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Novel Roles of Replication Protein A Phosphorylation in Cellular Response to DNA Damage

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Novel Roles of Replication Protein A Phosphorylation in the Cellular Response to DNA Damage

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by

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ABSTRACT

Novel Roles of Replication Protein A Phosphorylation in Cellular Response to DNA Damage

by

Moises Alejandro Serrano

Human replication protein A (RPA) is an eukaryotic single-stranded DNA binding protein

directly involved in a variety of DNA metabolic pathways including replication, recombination,

DNA damage checkpoints and signaling, as well as all DNA repair pathways. This project

presents 2 novel roles of RPA in the cellular response to DNA damage. The first elucidates the

regulation of RPA and p53 interaction by DNA-dependent protein kinase (DNA-PK), ataxia

telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) in homologous recombination

(HR). HR and nonhomologous end joining (NHEJ) are 2 distinct DNA double-stranded break

(DSB) repair pathways. Here, we report that DNA-PK, the core component of NHEJ, partners

with DNA-damage checkpoint kinases ATM, and ATR to synergistically regulate HR repair of

DSBs. The regulation was accomplished through modulation of the p53-RPA interaction. We

show that upon DNA damage p53 and RPA are freed from the p53–RPA complex. This is done

through simultaneous phosphorylation of RPA by DNA-PK, and p53 by ATR and ATM. Neither

the phosphorylation of RPA nor that of p53 alone could dissociate the p53-RPA complex;

furthermore, disruption of the release significantly compromised HR repair of DSBs. Our results

reveal a mechanism for the crosstalk between HR and NHEJ repair through the coregulation of

p53–RPA interaction by DNA-PK, ATM and ATR.

The second part of this project reveals a novel role of RPA32 phosphorylation in suppressing the

signaling of programmed cell death, also known as apoptosis. Our results show that deficiency in

RPA32 phosphorylation leads to increased apoptosis after genotoxic stress. Specifically, PARP-1 cleavage, Caspase-3 activation, sub-G1 cell population, annexin V staining and the loss of mitochondrial membrane potential were significantly increased in the phospho-deficient RPA32 cells (PD-RPA32). The lack of RPA phosphorylation also promoted activation of initiator Caspase-9 and effector Caspase-3 and -7. This regulation is dependent on the kinase activity of DNA-PK and is mediated by PUMA through the ATM-p53 pathway. Our results suggest a novel role of RPA phosphorylation in apoptosis that illuminates a new target that lies on the crossroads of DNA repair and cell death, a pivotal point that could be of importance for sensitizing cancer cells to chemotherapy.

I dedicate this manuscript to my family whose support and solace has made this achievement possible. Without the love, encouragement and understanding of my wife Linda, my parents Samuel and Zulma, my brothers Sebastian and Esteban, and my aunt Leonor, I would not have been able to achieve this goal.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

The DNA Damage Response

The genetic information carried by DNA is extremely precious because it presents the molecular unit of heredity of living organisms; nevertheless, various endogenous and environmental stresses such as exposure to ultraviolet radiation and tobacco smoke constantly threaten the integrity of our DNA. Different repair machineries have developed in cells to deal with a variety of DNA lesions (Figure 1-1). Single-strand break repair (SSBR) restores the sugar backbone of the broken single-stranded DNA filament [1]. Base excision repair (BER) corrects lesions arising from oxidation, alkylation, deamination, and depurination/depyrimidation reactions [2]. Bulky and helix-distorting lesions such as pyrimidine dimers and 6,4 photoproducts are corrected by nucleotide excision repair (NER) [3]. Mismatch repair (MMR) takes action when replication and recombination machinery causes a mismatch of a base or the insertion of a deletion loop (IDL) [4]. Finally, double-strand break repair (DSBR) is assigned to repair the sugar backbone of both DNA filaments after both strands of DNA break [5,6]. Double stranded breaks (DSBs) are the most lethal form of DNA damage and are of particular interest in this research project.
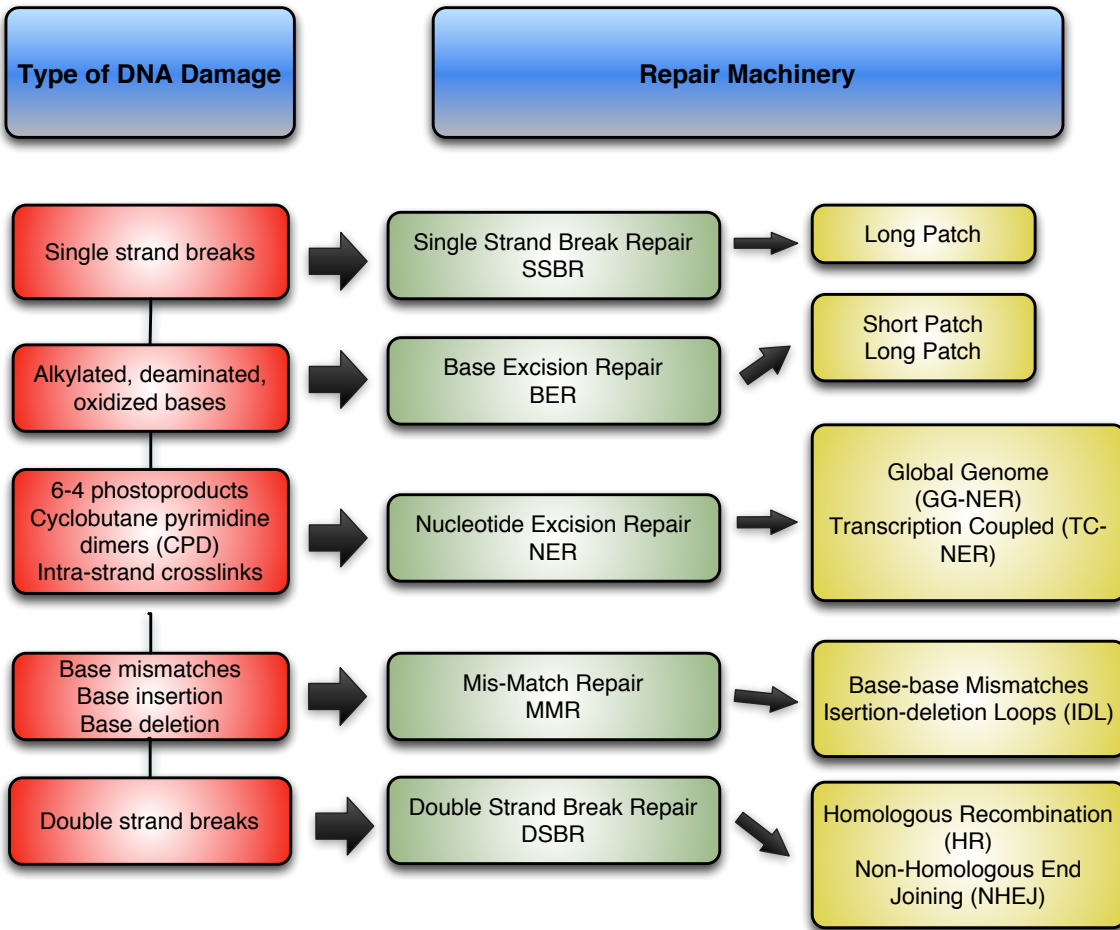
Figure 1-1. The DNA Repair Machinery. The DNA repair machinery has evolved into separate systems that specialize in the repair of different DNA lesions.

DNA Double-Strand Break Repair

DSBs are formed when both strands of the double helix DNA are broken. DSBs can be beneficial when they occur in a managed manner, such as during development of the immune system and generation of genetic diversity in meiosis [7-9]; however, DSBs can also be detrimental. Such detrimental effects can be produced spontaneously during normal DNA metabolism by external factors like ionizing radiation (IR) and tobacco smoke or by certain classes of chemicals. Regardless of their source DSBs if not repaired are the most toxic form of DNA damage and can cause various developmental, immunological, and neurological disorders as well

as genome rearrangement and genome instability, 2 main drivers of cancer [10,11]. The major

endogenous source of DSBs occurs when DNA replication forks encounter unrepaired DNA

lesions and subsequently trigger the collapse of replication forks (Figure 1-2) [12]. Agents currently

used in the treatment of cancer such as camptothecin (CPT) analogous take advantage of

replication to generate DSBs and induce cell death. CPT is a topoisomerase I inhibitor that

arrests the topoisomerase I-nicked DNA intermediate. The mechanism of topoisomerase I

poisoning is mediated by CPT's capacity to stabilize the covalent enzyme-DNA complex and

block re-ligation of the 2 broken DNA ends. In rapidly dividing cells the cytotoxic effects of

CPT are enhanced by the collision of DNA replication forks with trapped Top I-DNA

complexes, converting DNA single-strand breaks into potentially lethal, irreversible double

strand DNA breaks [13,14].
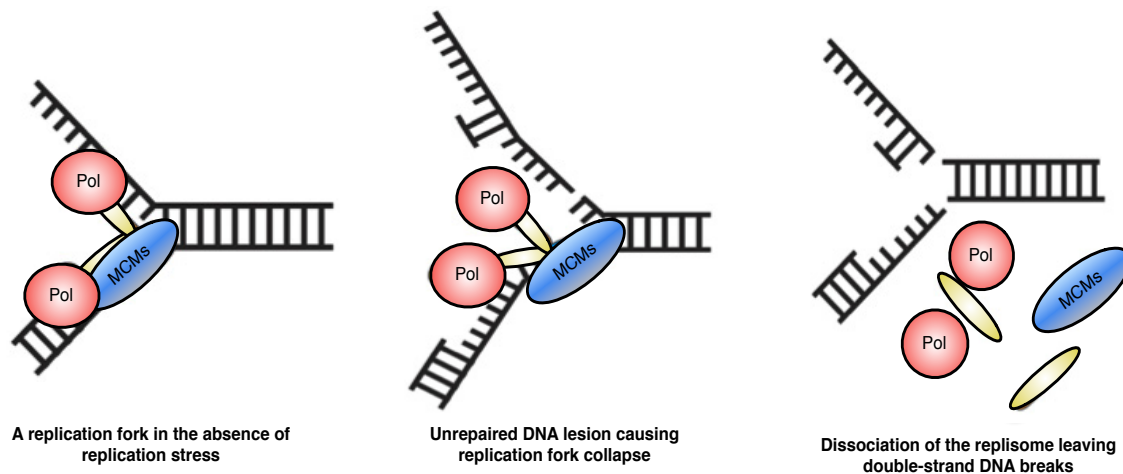
Figure 1-2. Replication Fork Collapse. The collision of unrepaired DNA lesions with the
replication machinery renders the replication-induced DSB. Pol: DNA polymerases, MCMs:
Mini-chromosome maintenance complex 2-7 (MCM2-7), yellow ovals: Other coupling proteins
involve in DNA replication. (Adapted from Berens et al. 2012 [15])

Two major DSB repair pathways have evolved: homologous recombination (HR) and

nonhomologous end joining (NHEJ) (Figure 1-3). NHEJ repairs DNA breaks using very limited

or no sequence homology to rejoin the juxtaposed ends in a manner that is error prone. The

initial step of NHEJ occurs when the Ku70/Ku80 complex binds to the ends of the break. The

Ku70/Ku80 complex then loads DNA-protein kinase (DNA-PK). The DNA-PK complex recruits

XRCC4 (X-ray repair cross-complementin protein 4), DNA ligase IV and DNA polymerase to

fill the gaps and ligate the ends (Figure 1-3 right). Because NHEJ promotes the direct ligation of

the DSB ends, this pathway frequently results in insertions, deletions, substitutions of bases at

the break site, and possible translocations if DSBs from different parts of the genome are joined

[16]. In contrast to NHEJ, HR is error free and is initiated when the DSB is resected by the

nucleases and helicases Mre11/Rad50/NbsI (MRN complex). This generates two 30-nucleotide

single-stranded DNA (ssDNA) overhangs onto which the Rad51, RPA (Replication Protein A),

Rad52, and Rad54 assemble the nucleoprotein filament. This structure can invade the

homologous duplex DNA of the sister chromatid which is then used as a template for error-free

DNA synthesis by DNA polymerase [17]. Although NHEJ can be activated throughout every phase

of the cell cycle and is favored in G1 cells, HR is predominant during DNA replication because

an identical sister chromatid is closely available for repair.
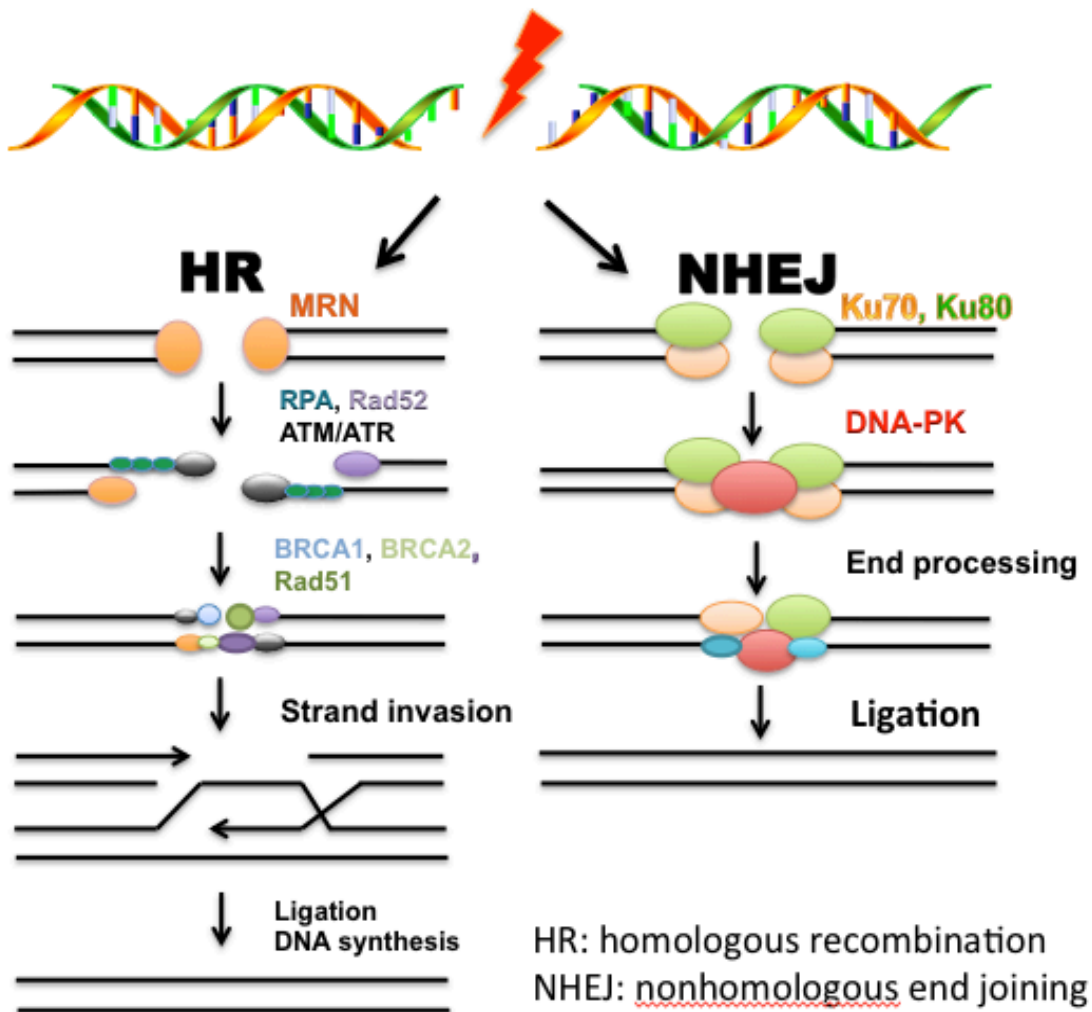
Figure 1-2. Double-Strand DNA Break Repair by HR and NHEJ. HR (left): One of 2 sister chromatids has suffered a DSB. Initial processing of the break results in single-stranded tails. The tails are a substrate for filament formation by direct homology recognition and DNA strand invasion to facilitate joining of the broken DNA strand with the intact sister chromatid. DNA strand ligation and DNA synthesis are followed to complete error-free repair. NHEJ (right): The DNA ends after the break are substrates for binding of the Ku70/Ku80 heterotrimer. DNA-PK proceeds to promote juxtaposition and to recruit other core components of NHEJ to complete the error-prone rejoining reaction. MRN: Mre11/Rad50/NbsI complex, BRCA1: Breast cancer type 1 susceptibility protein, BRCA2: Breast cancer type 2 susceptibility protein. Proteins are color-coded (Adapted from Binz et al. 2004 [18])

