depleted HeLa cells were treated with 3 μM cisplatin for 1 hour and then fixed 24 or 48 hours later. Cells were then stained for S1981P-ATM (green), and 53BP1 (red) as surrogate markers of DNA double strand breaks (DSBs). (B) The graph represents the average percentage of cells exhibiting greater than 10 colocalized foci containing both phospho-ATM and 53BP1 per cell at 24 and 48 hours after cisplatin exposure. Data represent the mean ± S.E.M of three independent experiments where >100 cells were counted in each experiment. (C) HeLa cells were transfected with siRNA targeting the *RAD51* gene product as described in Materials and Methods. Forty-eight hours later whole cell lysates were collected, separated by SDS-PAGE and subjected to immunoblot analysis using a primary antibody against RAD51 and tubulin as a loading control. Rad51 depleted HeLa cells did not resolve cisplatin-induced DSBs in a timely manner, consistent the with model that homologous recombination plays an important role in repair of replication-associated DSBs during interstrand cross-link repair.

The REV1-polymerase zeta functional complex is necessary for protection against mitomycin C induced cytotoxicity and chromosomal aberrations

The observation that REV1, REV3, or REV7-depleted HeLa cells accumulated or failed to resolve greater numbers of cisplatin-induced double strand breaks could be explained by increased replication fork instability and collapse due to the inability to complete replicative bypass of cisplatin intrastrand cross-links rather than deficient interstrand DNA cross-link repair. Therefore REV1 or polymerase zeta depleted cells were tested to determine whether they are uniquely sensitive to mitomycin C, a clinically relevant anticancer agent that produces monoadducts, intrastrand cross-links, and a far greater percentage of interstrand DNA cross-links (20%) as compared to cisplatin [47]. As shown in Figure 3.5A, REV1, REV3, or REV7 siRNA-transfected HeLa cells are significantly more sensitive to loss in clonogenic cell survival as compared to RAD18 or polymerase eta siRNA transfected cells. Similarly, *REV3L* knockout BL2 cells were also more sensitive to loss in viability after exposure to relatively low doses of mitomycin C when compared with polymerase eta or polymerase ίota knockout cells (Fig. 3.5B). Consistent with a deficiency in the repair of interstrand DNA cross-links, REV1, REV3, or REV7-depleted HeLa cells displayed greater numbers of mitomycin C-induced chromosomal aberrations per metaphase that primarily consisted of chromatid gaps and breaks, as compared to polymerase eta-depleted cells (Fig. 3.5C). Representative images of chromosomal aberrations observed in mitotic spreads isolated from siRNA-transfected HeLa cells treated with mitomycin C are displayed in Figure 3.4E and supplemental data Figure S3.5.

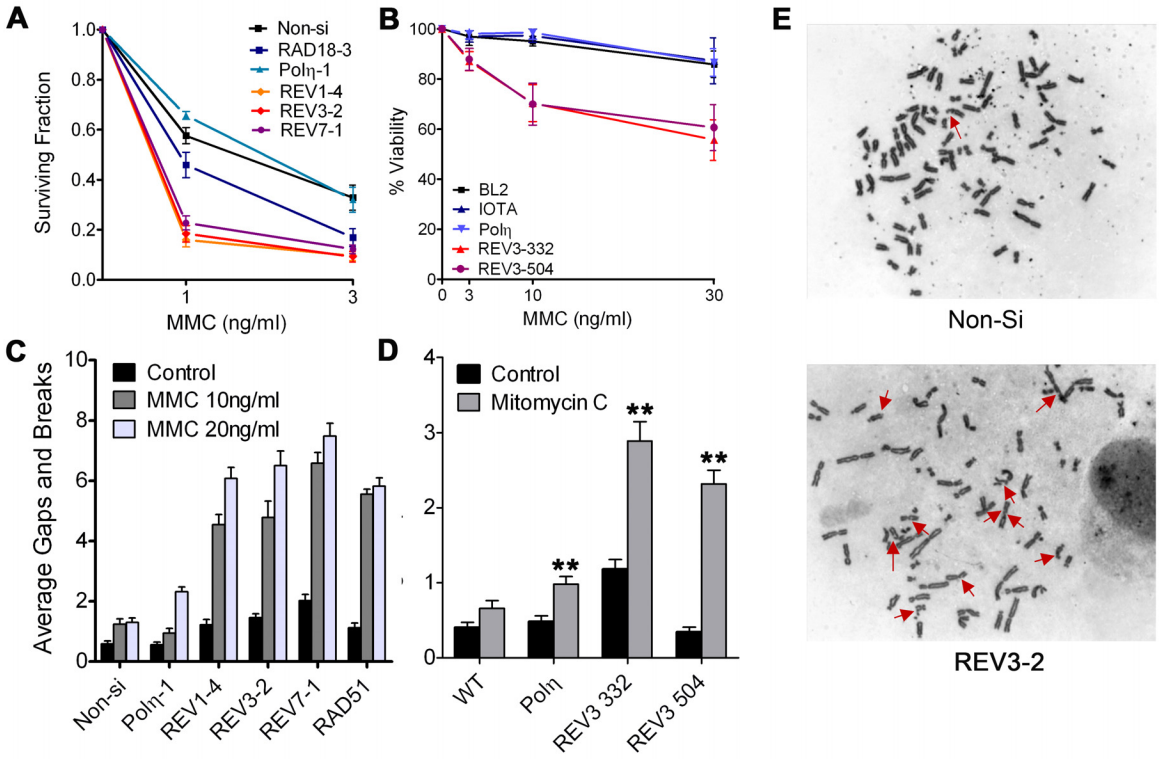


Figure 3.5 The REV1/polymerase zeta functional complex protects cells from mitomycin C-induced cytotoxicity and genomic instability. (A) HeLa cells depleted of RAD18, Polη, REV1, REV3 or REV7 were treated with mitomycin C (MMC) for 24 hrs at the indicated doses, and then subjected to the clonogenic survival assay. The average surviving fraction of at least three independent experiments are shown. Error bars represent S.E.M. (B) Wild type BL2 or BL2 cells deficient in polymerase ιota (Polι), polymerase eta (Polη), or REV3 (clones 332 and 504) were treated with MMC for 48 hours and then subjected to the trypan blue exlusion assay. The average percentage of cells excluding trypan blue is shown (n≥3). Error bars represent S.E.M. (C) Control (Non-si) or Polη, REV1, REV3, REV7 or RAD51-depleted HeLa cells were treated with 10 or 20 ng/ml MMC for 24 hrs, fixed, and then analyzed for chromosomal aberrations. (D) BL2 or BL2 knockout cells were treated with 40 ng/ml MMC for 24 hrs and then analyzed for chromosomal aberrations. Data is expressed as the average gaps and breaks per metaphase ± S.E.M. (n=50). *p* values were calculated by the Students *t* test, where ** represents p values <0.001. (E) Representative images of chromosomal aberrations observed in HeLa cells treated with 10 ng/ml MMC. Red arrows point to chromatid gaps and breaks.

For comparative purposes, HeLa cells were depleted of RAD51 via siRNA, a protein essential for homologous recombination repair (Fig. 3.4C). As expected, Rad51-depleted cells exhibited similar numbers of chromatid gaps and breaks per metaphase as compared to REV1, REV3, or REV7-depleted cells (Fig. 3.5C) consistent with the model

that these proteins are necessary for efficient recombination-dependent repair of interstrand DNA cross-links. The requirement of REV3 was also confirmed for protection against mitomycin C-induced chromosome instability in the BL2 model system where greater numbers of chromosomal aberrations in *REV3L* knockout cells were consistently observed as compared to wild type or *POLH* knockout cells (Fig. 3.5D).

The REV1-polymerase zeta functional complex is necessary for efficient repair of mitomycin C interstrand cross-links.

To further test the hypothesis that REV1 and polymerase zeta are necessary for efficient interstrand DNA cross-link repair, I assessed whether siRNA-transfected HeLa cells exhibit residual double strand breaks after a brief exposure to mitomycin C. At 24 hours, all mitomycin C-treated cells accumulated similar numbers of foci containing S1981P-ATM and 53BP1 regardless of which gene the siRNA was targeting (including the non-specific Non-si siRNA) (supplementary data Fig. S3.6). Consistent with the results shown in Figure 3.3 examining the resolution of DNA double strand breaks marked by S1981P-ATM and 53BP1 in response to cisplatin treatment, REV1, REV3 or REV7-depleted HeLa cells, but not RAD18 or polymerase eta-depleted cells, were deficient in resolving DNA double strand breaks marked by foci containing S1981P-ATM colocalized with 53BP1 48 hours after mitomycin C treatment (Fig. 3.6A and B). Similar results were found using two additional siRNA sequences to deplete Rad18, polymerase eta, REV1, REV3 or REV7 protein confirming that deficiencies in resolving mitomycin C-induced DSBs in REV1, REV3, or REV7-depleted HeLa cells are not due to off-target effects of the siRNA (supplemental data Fig. S3.7 and S3.8). Again, I validated this assay by demonstrating that foci retention following mitomycin C treatment is also a major phenotype in RAD51-depleted cells (Fig. 3.6C and supplemental data Fig. S3.9).

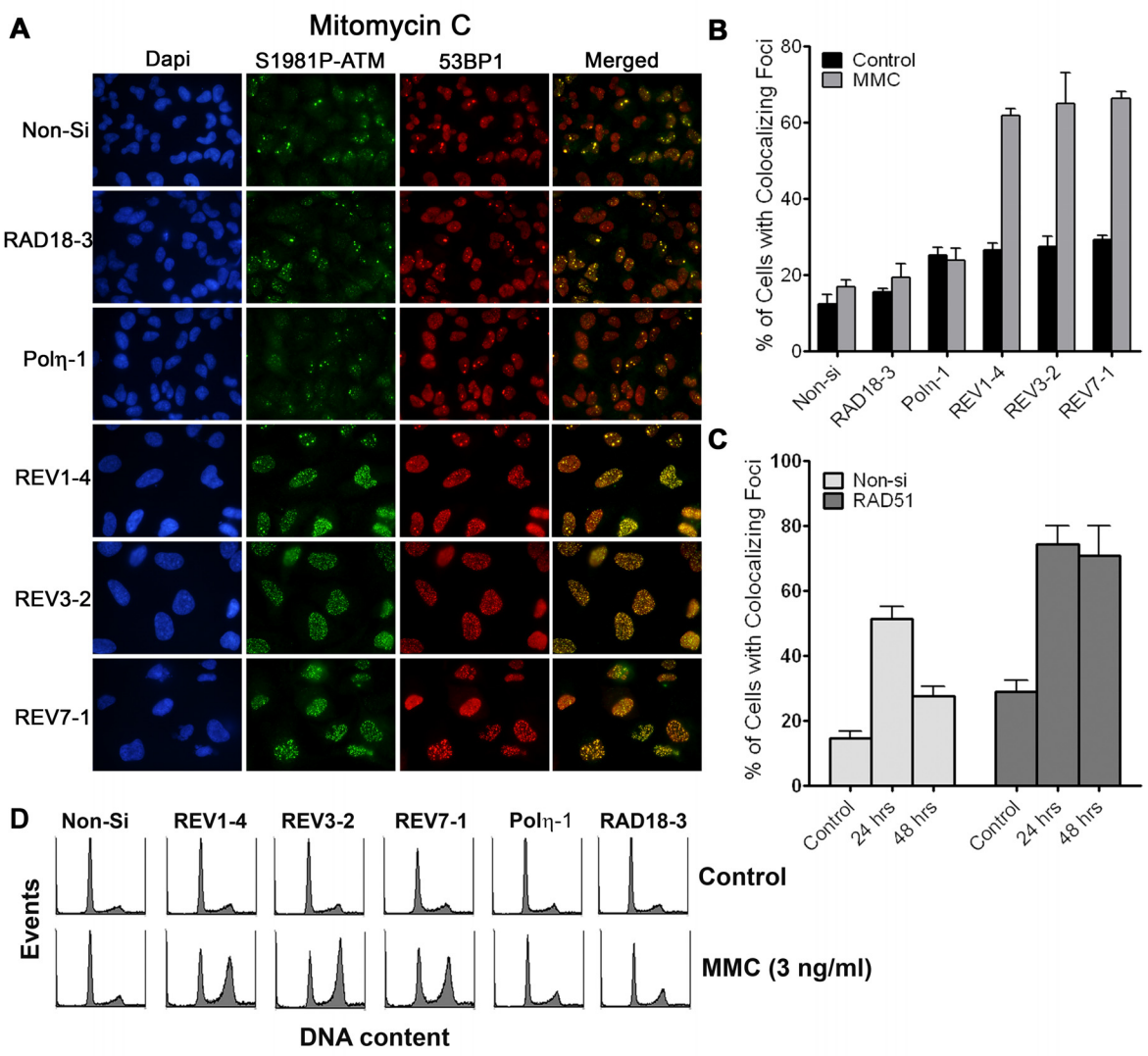


Figure 3.6 The REV1/polymerase zeta functional complex is necessary for efficient repair of interstrand DNA cross-links induced by mitomycin C. (A) RAD18, polymerase eta (Polη), REV1, REV3 or REV7-depleted HeLa cells grown on coverslips were treated with 30 ng/ml mitomycin C (MMC) for 1 hr and then allowed to recover for 48 hrs. Cells were fixed in 100% methanol and then stained for S1981P-ATM (green) and 53BP1 (red) as surrogate markers of DNA double strand breaks (DSBs). Nuclear DNA was stained with DAPI (blue). (B) The graph represents the average percentage of cells exhibiting 10 or more foci per cell containing both phospho-ATM and 53BP1. Error bars represent S.E.M. (n=3). (C) Inhibition of homologous recombination repair leads to phospho-ATM and 53BP1 foci retention in mitomycin C treated cells. Control or RAD51-depleted HeLa cells were treated with 30 ng/ml MMC for 1 hour and then fixed 24 or 48 hours later. The percentage of cells exhibiting 10 or more phospho-ATM and 53BP1 colocalized foci per cell are shown. Data represent the mean ± S.E.M of three independent experiments. (D) siRNA-transfected HeLa cells were treated with 3 ng/ml MMC for 48 hrs. Cells were fixed, stained for DNA content with propidium iodide, and then analyzed by flow cytometry. REV1, REV3, or REV7-depleted cells exhibit a

prolonged G2 cell cycle checkpoint suggestive of a defect in interstrand DNA cross-link repair.

Furthermore, HeLa cells deficient in REV1, REV3, or REV7, but not RAD18 or polymerase eta, displayed prominent cell cycle arrests in late S and G2 phases of the cell cycle (Fig. 3.6D). The result is indicative of prolonged cell cycle checkpoint induction after treatment with a relatively low dose of mitomycin C (3 ng/ml), a phenotype characteristically displayed by cells deficient in components of the FA pathway. Together, these results suggest that REV1 and polymerase zeta are necessary for resistance to agents that introduce DNA interstrand cross-links and their activities are important for efficient resolution of DNA double strand breaks, an intermediate formed during DNA replication-dependent interstrand DNA cross-link repair.

Discussion

In Chapter 2 data were presented to support the hypothesis that the E3 ubiquitin ligase RAD18 along with the translesion polymerases eta, REV1 and zeta are all necessary for synthesis past intrastrand cross-links induced by the chemotherapeutic agent cisplatin. However, as noted in Figure 2.8 HeLa cells depleted of REV1, REV3 or REV7 were uniquely more sensitive to cisplatin treatment than HeLa cells down-regulated for RAD18 or polymerase eta. The hypersensitivity observed in REV1, REV3 or REV7 knockdown cells suggest that REV1 and polymerase zeta may have additional biological roles beyond lesion bypass in HeLa cells exposed to cisplatin. Since translesion polymerases are implicated in the repair of DNA interstrand cross-links [9, 13-15], I investigated if the hypersensitivity observed in REV1, REV3 or REV7 depleted HeLa cells is due to failure to repair chemotherapeutic induced DNA interstrand cross-links. I also explored the possibility of polymerase eta contributing to interstrand cross-link repair and if RAD18 regulates translesion synthesis during interstrand cross-link repair.

Resistance to mitomycin C-induced chromosomal aberrations and cytotoxicity does not appear to require RAD18 in the experimental system suggesting that PCNA

monoubiquitination is not an important regulatory element in directing translesion synthesis during interstrand cross-link repair in S phase. This conclusion is consistent with the observation that mitomycin C is a relatively weak inducer of PCNA monoubiquitination (Fig. 2.4B). Though RAD18-mediated PCNA monoubiquitination may be necessary for translesion synthesis across cisplatin intrastrand cross-links or other distorting DNA adducts, depletion of RAD18 does not appear to have a significant impact on the resolution of replication-associated double strand breaks presumably associated with recombination-dependent interstrand DNA cross-link repair (Fig. 3.3 and 3.6).

The results highlight major differences between how interstrand DNA cross-link repair is proposed to be regulated in G1 versus DNA cross-link repair operational during S phase and reliant upon homologous recombination. In yeast and mammals, recombination-independent interstrand DNA cross-link repair involving polymerase zeta-dependent translesion synthesis is dependent upon RAD18-mediated PCNA monoubiquitination [17, 48]. Therefore, the moderate sensitivity observed in RAD18 depleted HeLa cells to mitomycin C (Fig. 3.5) may be contributed to disruption of recombination-independent repair of interstrand cross-links. The exact percentage of interstrand cross-links repaired in a recombination-independent manner versus a recombination-dependent manner is unknown. However, if recombination-independent repair of interstrand cross-links represented a major pathway of cross-link removal, the expectation would be a similar toxicity profile between RAD18 and polymerase zeta knockdown cells following mitomycin C exposure. The chromosomal instability and significantly greater hypersensitivity observed in REV1, REV3 or REV7 knockdown cells compared to RAD18 depleted cells following cisplatin or mitomycin C treatment demonstrates the predominance of recombination-dependent interstrand cross-link repair in human cells.

Depletion of the translesion polymerases REV1 and zeta are uniquely associated with protection against cisplatin and mitomycin C-induced chromosomal aberrations when compared to polymerase eta depleted HeLa cells. In addition, down-regulation of REV1 and polymerase zeta, but not polymerase eta, results in extraordinary mitomycin C

hypersensitive along with cell cycle arrest in late S and G2 phases of the cell cycle. REV1 and polymerase zeta were also found to be necessary for the timely resolution of DNA double strand breaks associated with repair of DNA interstrand cross-links. Polymerase eta is proposed to be necessary for extension of D-loops during homologous recombination [29]. Since cisplatin and mitomycin C-induced replication-associated DNA double strand breaks are resolved in a timely manner in polymerase eta deficient cells, polymerase eta is most likely not critical for recombination-dependent repair of interstrand cross-links (Fig 3.3 and Fig 3.6).