<u>Cell Cycle Checkpoints</u>

A successful DNA damage response is not only dependent on the efficient execution of the DNA repair mechanisms but also on the effectiveness of the cell-cycle checkpoint machinery. The cell cycle checkpoint machinery is a control mechanism that ensures cell division fidelity by attenuating cell cycle progression and facilitating DNA repair, thereby preventing DNA lesions from being converted to persistent mutations. The most important function of the checkpoints is to assess the presence and ensure the complete removal of DNA damage before allowing initiation of the next phase. The cell cycle checkpoint machinery surveys the structural integrity of genomic DNA, when damage is found, it signals attenuation of the cell cycle until repair is achieved, or if the damage is too extensive, the activation of cell destruction pathways. Checkpoints are equipped with sensors that detect DNA damage and generate distinct checkpoint signals primarily in the form of kinase-mediated protein phosphorylations (Figure 1-4). Such signals are processed to effect downstream targets (mediators) that coordinate with transducers to pass the response to effectors, as a result, the cell cycle is arrested. While arrested, DNA repair mechanisms react to restore the damage appropriately. If the genome integrity is too compromised, the cell is forced into death (apoptosis/necrosis). The key sensors in DNA damage recognition are the MRN complex and the phosphoinositide (PI) 3-kinases: ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-PK (reviewed previously in NHEJ pathway). The PI3 kinases phosphorylate a multitude of proteins and thus initiate the DNA damage response. Downstream of the sensor proteins are pathways that either stimulate survival (repair) or induce cell death. Studies from past decades have identified many proteins participating in the cell cycle checkpoint machinery, which have established its overall framework. However, many important questions remain

unanswered as new components of the checkpoint network continue to emerge. Here, we focus on the molecular network of 2 important proteins, RPA and p53, which lie downstream of the sensor checkpoint kinases and play key roles for the development of this project.
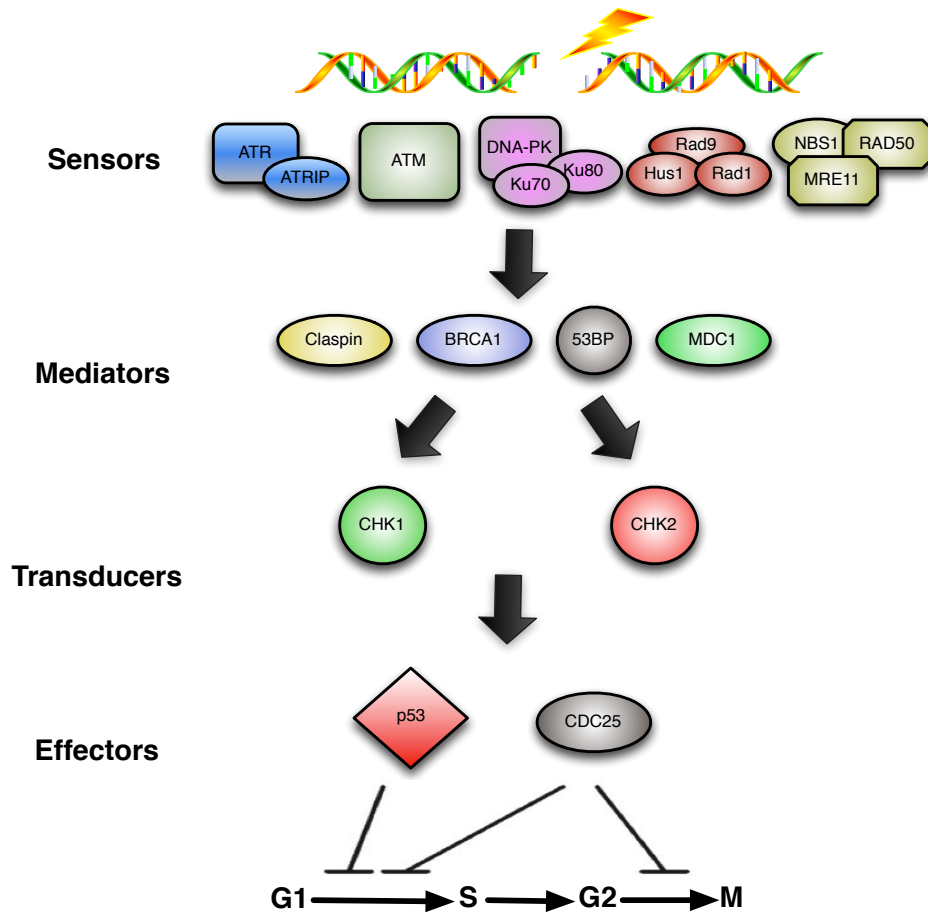
Figure 1-3. Signal Transduction Cascade that Leads to Cell Cycle Arrest. Cells are equipped with sensors that detect DNA damage and coordinate with mediators and transducers to pass the signal via kinases to effectors. Finally, effectors evoke the inhibition of the G1/S, S or G2/M progression (Adapted from Sancar et al. 2004 [19])

Persistent DNA Lesions Trigger Cell Death

DNA is the target for most, if not all, genotoxic agents. Some of the most common agents include UV light, ionizing radiation (X-rays), and common anticancer drugs such as cisplatin, adriamycin, CPT, and others. It has been well established that if damage to DNA is not repaired

harmful effects such as chromosomal aberrations, recombination, gene mutations, and cancer can emerge (Figure 1-5); however, persistent DNA lesions, can also trigger cell death. The data supporting this idea come mainly from studies of cells that are defective in DNA repair genes. Recent data have shown that nearly all cells that either have mutations, or a knockdown of essential repair proteins are hypersensitive to killing by genotoxins [20]. However, the mechanisms that decide whether a particular cell should stop dividing and enter the programmed self-death pathway remain largely obscure.
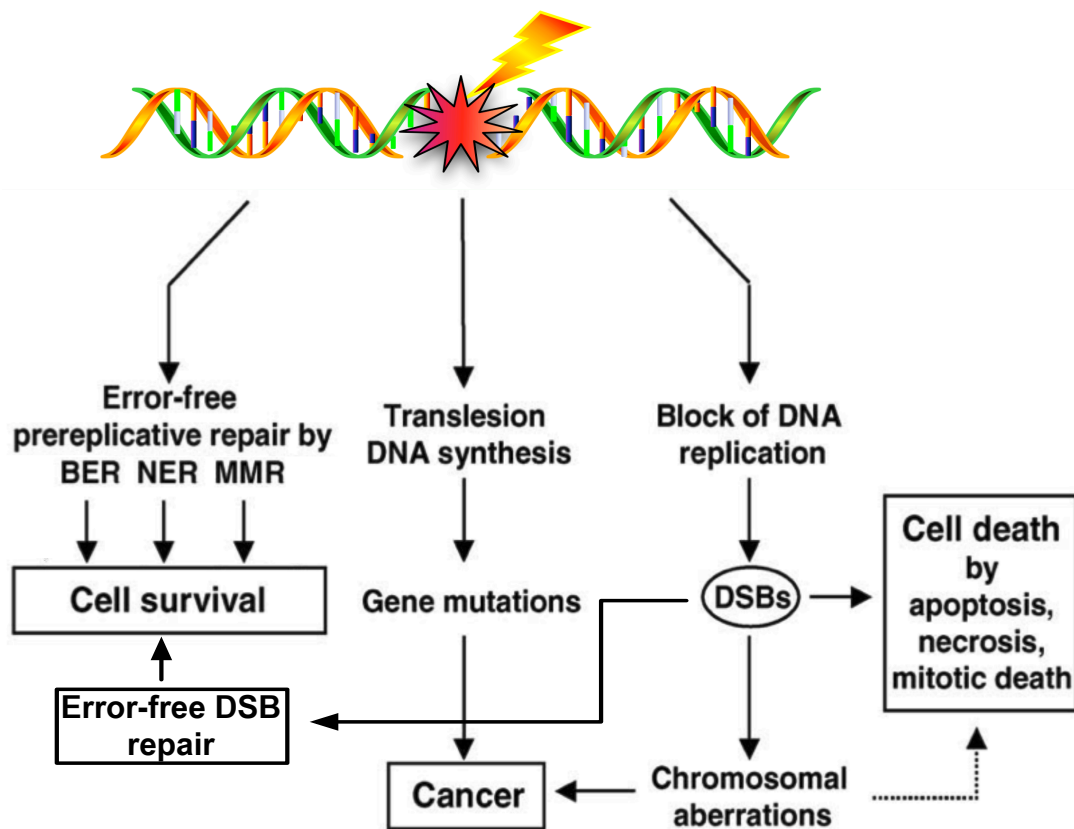
Figure 1-5. DNA Damage-Induced Endpoints. DNA lesions are repaired by different mechanisms (BER, NER, MMR and DSBR) that allow cells to survive and prevent mutations. If the damage is not properly repaired, gene mutations and chromosomal aberrations leading to cancer or cell death may follow.

Apoptosis

Apoptosis is a specific mode of cell death that is performed in a regulated manner. Apoptosis is used as a defense mechanism against stressful changes in internal and external environments as well as during proper cell development. Maintaining a balance between the degree of cell proliferation and cell death is critical for the conservation of normal physiological processes and, more important for this project, for the restraining of damaged cells that could become cancerous [21,22]. The formation of cancer cells is a complex multistep process that requires a sequence of alterations to the genome and breaching of a series of intracellular mechanisms. In the last century extensive research in the cancer field has investigated infringing of these mechanisms. Of particular interest is the mechanism that allows cancer cells to avoid death and confer survival advantage, thus understanding the mechanisms of apoptosis avoidance will improve therapeutic efficacy and will significantly improve cancer therapies [23].

Replication Protein A

Structure of RPA

Human RPA is a heterotrimer consisting of 3 subunits: 70, 32, and 14 kDa (referred to as RPA70, RPA32, and RPA14). Figure 1-6 represents the domain arrangement of RPA. RPA was initially discovered during studies of the simian vacuolating virus 40 (SV40) where it was found to be essential for replication. RPA is a major eukaryotic single-stranded DNA (ssDNA) binding protein and consists of a series of six DNA-binding domains (DBDs). Each DBD consists of a structural domain known as oligonucleotide/oligosaccharide binding fold (OB-fold). The OB-fold is a folding motif comprised of one 5-stranded β sheet coiled to form a β barrel and is capped by an α-helix located between the third and the fourth β strand. There are 4 OB-folds in the RPA70 subunit: DBD-F (amino acids 1-110), DBD-A (amino acids 181-290), DBD-B

(amino acids 300-420), and DBD-C (amino acids 436-616) [24]. RPA32 contains only one OB-

fold, DBD-D (amino acids 43-170), which is flanked at the N terminus by an unstructured tail

that can be heavily phosphorylated, and at the C terminus by a helix-turn-helix (HtH) domain

(amino acids 200-270). RPA14 has a single OB-fold (amino acids 1-114), referred to as DBD-E,

that is structurally important for the trimerization of RPA, but lacks DNA binding activity [25].
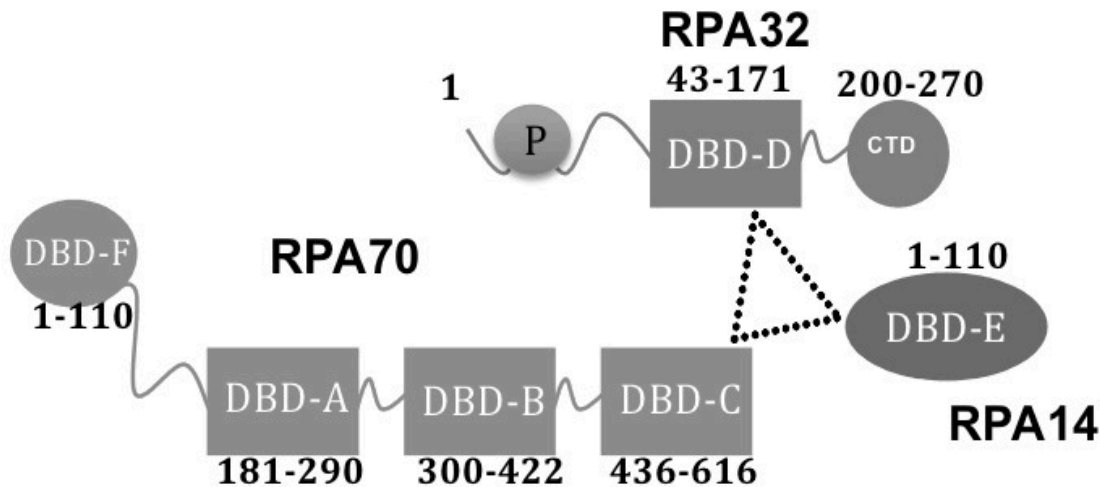
Figure 1-6. Schematic Representation of Replication Protein A. DBD A-F: DNA binding
domain A-F). CTD: C-terminal domain. Numbers represent the amino acids sequence in each
domain structure.

RPA Interaction with ssDNA

RPA binds ssDNA using only 4 of the 6 OB-fold domains: DBD-A, DBD-B, DBD-C (in

the RPA70 subunit), and DBD-D (in the RPA32 subunit). It is thought that the binding follows a

sequential assembly in which DBD-A through DBD-D bind to ssDNA 5' to 3' (Figure 1-7) [26,27].

During the RPA-ssDNA interaction, 3 distinct modes of binding occur according to the length of

ssDNA. The first is the 8-10 nucleotide-binding mode that consists of the serial binding of DBD-

A and DBD-B to their cognate elements [28]. The second is the 13-14 nucleotide-binding mode

where only the RPA70 subunit contacts the DNA through the OB-fold domains of DBD-A,

DBD-B, and DBD-C [29]. And in the third mode of binding all four domains, the 3 in RPA70 and the DBD-D in RPA32, mediate the 30-nucleotide mode [30].
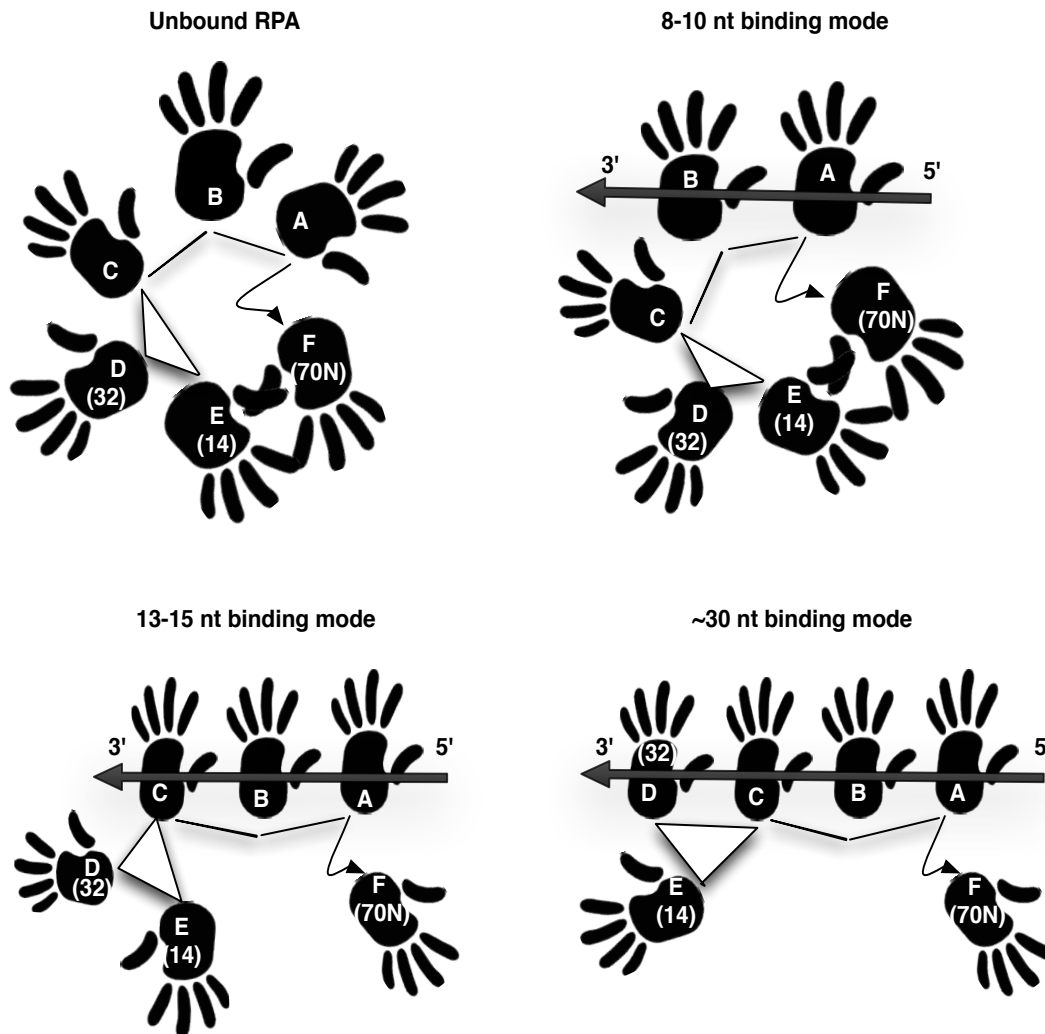
Figure 1-7. Proposed Model for RPA Binding to ssDNA (Adapted from Bochkarev et al. 2004 [24]). RPA binds to ssDNA through 3 sequential steps that depend on the length of ssDNA. Palms represent the OB-folds, DBDs are labeled A-F, 70N represents the N-terminus of RPA70, 32 represents RPA32 subunit and 14 represents the RPA14 subunit. Lines represent flexible linkers. ssDNA is represented with an arrow and the triangle represents the 3-helix subunit interface.

Because of its affinity towards ssDNA, RPA is directly involved in a large variety of DNA metabolic pathways such as replication, transcription, recombination, and all DNA repair pathways [31]. During replication, in studies performed on the SV-40 virus, it was shown that in the first step of initiation RPA was required for the unwinding of the DNA at origins; however, this function was not specific. RPA mutants that bind to ssDNA with high affinity and most other single-stranded binding proteins support the unwinding reaction [32]. Nevertheless, in the very next step of initiation the DNA polymerase alpha/primase complex recognizes the partially unwound DNA bound to RPA and the RNA primer is synthesized, a step where RPA is indispensable [33,34]. During this step RPA70 is primarily responsible for the interaction; however, RPA32 and RPA14 are also needed. Isolated RPA70 alone and other mutant forms of RPA missing the smaller 2 subunits were not able to support DNA replication [35-37]. In addition, RPA is important for DNA elongation once replication forks are established RPA remains associated with the replication machinery [38]. Therefore, cells in S phase show distinct RPA colocalization with the replication foci [39].

During DNA repair, RPA participates in DNA damage recognition, excision, and resynthesis of DNA at the site of damage [31]. During DSB repair pathways, the focus of repair mechanism of this project, RPA interacts with multiple key proteins. In the HR pathway RPA associates with 2 Rad52 epistasis group proteins, Rad51 and Rad52, and modulates their activities by dictating their binding order to the DNA lesion. RPA promotes Rad51 presynaptic filament assembly by reducing secondary structure in the long ssDNA regions but also suppresses the assembly by occluding the DNA. The inhibitory effect of RPA, nevertheless, can be overcome significantly in the presence of Rad52 because Rad52 recognizes RPA-bound

ssDNA, and this activity of Rad52 allows the Rad51–Rad52 complex to gain access to ssDNA already covered with RPA [40] (Figure 1-3). In NHEJ, RPA has been observed to interact with DNA-PK and to colocalize with the MRN complex [41]; however RPA's role in NHEJ is not well defined.

RPA in Cell Cycle Checkpoints

There are substantial data suggesting a role of RPA in the regulation of cell cycle checkpoints after DNA damage. In budding and fission yeast, different mutations in RPA32 and RPA70 caused hypersensitivity of cells to genotoxic agents, defective G1/S and intra-S checkpoint activation and prevented the downstream phosphorylation of checkpoint sensors ATR and ATM [42-44]. Cells containing the rfa1-t11 mutation (RPA70-K54E) [45] had decreased Rad53 phosphorylation (Chk2, checkpoint kinase 2) and failed to arrest at the G2/M checkpoint when DSBs were introduced [42,44]. A more recent report has shown that the coating of ssDNA by RPA is recognized by ATR-interacting protein (ATRIP) and that RPA is indispensable for the activation of checkpoint kinase 1 (Chk1) [46]. This report suggests that RPA is important for the localization of ATR to sites of DNA damage and activation of ATR kinase activity. In addition to ATR/ATRIP, RPA has been shown to be required for the loading of 2 other well-established checkpoint sensors: the Rad9-Rad1-Hus1 (9-1-1) complex and the Rad17-Rfc complex. These complexes are homologous to replication factor C (RFC) and PCNA respectively, which are required for DNA replication [47,48]. RPA also interacts with a number of well-known regulatory proteins and tumor suppressors such as p53, BRCA1, BRCA2, and PI3 kinases: ATR, ATM, and DNA-PK [41,46,49-54].

Phosphorylation of RPA

In the absence of DNA damage, RPA is phosphorylated in a cell-cycle dependent manner. During S phase 40-50% of endogenous RPA is phosphorylated and the timing of phosphorylation correlates with the initiation of replication [55]. RPA32 is phosphorylated at Ser-23 and Ser-29 during the G1/S transition and M-phase and then dephosphorylated at the completion of M-phase. These phosphorylations during an unperturbed cell cycle are primarily carried out by cyclin-CDKs [56-59]. In response to DNA damage, RPA32 is phosphorylated at several additional sites. Although it stills remains unclear how many and which of these residues are concurrently phosphorylated on each RPA molecule, the hyper-phosphorylated RPA has between five and nine sites of phosphorylation within RPA32N. These sites include Ser-4, Ser-8, Ser-11/Ser-12/Ser-13, Thr-21, Ser-23, Ser-29 and Ser-33. The DNAdamage-induced hyperphosphorylation of RPA is thought to be carried out by three members of the PIKK family: ATR, ATM and DNA-PK, likely dependent on the types of DNA damage. Given the important role of ATR, ATM and DNA-PK in DNA damage response and the regulation of cell cycle checkpoints, it is likely that RPA hyperphosphorylation is required for the regulation of these cell pathways.

It has been suggested that RPA undergoes a structural transformation when the RPA32N becomes hyper-phosphorylated. This structural transformation occurs via the inter-subunit interaction between the hyperphosphorylated RPA32N (hyp-RPA32N) and the DBD-B of RPA70 subunit. Results suggest that such structural alteration reduces the binding affinity of RPA to other binding partners and to short ssDNA; this is likely due to the blockage of DBD-B by hyp-RPA32N [60]. In agreement, the phosphorylation of RPA32 has been shown to modulate several RPA interactions including the interaction with 9-1-1 complex [61] and with 2 important

DSB repair proteins, Rad51 and Rad52 [62]. Additionally, the hyperphosphorylation of RPA32 has been shown to disrupt the RPA interaction with DNA polymerase α *in vitro* [63] and a RPA32 mutant that mimics the hyperphosphorylation by substitution of Ser-8, Ser-11, Ser-12, Ser-13, Thr-21, and Ser-33 with aspartic acid fails to localize to replication centers in cells [64].

In summary, RPA has a role in recognition of DNA damage for repair and for initiation of cell cycle checkpoints, a necessary role during initiation of DNA replication and an important role in all DNA repair pathways. In addition, RPA has been shown to be a regulatory protein by undergoing hyperphosphorylation in response to genotoxic stress. These together allude to RPA's potential role as a pivotal orchestrator between stoppage of DNA replication, initiation of DNA repair, and activation of cell cycle checkpoints in response to DNA damage.

<u>Protein p53</u>

p53 is a tumor suppressor whose inactivation is a key step of carcinogenesis for over half of human cancers. As 'the guardian of the genome', p53 is a key regulator of genome stabilization through its roles in cell cycle checkpoints, apoptosis, and DNA repair. p53 under unperturbed conditions is inactive due to its rapid degradation by its counterpart, the ubiquitin ligase Mdm2. However, upon the infliction of almost any cellular stress, Mdm2 is contained and p53 becomes highly abundant in the cell becoming a dominating transcription factor. Despite massive research efforts and the very impressive progress made over the past several decades on p53, it is increasingly evident that our current knowledge is incomplete. The constant discoveries of vast and different transcriptional targets controlled by p53 raises new questions about how these regulated networks interlock to promote tumor suppression. The significance of p53 in tumor suppression in humans is highlighted by the dramatic cancer predisposition of individuals

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with Li-Fraumeni syndrome (LFS), who inherit a p53 allele mutated, in most cases, between exons 5 and 8 of the p53 gene. Persons with LFS have a 25-fold increase predisposition to acquire a malignant tumor by the age of 50 [65].

Most tumor suppressor genes found to be mutated in cancer are inactivated by truncating mutations unlike them, the *TP53* gene in most human tumors is found to undergo a missense mutation that causes deficient transcription of p53's canonical targets. Most p53 mutations occur in exons 4-9, which encode the DBD of the protein, and 30% of these mutations fall within residues R175, G245, R248, R249, R273, and R282 (Figure 1-8) [66]. In addition, many p53 mutants can antagonize p53 tumor suppression roles by becoming dominant negative (DN) regulators. Because the active form of p53 functions only as a tetramer, a single DN p53 can heavily interfere with the proper activity of p53 as a tumor suppressor [67,68]. Furthermore, *TP53* mutations are often followed by loss of heterozygosity (LOH), where a particular locus heterozygous for a mutant and a wild-type allele has the wild-type allele either deleted or mutated [69,70]. Evidence also supports a gain-of-function (GOF) mechanism whereby many mutant p53 isoforms can acquire oncogenic properties [70]. DN, LOH, GOF effects and the tumor suppressor abilities of p53 may be one major weakness of our genome and the reasoning for its mutation in over half of human cancers.

Phosphorylation of p53 Transactivational Domain

Because of p53's importance in tumorigenesis, there have been extensive studies of the signaling cascades that connect DNA damage with p53. Phosphorylation in p53 is largely clustered in the transactivational domain (TAD), linker and the regulatory domain (REG); however focus will be placed on the TAD phosphorylation sites because this region is of importance to the development of this project. Mutations in the TAD1 and TAD2

phosphorylation sites generally result in changes in p53 stabilization and activation of its canonical targets. The alanine substitution of all phosphorylation residues in the TAD1 and TAD2 domain leads to a significant loss of p53 transactivational activity [71]. Additionally, studies have identified a variety of kinases that phosphorylate p53: ATR, ATM, Chk1 and Chk2 in the TAD region (Figure 1-8). Recent characterization of genetically manipulated mice incapable of p53 phosphorylation at Ser-18 and Ser-23 (Ser-15 and Ser-20 in humans), the 2 main sites targeted by the ATR/ATM/Chk1/Chk2 kinases, indicated the important role of these phosphorylation residues in the DNA damage induced p53-dependent responses [72]. The Ser-18/23 mutated mice were severely compromised in p53-dependent apoptosis with overall levels of apoptosis in γ-irradiated thymocytes similar to those in p53$^{-/-}$ while the Ser-18 mutated mice showed impaired G1/S cell cycle arrest following UV irradiation in mouse embryonic fibroblast (MEF) and diminished p53-dependent apoptosis in thymocytes following IR exposure [72-74].

Knock-in mice containing a single p53 allele with Thr-21 and Ser-23 mutated to aspartic acid (p53$^{T21D,S23D/-}$), which mimics the constitutively phosphorylated p53, showed premature aging and a significantly reduced life span [75]. Moreover, untreated cells from these mice showed increased p53-dependent transcription and apoptosis as compared with the p53$^{+/-}$ cells, but this activity was unaffected or even lowered when DNA damaged was impinged. A human p53 knock-in mouse (HUPKI) was generated to introduce a Ser-46 to alanine mutation, as this residue is not conserved in mice. The HUPKI mice showed decreased p53 levels after UV and IR exposure accompanied by a modest decrease in transactivation of p53 targets, such as Noxa and PUMA, along with an overall mild reduction of p53-dependent apoptosis [76]. Thus, results in the genetically modified p53-mice are concordant with other models in demonstrating the importance of phosphorylation in the TAD1 and TAD2 domains of p53 in the modulation of

many protein-protein interactions that either change the stability of p53, change its subcellular

localization or direct its function as a transcription factor.
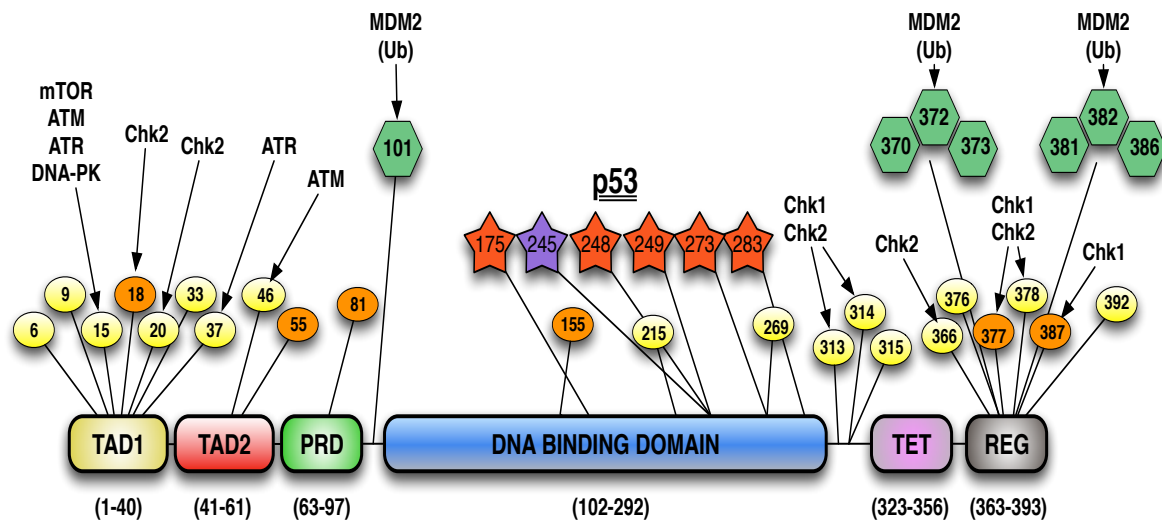
Figure 1-8. Schematic Representation of p53. Domain structure of p53 showing various sites of posttranslational modifications and mutations. Ovals represent phosphorylation sites, serines are yellow and threonines are orange. Hexagons represent sites ubiquitinated by Mdm2. Stars represent most commonly known mutations in the p53 protein in human cancers. TAD: Transactivational domain, PRD: Proline-rich domain, TET: Tetramerization domain, REG: Regulatory domain (Adapted from Jenking et al. 2012 [77]).

Interacting Partners of p53 Transactivational Domain

To date structures of p53 transactivational domain (TAD) in complex with 6 different

partner proteins have been reported [77]. The first, and most studied, is the complex with the

negative p53 regulator Mdm2. Mdm2 is the main regulator of p53 and restrains p53 by 2 main

mechanisms: 1) the binding of Mdm2 to the N-terminal of p53 masks the TAD required for p53

transcriptional activity and 2) targeting p53 for degradation via the ubiquitin-proteasome system

by the ubiquitin ligase activity of Mdm2 (Figure 1-8) [78]. In the Mdm2-p53 complex 3 highly-

conserved hydrophobic residues (Phe-19, Trp-23, and Leu-26) align along one face of the TAD1

helix and are packed deeply in a hydrophobic cleft of Mdm2 forming a relative strong complex

(Kd~100 nM) [79]. On the other hand, Mdm2 also has been shown to be a positive regulator of p53. A recent report demostrated that, upon DNA damage, ATM phosphorylates Mdm2 at Ser-395 that promotes the remodeling of the Mdm2 RING domain; the new conformation of Mdm2 is then able to bind p53 mRNA and promote p53 translation [80]. As a result, both p53 and Mdm2 participate in an auto-regulatory feedback loop that under normal conditions restrains p53 functions and under stressed conditions drives p53 activation.

The only other structures in complex with the p53 TAD include the CBP and p300 proteins, 2 homologous histone acetyltransferases that facilitate p53 transcriptional activity by promoting chromatin unwinding near the promoters of target genes [81], the p53 TAD-MdmX complex, the p53 TAD-p62, and the p53 TAD-RPA70N complex. The p53 TAD-RPA70N complex, most important for this project, represents a separate class of p53-interacting proteins that recognize the TAD as if it were ssDNA. In this complex TAD binds to the OB folds of RPA70 directly. The RPA70N -TAD binding competes with ssDNA-TAD allowing RPA to repress the transcriptional activity of p53 [53]. The p53 TAD interaction with ssDNA-binding proteins reveals a distinctive type of interaction in which acidic residues in TAD compete with negatively-charged phosphate groups of ssDNA.

p53 in Apoptosis

p53 can activate apoptosis via both transcription-dependent and transcription-independent mechanisms [82]. In the transcription-independent pathway, it has been shown that, following apoptotic stimuli, a portion of p53 localizes directly to the mitochondria where it physically interacts with Bcl-2 and/or Bcl-XL and antagonizes their antiapoptotic function at the outer mitochondrial membrane [83]. p53 also has been shown to directly activate Bax, thereby leading to

mitochondrial outer membrane permeabilization (MOMP); this facilitates the release of cytochrome c leading to apoptosis [83-87].

In the transcription-dependent pathway, p53 can activate both the intrinsic and the extrinsic signaling pathways that converge at the level of Caspase activation but differ in the upstream cascade. The Bcl-2 family members regulate the intrinsic pathway by the ratio of proapoptotic (Bax and Bak) to prosurvival (Bcl-2, Bcl-XL and Mcl-1) Bcl-2-family effector proteins. Proapoptotic Bcl-2 effector proteins oligomerize at the mitochondrial outer membrane resulting in MOMP and eventually activating effector caspases. The prosurvival Bcl-2 family members bind directly to the proapoptotic members, thus inhibiting MOMP. p53 can activate the intrinsic apoptotic pathway by transcriptionally activating Bax, PUMA, and Noxa. The extrinsic apoptotic pathway is triggered by the transmembrane death-domain at the surface of cells and p53 is involved by the direct activation of receptors and ligands such as Fas, Killer/Dr5 and Pidd.

Questions to be Answered in These Studies

The requirement of RPA in such a broad range of cellular processes including initiation of replication, recognition of DNA lesions, efficient DNA repair, and cell cycle checkpoint signaling allude to its potential role in the coordination and regulation of these cellular processes; however, the mechanistic details are still poorly understood. Recent findings from our laboratory and others have shown that RPA undergoes hyperphosphorylation mediated by DNA damage checkpoint kinases and that this hyperphosphorylation is crucial for the cellular responses to DNA damage [60-64,88]. We therefore hypothesize that the checkpoint-mediated hyperphosphorylation alters the interacting activity of RPA with ssDNA and proteins resulting in the facilitation and modulation of DNA damage responses. We propose that the modulation of the DNA damage response is achieved by 2 possible mechanisms: 1) hyperphosphorylation induces precise structural transformation of RPA that in turn modifies its biochemical activity towards ssDNA and proteins, and 2) recognition of the hyperphosphorylated motif of RPA by hyperphospho-binding proteins. In this way, hyperphosphorylation of RPA may constitute a regulatory mechanism by which some DNA damage responses are inhibited while others are activated owing to the failure or success of the molecular interaction with the hyperphosphorylated RPA.

Among the RPA–protein interactions, the RPA-p53 interaction is of particular interest as p53 is thought to be a main tumor suppressor. The interaction of RPA and p53 has been known for quite some time; however, the mechanisms that modulate the RPA-p53 binding as well as the importance of the association/dissociation of the RPA-p53 complex in the cellular response to DNA damage remains far from clear. In the study published in Oncogene (2013 vol.32 (19) pp.

2452-2462), and presented here in Chapter 2 [89], we reveal a detailed mechanism for the regulation of the RPA-p53 interaction.

DSBs are the most toxic form of DNA damage and can cause genome rearrangement, making them a major driver of cancer. Two major DSB repair machineries have evolved: homologous recombination (HR) and nonhomologous end joining (NHEJ) [90]. At first these 2 major repair machineries were thought to function independently of one another; however, in recent years there has been evidence for the crosstalk between these 2 pathways [91,92]. In Chapter 2, we provide for the first time, mechanistic details of a crosstalk between HR and NHEJ repair machineries, involving coordinated interactions between p53, RPA, DNA-PK, ATM, and ATR in the DNA damage response.

Various reports suggest the involvement of RPA phosphorylation in the coordination of DNA repair, replication and cell cycle checkpoints; however, very little is currently known about the role of RPA in the regulation of apoptosis. In the study presented in Chapter 3, we tested the hypothesis that the lack of RPA phosphorylation after DNA damage promotes cell death. In order to test this hypothesis, we assayed common apoptotic markers in RPA phosphorylation-deficient cells after the induction of genotoxic stresses. Our findings reveal a novel role of RPA phosphorylation in the induction of apoptosis that illuminates a novel target for chemotherapy.

CHAPTER 2

DNA-PK, ATM AND ATR COLLABORATIVELY REGULATE p53–RPA

INTERACTION TO FACILITATE HOMOLOGOUS RECOMBINATION DNA REPAIR.