Treatments which predominantly introduce interstrand cross-links (*e.g.* mitomycin C) do not effectively induce PCNA monoubiquitination or translesion synthesis polymerase focus formation (Fig. 2.4B) [49-51]. Therefore a major question is how the REV1/polymerase zeta functional complex is regulated with respect to its recruitment to an active replication fork blocked by an interstrand DNA cross-link and the subsequent bypass across an unhooked interstrand DNA cross-link adduct. Mirchandani *et al.* demonstrated the Fanconi anemia core complex, independent of FANCD2, is crucial for REV1 foci formation following exposure to UV [23]. Data provided in chapter 2 confirms that the Fanconi anemia core complex regulates translesion synthesis by promoting the formation of REV1 foci at sites of replication stress in cisplatin treated cells, and the recruitment is independent of FANCD2 (Fig. 2.7). In contrast to translesion synthesis located at stalled replication forks due to adducted DNA, recent evidence suggests that recombination-dependent interstrand DNA cross-link repair, as measured by a sophisticated reporter system in mammalian cells, requires both the Fanconi anemia core complex and FANCD2, as well as polymerase zeta [12].

Therefore it is speculated that the Fanconi anemia core complex performs at least two distinct functions when responding to replication stress: 1) Fanconi anemia core proteins regulate REV1-dependent translesion synthesis in response to replication fork stalling via a FANCD2-independent process that is parallel to Rad18-mediated monoubiquitination of PCNA, and 2) Fanconi anemia core proteins promote REV1 and polymerase zeta-dependent translesion synthesis during replication-associated interstrand cross-link repair in a FANCD2-dependent manner. Since FANCD2 is ubiquitinated via

the Fanconi anemia core complex in response to replication-associated interstrand cross-links, and PCNA is not, this may be an important signal for the REV1/polymerase zeta functional complex to localize to the site of interstrand cross-links.

To further complicate the relationship between the Fanconi anemia pathway and translesion synthesis, a recently proposed model offers a drastically different view of how the Fanconi anemia pathway coordinates interstrand cross-link repair. Shen *et al*. suggest the Fanconi anemia core complex along with the FANCD2-I complex is necessary for recombination-independent cross-link repair while FANCD1/BRAC2, FANCN and FANCJ are necessary for recombination-dependent cross-link repair [25]. Incorporating this model with previously published reports, the Fanconi anemia core complex, in a FANCD2-dependent manner, along with RAD18 via monoubiquitination of PCNA would regulate translesion synthesis at sites of recombination-independent repair of interstrand cross-links. The possibility would then exist for translesion synthesis to be regulated by FANCD1/BRAC2, FANCN and/or FANCJ during recombination-dependent repair of interstrand cross-links. Nonetheless, much work is needed to determine how translesion synthesis is regulated at sites of recombination-independent and recombination-dependent interstrand DNA cross-link repair.

Together, the findings indicate that REV1 and polymerase zeta facilitate recombination-dependent repair of DNA interstrand cross-links independently of PCNA monoubiquitination and polymerase eta. The observations reported here and elsewhere [11, 52] provide novel insight into the crucial role REV1 and polymerase zeta play in providing tolerance to a variety of DNA lesions during replication of genomic DNA and in facilitating repair of interstrand DNA cross-links. Understanding how REV1 and polymerase zeta contribute to bypass and repair of DNA damaged induced by chemotherapeutic agents may lead to the discovery of new drug targets to treat cancers resistant to DNA cross-linking agents. Taking into account the data presented in Chapter

2, the results clearly demonstrate differential roles of translesion polymerase in terms of what tolerance pathways they contribute to in response to intrastrand or interstrand DNA cross-links.

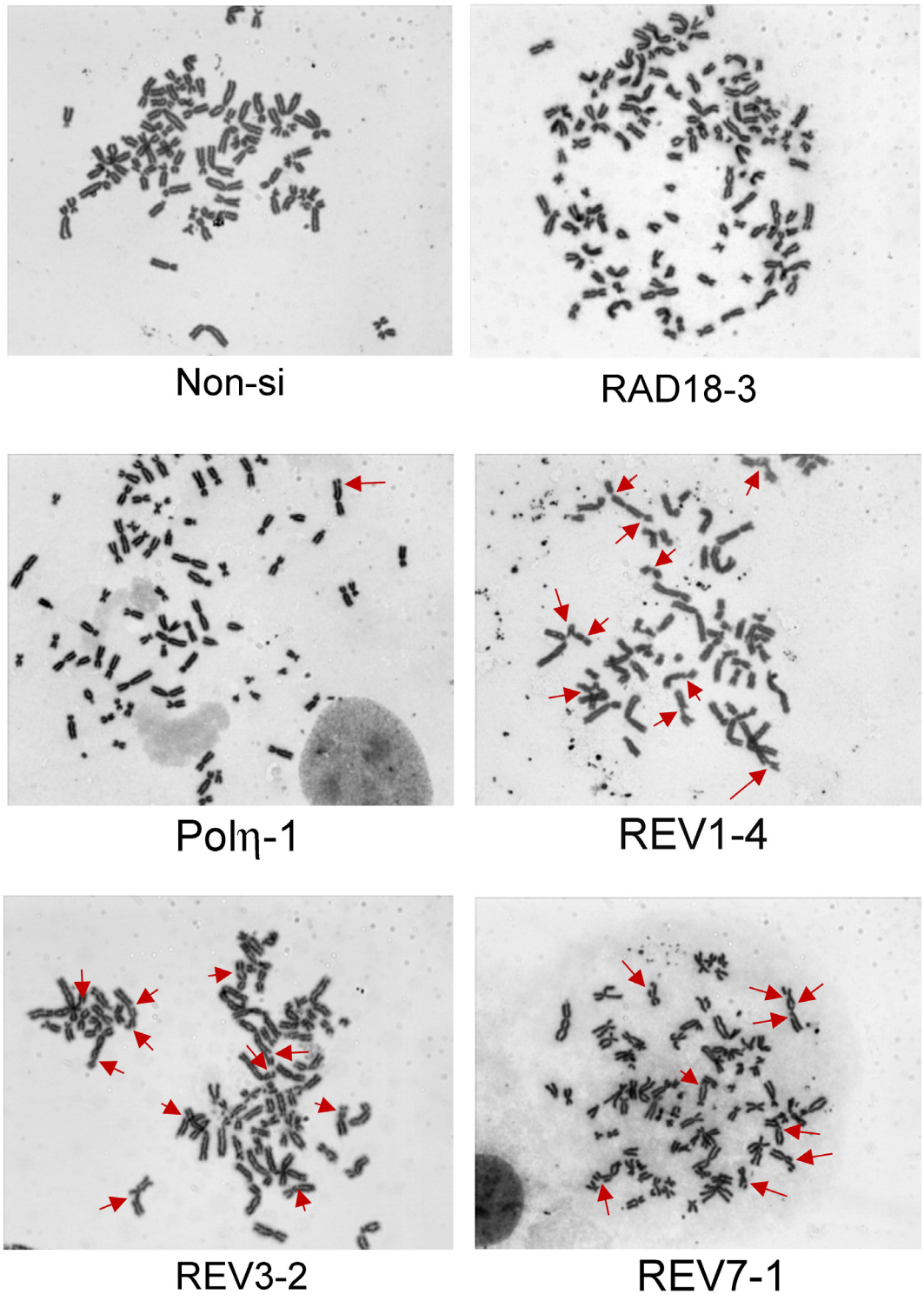


Figure S3.1 Representative images showing cisplatin-induced chromosomal aberrations in siRNA-transfected HeLa cells.

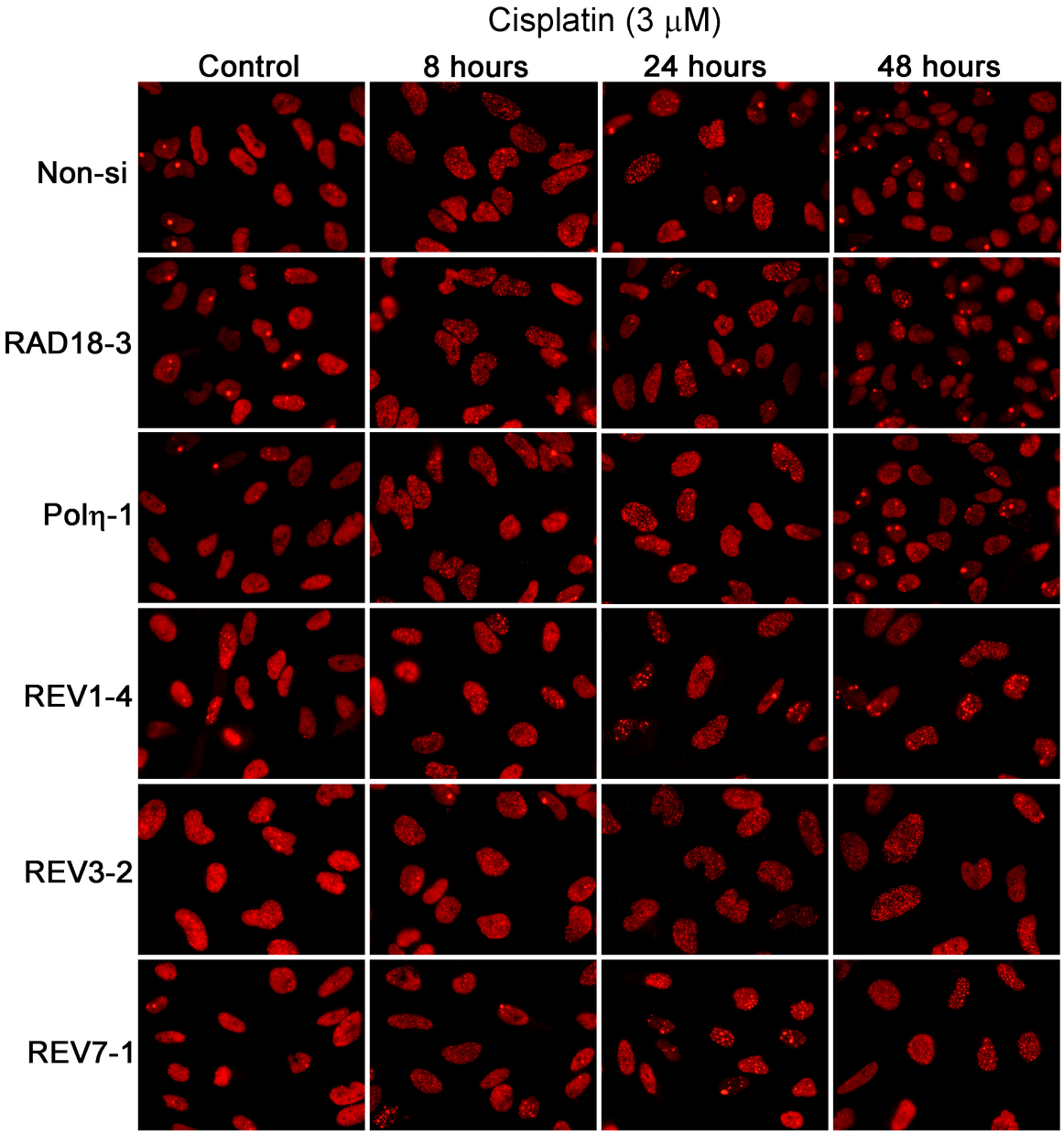


Figure S3.2 Time course demonstrating the appearance and resolution of 53BP1 foci over 48 hours after a one hour treatment with 3 μM cisplatin. HeLa cells exhibited cisplatin-induced 53BP1 foci, a surrogate marker of DSBs, between 8 and 24 hours. These foci resolve by 48 hours. Cells depleted of REV1, REV3 or REV7 retain 53BP1 foci suggesting deficiency in DNA repair.

Figure S3.3 Depletion of RAD18 or polymerase eta (Polη) has little impact on the ability of HeLa cells to resolve DNA double-stranded breaks (DSBs) associated with interstrand DNA cross-link repair. HeLa cells were independently transfected with one of three different siRNA sequences targeting RAD18 or Polη. Cells were then treated with 3 μM cisplatin for 1 hr, fixed 48 hours later, and then stained with antibodies specific for S1981P-ATM (green) and 53BP1 (red). Colocalized foci indicate the presence of DSBs thought to be an intermediate of interstrand cross-link repair. The data supports the conclusion that RAD18 and Polη are not necessary to resolve cisplatin interstrand cross-links.

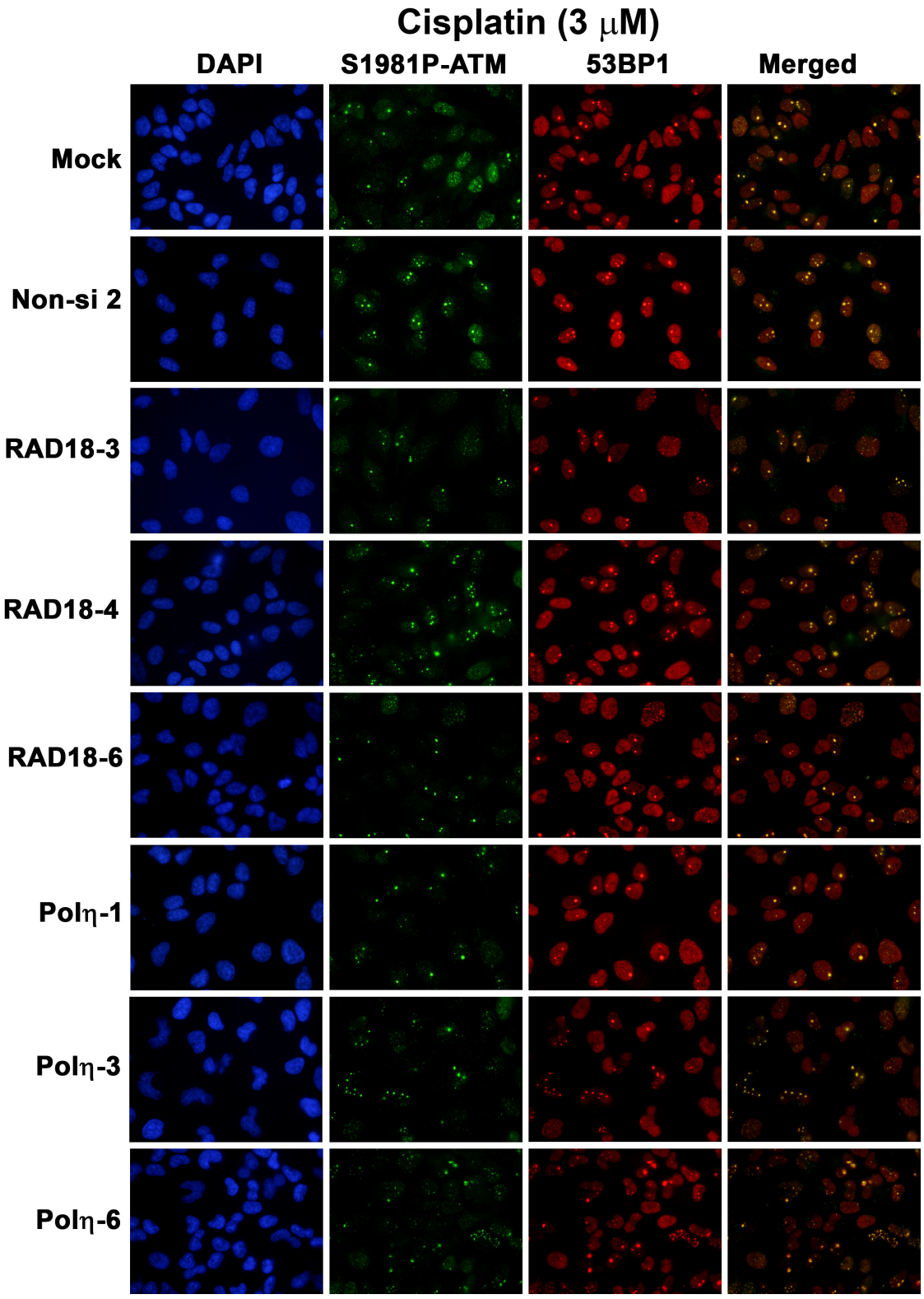
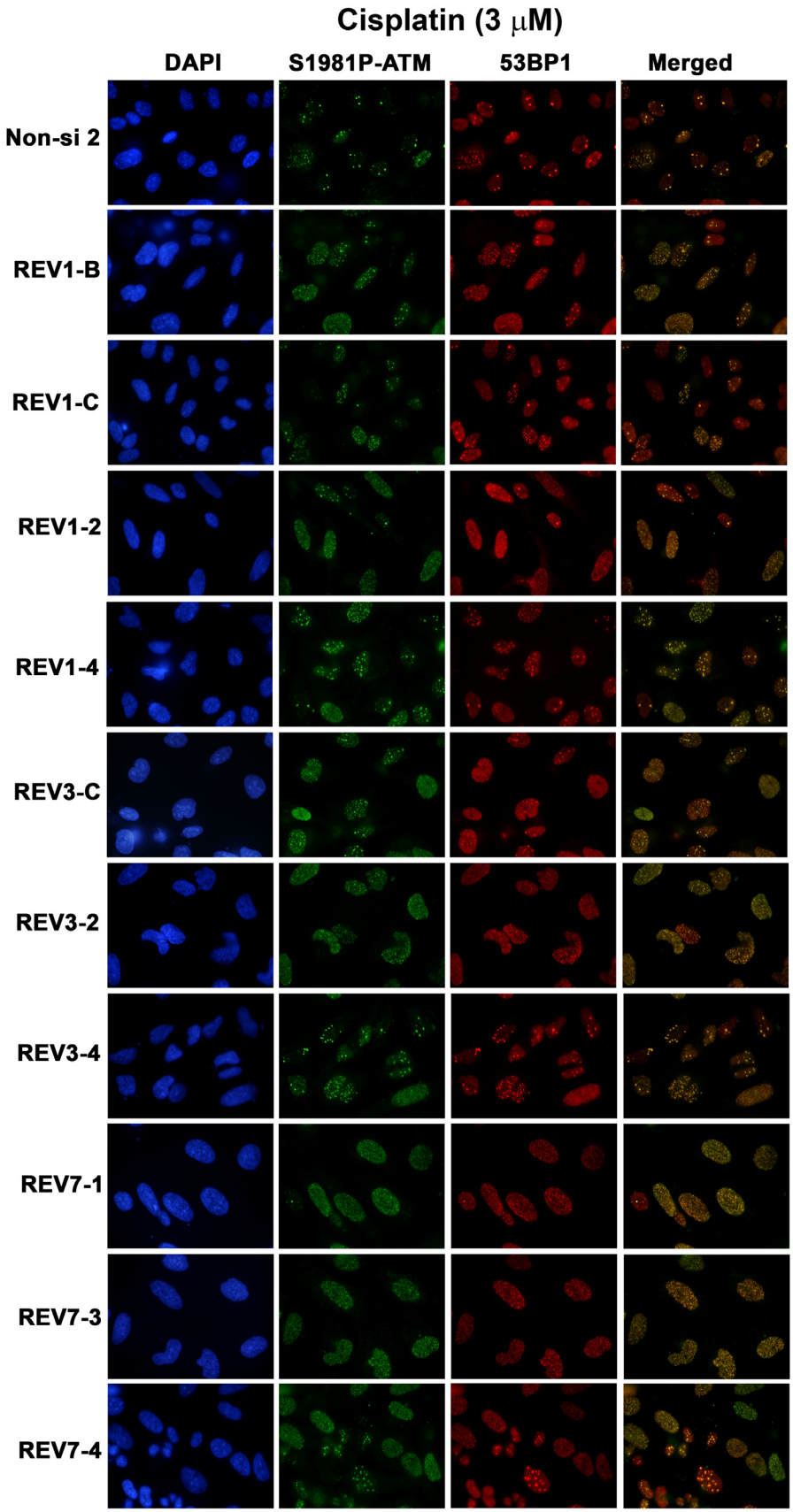