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Abstract

Homologous recombination (HR) and nonhomologous end joining (NHEJ) are two distinct DNA

double-stranded break (DSB) repair pathways. Here, we report that DNA-dependent protein

kinase (DNA-PK), the core component of NHEJ, partnering with DNA-damage checkpoint

kinases ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR), regulates HR

repair of DSBs. The regulation was accomplished through modulation of the p53 and replication

protein A (RPA) interaction. We show that upon DNA damage, p53 and RPA were freed from a

p53–RPA complex by simultaneous phosphorylations of RPA at the N-terminus of RPA32

subunit by DNA-PK and of p53 at Ser37 and Ser46 in a Chk1/Chk2-independent manner by

ATR and ATM, respectively. Neither the phosphorylation of RPA nor of p53 alone could

dissociate p53 and RPA. Furthermore, disruption of the release significantly compromised HR

repair of DSBs. Our results reveal a mechanism for the crosstalk between HR repair and NHEJ

through the co-regulation of p53–RPA interaction by DNA-PK, ATM and ATR.

DNA damage is a major cause of genome instability and, thus, the development of human cancer. In cells, DNA damage is removed by DNA repair pathways in coordination with DNA damage checkpoints. The latter halts cell cycle progression to allow time for DNA repair before cell cycling can resume [1-6]. DNA double- stranded breaks (DSBs) are the most lethal form of DNA damage and mainly are repaired by homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways in mammalian cells. NHEJ repairs the DSBs induced by genotoxic agents such as ionizing radiation. By contrast, HR repairs DSBs induced by genotoxins such as camptothecin (CPT). CPT is a topoisomerase I inhibitor that arrests the topoisomerase I-nicked DNA intermediate complex and leads to replication fork collapse at the nicked site to form DSBs [7, 8]. Although crosstalk may occur between HR and NHEJ [9, 10], the molecular mechanism remains unknown.

DNA-dependent protein kinase (DNA-PK) has a key role in NHEJ by recognizing DSBs, initiating NHEJ repair and assembling the repair machinery. DNA-PK is a 615 kDa heterotrimeric complex consisting of the catalytic subunit of DNA protein kinase, plus Ku70 and Ku80. As a member of the phosphatidylinositol 3-kinase- related kinase (PIKK) family, DNA-PK also phosphorylates proteins, such as H2AX, replication protein A (RPA), p53, XRCC4, Ku70 (XRCC6) and Ku80 (XRCC5) involved in DNA damage responses [11, 12]. Of those proteins, RPA is the major eukaryotic single-stranded DNA (ssDNA) binding protein and is a heterotrimer containing RPA70, RPA32 and RPA14 subunits. In addition to binding ssDNA, RPA also interacts with other proteins during the DNA damage response [5, 13-25] and is involved in almost all DNA metabolic pathways including the HR repair pathway. A mutation in RPA also is implicated in cancer [26, 27]. A remarkable fact about RPA is that upon DNA damage, the N-

terminus of RPA32 is hyper-phosphorylated by PIKK kinases [28]. We and others have presented evidence supporting a role of RPA in coordinating DDR pathways via the RPA32 hyperphosphorylation. [13, 14, 29-35] We have shown that upon hyperphosphorylation RPA undergoes a structural reorganization. [32]

Among RPA-protein interactions, the p53-RPA interaction [24, 36-41] is of particular interest as p53 is a tumor suppressor whose inactivation is a key step of carcinogenesis for over half of human cancers [42, 43]. As "the guardian of the genome" p53 is a key regulator of genome stabilization through its roles in cell cycle checkpoints, apoptosis and facilitating DNA repair [44]. It is well known that phosphorylation of p53 plays a critical role in regulating p53 activities in various DNA DAMAGE REPONSE pathways. Almost all the post-translational modifications on p53 occur in the unstructured region of the protein formed by the transactivation domain (TAD), the linker between the DNA-binding and TET domains, and the C-terminal 30 residues [45]. These same regions are involved in the p53 interaction with RPA dadad[24, 37, 45]. However, how the p53-RPA interaction is modulated and affects DNA damage responses is poorly understood. In the present study, we determined the mechanism by which the p53-RPA interaction is modulated as well as the impacts of the regulation on HR repair. We found that the p53-RPA complex was disassembled upon the phosphorylations of RPA and p53 by DNA-PK and ATM/ATR, respectively, in a synergistic manner. While phosphorylation of RPA or p53 alone showed no effect, phosphorylation deficiency of either p53 or RPA inhibited the dissociation of p53 and RPA. Also, the inhibition of phosphorylation significantly reduced the efficiency of HR repair. Our results unveil the mechanistic details of a crosstalk between HR and NHEJ repair machineries which involves highly coordinated interactions between p53, RPA, DNA-PK, ATM and ATR in the DNA damage response.

Materials and Methods

Cells, Cell Culture, Proteins and Antibodies

A549 cells were maintained at 37°C under a humidified atmosphere of 5% CO_2 in

Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine

serum (FBS; HyClone), 1% penicillin/streptomycin. U2OS cells expressing RPA32 wild-type

(WT-RPA) or a hyperphosphorylation-deficient mutant (PD-RPA) (kindly provided by Dr.

Xiaohua Wu; Scripps Research Institute, La Jolla, California, USA) were maintained in DMEM

supplemented with 10% FBS and antibiotics as described above. These U2OS cells were grown

in hygromycin (200 µg/mL) and puromycin (1 µg/mL) to maintain plasmid expression. These

are stable cell lines in which the endogenous RPA32 was stably knocked down while

recombinant WT-RPA or PD-RPA were stably produced [34]. The HCT-116 [ATR-/-] cells, also

known as ATR[flox/-] cells (kindly provided by Dr. Stephen Elledge, Harvard University), were

grown as described above using McCoy's 5A medium (ATCC).

Recombinant human RPA was expressed and purified as described (Yang *et al*., 2002 [17]).

Hyp-RPA was purified using previous procedures (Patrick *et al*., 2005 [30]). In addition,

hyperphosphorylation of RPA by purified DNA-PK (Promega, Madison, WI, U.S.A.) was

carried out as described (Liu *et al*., 2005 [32]).

Antibodies used in this study include anti-RPA32 (Sigma R3280), anti-p53 (Invitrogen

AHO0142 or Santa Cruz sc-6243), anti-phospho-p53(pSer15) (R&D AF1043 and Cell Signaling

9286), anti-phospho-p53(pSer20) (AnaSpec 54428), anti-phospho-p53(pSer37) (Santa Cruz sc-

135633), anti-phospho-p53(Ser46) (Cell Signaling 2521), anti-DNA-PK (Santa Cruz sc-9051),

anti-ATM (Bethyl Lab A300-299A), anti-ATR (Bethyl Lab A300-138A) and anti-RAD51 (Santa Cruz sc-8349).

S phase Cell Synchronization

To optimize RPA32 hyperphosphorylation in response to CPT treatment cells were synchronized in S phase by incubating with aphidicolin (APH) (1 µg/mL) for 18 hours before release into fresh media for 2 hrs. Synchronized cells were then treated with CPT.

Co-Immunoprecipitation

Immunoprecipitation (IP) in U2OS cell lysates was done after subcellular fractionation: cells were collected with a policeman and resuspended in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl$_2$, 1 mM EGTA, 0.1% Triton X-100, phosphatases and protease inhibitors) and incubated at 4 °C for 5 min. Low speed centrifugation (1,300xg/5 min) separated cytoplasmic proteins from pelleted nuclei. Isolated nuclei were lysed in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, phosphatase and protease inhibitors). Chromatin-bound proteins were collected (1500xg centrifugation/5 min), and resuspended in IP buffer (20 mM Tris-HCl, pH 7.8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1% NP-40) and subjected to DNase I digestion. Lysates were cleared (centrifugation at 13,000xg/15 min, 4 °C) and received 3 µg of anti-p53 antibodies for immunoprecipitation and incubated overnight at 4 °C, followed by incubation with protein G beads (Invitrogen 10-1242) for 2 hrs. Immune complexes were collected by centrifugation at 1,000xg.

Pull-Down Assays

Recombinant GST-tagged p53 protein (SignalChem P05-30BG) was incubated with purified RPA or hyp-RPA in RPA binding buffer at 4 °C overnight. 10% of the sample was loaded as "Input". Pre-equilibrated GST-agarose beads (GE) were added to the remaining sample

and incubated at 4 °C for 2 hrs. Immune complexes were collected by centrifugation at 1000 x g, washed 3X with RPA binding buffer and analyzed by western blotting.

For the p53-RPA interaction involving ssDNA, purified RPA or hyp-RPA was incubated with 5'-biotinylated ssDNA (dT30mer or dT90mer) at indicated ratios for 30 min at 25 °C in RPA binding buffer. Pre-equilibrated streptavidin beads were supplied and the samples incubated for 2 hrs at 4 °C. DNA-streptavidin complexes were collected, and then washed twice with RPA binding buffer to remove unbound RPA. Subsequently, recombinant p53 was supplied and the mixture incubated overnight at 4 °C. Complexes were collected at 1000 x g, washed 3X with RPA binding buffer and analyzed by western blotting.

siRNA and Plasmid Constructs Transfections

Cells were transfected with siRNA for 72 hrs using INTERFERin transfection reagent (Polyplus 409-10) following the manufacturer instructions. The siRNAs include ATM: CAUACUACUCAAAGACAUUTT, AAUGUCUUUGAGUAGUAUGTT, ATR: CCUCCGUGAUGUUGCUUGATT, UCAAGCAACAUCACGGAGGTT, DNA-PK: AGGGCCAAGCUGUCACUCUTT, AGAGUGACAGCUUGGCCCUTT. The pCB6-p53-WT and pCB6-p53-S15A expression constructs (kindly provided by Dr. Karen Vousden, Beatson Institute for Cancer, Bearsden, Glasgow, UK) were transfected into cells using JetPI transfection reagent (Polyplus 101-10) according to the manufacturer's instructions for 72 hrs. Similar transfections were performed with pCAG3.1-p53-WT, -S15A, -S20A, -S37A and -S46A expression vectors (kindly provided by Dr. Carl W. Anderson, Biology Department, Brookhaven National Laboratory, Upton, New York).

Comet Assay

U2OS cells stably expressing RPA32-WT or PD-RPA were treated with increasing doses of CPT for 2 hrs. Then, neutral comet assays were carried out using the Comet Assay System (Trevigen) according to the manufacturer's instructions. Fluorescence images were captured using a Nikon inverted fluorescent microscope with attached CCD camera at 100X magnification and the comet tail moment was measured using Comet Assay IV software (Perceptive). At least 50 cells were assessed per treatment. In parallel with the comet assay, cell cultures with the same treatments were harvested for co-immunoprecipitation and the proteins analyzed by western blotting.

Homologous Recombination Assays

H1299 (p53$^{-/-}$) or A549 cells were transfected with the HR reporter pDR-GFP (a gift of Maria Jasin, Addgene plasmid #26475) for 48 hrs. H1299 cells also were transfected simultaneously with the p53-expression constructs (WT, S37A and S46A), while A549 cells were treated with ATM and/or ATR inhibitors. Then, cells were either treated with 5 μM CPT for 24 hrs to induce phosphorylation of RPA and p53 and DNA double-strand breaks or transfected for 36 hrs (control) with an I-SceI expression vector (pCBASceI, a gift of Maria Jasin, Addgene plasmid #26477). Following the treatments, cells were visualized in phase contract or for green fluorescence using fluorescence microscopy. At least 100 cells were scored for GFP positive in three independent experiments.

Interaction of RPA with p53 in Cells

In order to address the functional implications of the p53-RPA interaction, we examined the ability of p53 to bind to the hyperphosphorylated form of RPA32 in cells by co-immunoprecipitation (co-IP). Cells expressing phosphorylation-deficient RPA32 (PD-RPA) and wild-type RPA32 [34], respectively, were treated with CPT for 3 hrs. CPT is a DNA DSB inducer and was able to induce RPA hyperphosphorylation in cells as indicated by the bands of hyperphosphorylated RPA32 (hyp-RPA32) which migrate slower than the nonphosphorylated RPA32 band on SDS-PAGE (2-1A). In contrast, as expected, CPT treatment resulted in no hyperphosphorylation of RPA32 in the PD-RPA cells. As shown in Figure 2-1A, the association of p53 with RPA predominately occurred between p53 and the unphosphorylated RPA with little or no hyp-RPA32 associating with p53. This suggests that RPA hyperphosphorylation may have disrupted the p53-RPA association. Note that a DNase I pretreatment of the cell lysate precludes a DNA linkage between RPA and p53 as an explanation for these immunoprecipitation (IP) results. To confirm that the preferential binding of p53 to unphosphorylated RPA was due to direct protein-protein interaction, we used purified recombinant RPA [17] which had been hyperphosphorylated [30]. After p53 IP from cell lysates with anti-p53 antibody, the immunoprecipitates were washed with buffer containing concentrations of NaCl up to 1 M to remove possible p53-associated proteins (Figure 2-1B). The wash was efficient as indicated by the removal of bound endogenous RPA. Then, an equimolar mixture of purified RPA and hyp-RPA was supplied to allow for interaction with the immunoprecipitated endogenous p53.

Subsequent blotting analysis of the co-immunoprecipitates confirmed that p53 directly interacted with the nonphosphorylated RPA while having little or no affinity to the hyp-RPA (Figure 2-1C).
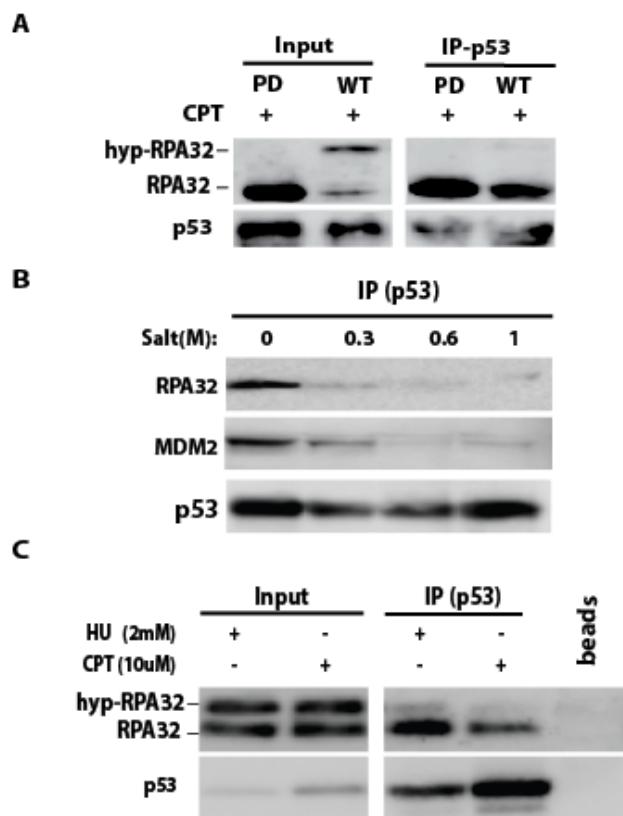
Figure 2-1. Hyperphosphorylated RPA is Unable to Interact with Endogenous p53. (**A**) Stable U2OS cells expressing WT- or PD-RPA32 were treated with 10 μM CPT for 3 hrs to induce RPA hyperphosphorylation. Cells were harvested and chromatin-bound proteins were isolated. Chromatin was subjected to DNase I digestion and 10% of the sample was loaded onto the gel (INPUT). The remaining lysate was immunoprecipitated using anti-p53 antibody. Samples were analyzed by western blotting. (**B**) IP was performed in A549 cell lysates using anti-p53 antibody. Immunoprecipitates were washed with buffer of increasing concentrations of salt (0.2-1.0 M) to remove proteins bound to p53, including endogenous RPA. Washed immunoprecipitates were analyzed by western blotting. (**C**) p53 from A549 cell lysates treated with 10 μM CPT for 2 hrs or 2 mM HU for 24 hrs was isolated by IP with anti-p53 antibody, followed by a 1 M salt buffer wash. Equimolar amounts of purified RPA and hyp-RPA were added and the proteins were allowed to interact for 6 hrs. Then, the p53 complexes were pulled down, washed and analyzed by western blotting.

In vitro Interaction of p53 with Native and Hyperphosphorylated RPA in the Presence or Absence of ssDNA

To further describe the p53-RPA interaction, co-IP assays with purified RPA/hyp-RPA and p53 proteins were performed. Surprisingly, the binding of recombinant p53 to the hyp-RPA is greater than that to native RPA (Figure 2-2A), contradicting the cellular results shown in Figure 2-1. The same experiment also was performed with RPA hyperphosphorylated by purified DNA-PK (Promega Corp., Madison, WI) [32] and a similar preference for hyp-RPA was obtained (Figure 2-2B). To investigate this discrepancy, similar *in vitro* immunoprecipitation was conducted in the presence of ssDNA as the binding to ssDNA is a major function of RPA in cells. After RPA pre-incubation with 5'-biotinylated dT30mer or dT90mer ssDNA, the ssDNA-bound RPA was pulled down with streptavidin-agarose beads, and then the RPA-ssDNA complex was incubated with purified recombinant p53. Recombinant p53 still bound more efficiently to hyp-RPA than native RPA in the presence of dT30mer or dT90mer (Figures 2-2A, 2-2B and 2-2C). To further determine the possible effect of ssDNA on the p53 interaction with native RPA and hyp-RPA, the immunoprecipitated p53-RPA complex of purified proteins was titrated with increasing concentrations of dT30mer ssDNA (Figure 2-2D). The ssDNA had little or no effect on the p53-RPA binding when the ssDNA had a 1:1 molar ratio to the proteins, but did competed with p53 for hyp-RPA at significantly higher ssDNA-to-protein ratios. Alternatively, RPA also was pre-incubated with various concentrations of ssDNA and then p53 was supplied. Similar results were obtained although native RPA binding to p53 also was affected at high ssDNA-to-protein ratios (Figure 2-2E). These data indicate that 1) hyperphosphorylation of RPA does not disrupt the RPA interaction with recombinant p53 *in vitro*; and 2) ssDNA does not play a significant role in mediating the phosphorylation-induced

disruption of cellular p53-RPA interaction observed in Figure 2-1. Thus, the

hyperphosphorylation of RPA alone may not be sufficient to substantially impact the p53-RPA

interaction; the post-translational modifications on p53 also may be important.
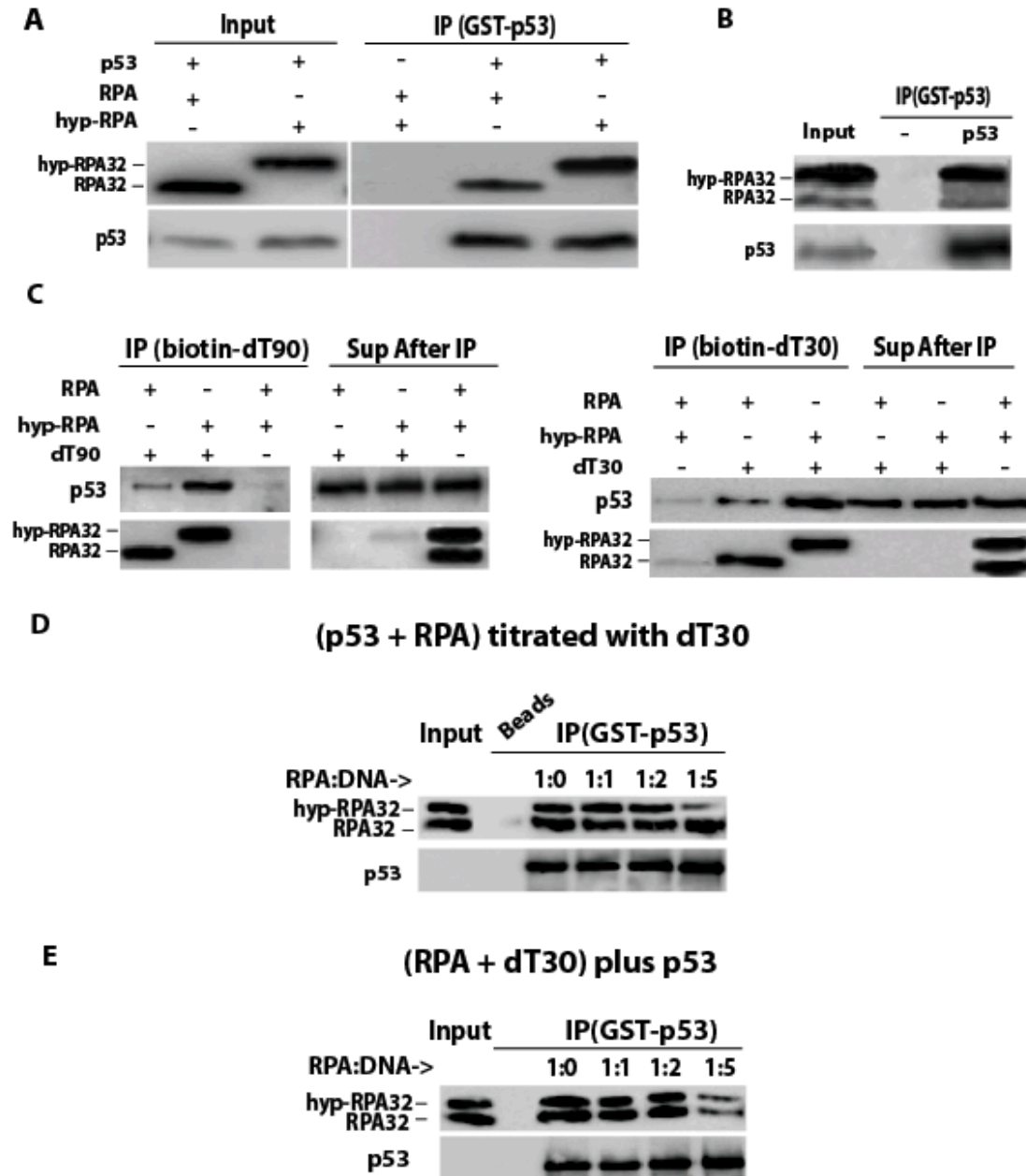
Figure 2-2. *In vitro* p53-RPA Interaction with and without ssDNA. (A) Recombinant
GST-tagged p53 protein was incubated with either recombinant RPA or hyp-RPA. Except for the

10% of sample volume loaded for input, samples were incubated with GST-agarose beads, collected by centrifugation, washed and analyzed by western blotting. (B) Recombinant RPA protein was phosphorylated *in vitro* using DNA-PK kinase, and then incubated with GST-p53, followed by IP as in (A). (C) Recombinant RPA or hyp-RPA was incubated with either dT90 or dT30 ssDNA. Recombinant p53 was supplied in excess to the RPA-ssDNA complex and incubated overnight. Biotinylated DNA complexes were collected by centrifugation, washed and analyzed by western blotting. (D) Recombinant RPA and hyp-RPA were mixed followed by incubation with p53 for 6 hrs. The ssDNA (dT30) was added in increasing molar ratios of DNA-to-RPA. The samples were subjected to immunoprecipitation with GST beads and analyzed by western blotting. (E) Recombinant RPA and hyp-RPA were mixed and incubated with increasing molar ratios of ssDNA (dT30). Purified p53 protein then was supplied at an equimolar RPA amount for binding for 6 hrs. The samples were subjected to p53 immunoprecipitation with GST beads and analyzed by western blotting.

Effect of p53 Phosphorylation on p53-RPA Interaction

To determine whether post-translational modifications of p53 are involved in the modulation of p53-RPA interactions, cells were treated with CPT followed by immunoprecipitation of p53 from the nuclear lysates. The p53 immunoprecipitates were washed with the 1M NaCl buffer to remove p53-associated proteins (Figure 2-1B). A portion of the endogenous p53 was treated with Calf Intestinal Alkaline Phosphatase (CIAP) to remove the endogenous phosphorylations. Then, recombinant RPA and hyp-RPA were supplied as an equimolar mix to allow for the interaction with p53. Western blotting analysis of the samples is shown in Figure 2-3 where the endogenous p53 predominately bound to the unphosphorylated form of RPA (lane 5). However the binding preference was reversed after the same endogenous p53 was de-phosphorylated with CIAP, then the p53-hypRPA interaction is favored (lane 4). The results indicated that phosphorylation of p53 also is involved in the modulation of the p53-RPA interaction.
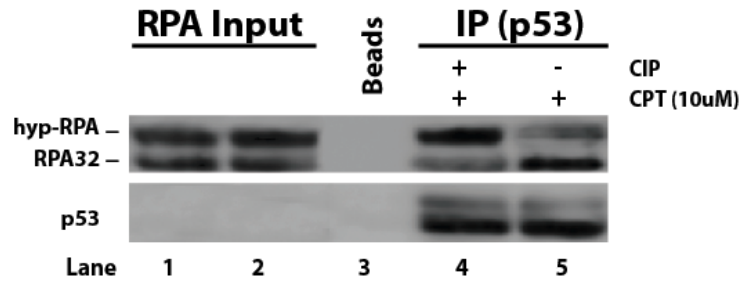
Figure 2-3. p53 Phosphorylation is Required for Regulation of p53-RPA Binding. p53 is immunoprecipitated from A549 cell lysates using anti-p53 antibody. Samples were washed with 1 M salt buffer and incubated with Calf Intestinal Alkaline Phosphatase (CIP). After washing, recombinant RPA and hyp-RPA were added in equal molar amounts and incubated with the endogenous p53 overnight. Samples were then spun down, washed and analyze by western blotting.

Modulation of p53-RPA Binding upon CPT Treatment is DNA-PK, ATR and ATM Dependent

Hyperphosphorylation of RPA in response to DNA damage is carried out by members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family which includes ATM, ATR and DNA-PK [5, 39, 46]. To identify the protein kinases involved in the phosphorylation-mediated regulation of the cellular p53-RPA interaction in response to CPT treatment, RPA hyperphosphorylation was evaluated in the cells treated with protein kinase inhibitors (Figures 2-4A and 2-4B), or depleted of ATR, ATM or DNA-PK by siRNAs (Figure 2-4C). The kinase activities of ATR and ATM were efficiently inhibited by caffeine, an inhibitor of ATR and ATM, as demonstrated by the inhibition of p53 phosphorylation at Ser15, a downstream DNA damage signaling event in the ATR and ATM checkpoint pathways (Figure 2-4A, left). The caffeine treatment inhibited the release of hyp-RPA from p53 since the hyp-RPA remained bound efficiently to p53 as compared with native RPA following DNA damage (Figure 2-4A, right). The results were further confirmed by the more specific ATM and ATR inhibitors

Ku55933 and Nu6027, respectively (Figure 2-4B). Consistent results were also obtained with ATR-deficient cells (Figure S2-1). To further assess the effect of individual PIKK proteins on modulation of p53-RPA interaction, siRNAs were used to knockdown ATR, ATM, or DNA-PK (Figure 2-4C). Subsequent co-immunoprecipitation assays of cell lysates indicated that in agreement with the results of inhibitor treatments, depletion of ATR or ATM significantly increased the level of hyp-RPA binding to p53 versus control siRNA (Figure 2-4C). In addition, we found that DNA-PK was required for the CPT-induced RPA hyperphosphorylation while ATM and ATR are not, which is consistent with the previous reports [39, 46-48]. As expected, knockdown of DNA-PK kept RPA bound to p53 (Figure 2-4C).
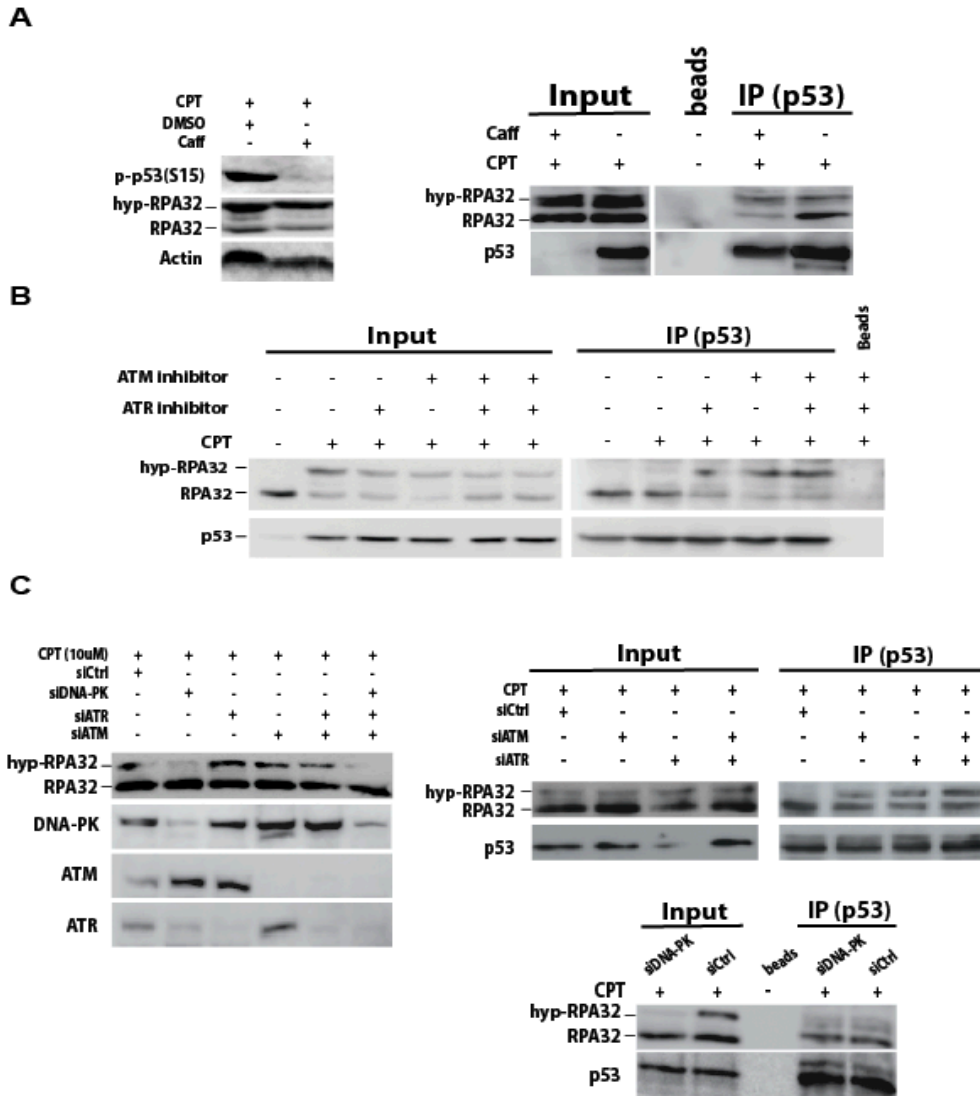
Figure 2-4. Modulation of p53-RPA Binding is Dependent on DNA-PK as well as ATM and ATR. (A) A549 cells were treated with caffeine to inhibit ATM and ATR activities prior to the CPT treatment. Whole cell lysates were loaded in a 10% SDS page (left). Nuclear lysates then were isolated and subjected to DNase I digestion and 10% of sample was loaded as input. IP was subsequently performed using anti-p53 antibody and co-immunoprecipitated proteins were analyzed by western blotting with the indicated antibodies (B) A549 cells were transfected with ATM siRNA, ATR siRNA, DNA-PK siRNA or their combinations. Cells were treated with CPT. Whole cell lysates were collected and analyzed by western blotting with indicated antibodies. Nuclear lysates then were isolated and subjected to DNase I digestion. Complexes

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with p53 were isolated by IP using anti-p53 antibody and the co-immunoprecipitated proteins were analyzed by western blotting.

Phosphorylation of p53 at Ser37 and Ser46 is Important for Regulation of p53-RPA binding

Since phosphorylation of p53 at serine 15 is involved in DNA damage checkpoint signaling, it is of interest to determine if phosphorylation of this site is involved in modulating the p53-RPA interaction. We therefore transfected constructs for expressing wild type and mutant p53 in which the serine was replaced with an alanine (S15A), respectively, into H1299 cells (p53$^{-/-}$). After transfection cells were treated with CPT, nuclear lysates were prepared, and co-immunoprecipitation performed using anti-p53 antibody. In agreement with our *in vivo* data described above, we found that only non-phosphorylated RPA32 was able to be co-immunoprecipitated with p53 and that the S15A mutation did not affect the p53 binding to RPA (Figure 2-5A). To confirm the results, the same immunoprecipitates were washed with 1 M NaCl buffer to remove p53-associated proteins. Then, an equimolar amount of recombinant RPA and hyp-RPA proteins were added. As shown in Figure 2-5B, the mutation at Ser15 in p53 did not affect p53-RPA binding.

To identify the phosphorylation site(s) of p53 important for regulation of the p53-RPA interaction, we transfected H1299 cells with a series of p53 mutant expression constructs in which one single serine had been mutated to alanine. The mutations were all localized in the N-terminus of p53 (S15A, S20A, S37A, S46A). The transfected H1299 cells were treated with CPT to induce phosphorylation of p53 (Figure 2-5C). Anti-p53 antibody then was used to pull-down the p53. After washing with 1 M salt buffer, the immunoprecipitates were mixed with equimolar amounts of recombinant RPA and hyp-RPA to test their interactions with the p53 proteins. The S37A and S46A mutations prevented p53 dissociation from hyp-RPA relative to WT-p53,

indicating that phosphorylations at Ser37 and Ser46 of p53 are required for release of RPA upon

phosphorylation of RPA32 (Figure 2-5D). These observations suggest that the two particular

serines are involved in regulating p53-RPA complex formation and stability in the CPT-induced

DNA DAMAGE REPONSE. Furthermore, individual knockdown of ATR and ATM identify the

checkpoint kinases responsible for specific serine phosphorylation: the CPT-induced

phosphorylation of p53 at Ser37 is primarily dependent on ATR while the phosphorylation at