Cisplatin (3 μM)
DAPI
S1981P-ATM
53BP1
Merged
Mock
Non-si 2
RAD18-3
RAD18-4
RAD18-6
Polη-1
Polη-3
Polη-6

Figure S3.4 REV1, REV3 and REV7 are required for efficient resolution of cisplatin interstrand cross-link associated DNA double strand breaks. HeLa cells were independently transfected with one of at least three different siRNAs targeting REV1, REV3, or REV7 mRNA. Cells were treated with 3 μM cisplatin for 1 hr, fixed 48 hours later, and then stained with antibodies specific for S1981P-ATM (green) and 53BP1 (red). Colocalized foci indicate the presence of DNA double-stranded DNA breaks (DSBs). Depleting REV1, REV3 or REV7 with each individual siRNA sequence resulted in a significant increase of S1981P-ATM foci which colocalize with 53BP1 foci 48 hours after cisplatin treatment when compared to controls as well as Polη or RAD18 depleted cells). The data support the conclusion that REV1, REV3 and REV7 are necessary to efficiently resolve cisplatin-induced DNA DSBs, which are thought to arise as intermediates during interstrand cross-link repair.


Cisplatin (3 μM)
DAPI
S1981P-ATM
53BP1
Merged
Non-si 2
REV1-B
REV1-C
REV1-2
REV1-4
REV3-C
REV3-2
REV3-4
REV7-1
REV7-3
REV7-4

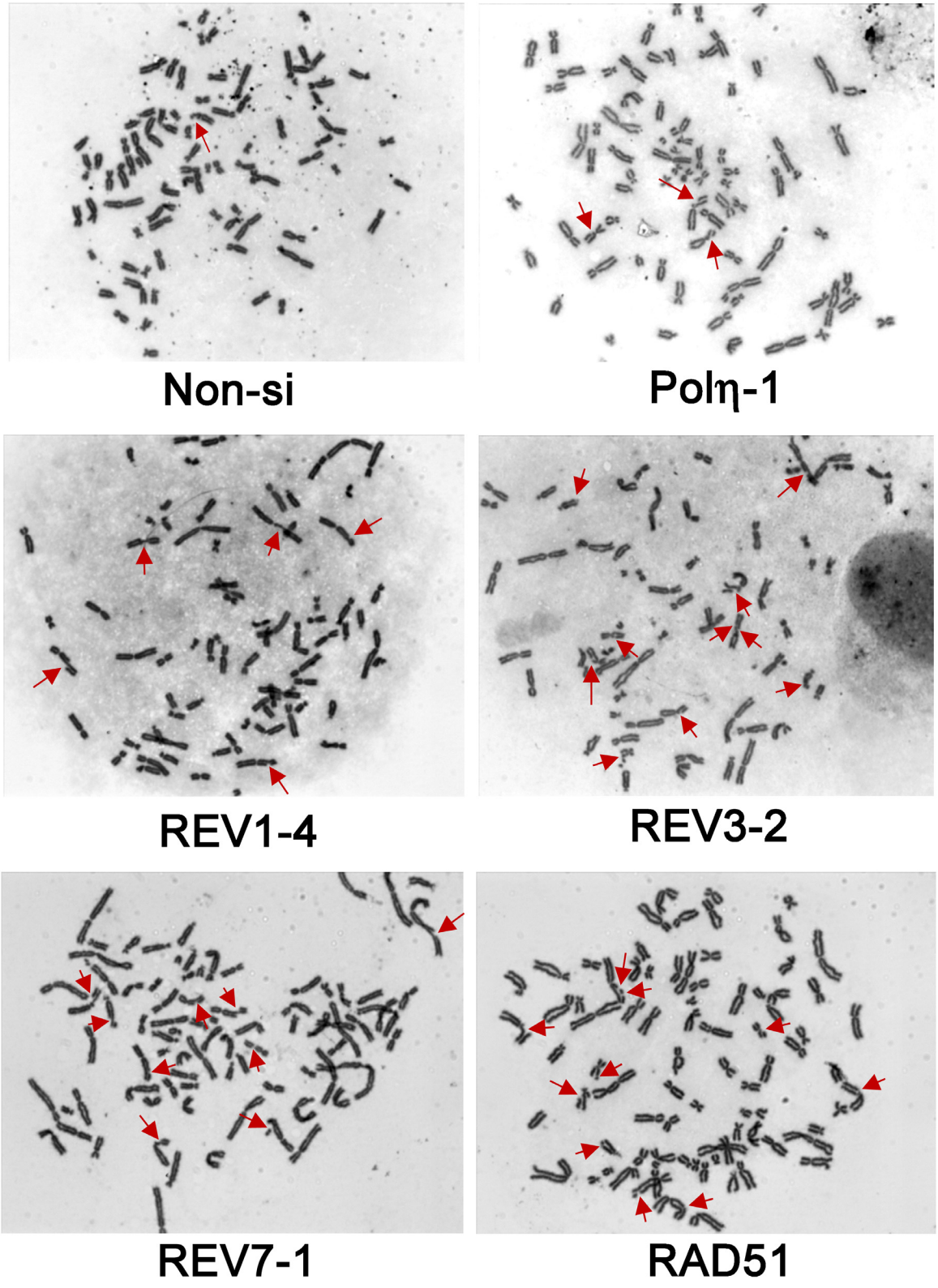


Figure S3.5 Representative images showing MMC-induced chromosomal aberrations in siRNA-transfected HeLa cells.

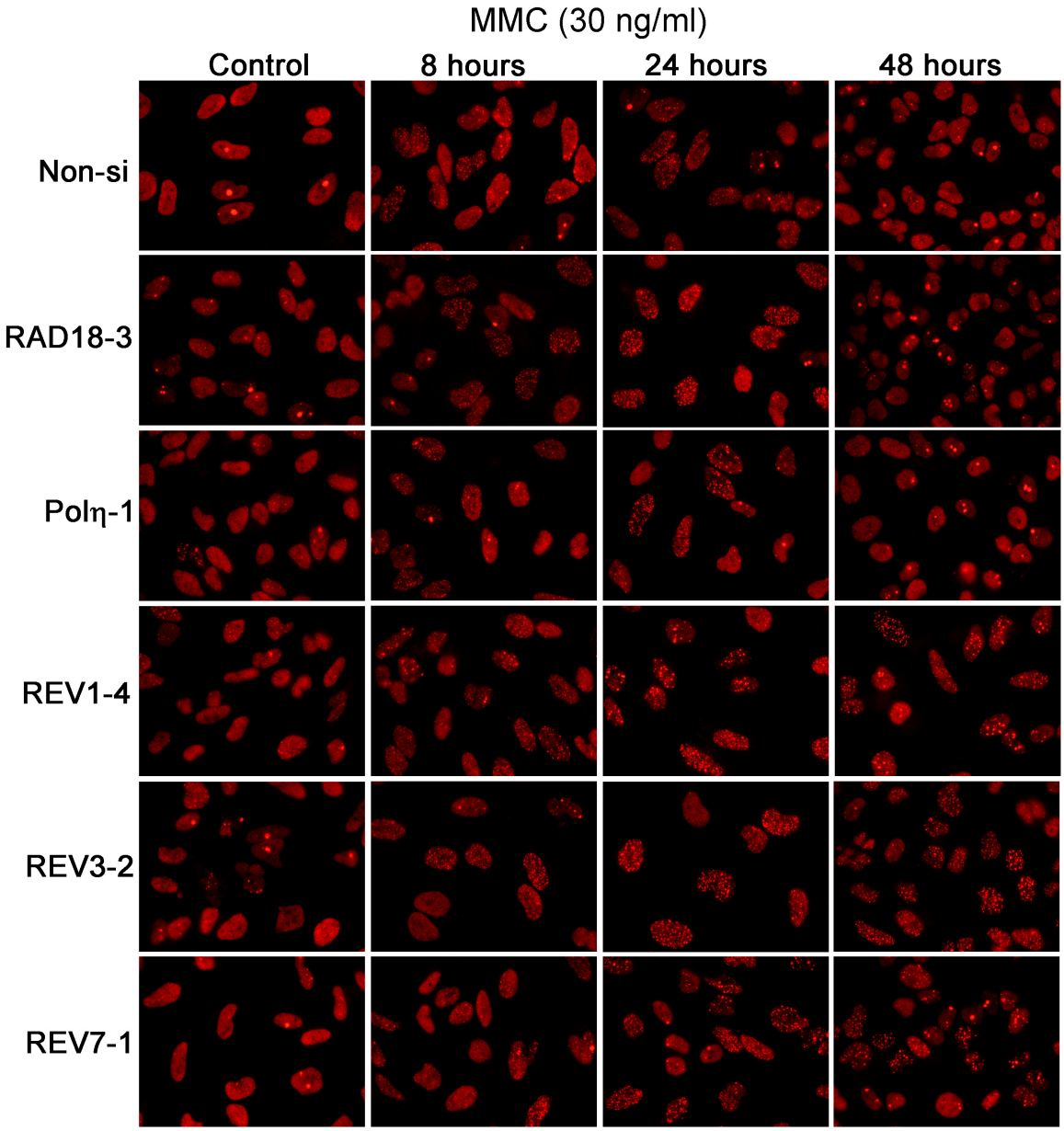


Figure S3.6 Time course showing the appearance and resolution of 53BP1 foci over 48 hours after a one hour treatment with 30 ng/ml mitomycin C. HeLa cells exhibited mitomycin C-induced 53BP1 foci, a surrogate marker of DNA double strand breaks, between 8 and 24 hours. These foci typically resolve by 48 hours. Cells depleted of REV1, REV3 or REV7 retain these 53BP1 foci suggesting deficiency in DNA repair.

Figure S3.7 Depletion of RAD18 or polymerase eta (Polη) has little impact on the ability of HeLa cells to resolve DNA double strand breaks (DSBs) associated with interstrand DNA cross-link repair. HeLa cells were independently transfected with one of three different siRNA sequences targeting RAD18 or Polη. Cells were then treated with 30 ng/ml mitomycin C (MMC) for 1 hr, fixed 48 hours later, and then stained with antibodies specific for S1981P-ATM (green) and 53BP1 (red). Colocalized foci indicate the presence of DSBs thought to be intermediates of interstrand cross-link repair. The data support the conclusion that RAD18 and Polη are not necessary to resolve MMC interstrand cross-links.

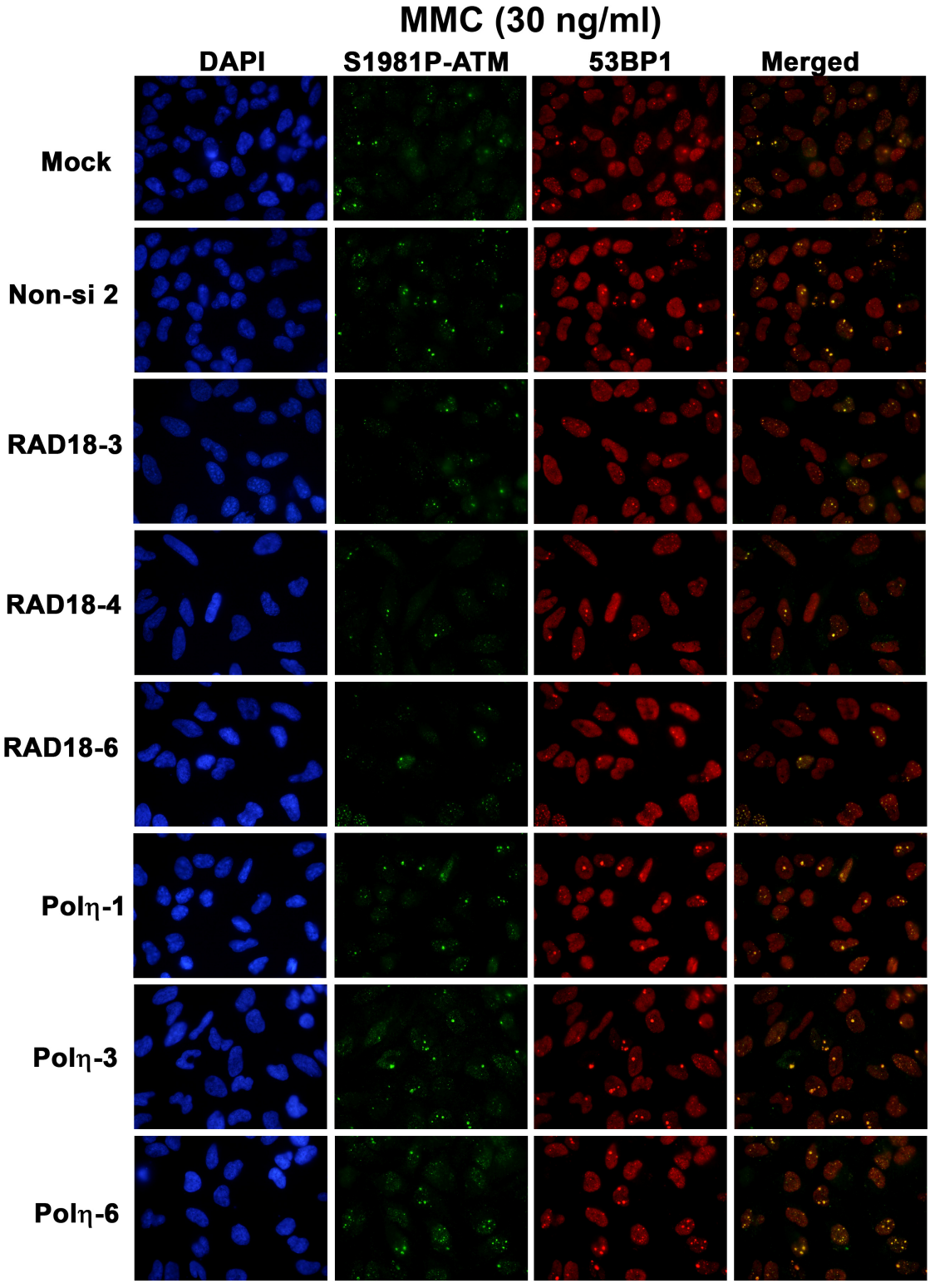
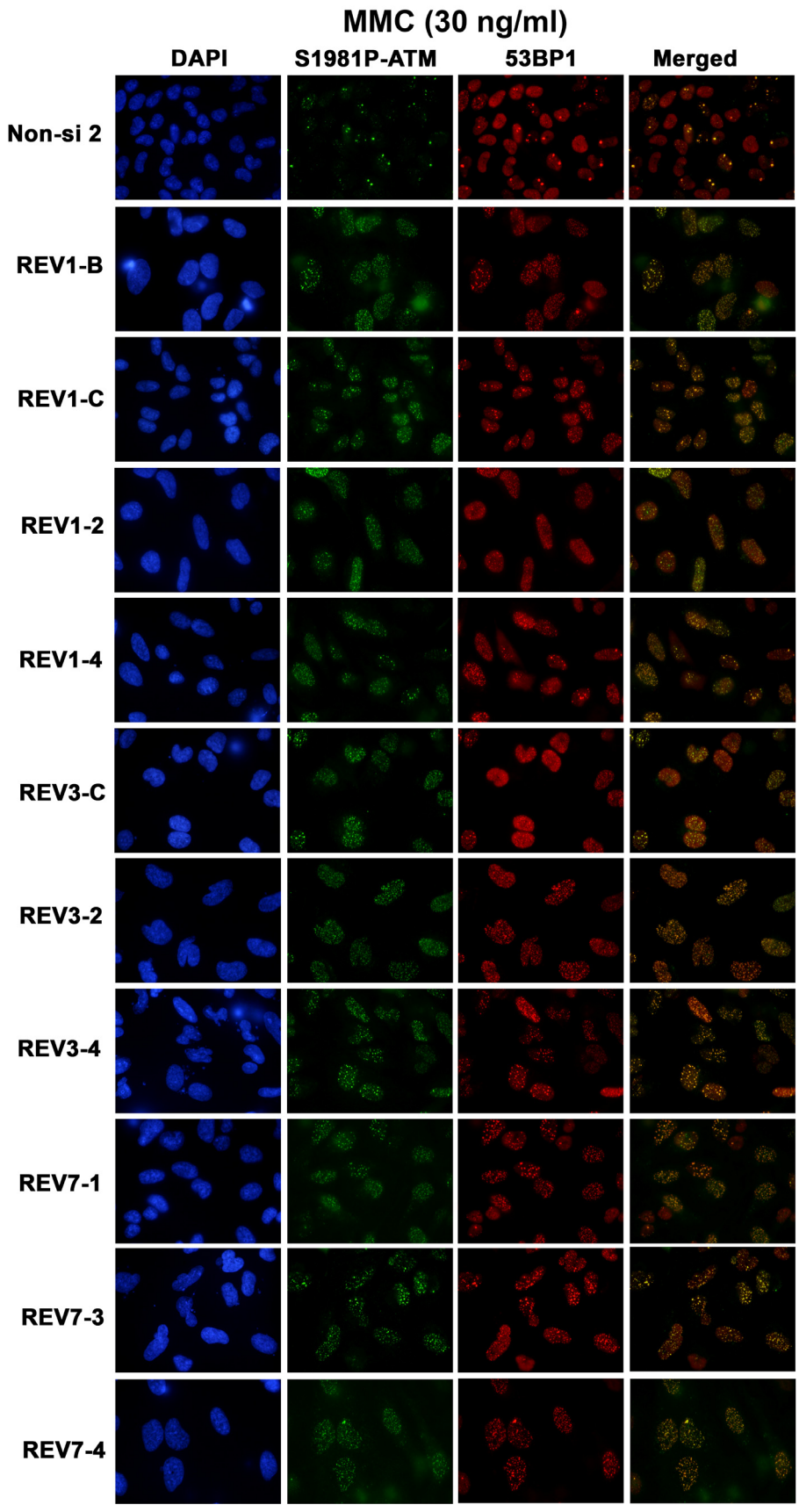