Ser46 depends on ATM.
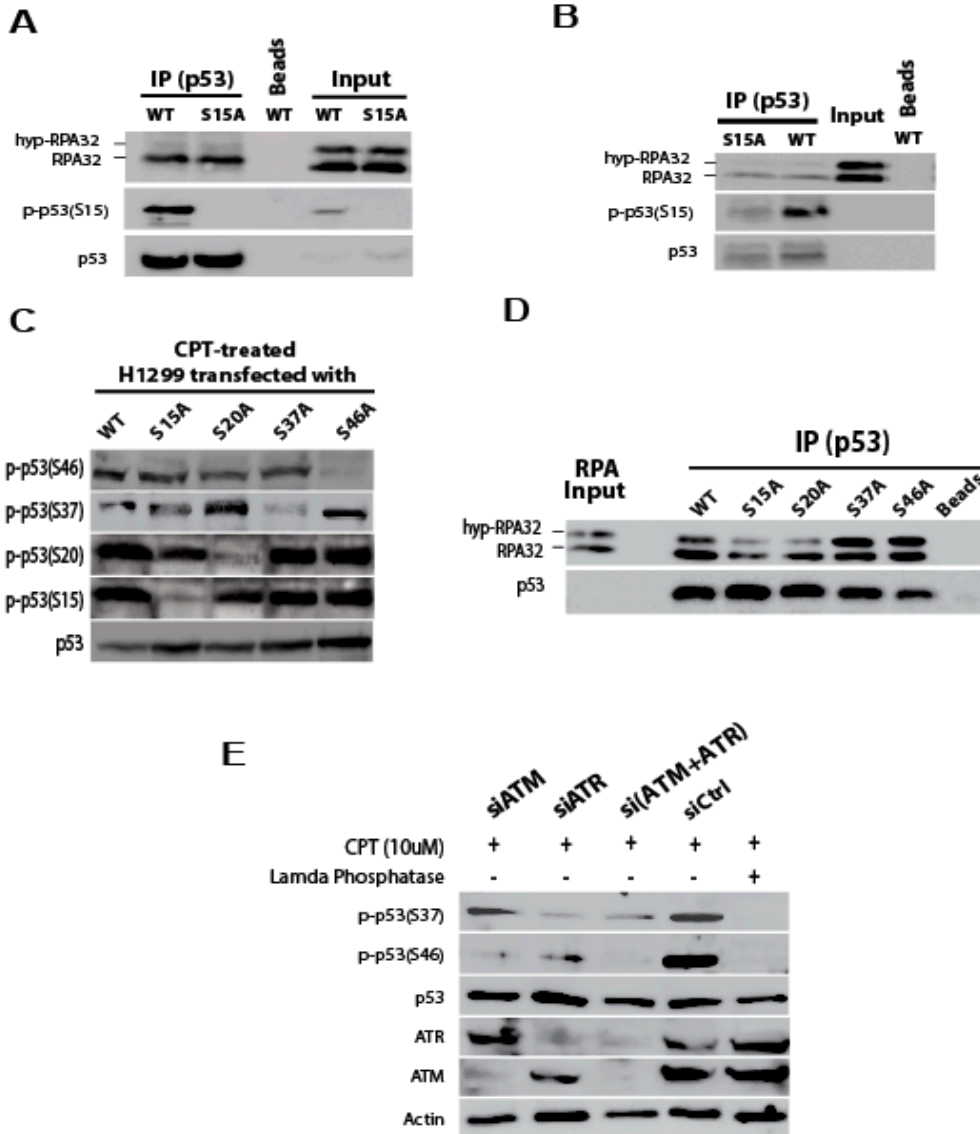
Figure 2-5. Phosphorylations of Ser37 and Ser46 of p53 are Important for Regulation of p53-RPA Binding. (A) H1299 cells (p53$^{-/-}$) were transfected with p53 wild type (WT) and p53 S15A constructs for 72 hrs. The cells were synchronized with APH before treatment with 10 μM CPT for 2 hrs. Nuclear fractions were isolated, treated with DNase I, followed by immunoprecipitation with anti-p53 antibody. The co-immunoprecipitated proteins were analyzed by western blotting using indicated antibodies. (B) The immunoprecipitates generated as in (A) were washed with 1 M salt buffer to remove co-immunoprecipitated proteins. Then equimolar quantities of RPA and hyp-RPA were supplied to allow interaction with the immunoprecipitated p53. The p53-RPA interaction was analyzed by collecting the p53 immunoprecipitates for analysis by western blotting. (C) H1299 cells were transfected with four different p53 constructs

in which one single serine was mutated to alanine on the N terminus of p53 (WT, S15A, S20A, S37A, S46A). Transfections were done for 72 hrs, followed by treatment with CPT to induce p53 phosphorylation. (D) Whole cell lysates were prepared and subjected to IP with anti-p53 antibody. The immunoprecipitates were washed with 1 M salt buffer, and then supplied with equimolar mixture of purified RPA and hyp-RPA. The p53-RPA complex formation was analyzed by western blotting. (E) Cells were transfected with ATM siRNA, ATR siRNA, or a combination of both, followed by CPT treatment. Prepared whole cell lysates with or without lambda phosphatase treatment were analyzed by western blotting.

Loss of Hyperphosphorylation of RPA Compromises DSB Repair

DNA damage-induced hyperphosphorylation of RPA stimulates RPA localization to DSB repair and checkpoint complexes [13, 14], thus likely enhancing DSB repair. Also, the interaction of p53 with RPA mediates suppression of HR [24]. Therefore, it is of interest to determine if phosphorylation-mediated regulation of the p53-RPA interaction plays a role in modulating DSB repair. Neutral comet assays were performed to assess the HR repair of CPT-induced DSBs in cells expressing PD-RPA versus cells expressing WT-RPA32. As shown in Figures 2-6A and 2-6B, repair of CPT-induced DSBs was significantly compromised in cells with PD-RPA in comparison to cells with WT-RPA. Consistently, in parallel experiments unphosphorylated RPA was efficiently co-immunoprecipitated with p53 in the cells expressing PD-RPA, while most hyp-RPA in the cells expressing wt-RPA was incapable of co-immunoprecipitation with p53 (Figure 2-6C, compare hyp-RPA to RPA ratios in lanes 6- 8 with lanes 14-16, respectively). These data suggest that RPA was unphosphorylated and, thus, sequestered in a p53-RPA complex in PD-RPA cells, inhibiting HR repair of CTP-induced DSBs. By contrast, RPA was extensively hyperphosphorylated and mostly free of binding to p53 in WT-RPA cells, making them available for HR repair.

We reasoned that RPA released from p53 sequestration by RPA32 phosphorylation would remain in the supernatant after IP pull-down of p53 and show association with DSB repair proteins. To test this, lysates from CPT-treated A549 cells were subjected to two consecutive immunoprecipitation steps in which p53 was immunoprecipitated first and then Rad51 was immunoprecipitated from the remaining supernatant. Although native RPA was efficiently sequestered by p53, little hyp-RPA was bound to the p53 in CPT-treated or -untreated cells (Figure 2-6D, lanes 3 and 4). Subsequently, anti-Rad51 antibody co-immunoprecipitated Rad51 and hyp-RPA from the remaining supernatant (lane 7) while little non-phosphorylated RPA was co-immunoprecipitated with Rad51. Similar results were obtained with U2OS cells expressing PD-RPA32 as compared with WT-RPA (Figure S2-2). Furthermore, CPT-induced nuclear focus formation of Rad52 was significantly reduced in cells expressing PD-RPA32 than those expressing wild-type RPA32 (Figures 2-6E and 2-6F). Rad51 interaction with ssDNA-bound RPA plays an important role in promoting Rad51 presynaptic filament assembling at DSBs [49-51], Thus, a significant amount of cellular RPA is sequestered in a p53-RPA complex under normal conditions and upon DNA damage, phosphorylation releases RPA or prevents hyp-RPA from binding to p53, promoting DSB repair.
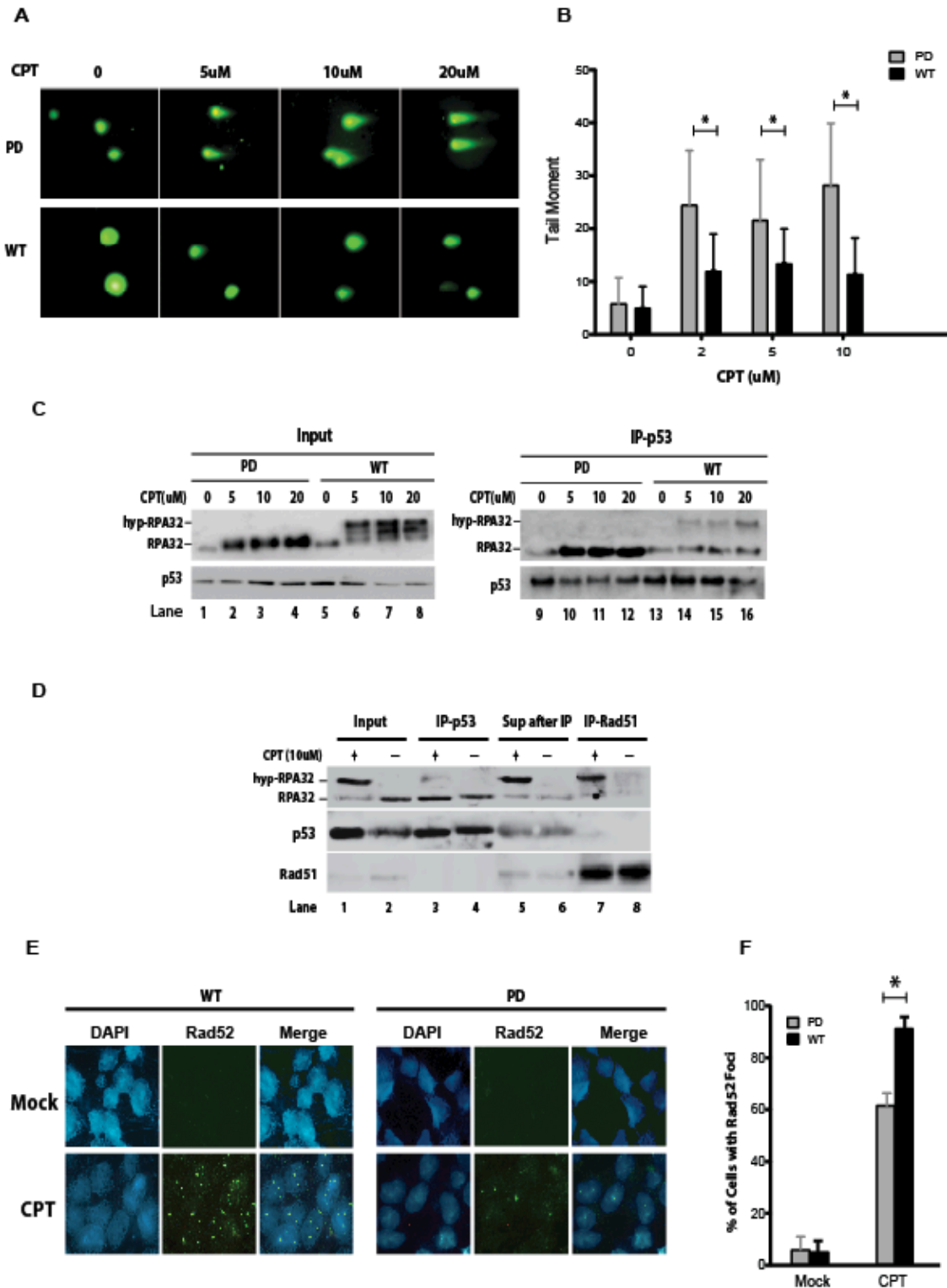
Figure 2-6. Phosphorylation-Mediated Regulation of p53-RPA Binding is required for DSB Repair. (A) Stable U2OS cells expressing WT- or PD-RPA32 were treated with CPT in a dose-dependent manner for 2 hrs. Comet assay under neutral conditions was performed to assess

the efficiency of DSB repair. (B) Tail moment was measured using the Comet Assay IV software (Perceptive). At least 50 cells were assessed per treatment (* represents a p-value less than 0.001). (C) Co-immunoprecipitation assay was performed simultaneously using duplicate cell cultures. Nuclear lysates were isolated and anti-p53 antibody was used for immunoprecipitation; samples were then analyzed by western blotting using the indicated antibodies. (D) A549 cells were treated with CPT or mock treated, followed by nuclear fractionation and incubation with DNase I. Soluble fractions were incubated with anti-p53 antibodies for co-immunoprecipitation (lanes 3-4). The supernatant after p53 IP then was immunoprecipitated again using Rad51 antibodies (lanes 7-8). (E) Cells expressing WT- or PD-RPA32 were treated with CPT and subjected to immunofluorescence microscopic determination of nuclear focus formation of Rad52. (F) Quantitative analysis of the data from (E). 100 cells were randomly selected in three separate experiments. Cells with at least one focus were counted (* represents a p-value less than 0.001).

Phosphorylation of Ser37 and Ser46 of p53 are Important for Homologous Recombination Repair

To further confirm the above results, constructs for expression of p53 with S37A or S46A mutation were generated. Then, we performed the pDR-GFP-based HR assays [52, 53] in H1299 cells transfected with the S37A or S46A p53 constructs in the presence or absence of CPT. As shown in Figures 2-7A and 2-7B, homologous recombination repair of the CPT-induced DSBs, as indicated by the cells emitting green fluorescence, was significantly compromised in cells expressing the S37A or the S46A p53 constructs in comparison to the cells expressing WT p53.

ATM and ATM Inhibition Impairs Homologous Recombination Repair

The same pDR-GFP-based HR assays also were performed with cells treated with ATM and ATR inhibitors KU55933 and NU6027, respectively.   Figures 2-7C and 2-7D show that the inhibition of ATR kinase significantly reduced HR efficiency in cells treated with CPT.

Furthermore, in the cells treated with the ATM inhibitor, the HR activity was also reduced, though not statistically significant ($p = 0.08$), as compared to the mock-treated cells. Consistently, when both inhibitors were used, the HR rate was significantly reduced in the inhibitor-treated versus mock-treated cells. Together, these results support a role of ATM and ATR kinases in regulation of HR, at least partially through their regulation of the p53-RPA interaction.

A

B

C

D

E

57
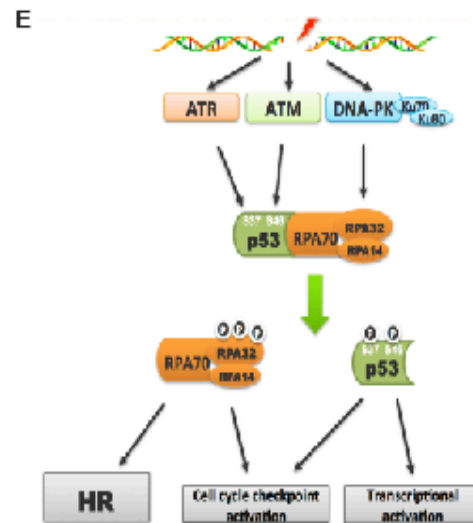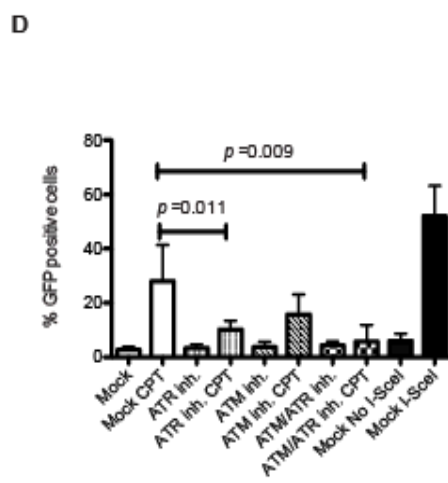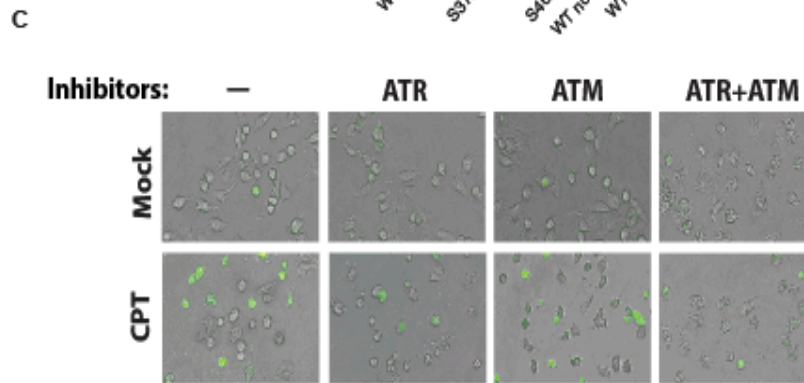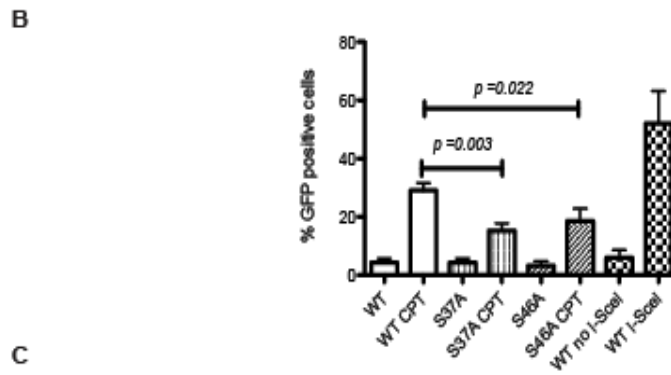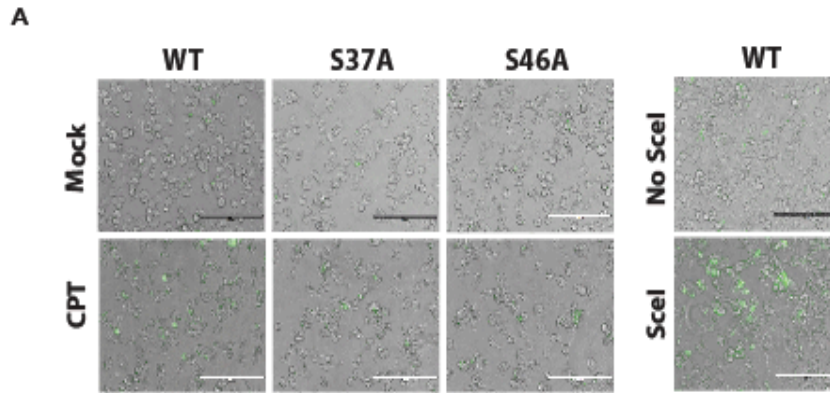
Figure 2-7. ATM- and ATR-Dependent Phosphorylation of Ser37 and Ser46 of p53 is Important for Efficient HR Repair of DSBs. (A) H1299 cells (p53$^{-/-}$) were transfected simultaneously with the HR reporter pDR-GFP and a p53 construct (WT, S37A or S46A) for 48 hrs. The cells then were either treated with 5 μM CPT for 24 hrs to induce phosphorylation of p53 and DNA double-strand breaks or transfected with an I-SceI endonuclease expression vector for 36 hrs as a positive control. Phase contrast microscopy was used to visualize cells and GFP expressing cells were scored by fluorescence microscopy. Cells with green fluorescence indicated functional HR. (B) Percentage of GFP-positive cells was measured from a random selection of 100 cells in three separate experiments. (C) A549 cells were transfected with the HR reporter pDR-GFP for 48 hrs. Cells then were treated with 10 μM ATM and/or ATR inhibitors for 1 hr prior to CPT treatment (5 μM for 24 hrs). (D) Percentage of GFP positive cells was measured and analyzed as above. (E) Proposed mechanism.

Discussion

Cellular DDRs are a complex defense system against genome instability and involves multiple biochemical pathways. In particular, HR and NHEJ repair pathways and ATM and ATR checkpoints play pivotal roles in cellular response to DSB damage. This study addresses important questions concerning how these pathways are regulated and coordinated with one another, important information for our understanding of the mechanisms of DDRs. We provide evidence that DNA-PK, the hallmark protein of NHEJ, together with ATR and ATM plays a regulatory role in the repair of CPT-induced DSBs, and this regulation is mediated by synergistic phosphorylations of both p53 and RPA. This finding reveals a novel crosstalk mechanism between HR and NHEJ pathways and coordination between ATM/ATR/p53 checkpoints and DNA-PK.

The complex mechanism unveiled in this study is centered on the regulation of p53-RPA interaction via site-specific post-translational modifications of p53 and RPA. Remarkably, the

regulation requires participation of all three major PIKK family members involved in DDRs, DNA-PK, ATM and ATR. Upon DNA damage, each kinase phosphorylates specific sites of p53 or RPA to make a synergistic contribution to inducing p53-RPA dissociation. Specifically, DNA-PK hyperphosphorylates RPA at multiple sites in the N-terminal domain of RPA32, while ATR and ATM phosphorylate p53 at Ser37 and Ser46, respectively (Figure 2-5). Surprisingly, phosphorylation of p53 at Ser15, well known for its role in ATR/ATM-dependent checkpoint activation and DDRs [54-57] is not required (Figure 2-5). In addition, phosphorylation of p53 at Ser20 by Chk2 [58] does not participate either (Figure 2-5) consistent with the lack of effect of Chk2 or Chk1 on p53-RPA interaction (Figure S2-3). These data suggest that p53 phosphorylations involved in modulating p53-RPA interactions are carried out directly by ATR and ATM in a Chk1/Chk2-independent manner. Although excess ssDNA interfered with RPA-p53 complex formation [38], we found that equimolar ssDNA did not substantially inhibit the p53-RPA interaction (Figures 2-2C and 2-2D).

The impact of p53-RPA association/dissociation on their cellular functions could occur at multiple levels. Normally, RPA expression is constant at a relatively abundant level during cell cycle transit [59]. It is known that p53 interacts with RPA via p53's N-terminal domain containing the transactivation and trans-repression functions of the protein [60]. Since a basal level of p53 is required for antioxidant activities in normal cell growth [61], the p53-RPA complex formation may serve to mask this p53 domain and prevent the above-basal levels of free p53 from interrupting normal cellular functions, complementing the Mdm2 function of sequestering and inactivating p53. With significant DNA damage, however, cellular p53 is elevated while expression of RPA remains unaffected [62]. Here, disruption of the p53-RPA complex may be necessary to free RPA for functioning in DDRs as RPA plays indispensable roles in DNA damage checkpoint and

repair pathways. Indeed, our results indicate that a deficiency in RPA phosphorylation and release from the p53-RPA complex significantly reduces repair efficiency of DSBs induced by CPT (Figures 2-6 and 2-7). The released hyp-RPA binds much more efficiently to Rad51 than does native RPA (Figure 2-6D) [13]. These observations suggest that the phosphorylations of RPA and p53 not only frees RPA during the DNA damage response, but also allows RPA to more efficiently recruit Rad51 to the DSB sites during an early step of HR, thus promoting the repair process [13, 33]. In addition, the phosphorylations may serve to prevent RPA sequestration by increasing amounts of p53. Furthermore, although p53 is highly expressed in cells following DNA damage, it is also possible that released phosphorylated p53 could enhance the DNA damage checkpoints and transcriptional activation of genes involved in DDRs. In this enhancement, RPA might be a regulatory element ensuring that p53 would be available only after DNA damage.

The multiple diverse functions for both RPA and p53 imply that the DNA-PK/ATM/ATR modulation of the p53-RPA interaction may have multiple, varied impacts on the DDRs beyond HR repair. Activation of tumor suppressor protein p53 orchestrates multiple cellular responses involved in cell cycle control and apoptosis [42, 43]. Also, RPA is involved in almost every, if not all, DNA damage response pathways, from damage signaling, checkpoint activation through DNA repair [5]. Also, hyp-RPA is more efficient in recruiting the checkpoint complex Rad9/Rad1/Hus1 [14], preventing its association with replication centers [29], facilitating mitotic exit in response to mitotic DNA damage[63], and regulating mismatch repair [31]. These potential hyp-RPA activities in the DNA damage response network depend on the stability of the p53-RPA interaction regulated by the PIKK members.

Given that p53 interacts with RPA via its N-terminal domain [60] and that the phosphorylation at S37 and S46 in the N-terminus of p53 by ATR/ATM disrupted p53-RPA interactions (Figure 2-5), these phosphorylations may interfere with RPA binding to the N-terminus of p53. This disruption of the p53-RPA complex requires the concomitant hyperphosphorylation of RPA32. As reported previously, hyperphosphorylation alters RPA conformation [32]. Thus, this may structurally change the p53-binding domain/motif of RPA although this change alone may not be sufficient to disrupt the formation of the p53-RPA complex. On the other hand, the phosphorylation at S37 and S46 in the N-terminal domain of p53 changes both the chemistry and structure of the domain. It is likely that combination of these changes with those in RPA due to hyperphosphorylation prevents RPA from binding to p53. However, revealing the details of the phosphorylation-induced structural changes is beyond the scope of the current study but deserves further investigation.

Taken together, we propose that under unstressed conditions, the low level of 'free' p53 is sequestered by the abundant RPA in cells. The sequestration not only prevents relatively high levels of p53 from interfering with normal cellular functions and cell cycle progression, but also may help to maintain a basal level of p53 for upregulation of a few genes for activities against DNA damage induced by endogenous reactive oxygen species in cells under normal growth conditions. Upon severe DNA damage, however, phosphorylation of p53 and RPA by ATM/ATR and DNA-PK, respectively, prevents RPA sequestration by the damage-induced high level accumulation of p53, freeing phosphorylated forms of both p53 and RPA for DNA damage response functions.

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CHAPTER 3

LACK OF PHOSPHORYLATION OF REPLICATION PROTEIN A FACILITATES DNA

DAMAGE-INDUCED APOPTOSIS

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Abstract

DNA damage triggers a diverse response of cellular processes in eukaryotic cells

including DNA repair, cell cycle checkpoints and apoptosis. Human replication protein A (RPA),

the major single-stranded DNA-binding protein, has been shown to be important for many

cellular DNA metabolic pathways; however, its role in apoptosis remains highly elusive. RPA is

a heterotrimer composed of RPA70, RPA32, and RPA14 subunits. The N-terminal region of

RPA32 undergoes hyperphosphorylation in response to DNA damage, which is believed to play

a role in modulating many cellular activities. In this study, using the U2OS cells expressing wild

type (WT-RPA32) or phospho-deficient RPA32 (PD-RPA32), we show that deficiency in

RPA32 phosphorylation led to a significant increase in apoptosis induced by genotoxic stress.

Specifically, PARP-1 cleavage, sub-G1 phase population, and loss of mitochondrial membrane

potential were significantly increased in PD-RPA32 vs. WT-RPA32 cells after DNA damage.

The lack of RPA phosphorylation promoted the activation of initiator Caspase-9 and effector

Caspase -3 and -7. Furthermore, we found that the effect of RPA phosphorylation on apoptosis

was dependent on the kinase activity of DNA-PK and was mediated by PUMA through the

ATM-p53 pathway. Our results suggest a novel role of RPA phosphorylation in regulation of

apoptosis which could be of importance in development of new therapeutic strategies for sensitization of cancer cells.

Introduction

The cellular DNA repair machinery plays a critical role in preventing detrimental mutations into our DNA. Defects in DNA repair underlie a number of human genetic diseases that affect a wide variety of body systems, most notably a predisposition to cancer. When DNA damage is excessive to the point that it overwhelms the DNA repair machinery, cells trigger their destruction by activating a stepwise process known as apoptosis. The evidence supporting that vast amounts of DNA lesions trigger apoptosis is conclusive; however, the molecular mechanisms that signal the initiation of apoptosis remain elusive. Drugs that favor apoptosis over DNA repair could be of great importance for the sensitization of tumor cells, as a result great attention has been brought to finding components capable of regulating these two pathways.

Replication protein A (RPA), a heterotrimer of RPA70/RPA32/RPA14, is the major eukaryotic single-stranded DNA binding protein and is involved in all aspects of DNA metabolism: replication, repair, and recombination. RPA32 is hyperphosphorylated in response to DNA damage and evidence suggests that, upon hyperphosphorylation, RPA undergoes a structural reorganization which provides a model for the coordination of different DNA damage response pathways [60-64,88,89,93,94]. Various reports involve RPA phosphorylation in the coordination of DNA repair and cell cycle checkpoints. Particularly, RPA phosphorylation has been shown to modulate interactions with the 9-1-1 complex [61], Rad51/ Rad52 [62] and p53 [89].

However, little is currently known about the role of RPA phosphorylation in the initiation of apoptosis.

p53 is a key regulator of genome instability and is one of the most commonly inactivated genes in human cancers. p53 is a major regulator of cellular fate in response to genotoxic stresses because it can trigger several cellular events: cell-cycle arrest, senescence, differentiation and apoptosis. The option chosen is dependent on the circumstances under which p53 is activated [95-98]. Under some circumstances, p53 can contribute to repair which potentially allows the cell to recover back into the proliferating pool [99,100]. However, in most cases, activation of p53 leads to irreversible inhibition of cell proliferation, mostly accomplished by the activation of apoptosis by its transcriptional activity. There are a vast number of genes that can be activated by the transcriptional activity of p53; an important one encodes a protein called PUMA, or p53-Upregulated Modulator of Apoptosis. PUMA is a BH3-only family member that promotes apoptosis by antagonizing anti-apoptotic proteins through their BH3 domain interactions [101]. Specifically, PUMA is thought to bind Bcl-2 and Bcl-XL, therefore, promoting cytochrome c release [102,103].

DNA double-stranded breaks (DSBs) are the most lethal form of DNA damage and are repaired in mammalian cells by homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways. Camptothecin (CPT), a widely used chemotherapy drug in its analogous forms, is a topoisomerase I inhibitor that arrests topoisomerase I-nicked DNA intermediate complexes leading to replication fork collapse at the nicked site resulting in DSB formation [13,14]. Ataxia telangiectasia mutated (ATM), a member of the phosphatidylinositol 3-kinase- related kinase (PIKK) family, plays a key role in surveillance of the genome's integrity and is the main signal transducer in response to DSBs. ATM phosphorylates well known targets:

70

histone H2AX, the Mre11-Rad50-NBS1 (MRN) complex, Chk2, RPA and Mdm2. DNA-dependent protein kinase (DNA-PK), also a PIKK family member, has a key role in initiating NHEJ by recognizing DSBs and recruiting other members of NHEJ machinery. DNA-PK phosphorylates proteins involved in DNA damage responses such as H2AX, RPA, p53, XRCC4, Ku70 (XRCC6) and Ku80 (XRCC5) [104,105].

In the present study, we show a novel role of RPA phosphorylation in the inhibition of apoptosis. We found that the lack of RPA32 phosphorylation facilitates DNA damage-induced apoptosis and that the pro-apoptotic effects are dependent upon DNA-PK. We determined that, in response to CPT, ATM is responsible for carrying the signal that starts the p53-dependent apoptotic cascade resulting in the activation of PUMA. PUMA, in turn, promotes the activation of initiator Caspase-9 and effector Caspase -3 and -7. Overall this study reveals an important new role of RPA32 phosphorylation in the DNA damage response leading to programmed cell death. RPA32 could be of potential interest in the development of novel chemotherapies targeting the crossroads of two important mechanisms: DNA repair and apoptosis.

Materials and Methods

Cells, Cell Culture, Treatments and Antibodies

U2OS cells expressing RPA32 wild-type (WT-RPA32) or a hyperphosphorylation-deficient mutant (PD-RPA32) (kindly provided by Dr. Xiaohua Wu; Scripps Research Institute, La Jolla, CA, USA) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS: HyClone), 1% penicillin/streptomycin, hygromycin (200 mg/ml) and puromycin (1 mg/ml) to maintain plasmid expression. These are stable cell lines in which the

endogenous RPA32 was knocked down while recombinant WT-RPA32 or PD-RPA32 were produced [94]. UV-C irradiation was performed using a 254 nm lamp at 0.83 J/m^2/sec. CPT was purchased from Sigma Chemical Co. (C9911). Stock solutions of CPT were made in dimethyl sulfoxide (DMSO) before addition to cell culture medium. The ATR kinase inhibitor, NU6027, the ATM kinase inhibitor, Ku55933, and the DNA-PK inhibitor, NU7441, were purchased from EMD Millipore and used at a final concentration of 10 µM in cell culture medium. Inhibitors were added 1h before CPT treatment.

Antibodies used in this study include RPA32 (Sigma R3280), phospho-RPA32-Ser4/8 (Bethyl A300-245A), p53 (Santa Cruz sc-6243), phospho-p53 (pSer15) (Cell Signaling 9286), DNA-PK (Santa Cruz sc-9051), ATM (Bethyl Lab A300-299A), ATR (Bethyl Lab A300-138A), cleaved-Caspase-3 (Cell Signaling 9664), PARP-1 (Santa Cruz sc-8007), PUMA (Cell Signaling 4976), MHSP70 (MA3-028; Thermo Scientific), pChk2 (Thr68) (Cell Signaling 2661) and a FITC-conjugated β-Actin antibody (Sigma Chemical Co).

Sub-G1 Population Analysis

Nuclear DNA was propidium iodide stained as described previously [106]. Cells were grown in dishes to reach 70-80% confluence, trypsin-harvested, fixed with 70% ethanol at 4°C, and then pelleted and re-suspended in PBS containing 20 µg/mL of propidium iodide and 100 U RNase. Samples were then incubated for 30 minutes at 37°C. After staining, 30,000 cells were counted using the Accuri C6 flow cytometer to measure the DNA content.

Mitochondrial Membrane Potential Analysis

MitoTracker Red CMXRos (Life Sciences M7512) is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon an intact mitochondrial membrane potential. Cells were seeded at a confluence of 70% one day ahead of DNA damage

treatments. To prepare a stock solution, MitoTracker Red CMXRos was dissolved in DMSO to a final concentration of 1 mM. Right before use, the dye was diluted to 200 nM in growth medium and incubated 30 minutes in the dark with the cells. Both the adherent and non-adherent cells were harvested, washed with PBS, and analyzed by both flow cytometer and fluorescence microscopy.

S phase Cell Synchronization

To optimize RPA32 hyperphosphorylation in response to CPT treatment cells were synchronized in S phase by incubating with aphidicolin (APH) (1 ug/ml) for 18 h before release into fresh media for 2 h. Synchronized cells were treated with 10 μM CPT for the indicated times.

Cell Fractionation

Mitochondrial isolation was performed using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific 89874) according to the manufacturer's instructions. Cells were suspended in lysis buffer to disrupt the plasma membrane. Plasma membranes and compartmentalized organelles, such as endoplasmic reticulum, mitochondria, and nuclei, remained intact and were collected by centrifugation at 1000xg for 10 min. The resulting pellet was resuspended in disruption buffer, repeatedly passed through a 21-gauge needle, and re-centrifuged at 1000xg for 10 min to pellet nuclei, cell debris, and unbroken cells. The supernatant that contains mitochondria was re-centrifuged at 6000xg for 10 min to pellet mitochondria. After removal of the supernatant, mitochondria were lysed with 1X SDS loading buffer and analyzed by SDS-PAGE and western blotting.

<u>Caspase-3/7 Activation Assay</u>

Caspase-3 and Caspase-7 activities were measured using a Caspase-Glo assay kit (Promega G8090) according to the manufacturer's instructions. Cells were grown in a 96-well white-walled plate and treated accordingly. Then the activity of Caspase-3/7 was measured by adding a luminescent substrate containing the DEVD sequence, which upon Caspase-3/7 cleavage, releases the luciferase aminoluciferin. The luminescent signal was then measured in a plate-reader luminometer.

<u>siRNA and Plasmid Transfections</u>

Cells were transfected with siRNA for 48 h using INTERFERin transfection reagent (409-10, Polyplus-transfection Inc., New York, NY, USA) following the manufacturer's instructions. The siRNAs include ATM: CAUACUACU CAAAGACAUUTT, AAUGUCUUUGAGUAGUAUGTT, ATR: CCUCCGUGAUG UUGCUUGATT, UCAAGCAACAUCACGGAGGTT, DNA-PK: AGGGCCAAGCUG UCACUCUTT, AGAGUGACAGCUUGGCCCUTT, and Chk1: ACAGUAUUUCGGUAUAAUATT. The pCB6-p53-WT expression construct (kindly provided by Dr. Karen Vousden, Beatson Institute for Cancer, Bearsden, Glasgow, UK) were transfected into cells using JetPI transfection reagent (Polyplus 101-10) according to the manufacturer's instructions for 48 h.

<u>Apoptosis Analysis by Annexin V Staining</u>

Phosphatidylserine exposure on the outer leaflet of the plasma membrane was detected by the FITC-annexin V apoptosis detection Kit II (Invitrogen V13242) according to the manufacturer's instructions. Cells were pelleted following treatment and washed in PBS. Cells were re-suspended in binding buffer at 1×10^6 cells/ml, mixed with FITC-annexin V and

incubated at room temperature for 15 minutes in the dark. The annexin V– positive cells were analyzed by flow cytometry.