MMC (30 ng/ml)
DAPI
S1981P-ATM
53BP1
Merged
Mock
Non-si 2
RAD18-3
RAD18-4
RAD18-6
Polη-1
Polη-3
Polη-6

Figure S3.8 REV1, REV3 and REV7 are required for efficient resolution of DNA double strand breaks associated with repair of interstrand cross-links induced by mitomycin C. HeLa cells were transfected with one of at least three different siRNAs targeting REV1, REV3, or REV7 mRNA. Cells were treated with 30 ng/ml mitomycin C (MMC) for 1 hr, fixed 48 hours later, and then stained with antibodies specific for S1981P-ATM (green) and 53BP1 (red). Colocalized foci indicate the presence of DNA double-strand breaks (DSBs). Depleting REV1, REV3 or REV7 with each individual siRNA sequence resulted in a significant increase of S1981P-ATM foci which colocalize with 53BP1 foci 48 hours after MMC treatment when compared to controls as well as Polη or RAD18 depleted cells. The data support the conclusion that REV1, REV3 and REV7 are necessary to efficiently resolve MMC-induced DSBs, which are thought to arise as intermediates during interstrand cross-link repair.


MMC (30 ng/ml)
DAPI
S1981P-ATM
53BP1
Merged
Non-si 2
REV1-B
REV1-C
REV1-2
REV1-4
REV3-C
REV3-2
REV3-4
REV7-1
REV7-3
REV7-4

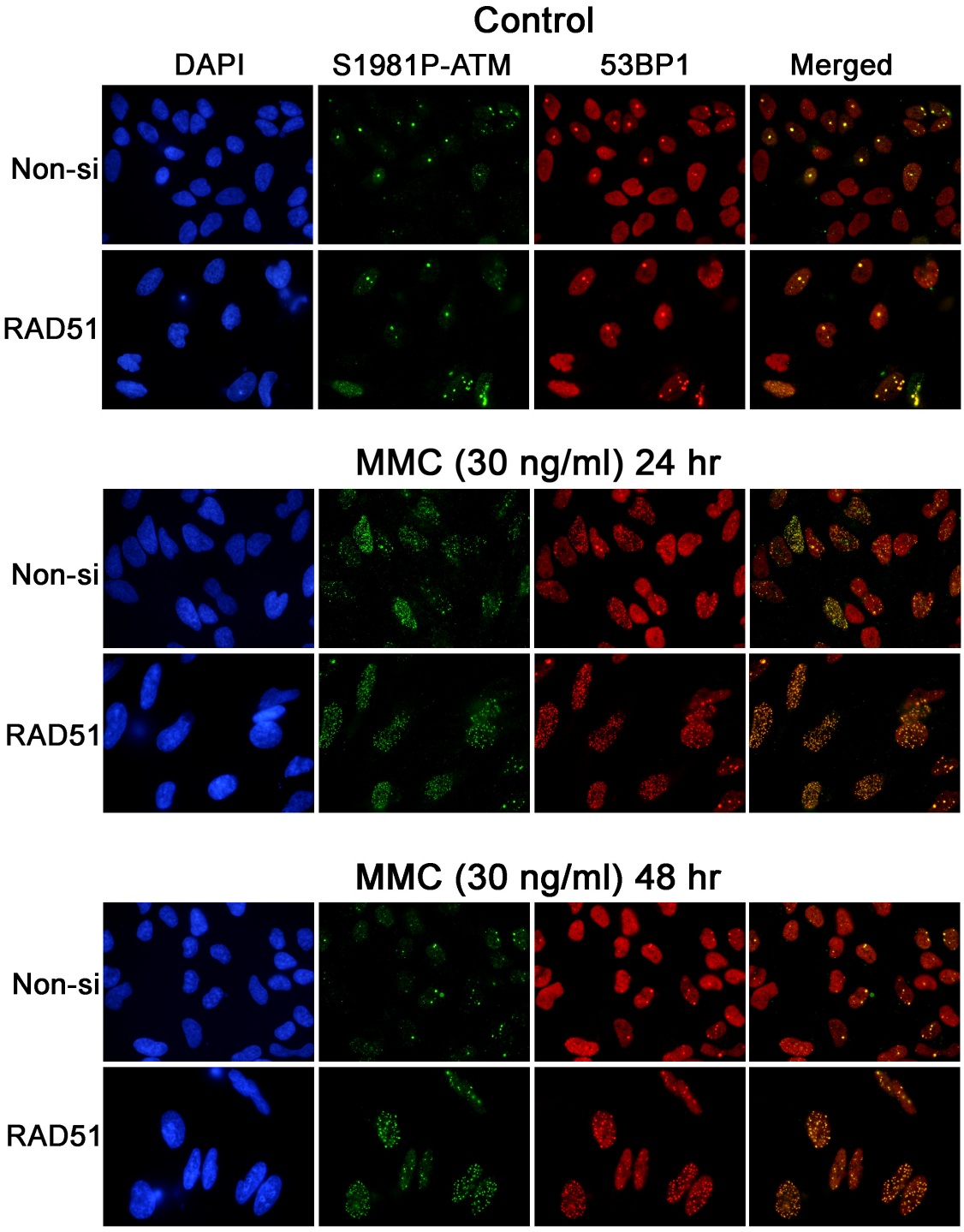


Figure S3.9 RAD51-deficient HeLa cells fail to resolve mitomycin C-induced DSBs in a timely manner. siRNA transfected HeLa cells were treated with 30 ng/ml mitomycin C for 1 hour and then fixed 24 or 48 hours later. RAD51-deficient cells failed to resolve mitomycin C-induced DSBs; consistent with the model that homologous recombination plays an important role in the repair of replication-associated DSBs during interstrand cross-link repair

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CHAPTER 4

A Role for the Translesion Polymerases REV1 and Zeta in Protection against Ionizing Radiation-Induced Cytotoxicity

Summary

Translesion polymerases have the capacity to replicate past DNA adducts, thus preventing replication stress and allowing the cell more time to cope with the damaged DNA until it can repaired. Recently, translesion polymerases have been implicated in the repair of DNA damage as well. Here I addressed if the translesion polymerases eta, REV1 and zeta have a biological role in response to ionizing radiation, specifically if these translesion polymerases are necessary for DNA double strand break repair. Depletion of REV1 or polymerase zeta in HeLa cells resulted in a prolonged G2 cell cycle arrest following exposure to ionizing radiation suggestive of a defect in repair of double strand breaks. In addition, enhanced radiosensitivity and chromosomal aberration formation was observed in REV1 and polymerase zeta depleted cells. REV1 and polymerase zeta were also found to be necessary to either prevent formation of DNA double strand breaks or repair DNA double strand breaks in response to ionizing radiation-induced DNA damage. However, polymerase eta appeared to have no cellular role in protection against cytotoxicity induced by ionizing radiation. Furthermore, RAD18 was found to have no major role in protecting cells against ionizing radiation-induced cytotoxicity. Lastly, REV1 and polymerase zeta depleted HeLa cells were demonstrated to have a mild defect in homologous recombination as measured by a homologous recombination reporter assay.

Introduction

Translesion DNA polymerases are capable of preventing prolonged replication stress by replicating past DNA adducts capable of stalling high fidelity polymerases [1]. In mammalian cells, the translesion polymerases eta, REV1 and zeta are thought to cooperate, in an insertion and extension model, to bypass multiple types of DNA damage including chemotherapeutic drug-induced DNA damage [2-5]. In addition to facilitating direct lesion bypass, polymerase eta, REV1 and polymerase zeta (REV3 and REV7) may also play an important role in repair of DNA damage [6-11]. The translesion polymerases eta, REV1 and zeta have been proposed to be necessary for either translesion synthesis across ionizing radiation-induced DNA damage or repair of DNA damage caused by ionizing radiation. *Rev1*, *Rev3* or *Rev7* knockout *Saccharomyces cerevisiae* are sensitive to ionizing radiation, and display a decrease in ionizing radiation-associated mutagenesis implying these translesion polymerases have a cellular role in response to ionizing radiation [12]. In addition, REV7 depleted nasopharyngeal carcinoma cells are hypersensitive to ionizing radiation, while *REV1*$^{-/-}$ and *REV3*$^{-/-}$ chicken DT40 cells are radiosensitive and display an increase of ionizing radiation-induced chromosomal aberrations [10-11, 13]. Furthermore, polymerase eta has been proposed to directly interact with RAD51 and to be necessary for D-loop extension during homologous recombination [14].

Currently, the biological role translesion polymerases have in response to ionizing radiation is poorly understood. Following exposure to ionizing radiation several different DNA lesions are formed including DNA adducts such as 7,8-dihydro-8-oxoguanine, abasic sites, DNA single strand breaks and DNA double strand breaks [15]. The translesion polymerases eta, REV1 and zeta may be necessary to bypass DNA abasic sites or DNA adducts formed by ionizing radiation. Polymerase eta is known to synthesize across from 7,8-dihydro-8-oxoguanine in a error free manner, while REV1 is thought to be capable of inserting a cytosine across from abasic sites [16-20]. Translesion polymerases may also be necessary for repair of DNA double strand breaks by participating in either non-homologous end-joining or homologous recombination [1, 14, 21-23]. Here, I tested the hypothesis that the translesion polymerases eta, REV1 and zeta have a biological role in

response to ionizing radiation in a human cancer cell line. I also investigated what cellular pathways these polymerases may participate in.

Depletion of REV1 or polymerase zeta in HeLa cells resulted in a prolonged G2 cell cycle arrest following exposure to ionizing radiation, suggestive of a repair defect. The REV1/polymerase zeta functional complex also protected the cells against ionizing-radiation induced cytotoxicity, noted by enhanced radiosensitivity and chromosomal aberration formation in REV1 or polymerase zeta down-regulated cells. REV1 and polymerase zeta were also found to be necessary to either prevent formation of replication-associated DNA double strand breaks or repair DNA double strand breaks in response to ionizing radiation-induced DNA damage. Polymerase eta, though, appeared to have no cellular role in protection against cytotoxicity induced by ionizing radiation.

To address what biological pathways REV1 and polymerase zeta may participate in; RAD18 was down-regulated in HeLa cells to determine if disruption of PCNA monoubiquitination-dependent translesion synthesis resulted in similar phenotypes observed in REV1 or polymerase zeta-depleted HeLa cells following ionizing radiation. RAD18-depleted HeLa cells displayed similar phenotypes as Non-si transfected HeLa cells following exposure to ionizing radiation. The results suggest translesion synthesis dependent upon PCNA monoubiquitination does not have a major role in protecting cells against ionizing radiation-induced cytotoxicity. However, the phenotypes observed in RAD51 depleted HeLa cells were comparable to the phenotypes found in REV1 or polymerase zeta knockdown cells implying these translesion polymerase may play a role in repair of DNA double strand breaks. REV1 and polymerase zeta depleted HeLa cells were also demonstrated to have a mild defect in homologous recombination as measured by a homologous recombination reporter assay.

Multiple anti-cancer agents have a mechanism of action that results in DNA double stand break formation including ionizing radiation, bleomycin, the topoisomerase I poisons such as camptothecin and the topoisomerase II poisons including etoposide and doxorubicin. Even though DNA double strand breaks are exceptionally lethal, cancer cells can become refractory to the damage [24]. The ability to repair DNA double strand breaks, thus lessening the cytotoxic effects, may contribute to resistance of double strand

break-inducing agents [25-27]. Evidence provided here suggests that the translesion polymerases REV1 and zeta have a cellular role in response to ionizing radiation, and that these polymerases may contribute to repair of ionizing radiation-induced DNA double strand breaks in HeLa cells. Participation of REV1 and polymerase zeta in double strand break repair may allow for the discovery of new drug targets and treatments for cancers resistant to double strand break-inducing agents.

Methods and Materials

Cell lines and culture conditions. HeLa cells obtained from ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Construction of *POLI*, *POLH*, and *REV3L* $^{-/-}$ BL2 lines, cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, have been described [28-29].

RNA interference. HeLa cells were seeded in 6 well plates at a density of $1x10^5$ per well. siRNA transfections were carried out with X-tremeGENE (Roche), using 50 nM siRNA plus 5µl X-tremeGENE per well. Transfections were allowed to go overnight in the presence of serum followed by a change in medium the next morning. When delivering siRNA into cells cultured in 12 well plates, the conditions for transfection was reduced by one half. The non-specific (Non-si) siRNA as well as the siRNAs directed against RAD18, polymerase eta (Polη), REV1, polymerase zeta (REV3 and REV7), and RAD51 were obtained from Qiagen. The gene-specific target sequences are as follows: RAD18-3 (GAG CAT GGA TTA TCT ATT CAA), Polη-1 (CTG GTT GTG AGC ATT CGT GTA), REV1-4 (ATC GGT GGA ATC GGT TTG GAA), REV3-2 (CCC ACT GGA ATT AAT GCA CAA), REV7-1 (GTG GAA GAG CGC GCT CAT AAA), RAD51 (AAG CTG AAG CTA TGT TCG CCA) and Non-si (AAT TCT CCG AAC GTG TCA CGT). Refer to Figure 2.1 and supplemental data Figures S2.1 and S2.2 for further validation of siRNA sequences.

Antibodies. The rabbit polyclonal antibody anti-53BP1 (H-300) was purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-phospho-ATM (Ser 1981) antibody was obtained from Rockland. The anti-phospho-histone H2A.X (Ser139, clone JBW301)

antibody was purchased from Millipore and the mouse anti-BrdU antibody was purchased from BD Pharmigen.

Assessment of cell viability. For clonogenic survival assays, HeLa cells were transfected overnight with siRNA as described above and allowed to recover the following day. Forty-hours after the initiation of the siRNA transfections, cells were exposed to varying doses of ionizing radiation and then seeded at known cell densities for colony formation. Twelve days later, colonies were stained and fixed in a solution containing 3:1 methanol and glacial acetic acid plus 1% trypan blue (Sigma). Colonies of 50 cells or greater were counted and the surviving fractions for each siRNA treatment group represent the plating efficiency for each treatment divided by the plating efficiency of the corresponding untreated control. The BL2 lymphoma lines were exposed to ionizing radiation at varying doses. Forty-eight hours following exposure to ionizing radiation, the cells were washed with PBS and assessed as to their ability to exclude trypan blue as a measure of viability.

Cell proliferation. HeLa cells with stable incorporation of the DR-GFP plasmid were down-regulated for RAD18, Polη, REV1, REV3, REV7 or RAD51 as described above. Twenty-four hours after initiation of siRNA transfections, the cells were infected with adenovirus containing I-Sce1 overnight. The next morning cells were washed once with PBS. Forty-eight hours later the cells were incubated in media containing 33.3 μM BrdU (Fisher) for 17 minutes then collected and fixed in ice-cold 70% ethanol. The cells were resuspended in PBS containing RNaseA (Roche) and incubated at 37°C for 30 minutes. After spinning down the cells, they were resuspended in 1 ml 0.1 N HCl containing 0.7% Triton X-100, and incubated on ice for 10 minutes. One ml PBS was added to the cells, aspirated, and 1 ml sterile water was added followed by incubation at 97°C for 15 minutes. The samples were placed on ice for 15 minutes, spun down, and resuspended in 100 μl blocking buffer (5% FBS, 1% goat serum, and 0.05% tween-20 in PBS). Mouse anti-BrdU antibody (BD Pharmigen) was diluted 1:100 in the blocking solution and 100 μl added to the samples for 30 minutes. The cells were washed and 150 μl blocking solution containing a 1:20 dilution of anti-mouse IgG-FITC (Sigma) was added to the

cells for 30 minutes. The cells were washed and resuspended in 500 µl PBS containing propidium iodide and acquired by flow cytometry.

Homologous recombination plasmid reporter assay. The puromycin resistant homologous recombination (HR) plasmid reporter (pDR-GFP) was a generous gift from Dr. Maria Jasin. HeLa cells were plated at a density of 3 x10^5 cells per well of a six well plate and transfected with pDR-GFP utilizing 6 µl of Lipofectamine 2000 (Invitrogen) plus 2 µg of pDR-GFP per well for a total of 6 hours in serum free Opti-Mem (Gibco). HeLa cells with stable incorporation of the plasmid were selected for by plating the cells at a density of 1 cell per well in a 96 well plate and treating with 2 µg/ml puromycin. Puromycin resistant clones were characterized by introducing I-Sce1 into the cells and measuring GFP expression by flow cytometry. Clones expressing GFP after introduction of I-Sce1 were deemed to have stable incorporation of pDR-GFP. Twenty-four hours after the initiation of siRNA transfections, adenovirus containing I-Sce1 was added to the cells overnight. The next morning cells were washed once with PBS. Forty-eight hours following adenovirus infection, the cells were collected and analyzed for GFP expression as detailed below. The I-Sce1 adenovirus was a gift from the University of Michigan Vector Core.

Flow cytometry. For cell cycle analysis, cells were fixed with ice cold 70% methanol and then resuspended in PBS containing RNAse A and propidium iodide to determine DNA content. For acquisition of GFP expression, HeLa cells were fixed with 3.7% para-formaldehyde solution and on the day of analysis washed once with PBS before analysis. Cells were acquired on a BD FACSCaliber system using CellQuest software.

Immunofluorescence. For S1981P-ATM and 53BP1 immunofluorescence along with γ-H2AX staining, cells were cultured and transfected on glass coverslips. Before fixing in ice cold 100% methanol, the cells were washed in phosphate buffered saline (PBS) once. Samples were blocked with 5% fetal bovine serum, 0.05% Triton X-100, and 1% goat serum for 1 hour. Following the block, cells were incubated with the appropriate primary antibody listed above for 45 minutes then washed three times with PBS and incubated with the appropriate secondary goat anti-rabbit or goat anti-mouse Alexa Fluor dye conjugated secondary antibody (Molecular Probes) for 45 minutes. The cells were then

washed with PBS, counterstained with DAPI to visualize nuclear DNA, and then mounted onto slides with ProLong Gold antifade reagent (Invitrogen).

Analysis of chromosomal aberrations. HeLa cells were exposed to 4GY ionizing radiation, and 24 hrs later mitotic cells were enriched through the addition of 50 ng/mL colcemid (Gibco) for 45 minutes. After colcemid exposure, the cells were treated for 18 minute at 37°C with a hypotonic solution consisting of 0.075 M KCl and then fixed in Carnoy's fixative (3:1 methanol:glacial acetic acid). Cells were dropped onto microscope slides and allowed to dry. Prior to analysis chromosomes were stained with Giemsa as described [30]. Fifty mitotic spreads were analyzed for each condition. For comparisons of the average number of gaps and breaks per cell between control and ionizing radiation exposed groups, the student's T-test was employed.

Results

Differential roles for the translesion polymerases eta (Polη), REV1 and zeta (Polζ) in response to ionizing radiation-induced cytotoxicity.

To investigate if the translesion polymerases eta, REV1 and zeta have a cellular role in response to ionizing radiation, HeLa cells depleted of these proteins were exposed to ionizing radiation or ultraviolet radiation for comparative purposes and analyzed for cell cycle distribution. HeLa cells down-regulated for polymerase eta, REV1, or polymerase zeta (REV3 and REV7) were exposed to either 4 J/m^2 ultraviolet radiation or 4 GY ionizing radiation and collected 24 hours later. Depletion of Polη, REV1, REV3 or REV7 individually in HeLa cells resulted in cell cycle arrest in S and G2 phases of the cell cycle following exposure to ultraviolet light (Fig. 4.1A). The cell cycle distribution following exposure to UV radiation in Polη, REV1, REV3 or REV7-depleted HeLa cells is very similar to the cell cycle profile observed following cisplatin treatment (Fig. 2.2C). Over the course of 24 hours Polη, REV1, REV3 or REV7 knockdown cells arrested in the S and G2 phases of the cell cycle while the Non-si transfected cells are able to escape and progress through the cell cycle and proceed to G1. The results are consistent with idea

that polymerase eta, REV1 and polymerase zeta are necessary for bypass of both cisplatin and ultraviolet radiation-induced DNA adducts as indicated by the inability of depleted cells to complete S phase.

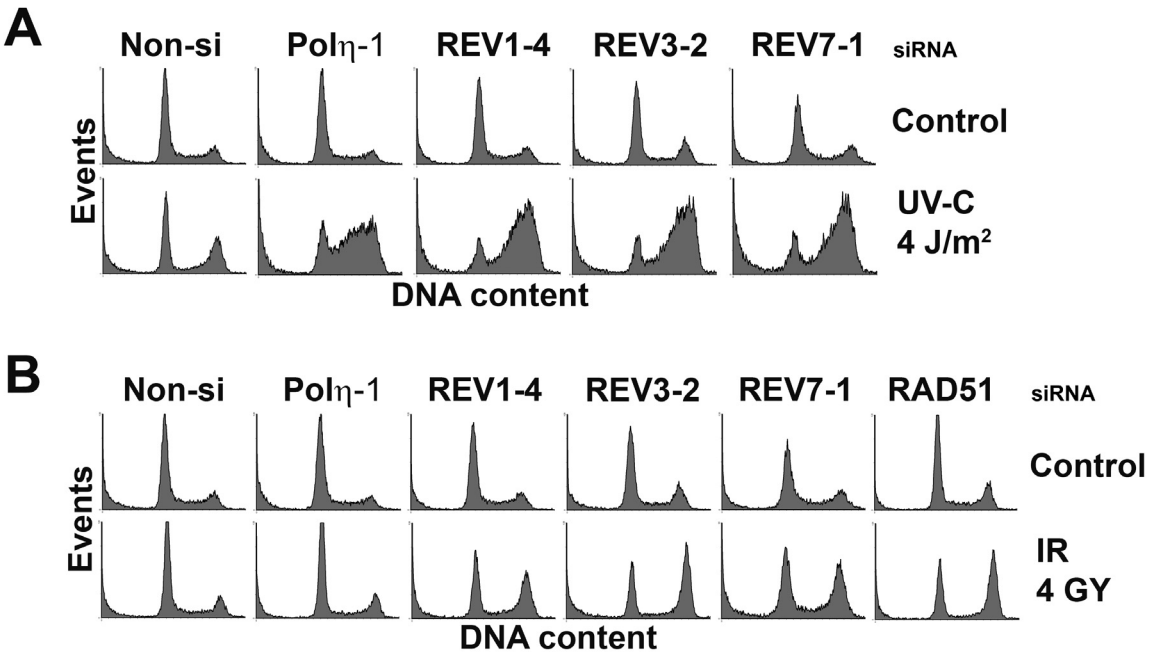

Figure 4.1 Differential roles of the translesion polymerases eta (Polη), REV1 and zeta (REV3 and REV7) in response to ultraviolet and ionizing radiation. HeLa cells transfected with the indicated siRNAs were exposed to either 4 J/m^2 ultraviolet (UV) radiation or 4GY ionizing radiation (IR). Twenty-four hours later the cells were fixed, stained for DNA content with propidium iodide and analyzed by flow cytometry. (A) Exposure of Polη, REV1, REV3 or REV7-depleted HeLa cells to 4 J/m^2 ultraviolet (UV) radiation resulted in accumulation of cells in S and G2 phases of the cell cycle, a phenotype associated with replication stress due to stalled replication forks. (B) REV1, REV3, or REV7 knockdown cells exhibit a prolonged G2 cell cycle checkpoint in response to IR. HeLa cells deficient of Polη demonstrated a similar cell cycle distribution as Non-si transfected cells in response to IR implying Polη does not have a biological role in the response to ionizing radiation.

HeLa cells deficient of REV1, REV3 or REV7, but not Polη, displayed a prolonged G2 cell cycle arrest 24 hours after ionizing radiation exposure, signifying Polη does not have a biological role in response to ionizing radiation (Fig. 4.1B). Approximately 8 to 16 hours after exposure to 4 GY ionizing radiation the Non-si, Polη, REV1, REV3 or REV7 siRNA transfected HeLa cells displayed a late S/G2 phase cell

cycle arrest (data not shown). The cell cycle arrest is attributed to the activation of cell cycle checkpoints due to DNA damage induced by ionizing radiation. By 24 hours Non-si and Polη siRNA transfected cells escaped G2 cell cycle arrest and proceeded into the G1 phase of the cell cycle, implying that the ionizing radiation-induced damage was repaired. The prolonged cell cycle arrest observed in the REV1, REV3 or REV7-depleted cells suggest that DNA damage in not repaired, and is a similar phenotype observed in cells with a defect in non-homologous end-joining or homologous recombination [31]. Depletion of translesion synthesis proteins using several different siRNA sequences targeting Polη, REV1, REV3 or REV7 lead to similar cell cycle arrest profiles following ultraviolet or ionizing radiation exposure demonstrating that the effects observed here are gene-specific (supplemental data Fig. S4.1 and S4.2).

The cell cycle distribution of REV1, REV3 or REV7-depleted HeLa cells was directly compared to HeLa cells with a nonfunctional homologous recombination repair pathway via RAD51 down-regulation (Fig. 3.4C). The recombinase RAD51 forms a nucleoprotein filament on the 3´ DNA overhang located at the site of a DNA double strand break, and actively searches the DNA for a homologous repair template. In the absence of RAD51 the homologous recombination repair pathway is severely inhibited. RAD51-depleted HeLa cells were exposed to 4 GY ionizing radiation and collected 24 hours later for cell cycle analysis (Fig 4.1B). REV1, REV3 or REV7-depleted HeLa cells exhibited almost the same cell cycle redistribution as RAD51-depleted HeLa cells following ionizing radiation, suggesting REV1, REV3 or REV7 may have a role in repair of ionizing radiation-induced DNA double strand breaks possibly by participating in the homologous recombination repair pathway.

Due to the observed differences in cell cycle distribution following ionizing radiation between REV1, REV3 or REV7-depleted HeLa cells and Polη knockdown cells, a hypothesis was formed that REV1, REV3 and REV7 are necessary to protect cells against ionizing radiation-induced cytotoxicity while polymerase eta would not be essential for protection against DNA damage caused by ionizing radiation. To test this hypothesis, REV1 or polymerase zeta-depleted cells were subjected to a clonogenic survival assay to determine whether they are uniquely sensitive to ionizing radiation. As

expected, HeLa cells deficient of polymerase eta are not any more sensitive to ionizing radiation than HeLa cells transfected with a non-specific (Non-si) siRNA (Fig. 4.2A). REV1, REV3, or REV7 siRNA-transfected HeLa cells, on the other hand, are significantly more sensitive to loss in clonogenic cell survival as compared to polymerase eta-depleted cells (Fig. 4.2A). To confirm these results in a different model system, *POLH* or *REV3L* knockout Burkitt's lymphoma BL2 cells were exposed to varying doses of ionizing radiation and viability measured 48 hrs later via trypan blue exclusion. Similar to the results obtain from the clonogenic survival assay, *REV3L*$^{-/-}$ BL2 cells were more sensitive to loss in viability after exposure to ionizing radiation when compared with *POLH* knockout cells (Fig.4.2B). Based on the results obtained from the trypan blue exclusion assay, polymerase ίota is similar to polymerase eta in that this polymerase does not appear to have a biological role in protecting cells from ionizing radiation-induced cell death.

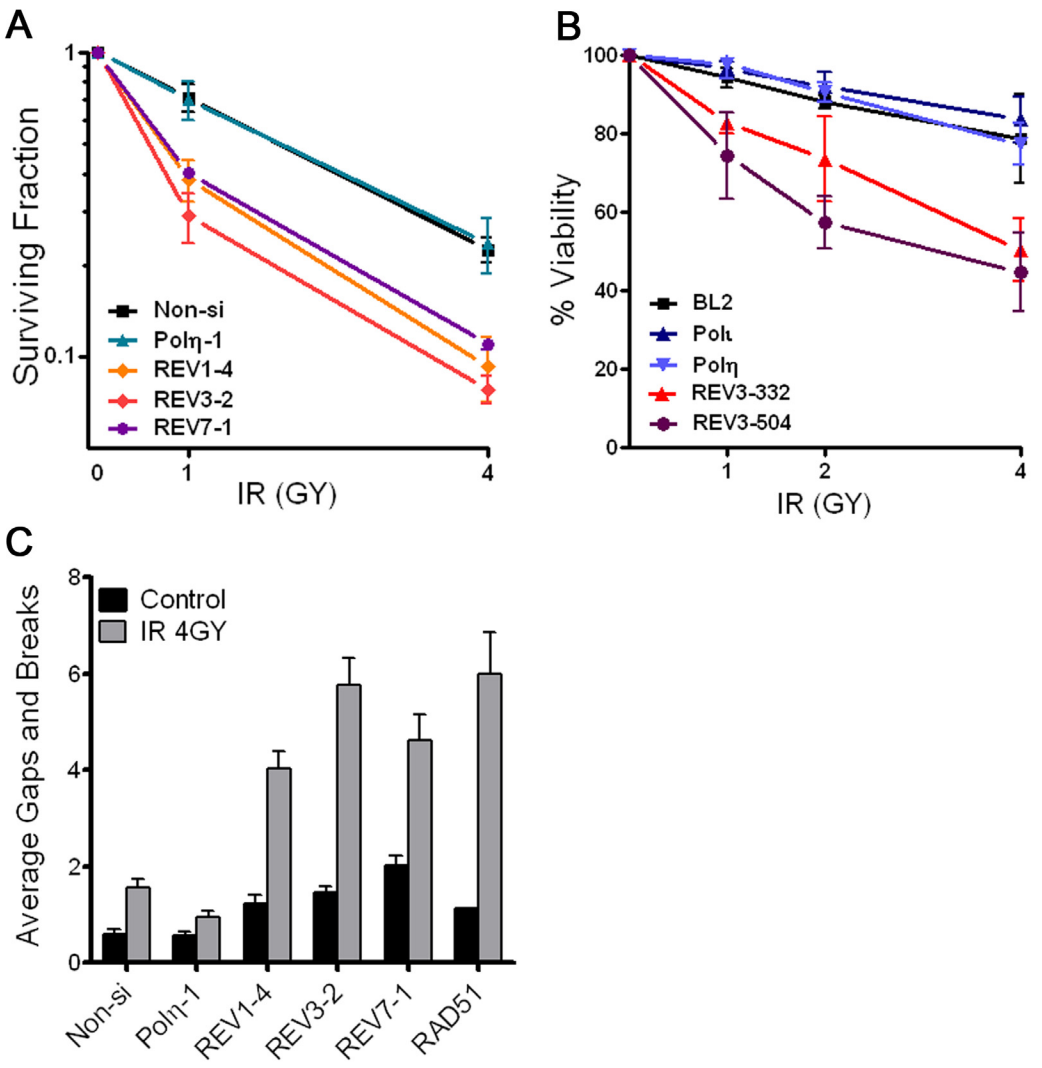

Figure 4.2 REV1, REV3 or REV7, but not Polη, protects against ionizing radiation-induced cytotoxicity. (A) HeLa cells were transfected with non-specific siRNA (Non-si) or siRNA targeting Polη, REV1, REV3 or REV7. Forty hours after the initiation of siRNA transfection, cells were exposed to varying doses of ionizing radiation, seeded at known densities, and then allowed to form colonies for approximately 12 days. Data is expressed as the average surviving fraction ± S.E.M. of at least three independent experiments for each dose of ionizing radiation (IR). (B) Wild type BL2 cells or BL2 polymerase ίota (Polι), polymerase eta (Polη), or REV3 (clones 332 and 504) knockout cells were treated with various doses of IR. Forty-eight hours later the cells were harvested and subjected to the trypan blue exclusion assay to assess viability. Data is expressed as the average percentage of cells stained negative for trypan blue, normalized to untreated control. At least three independent experiments were performed. Error bars represent S.E.M. (C) siRNA transfected HeLa cells exposed to 4GY IR were assessed for chromosomal gaps and breaks 24 hours later as described in Materials and Methods. Data is expressed as the average gaps and breaks per metaphase ± S.E.M. (n=50). REV1,

REV3, REV7 or RAD51-depleted cells displayed an increase of IR-induced chromosomal aberrations.

In response to ionizing radiation-induced DNA damage, chromosomal aberrations may arise due to failed repair of DNA double strand breaks leading to gaps and breaks in the chromosome or improper rejoining of broken ends resulting in distorted chromosomes [32]. To determine if REV1, REV3 or REV7 protect against ionizing radiation-induced chromosomal aberrations, HeLa cells depleted of these proteins were exposed to 4 GY ionizing radiation and collected 24 hrs later for chromosomal aberration analysis. Consistent with the loss in clonogenic survival, REV1, REV3, or REV7-depleted HeLa cells displayed a greater number of ionizing radiation-induced chromosomal aberrations per metaphase as compared to polymerase eta-depleted cells (Fig. 4.2C). RAD51-depleted HeLa cells exhibited similar numbers of chromosomal gaps and breaks as compared to REV1, REV3, or REV7-depleted cells (Fig. 4.2C). Similarities found between REV1, REV3 or REV7-depleted cells and RAD51-depleted cells suggest that chromosomal aberrations may arise due to defects in repair of DNA double strand breaks. However, the enhancement of chromosomal aberrations in REV1, REV3 or REV7-depleted HeLa cells following ionizing radiation may also be due to the inability to bypass abasic sites along with other altered DNA bases caused by exposure to ionizing radiation.

The REV1-polymerase zeta functional complex is necessary for efficient repair of DNA double strand breaks

The prolonged G2 phase cell cycle arrest of REV1, REV3 or REV7 deficient cells along with the hypersensitivity observed following ionizing radiation is consistent with a defect in repair of ionizing radiation-induced DNA double strand breaks. Moreover, REV1, REV3 or REV7-depleted cells displayed enhanced ionizing radiation-induced chromatid gaps and breaks, which may also be attributed to failure of efficient DNA double strand break repair. To investigate if REV1, REV3 or REV7 is necessary for

repair of ionizing radiation-induced DNA double strand breaks, HeLa cells depleted of these proteins were exposed to 2 GY ionizing radiation, and probed for unrepaired DNA double strand breaks. To quantitate unresolved double strand breaks, the cells were stained for phosphorylated ATM along with 53BP1 as surrogate markers of DNA double strand breaks. ATM, which undergoes autophosphorylation on serine 1981(S1981P) in response to DNA double strand breaks, localizes to sites of breaks forming foci that mark the location of the DNA double strand breaks [33-34]. 53BP1, a participant in DNA damage signaling and repair, also forms foci at sites of DNA double strand breaks [35-36].

As shown in Figure 4.3A, 15 minutes after exposing HeLa cells to 2 GY ionizing radiation, approximately 100% of the cells displayed greater than ten S1981P ATM-53BP1 containing foci per cell. By 24 hours the S1981P ATM-53BP1 foci were resolved implying the DNA double strand breaks were either repaired or in the process of being repaired (Fig.4.3A). The majority of the S1981 ATM-53BP1 containing foci were resolved by 24 hours in non-specific (Non-si) or polymerase eta siRNA transfected HeLa cells exposed to 2 GY ionizing radiation (Fig. 4.3B and C). However, greater than 60% of the REV1, REV3 or REV7 down-regulated HeLa cells displayed greater than ten S1981P ATM-53BP1 containing foci per cell 24 hours after exposure to 2 GY ionizing radiation. Approximately the same percentage of RAD51-depleted HeLa cells exhibited greater than ten S1981P ATM-53BP1 containing foci per cell as compared to REV1, REV3 or REV7 knockdown cells. The persistence of S1981P ATM-53BP1 containing foci suggests ionizing radiation-induced DNA double strand breaks were not being repaired efficiently in the absence of REV1, REV3 or REV7 (Fig.4.3B and C). However, the possibility cannot be ruled out that persistence of DNA double strand breaks is due to insufficient replicative bypass of ionizing radiation-induced base damage resulting in collapsed replication fork

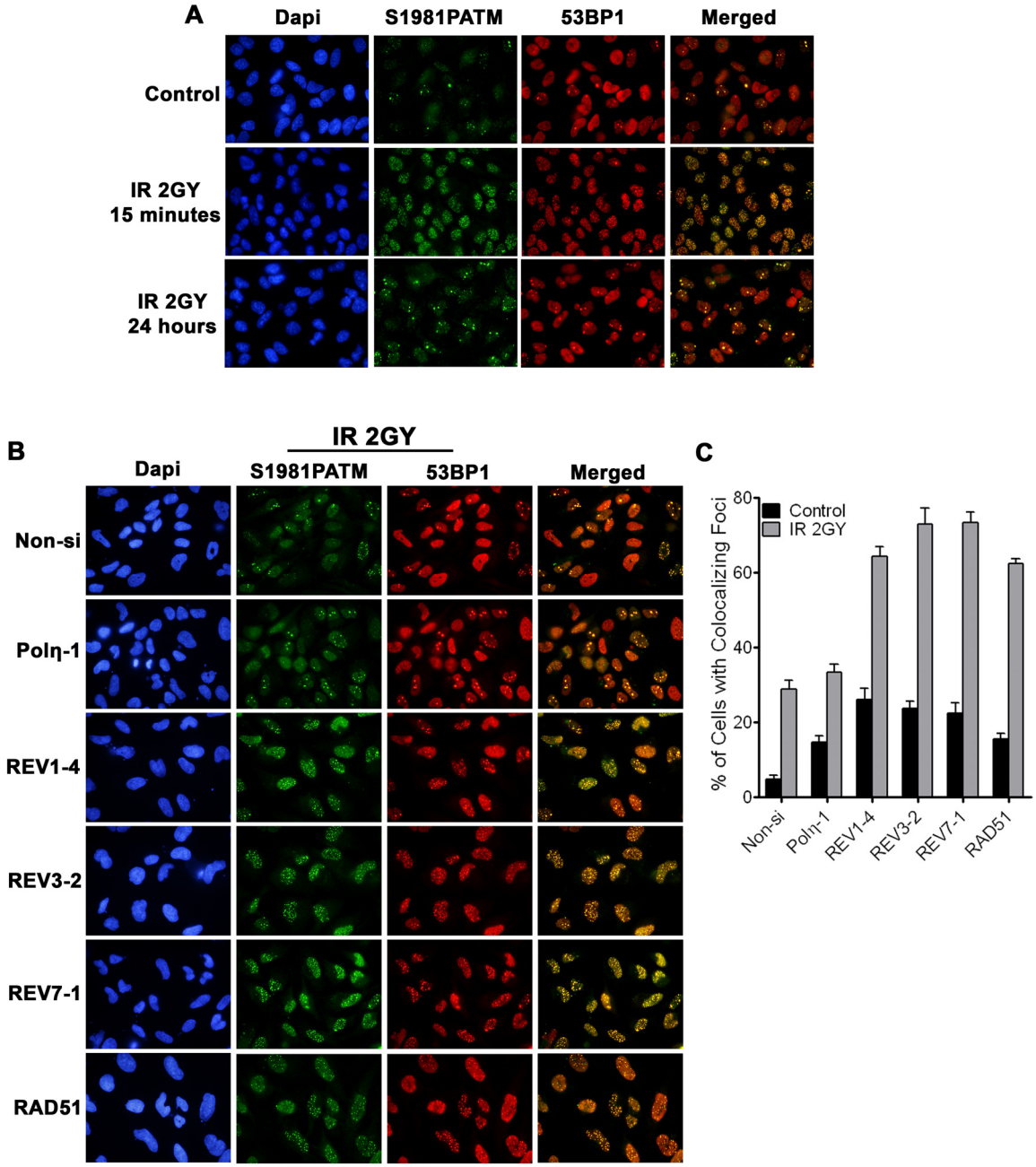

Figure 4.3 The REV1/polymerase zeta functional complex is necessary for efficient resolution of DNA double strand breaks induced by ionizing radiation (IR). HeLa cells were exposed to 2GY IR, then fixed in 100% methanol and stained for S1981P-ATM (green) along with 53BP1 (red) as surrogate markers of DNA double strand breaks (DSBs). Nuclear DNA was stained with DAPI (blue). (A) HeLa cells were fixed 15 minutes or 24 hours after exposure to IR. Approximately 100% of the cells contained greater than ten pATM colocalizing with 53BP1 per cell 15 minutes following IR exposure. (B) Polymerase eta (Polη), REV1, polymerase zeta (REV3 and REV7) or RAD51-depleted HeLa cells grown on coverslips were exposed to 2GY IR and allowed to recover for 24 hrs. HeLa cells deficient of REV1, REV3, REV7 or RAD51 did not

resolve IR-induced DSBs in a timely manner. (C) The graph represents the average percentage of cells exhibiting ten or more foci per cell containing both phospho-ATM and 53BP1. Error bars represent S.E.M. (n=3).

The REV1-polymerase zeta functional complex may contribute to homologous recombination repair but is not absolutely required

Unresolved S1981P ATM-53BP1 containing foci in REV1, REV3 or REV7 down-regulated HeLa cells following ionizing radiation may be explained by insufficient repair of double strand breaks induced by ionizing radiation. Since the phenotypes observed in REV1, REV3 or REV7-depleted HeLa cells are comparable to RAD51 deficient HeLa cells, REV1 and polymerase zeta may play role in homologous recombination. To determine if REV1 and polymerase zeta have a cellular role in homologous recombination, a homologous recombination reporter plasmid obtained from Maria Jasin was utilized. The homologous recombination reporter plasmid contains a full length *GFP* gene with premature stop codons flanking an I-Sce1 restriction enzyme site (Fig. 4.4A). Downstream of the full length *GFP* gene is an incomplete *GFP* gene that is used as a homologous repair template. I-Sce1 will cut at the restriction enzyme site in the full length *GFP* gene resulting in a DNA double strand break. If correct homologous recombination occurs to repair the I-Sce1-induced DNA double strand break by employing the incomplete *GFP* repair template, then the premature stop codons will be removed and the I-Sce1 site will be abolished resulting in expression of GFP.

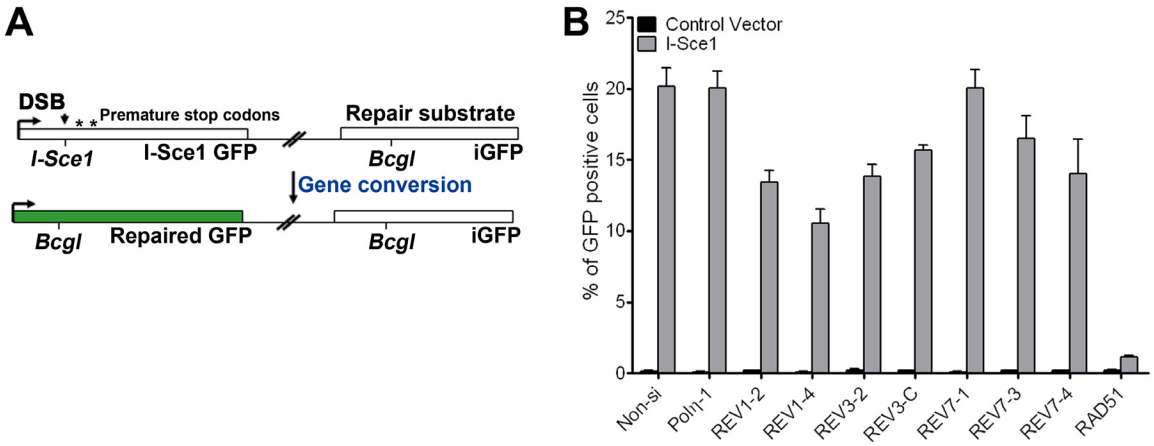

Figure 4.4 Depletion of the translesion polymerases REV1 and zeta results in a mild homologous recombination defect. (A) A depiction of the homologous recombination plasmid reporter assay developed in Maria Jasin's laboratory. The plasmid contains a full length *GFP* gene with premature stop codons flanking an I-Sce1 restriction enzyme site and an incomplete *GFP* gene utilized as a repair template. Introducing I-Sce1 into a cell containing the plasmid reporter results in the formation of a DNA double strand break at the restriction enzyme site. If correct homologous recombination occurs to repair the I-Sce1-induced DNA double strand break employing the incomplete *GFP* repair template, then the premature stop codons will be removed and the I-Sce1 site will be abolished resulting in the expression of GFP. (B) HeLa cells containing the homologous recombination reporter plasmid were depleted of polymerase eta (Polη), REV1, polymerase zeta (REV3 and REV7) or RAD51. I-Sce1 was introduced into the cells via adenovirus 24 hours after initiation of siRNA transfection. Forty-eight hours after adenovirus infection the cells were fixed with 3.7% para-formaldehyde and analyzed by flow cytometry for GFP positive cells. Depletion of REV1 or REV3 resulted in approximately 30-40% reduction of GFP positive cells as compared to Non-si transfected HeLa cells, while depletion of RAD51 resulted in approximately 90% reduction of GFP positive cells.

HeLa cells were transfected with the homologous recombination reporter plasmid (pDR-GFP) and selected for stable incorporation as described in Materials and Methods. HeLa cells containing the homologous recombination reporter plasmid were depleted of polymerase eta, REV1 and polymerase zeta (REV3 and REV7) and then infected with an adenovirus in order to deliver I-Sce1 into the cells at a high efficiency rate. Forty-eight hours after introduction of the restriction enzyme I-Sce1, the cells were fixed and analyzed for GFP expression by flow cytometry. RAD51 siRNA transfected HeLa cells were used a positive control for inhibition of homologous recombination. Down-regulation of RAD51 almost entirely abolished homologous recombination, as measured

by GFP expression. Depletion of polymerase eta, though, had no effect on GFP expression, implying that in HeLa cells this polymerase does not have a role in homologous recombination (Fig 4.4B). Knockdown of REV1 or REV3 did not completely inhibit GFP expression, but did result in approximately a 30-40% decrease of GFP positive cells. No major differences were observed in cell proliferation (Fig. S4.3), thus a decrease in REV1, REV3 or RAD51-depleted cells replicating DNA cannot explain the diminished homologous recombination repair efficiency. Therefore, depletion of REV1 or REV3 resulted in a mild homologous recombination defect. Surprisingly, depletion of REV7 did not appear to have as great of an effect upon homologous recombination as REV1 or REV3 knockdown.

RAD18 does not regulate the REV1/polymerase zeta function complex in HeLa cells after ionizing radiation exposure

Following exposure to ionizing radiation, several different DNA lesions are formed including DNA adducts such as 7,8-dihydro-8-oxoguanine and abasic sites [15]. REV1 and polymerase zeta may be necessary for bypass of DNA adducts and abasic sites induced by ionizing radiation. As explained in Chapter 1, RAD18 is thought to be essential for initiation of translesion synthesis by monoubiquitinating PCNA at sites of stalled replication forks where helicase and polymerase uncoupling occurs. To support this idea, inhibition of PCNA monoubiquitination results in diminished REV1 foci formation at sites of replication stress induced by cisplatin (Fig 2.6). Abasic sites and DNA adducts caused by ionizing radiation would not be expected to inhibit helicase and polymerase uncoupling. Therefore, if REV1 and polymerase zeta are necessary for replicating past DNA base damage induced by ionizing radiation, RAD18 would be expected to regulate this process by monoubiquitinating PCNA at sites of replication stress where helicase and polymerase uncoupling occurs.

To test whether RAD18 monoubiquitinates PCNA in response to ionizing radiation, HeLa cells were exposed to a relatively high dose of ionizing radiation and collected 24 hours later for immunoblot analysis. Exposure to 10 GY ionizing radiation did not induce PCNA monoubiquitination (Fig 2.4). Furthermore, PCNA does not localize to sites of DNA damage marked by H2AX phosphorylation following exposure

to ionizing radiation, suggesting a lack of replication fork stalling due to ionizing radiation-induced DNA damage (Fig. 2.4). The most likely explanation for this observation is that the majority DNA adducts and abasic sites caused by ionizing radiation are corrected shortly after exposure. It is possible, though, that the number of stalled replication forks where helicase and polymerase uncoupling occurs is significantly less in cells exposed to ionizing radiation when compared to cisplatin treated cells, thus rendering detection of monoubiquitinated PCNA difficult.

If RAD18 regulates the REV1/polymerase zeta functional complex via PCNA monoubiquitination, then cell cycle distribution of RAD18-depleted HeLa cells should be similar to REV1, REV3 or REV7 knockdown cells following ionizing radiation. RAD18-depleted HeLa cells exposed to 4 J/m^2 ultraviolet radiation were arrested in late S and G2 phase of the cell cycle 24 hours after exposure to ultraviolet light (Fig 4.5A). The cell cycle distribution following exposure to ultraviolet radiation was similar in RAD18, REV1, REV3 or REV7-depleted HeLa cells, suggesting RAD18 may have an important regulatory role in the cellular response of REV1and polymerase zeta to ultraviolet light (Fig. 4.1A and Fig 4.5A). Twenty-four hours after exposure to 4 GY ionizing radiation, RAD18-depleted cells exhibited a similar cell cycle distribution as non-specific (Non-si) transfected cells (Fig 4.5B). RAD18-depleted HeLa cells to do not display a similar prolonged G2 cell cycle arrest as observed in REV1, REV3 or REV7 down-regulated HeLa cells. The data suggest RAD18, via monoubiquitination of PCNA, does not regulate the REV1/polymerase zeta functional complex following ionizing radiation. Depletion RAD18 of using several different siRNA sequences lead to similar cell cycle arrest profiles following ultraviolet or ionizing radiation exposure demonstrating that the effects observed here are gene-specific (supplemental data Fig. S4.1 and S4.2).

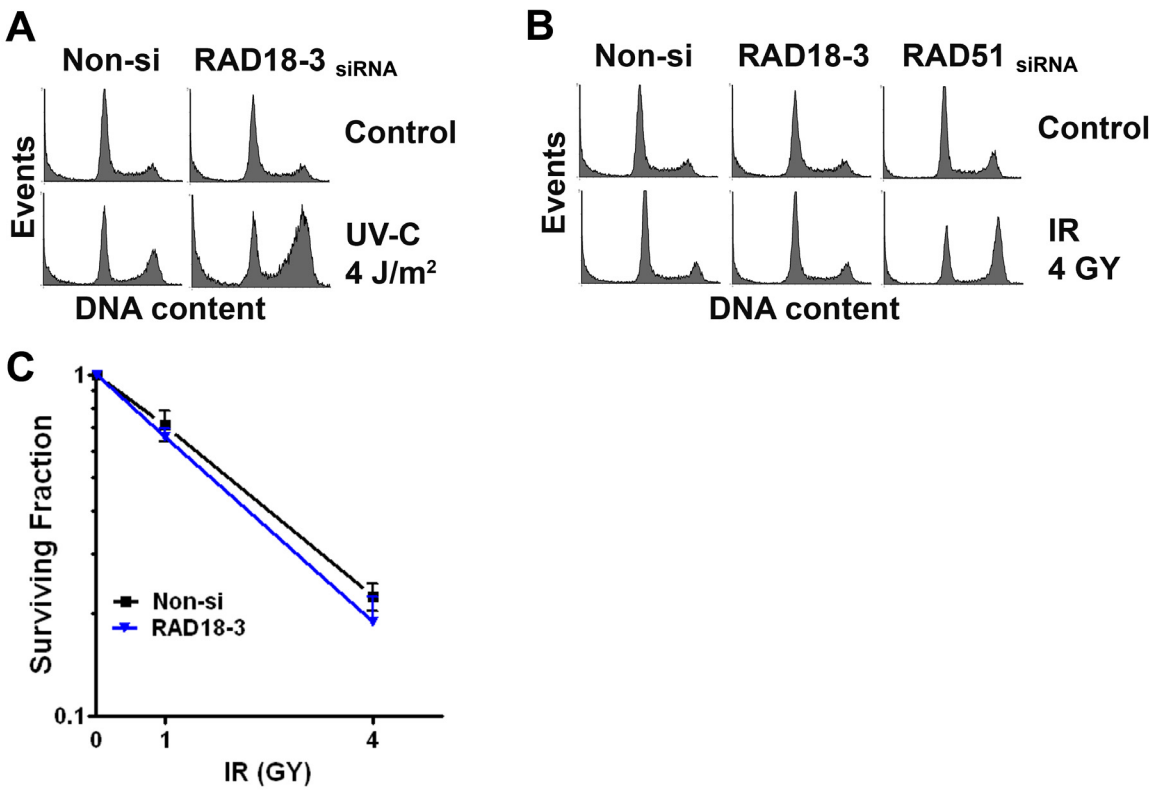

Figure 4.5 RAD18 does not regulate the REV1/polymerase (Polζ) zeta functional complex in response to ionizing radiation. (A and B) RAD18-depleted HeLa cells were exposed to either 4 J/m^2 ultraviolet (UV) radiation or 4 GY ionizing radiation (IR). Twenty-four hours later the cells were fixed, stained for DNA content with propidium iodide and analyzed by flow cytometry. Down-regulation of RAD18 resulted in accumulation of cells in S and G2 phases of the cell cycle after UV exposure (A), but no cell cycle checkpoint was observed 24 hours following IR exposure (B). (C) HeLa cells deficient of RAD18 were exposed to varying doses of ionizing radiation, seeded at known densities, and then allowed to form colonies for approximately 12 days. Data is expressed as the average surviving fraction ± S.E.M. of at least three independent experiments for each dose of ionizing radiation (IR).

A clonogenic survival assay was performed to determine if RAD18 deficient HeLa cells display a similar sensitivity to ionizing radiation as REV1, REV3 or REV7 down-regulated cells. RAD18 deficient cells did not display greater sensitivity to ionizing radiation than compared to Non-si transfected HeLa cells (Fig 4.5C). A lack of radiosensitivity greater than that observed in the Non-si transfected HeLa cells strongly suggest RAD18 has no biological role in protecting cells from ionizing radiation-induced cytotoxicity. Since RAD18-depleted cells do not show a similar sensitivity to ionizing

radiation as do REV1, REV3 or REV7 depleted cells, it is unlikely RAD18 regulates the REV1/polymerase zeta functional complex in response to ionizing radiation.

To confirm RAD18 has no biological role in response to ionizing radiation in HeLa cells, RAD18-depleted cells were exposed to 2 GY ionizing radiation and 24 hours later probed for the presence of unresolved DNA double strand breaks. RAD51-depleted cells, utilized as a positive control, did not resolve S1981 ATM-53BP1containing foci in a timely manner (Fig 4.6A). RAD18 depleted cells, though, appeared to resolve S1981 ATM-53BP1 containing foci within 24 hours of ionizing radiation exposure (Fig 4.6A and B). In addition, RAD18 was determined not to have a role in homologous recombination in HeLa cells. RAD18 was down-regulated in HeLa cells with the homologous recombination reporter plasmid stably integrated, and 24 hours later were infected with an adenovirus engineered to deliver the restriction enzyme I-Sce1 into the cells. In contrast to REV1 or REV3 depleted cells, no difference in homologous recombination, as measured by GFP expression, was noted in RAD18 deficient cells.

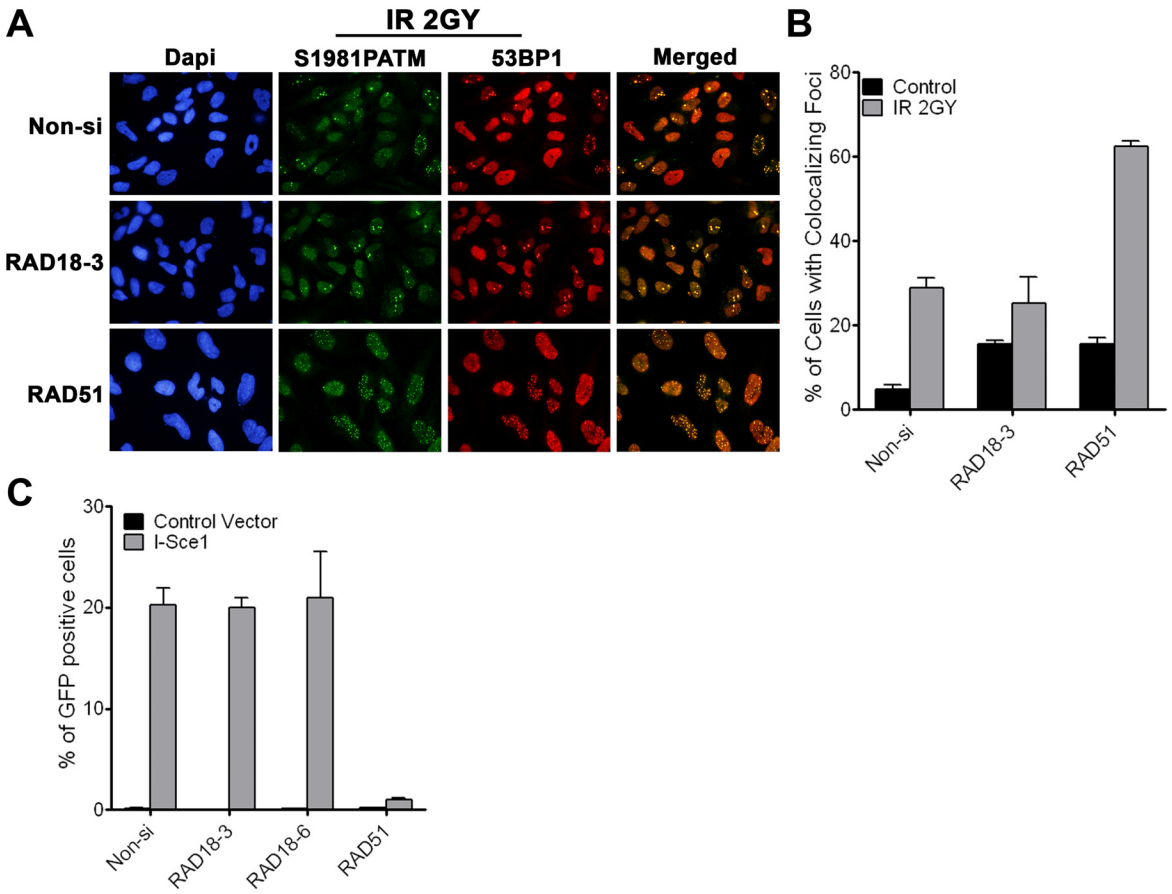

Figure 4.6 RAD18 does not contribute to repair of ionizing radiation-induced DNA double strand breaks in HeLa cells. (A) RAD18 or RAD51 deficient HeLa cells grown on coverslips were exposed to 2GY IR, then 24 hrs later fixed in 100% methanol and stained for S1981P-ATM (green) along with 53BP1 (red) as surrogate markers of DNA double strand breaks (DSBs). Nuclear DNA was stained with DAPI (blue). (B) The graph represents the average percentage of cells exhibiting 10 or more foci per cell containing both phospho-ATM and 53BP1. Error bars represent S.E.M. (n=3). (C) HeLa cells containing the homologous recombination reporter plasmid were depleted of RAD18 or RAD51. Two different siRNA sequences targeting RAD18 mRNA were transfected into the cells independently of each other. I-Sce1 was introduced into the cells via adenovirus 24 hours after initiation of siRNA transfection. Forty-eight hours after adenovirus infection the cells were fixed with 3.7% para-formaldehyde and analyzed by flow cytometry for GFP positive cells.

Based upon these results, the phenotypes observed in REV1, REV3 or REV7 depleted cells following ionizing radiation is unlikely due to lack of translesion synthesis past DNA adducts, assuming RAD18-dependent monoubiquitination of PCNA promotes replicative bypass of ionizing radiation-induced base damage. If the REV1/polymerase

zeta functional complex does have a role in bypass of ionizing radiation-induced base damage, the results obtained here are of great interest since no reports to date have suggested translesion polymerases replicate past DNA adducts in a monoubiquitinated PCNA-independent manner in intact human cells.

Discussion

Here, evidence was provided to support a biological role for the translesion polymerases REV1 and zeta in protection against ionizing radiation-induced cytotoxicity. REV1 or polymerase zeta (REV3 and REV7)-depleted HeLa cells exposed to ionizing radiation displayed a prolonged G2 cell cycle arrest suggestive of a deficiency in repair of DNA damage. In addition, REV1, REV3 or REV7-depleted cells were hypersensitive to ionizing radiation and exhibited an enhancement of chromosomal aberrations after exposure to ionizing radiation. REV1, REV3 or REV7 down-regulated HeLa cells did not resolve DNA double strand breaks marked by S1981 ATM foci colocalizing with 53BP1 foci in a timely manner after ionizing radiation exposure. Even though previous studies have proposed that polymerase eta may a cellular role in repair of DNA double strand breaks, polymerase eta did not appear to be necessary for protection against cytotoxicity induced by ionizing radiation in HeLa cells [14]. Irradiated RAD51-depleted HeLa cells exhibited similar phenotypes as HeLa cells deficient in REV1, REV3 or REV7 suggesting that these polymerases may play a role in homologous recombination. Nevertheless, the phenotypes observed in REV1, REV3 or REV7-depleted HeLa cells following ionizing radiation could be explained by a lack of translesion synthesis across ionizing radiation-induced DNA adducts, or a defect in either non-homologous end-joining or homologous recombination repair.

To address if REV1 or polymerase zeta have a cellular role in homologous recombination, a homologous recombination reporter plasmid was utilized. Depletion of REV3 or REV1 resulted in a decrease of homologous recombination, as measured by GFP expression, by approximately 30-40% respectively. The reason for the partial reduction of homologous recombination in REV1 or REV3-depleted cells is unclear. The

Fanconi anemia pathway is thought to have an integral role in homologous recombination, but similar to REV1 or REV3-depleted HeLa cells, Fanconi anemia cells display mild homologous recombination defects [37-40]. Thus, depletion of proteins implicated in the homologous recombination pathway does not always completely eliminate homologous recombination as measured by the homologous recombination reporter assay utilized in this study. One explanation may be that the type of double strand break formed by the I-Sce1 restriction enzyme may not represent the type of DNA double strand break translesion polymerases are needed to resolve. If the homologous repair template contains abasic sites or other types of altered bases due to ionizing radiation damage, then translesion synthesis may be critical for extension of D-loops. Nevertheless, the reduction in homologous recombination observed in REV1 or REV3-depleted HeLa cells suggest that REV1 and polymerase zeta may have a partial role in homologous recombination.

Surprisingly, depletion of REV7 did not appear to have as great of an effect upon homologous recombination as REV1 or REV3 knockdown. Since only one DNA double strand break is induced after introducing I-Sce1 into the cells, the level of protein depletion may of critical importance. REV7 expression in HeLa cells is significantly greater than compared to REV1 or REV3 expression. Even though immunoblot analysis suggest REV7 is depleted to a great extent in HeLa cells (Fig. 2.1 and S2.2) when compared to Non-si siRNA transfected HeLa cells, there may be enough REV7 protein present in the cells to participate in the repair of one DNA double strand break. To support this idea, there appeared to be some inconsistency in percentage of GFP positive cells between the different siRNA sequences targeting REV7 mRNA. Down-regulation of REV7 in HeLa cells containing the homologous recombination reporter plasmid via REV7-3 or REV7-4 siRNA sequences appeared to decrease homologous recombination, as measured by GFP expression, suggesting these two siRNA sequences may deplete REV7 to a greater extent. REV7 does not have catalytic activity, but rather contributes to translesion synthesis by supporting the catalytic activity of REV3 and possibly directing REV3 to sites of DNA damage. The possibility exists that for certain types of DNA damage REV7 is dispensable in terms of contributing to REV3's catalytic activity.

Based upon the data obtained in this study, RAD18 does not appear to have a major role in protecting cells against ionizing radiation. The results were somewhat surprising and bring up three intriguing points. First, RAD18-dependent translesion synthesis across DNA adducts or abasic sites caused by ionizing radiation appears not to be a major contributor to protection against ionizing radiation-induced cytotoxicity. Chapter 2 provided several lines of evidence demonstrating RAD18 is essential for translesion synthesis across DNA adducts induced by both cisplatin and ultraviolet radiation. RAD18 was also shown to be necessary for REV1 localization to sites of replication stress since U2OS cells depleted of RAD18 displayed diminished REV1 foci formation in response to cisplatin or ultraviolet radiation (data not presented) presumably due to lack of PCNA monoubiquitination (Fig 2.6). However, exposure of RAD18-depleted HeLa cells to ionizing radiation does not result in any phenotypes suggestive of a defect in translesion synthesis, and HeLa cells exposed to a relatively high dose of ionizing radiation do not exhibit monoubiquitinated PCNA as measured by immunoblot analysis (Fig. 2.4, 4.5 and 4.6).

Second, the biological role of RAD18 in response to ionizing radiation is poorly understood. Similar to the results presented here, *RAD18* knockout mice have no observable phenotypes suggestive of a defect in homologous recombination or non-homologous end-joining, and embryonic stem cells derived from these mice are not sensitive to ionizing radiation [41]. In contrast, Huang *et al.* recently proposed RAD18, via interactions with RAD51C and independently of it E3 ligase activity, is absolutely required for homologous recombination [42]. RAD18 has also been implicated in double strand break repair in the G1 phase of the cell cycle, and is thought to contribute to repair by interacting with and monoubiquitinating 53BP1 [43]. In addition, *RAD18*$^{-/-}$ mouse embryonic fibroblast have been shown to have no defect in homology-dependent repair, and in some cases even display an enhancement of sister chromatid exchanges [44-45]. Lastly, RAD18 has been suggested to suppress non-homologous end-joining in order to facilitate homologous recombination-mediated repair [46]. Clearly, drastically different views of how RAD18 contributes, or does not contribute, to repair of DNA double strand breaks have been proposed in the literature. Currently, it is poorly understood as to why results have varied so greatly. One explanation is that different experimental methods or

endpoints could contribute to the discrepancies. Also, the use of different cell lines between research groups could account for the diverse results, though many of the papers cited above utilized both *RAD18*$^{-/-}$ DT40 cells and *RAD18*$^{-/-}$ mouse embryonic fibroblast and still came to different conclusions. Nonetheless, much work remains to be done in order to clarify the biological role of RAD18 in terms of DNA double strand break repair and what role, if any, RAD18 has in protection against ionizing radiation-induced DNA damage.

Third, since depletion of RAD18 in HeLa cells has no obvious effect upon cellular phenotypes in response to ionizing radiation, it would be logical to assume RAD18, via its E3 ligase activity, has no regulatory control over the cellular roles of REV1 or polymerase zeta following ionizing radiation. This leads to the question, how are REV1 and polymerase zeta regulated in terms of response to ionizing radiation-induced DNA damage, or in other words what is signaling the REV1/polymerase zeta functional complex to sites of DNA damage caused by ionizing radiation. Since the Fanconi anemia pathway is known to regulate translesion synthesis, via promotion of REV1 to sites of replication stress (Fig. 2.7) [47], the possibility exist for the Fanconi anemia pathway to have a role in localization of the REV1/polymerase zeta functional complex to sites of ionizing radiation-induced DNA damage. In addition, the Fanconi anemia pathway may also promote the REV1/polymerase zeta functional complex to participate in the homologous recombination repair pathway. Similar to HeLa cells depleted of REV1 or REV3, cells deficient of proteins composing the Fanconi Anemia core complex, such as FANCA or FANCC, are known to have a mild defect in the homologous recombination repair pathway [38, 40, 48-49]. Besides the core complex, other Fanconi anemia proteins are involved in homologous recombination including FANCD1/BRAC2, FANCJ and FANCN/PALB2 [40, 50-54]. For these reasons, there is potential that in response to ionizing radiation the REV1/polymerase zeta function complex may be regulated by the Fanconi anemia pathway.

Taken as a whole, REV1 and polymerase zeta appear to have a biological role in the protection against ionizing radiation-induced cytotoxicity in a human cancer cell line, possibly by contributing to homologous recombination. Further understanding of how

Rev1 and polymerase zeta contribute to protection against ionizing radiation-induced cytotoxicity may reveal new drug targets allowing for the treatment of radioresistant cancers. In addition, RAD18 and polymerase eta appear to have no significant biological roles in protecting cells from ionizing radiation-induced cytotoxicity.

Supplemental Data

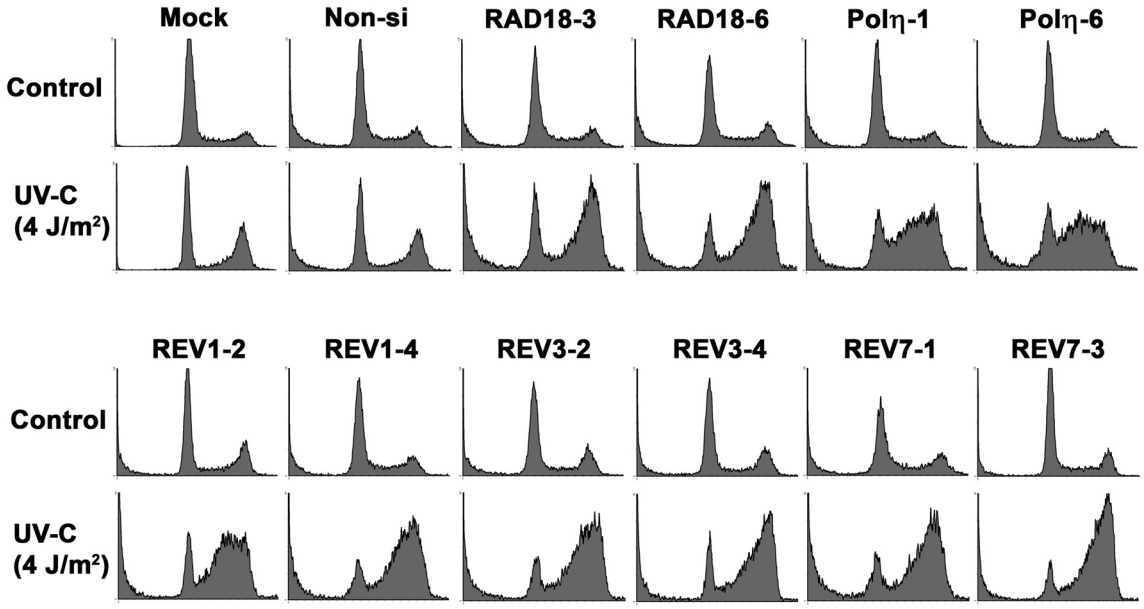

Figure S4.1 Depletion of RAD18, Polη, REV1, REV3 or REV7 leads to cell cycle arrest in S and G2 phases of the cell cycle following ultraviolet radiation (UV-C) exposure. HeLa cells were independently transfected with one of at least two different siRNA sequences targeting RAD18, Polη, REV1, REV3 or REV7 mRNA. Forty hours after transfection the cells were exposed to 4 J/m^2 UV-C. Twenty-four hours later, cells were fixed, resuspended in PBS containing RNAse A and propidium iodide, and then acquired on a BD FACSCaliber system using CellQuest software. The y-axis represents number of events. The x-axis reflects DNA content. Depletion of RAD18, Polη, REV1, REV3 or REV7 by each individual siRNA sequence resulted in similar cell cycle profiles following UV-C exposure. Mock transfected or Non-si siRNA-transfected cells were relatively non-perturbed by this treatment.

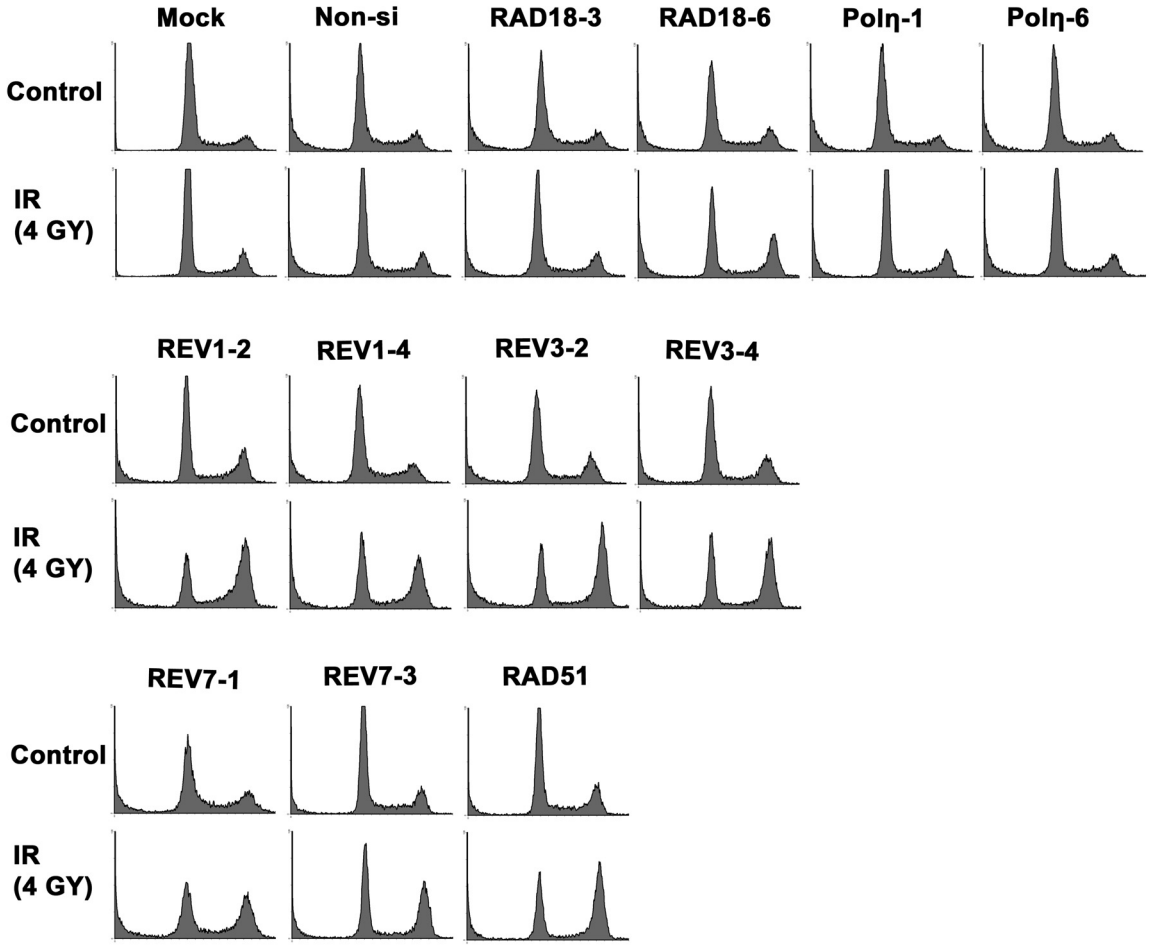

Figure S4.2 Depletion of REV1, REV3 or REV7 leads to cell cycle arrest in the G2 phase of the cell cycle following ionizing radiation (IR) exposure. HeLa cells were independently transfected with one of at least two different siRNA sequences targeting RAD18, Polη, REV1, REV3, or REV7 mRNA. Forty hours after transfection the cells were exposed to 4 GY IR. Twenty-four hours later, cells were fixed, resuspended in PBS containing RNAse A and propidium iodide, and then acquired on a BD FACSCaliber system using CellQuest software. The y-axis represents number of events. The x-axis reflects DNA content. RAD18 or Polη siRNA transfected HeLa cells were able to escape G2 phase arrest and progress into G1. REV1, REV3 or REV7 depletion by each individual siRNA sequence resulted in similar cell cycle profiles following IR exposure. A prolonged cell cycle arrest in G2 phase suggests an inability to repair ionizing radiation induced DNA damage. Mock transfected or Non-si siRNA-transfected cells were relatively non-perturbed by this treatment.

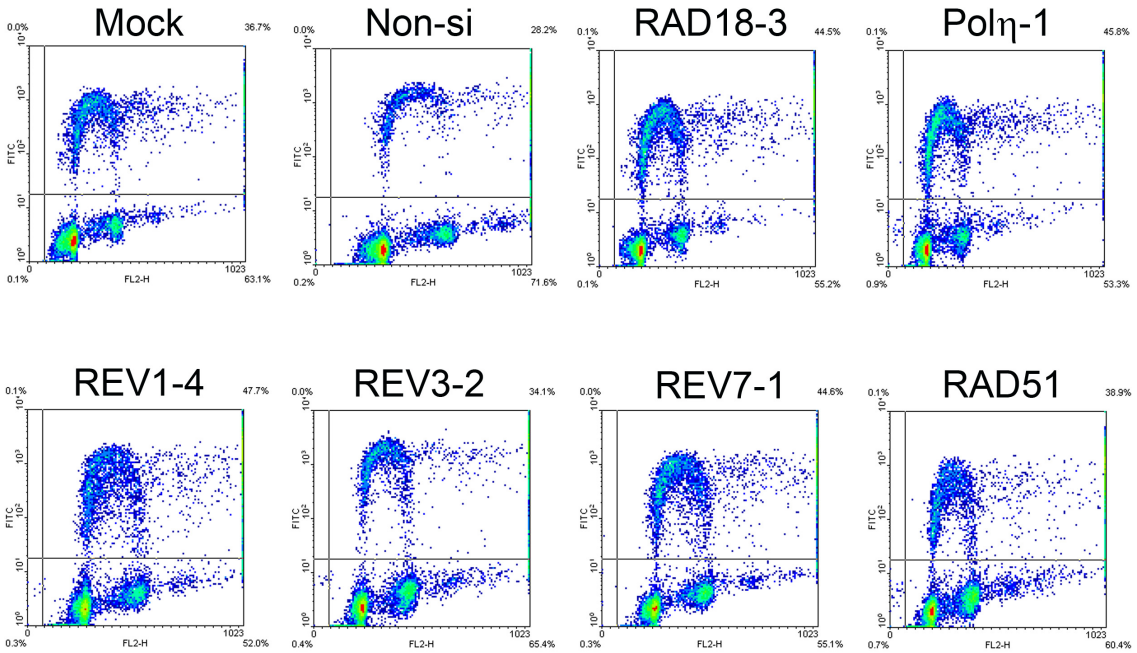

Figure S4.3 Depletion of RAD18, Polη, REV1, REV3 or REV7 does not alter cell proliferation. HeLa cells with stable integration of the homologous recombination reporter plasmid (pDR-GFP) were depleted of RAD18, Polη, REV1, REV3, REV7 or RAD51. Twenty-fours hours after the initiation of siRNA transfection, the cells were infected with an adenovirus containing the *I-Sce1* gene. Forty-eight hours later the cells were exposed to 2 mg/ml BrdU for 17 minutes. The cells were collected and analyzed for BrdU incorporation via flow cytometry. No major differences were noted in cell proliferation.

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CHAPTER 5

Conclusion

Based on the findings presented in this dissertation, the translesion DNA polymerases eta, REV1 and zeta appear to have differential cellular roles in response to DNA damage induced by chemotherapeutic agents. The translesion polymerases eta, REV1 and zeta all appear to be necessary for replicating past cisplatin-induced intrastrand cross-links, thus preventing replication stress and eventual replication fork collapse. REV1 and polymerase zeta, but not polymerase eta, may participate in repair of both interstrand cross-links induced by mitomycin C and DNA double strand breaks formed after exposure to ionizing radiation. Whether bypassing or participating in the repair of damaged DNA, the translesion polymerases were found to protect against cytotoxicity induced by cisplatin, mitomycin C or ionizing radiation. Therefore, developing inhibitors targeting translesion polymerases may allow for sensitization of cancer cells resistance to chemotherapeutic agents that induce DNA damage. In addition to inhibiting translesion polymerases, chemotherapeutic agents may be designed that produce adducts to bulky to be replicated across.

REV1 and polymerase zeta were found to be essential for bypassing cisplatin intrastrand cross-links along with repairing interstrand DNA cross-links induced by both cisplatin and mitomycin C. Treatment of HeLa cells depleted of REV1, REV3 or REV7 with cisplatin resulted in phenotypes suggestive of replication stress including cells accumulating in S and G2 phases of the cell cycle, pan-nuclear like pattern of γ-H2AX staining, and activation of ATR as measured by phosphorylation of CHK1. REV1, REV3 or REV7-depleted HeLa cells also displayed enhanced chromosomal aberrations and extreme sensitivity to cisplatin and mitomycin C, consist with the idea REV1 and polymerase zeta are necessary for repair of interstrand DNA cross-links. In addition, DNA double strand breaks, most likely formed during recombination-dependent repair of

interstrand cross-links, were not resolved in a timely manner in REV1, REV3 or REV7 knockdown cells. The inability to repair double strand breaks induced by recombination-dependent repair of interstrand cross-links strongly implies REV1 and polymerase zeta are crucial for recombination-dependent interstrand cross-link repair.

Furthermore, REV1 and polymerase zeta appeared to protect cells against ionizing radiation-induced cytotoxicity, as noted by a decrease in clonogenic survival and enhancement of chromosomal aberrations in HeLa cells depleted of REV1, REV3 or REV7. Down-regulation of REV1 or REV3 in HeLa cells resulted in a mild defect in homologous recombination, as measured by a homologous recombination reporter plasmid, suggesting REV1 and polymerase zeta may participate in recombination-dependent repair of DNA double strand breaks. However, it is unclear if REV1 and polymerase zeta protect cells from ionizing radiation-induced cytotoxicity by repairing DNA double strand breaks via homologous recombination, or if these polymerases bypass damaged DNA bases preventing replication stress. Also, the possibility cannot be ruled out that REV1 and polymerase zeta may participate in repair of DNA double strand breaks via the non-homologous end-joining pathway. Taken together, the REV1/polymerase zeta functional complex appears to be necessary for protection against cytotoxicity induced by multiple chemotherapeutic agents including cisplatin, mitomycin C, and ionizing radiation

Polymerase eta was found to have a more limited role in terms of protecting cells against chemotherapeutic-induced DNA damage. Similar to REV1 and polymerase zeta, depletion of polymerase eta resulted in phenotypes suggestive of replicative stress following cisplatin treatment. However, knockdown of polymerase eta did not lead to an enhancement of chromosomal aberrations in HeLa cells challenged with cisplatin, and did not result in as great of sensitivity to cisplatin as compared to REV1, REV3 or REV7 knockdown. Furthermore, polymerase eta siRNA transfected HeLa cells displayed similar phenotypes to Non-si siRNA transfected HeLa cells exposed to mitomycin C. These results suggest polymerase eta is essential for replicate bypass of cisplatin-induced intrastrand cross-links, but polymerase eta apparently has no cellular role in protecting cells against interstrand cross-links induced by both cisplatin and mitomycin C. A

previously published report indicated that polymerase eta is essential for D-loop extension during homologous recombination [1]. Surprisingly, polymerase eta was not found to have a cellular role in response to ionizing radiation. Thus, polymerase eta appears to be essential for bypass of cisplatin intrastrand cross-links, but unlike REV1 or polymerase zeta, does not protect cells against cytotoxicity induced by mitomycin C or ionizing radiation.

RAD18 and the Fanconi anemia core complex were found to have a regulatory role over translesion synthesis in terms of bypassing adducted DNA bases. RAD18, via monoubiquitination of PCNA, initiates translesion synthesis at sites of stalled replication forks where helicase and polymerase uncoupling occurs. RAD18-depleted U2OS cells failed to show efficient polymerase eta or REV1 foci formation following cisplatin treatment. In a parallel pathway, the Fanconi anemia core complex appeared to be essential for REV1 foci formation in response to cisplatin exposure. Interestingly, RAD18 did not regulate the cellular functions of REV1 or polymerase zeta in response to agents that induce interstrand DNA cross-links or DNA double strand breaks. The reason may be due to the fact interstrand cross-links and double strand breaks do not allow for uncoupling of the helicase and polymerase at sites of stalled replication forks. The uncoupling of the helicase and polymerase, thus leading to single stranded DNA, is thought to be essential for localization of RAD18 to sites of replication stress. If RAD18 does not regulate the cellular functions of REV1 or polymerase zeta in response to mitomycin C and ionizing radiation, then the question arises as to what is regulating the REV1/ polymerase zeta functional complex following exposure to these agents. One can speculate that the Fanconi anemia pathway may regulate the cellular roles of REV1 and polymerase zeta following exposure to mitomycin C and ionizing radiation.

A summary of the major conclusions are as follows:

1. The translesion polymerases eta, REV1 and zeta are all necessary for translesion synthesis past cisplatin intrastrand cross-links supporting an insertion and extension model in human cells. Both RAD18 and the Fanconi anemia core complex have a regulatory role during replicative bypass of cisplatin adducts.

2. The REV1/polymerase zeta functional complex, but not polymerase eta, appears to have a cellular role in the repair of interstrand DNA cross-links, signifying that the translesion polymerases eta, REV1 and zeta have differential roles in response to the type of DNA damage present.
3. The REV1/polymerase zeta functional complex, but not polymerase eta, protects from ionizing radiation-induced cytotoxicity possibly by participating in the repair of DNA double strand breaks via homologous recombination.
4. RAD18 is necessary to initiate translesion synthesis at sites of DNA adducts by monoubiquitinating PCNA on lysine 164, but RAD18 does not regulate the cellular functions of REV1 or polymerase zeta in response to DNA damage caused by mitomycin C or ionizing radiation.
5. The Fanconi anemia core complex influences translesion synthesis by promoting localization of REV1 to sites of cisplatin-induced replication stress. Although unproven, the Fanconi anemia pathway may have an important role in regulating the REV1/polymerase zeta functional complex in response to mitomycin C and ionizing radiation-induced DNA damage.

Although poorly understood, resistance to chemotherapeutic agents may be attributed to the ability of cells to bypass and/or repair damaged DNA, therefore lessening cytotoxic effects [2-9]. Inhibiting the ability to bypass or repair DNA damage may sensitize resistance cancers to chemotherapeutic agents. Here, I investigated if the translesion polymerases eta, REV1 and zeta contribute to the bypass and repair of DNA damage induced by the chemotherapeutic agents cisplatin, mitomycin C and ionizing radiation. The depletion of polymerase eta, REV1 or polymerase zeta sensitized cells to cisplatin while down-regulation of REV1 or polymerase zeta resulted in hypersensitivity to both mitomycin C and ionizing radiation. Based upon the data contained in this dissertation, the translesion polymerases eta, REV1 and zeta may be valid drug targets in order to sensitize cancer cells to chemotherapeutic agents. Furthermore, developing drugs that inhibit the activities of the REV1/polymerase zeta functional complex may have the most significant impact upon treating resistant tumors since REV1 and polymerase zeta may

protect against cytotoxicity induced by multiple chemotherapeutic agents including cisplatin, mitomycin C and ionizing radiation.

Translesion polymerases, due to there mutagenic properties, have been associated with cancer development [10-12]. It is speculated that secondary cancers can arise due to error prone translesion synthesis across chemotherapeutic-induced DNA damage. Inhibition of translesion polymerases may prevent mutagenic events from occurring during cancer treatment, thus reducing development of secondary tumors [13-14]. Targeting translesion polymerases in order to disrupt translesion synthesis holds the potential to not only sensitize resistance cancers to therapy, but prevent the development of resistance due to translesion synthesis associated mutagenesis [7, 9, 15-16]. Therefore, developing inhibitors targeting translesion polymerases may allow for prevention of cancer development, sensitization of cancer cells to chemotherapy, and inhibition of the emergence of resistance to anti-cancer agents.

It is unclear if disruption of translesion synthesis can be accomplished safely in humans. Translesion polymerases, such as polymerase zeta, may be important for maintaining chromosomal stability and preventing tumor development [17-18]. However, cancer cells may be more dependent upon translesion polymerases allowing for selectivity between normal cells and cancer cells, thus reducing any potential cytotoxic effects to non-cancer cells. Cancer cells lacking a functional G1 checkpoint could potentially be more dependent upon translesion polymerases since the cells cannot arrest in G1 allowing for repair of chemotherapeutic-induced DNA damage before progressing to S phase. In this particular situation, the cells deficient in a G1 checkpoint may be dependent upon translesion polymerases to bypass or repair any DNA damage present during DNA synthesis. Although poorly understood, p53 and p21 appear to regulate translesion synthesis, and in the absence of these proteins translesion synthesis is up-regulated [19-20]. It is estimated that approximately 50% of tumors are p53 deficient. Therefore, 50% of tumors may have up-regulated translesion synthesis perhaps leading to dependence upon this pathway for coping with DNA damage. If inhibitors targeting translesion polymerases cannot be developed, the possibility remains for designing chemotherapeutic agents that either produce DNA adducts too bulky to be bypassed or

cross-links that distort the DNA to such a degree translesion synthesis past the damage cannot occur.

In summary, I provided substantial evidence to support the hypothesis that polymerase eta, REV1 and polymerase zeta are all necessary for bypass of cisplatin intrastrand cross-links. RAD18 initiates translesion synthesis via monoubiquitination of PCNA, and in a parallel pathway the Fanconi anemia core complex regulates REV1 focus formation in response to cisplatin exposure. REV1 and polymerase zeta, but not polymerase eta, appear to be necessary for protection against cytotoxicity induced by mitomycin C and ionizing radiation. Data were presented to support the idea REV1 and polymerase zeta are essential for repair of interstrand DNA cross-links. The REV1/polymerase zeta functional complex may also be necessary for repair of DNA double strand breaks, and perhaps replicative bypass of ionizing radiation-induced base damage. Based on the data presented in this dissertation, translesion polymerases may protect cancer cells from the cytotoxic effects of anti-cancer agents, validating the translesion synthesis pathway as a feasible drug target for sensitizing cancer cells to chemotherapeutic agents.

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