<div align="center">Results</div>

Lack of RPA32 Hyperphosphorylation Results in PARP-1 Cleavage and Accumulation of Cells in Sub-G1

Cleavage of PARP-1 (poly(ADP-ribose) polymerase) has been suggested to be an important inactivation mechanism of DNA repair and is a widely used marker for cells undergoing apoptosis [25,26]. PARP-1 usually aids in DNA repair; however, during apoptosis PARP-1 is cleaved producing an 89 kDa C-terminal fragment containing the catalytic domain, and a 24 kDa N-terminal fragment harboring the DNA binding domain. The DNA-binding domain detached from the catalytic domain converts PARP-1 into a negative regulator of repair and an indicator of cell death. To investigate the effect of RPA phosphorylation on apoptosis, we examined the PARP-1 cleavage after genotoxic stress in phosphorylation-deficient RPA32 (PD-RPA32) cells and wild-type RPA32 (WT-RPA32) cells [94]. As shown in Figure 3-1A, cells were UV irradiated and let to recover for the indicated times or incubated with CPT for the indicated periods of time. Both UV- and CPT-treated cells were able to generate RPA32 phosphorylation in the WT-RPA32 cells as indicated by the bands of hyperphosphorylated RPA32 (hyp-RPA) which migrated slower than the non-phosphorylated RPA32 band on SDS–PAGE; however, the PD-RPA32 cells were incapable of undergoing phosphorylation in RPA32. As shown in Figure 3-1A, the DNA damage-induced cleavage of PARP was significantly more in the PD-RPA32 cells as compared to that in WT-RPA32 cells and the degradation was evident as early as 6 hours

after CPT or UV exposure. Furthermore, the induction of PARP cleavage is also higher in PD-RPA32 vs WT-RPA32 cells treated with etoposide (Etop), a topoisomerase II inhibitor, which causes DSBs by preventing the re-ligation of DNA strands (Figure 3-1B). Interestingly, although the induction of PARP degradation in PD-RPA32 cells appears to be independent of the type of DNA damaging agent, hydroxyurea-treated (HU) cells did not undergo PARP cleavage despite the RPA32 hyperphosphorylation. The results of HU-treated cells might be of importance and will be addressed in the discussion.

To confirm the role of RPA32 phosphorylation in cell death, we performed propidium iodide staining of fixed cells to ascertain the percentage of cells in the sub-G1 population. Our results indicate that CPT exposure resulted in a significant increase in the percentage of cells in the sub-G1 phase in the PD-RPA32 cells when compared to the WT-RPA32; however, as expected, in the absence of CPT no significant differences in sub-G1 distributions were observed (Figure 3-1C and 3-1D).
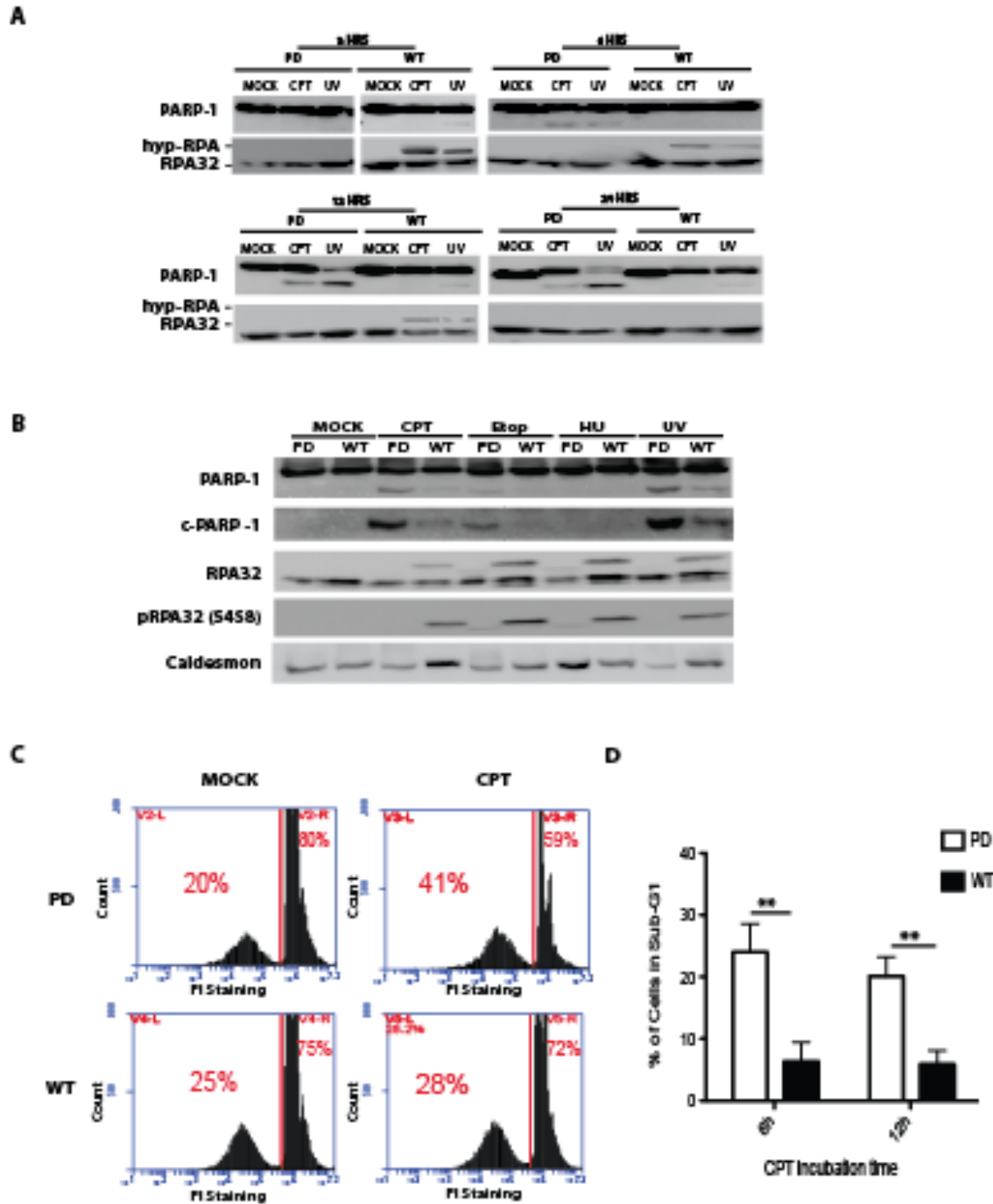
Figure 3-1. Lack of RPA Phosphorylation Results in PARP-1 Cleavage and Accumulation of Cells in Sub-G1. (A) Stable U2OS cells expressing WT- or PD- RPA32 were treated with 10 μM CPT for 3, 6, 12 and 24h or were irradiated with 30 J/m^2 and allowed to recovered for the indicated times. Whole-cell lysates were then analyzed by western blotting. (B) Cells were treated with 10 μM CPT, 100 μM etoposide, 2 mM HU for 12 hours or were exposed

to 30 J/m^2 UV and let to recover for 12 hours and analyzed as in (A). Caldesmon was used as loading control. (C) Cells were treated with 10 μM CPT for the indicated times, stained with propidium iodide (PI) and analyzed by flow cytometry. (D) The percentage of cells in sub-G1 was measured (left quadrant) and statistical significance was determined by student t-test for 3 independent samples. Bars represent SD; **, $P<0.002$.

Phosphorylation Deficiency of RPA32 Promotes Loss of Mitochondrial Membrane Potential and Cytochrome C Release

We reasoned that cells with extensive and persistent DNA damage might not be able to maintain mitochondrial membrane potential across the inner membrane which is believed to play a role in the apoptotic process. The integrity of the mitochondrial membrane potential was evaluated using MitoTracker Red CMXRos, a fluorophore labeling active mitochondria. Cells were treated with CPT for 6, 12 or 24 hours, incubated for 30 min with the fluorophore and then harvested and analyzed by flow cytometry. PD-RPA32 cells had a significantly greater loss of mitochondrial membrane potential at all time points when compared to the WT-RPA32 cells (Figure 3-2A and 3-2B). To further elucidate the cell death stimulated by PD-RPA32, we analyzed the release of cytochrome c. Cytochrome c acts as an important molecule at the early stages of the apoptosis pathway; its release from mitochondria into the cytosol leads to the activation of Caspase-9, which then converts Caspase-3 into its active form, resulting in apoptosis. To address this question, mitochondria and cytosol were isolated from WT-RPA32 and PD-RPA32 cells after CPT or UV exposure. As shown in Figure 3-2C, cytochrome c was released into the cytosol of PD-RPA32 cells, but little of WT-RPA32 cells. Additionally, corroborating the results from Figure 3-1, the nuclear fraction of the PD-RPA32 cells showed significantly more PARP cleavage when compared to the WT-RPA32 cells (Figure 3-2C). This indicates that cytochrome c participates in the executioner phase of the apoptotic cell death

cascade and that the regulation of RPA phosphorylation in apoptosis is upstream of

mitochondrial events.
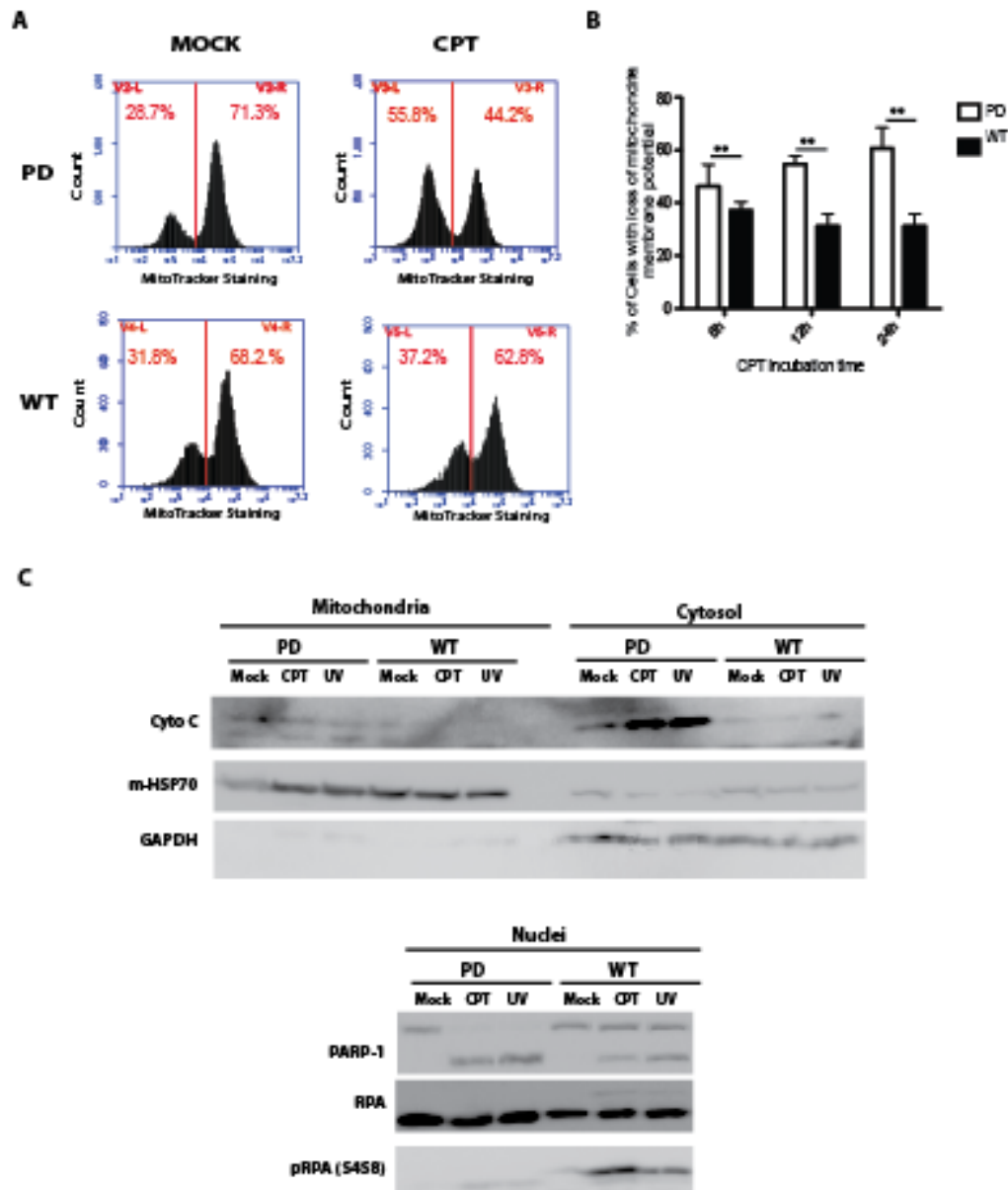
Figure 3-2. RPA32 Phosphorylation Deficiency Promotes Loss of Mitochondrial Membrane Potential and Cytochrome C Release. (A) Stable U2OS cells expressing WT- or PD-RPA32 were treated with 10 μM CPT for the indicated times and labeled using MitoTracker Red CMXRos. The percentages of cells with intact mitochondrial membrane potential (positively stained, right peak) were counted using flow cytometry. (B) Statistical significance was determined by student t-test for 3 independent samples. Bars represent SD; **, *P*<0.002. (C)

WT-or PD-RPA32 cells were treated with CPT (10 µM) or UV (30J/m^2), then mitochondria, cytosol and nuclei were isolated and analyzed by western blotting.

RPA32 Phosphorylation Deficiency Promotes Activation of Caspases

Release of cytochrome c into the cytosol induces oligomerization of apoptotic peptidase activating factor 1 (APAF1) to form the apoptosome. The apoptosome then activates pro-Caspase-9, the initiator Caspase in the intrinsic apoptosis pathway [107]. Active Caspase-9, in turn, triggers the cleavage and activation of the executioner Caspase-3 and Caspase-7. To address whether RPA phosphorylation has an effect in this cascade of events, we first analyzed the activation of Caspase-3. As shown in Figure 3A, executioner Caspase-3 was activated at a much higher rate in cells deficient in RPA32 phosphorylation. The activation of Caspase-3 was seen as early as 6 hours post-UV irradiation in PD-RPA32 cells. Additionally, Caspase-3 was activated with CPT and etoposide treatment, but not with HU (Figure 3-3B). Caspase-3/7 activities were measured by monitoring the cleavage of the tetra-peptide sequence DEVD, a Caspase-3/7 substrate. As Figure 3-3C shows, Caspase-3/7 activities were elevated in PD-RPA32 cells after etoposide treatment, but not in the WT-RPA32. To further elucidate the apoptotic pathway, Figure 3-3C shows that PD-RPA32 cells activated Caspase-9 and Caspase-7 after exposure to CPT and UV while WT-RPA32 had little Caspase-9/7 activities.
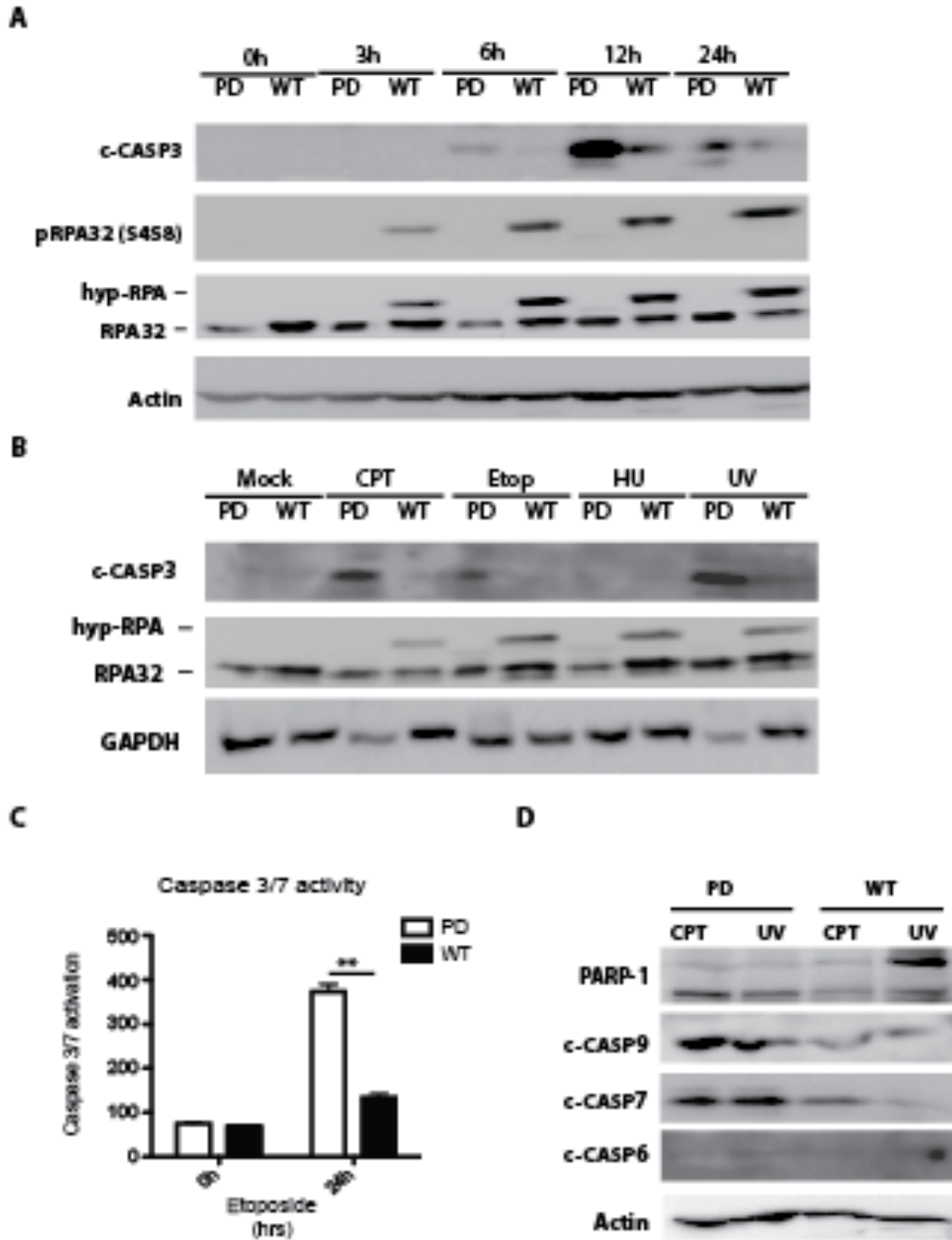
Figure 3-3. RPA32 Phosphorylation Deficiency Promotes Activation of Caspases. (A) Stable U2OS cells expressing WT- or PD- RPA32 were treated with 30J/m^2 UV and allowed to recover for the indicated times. Whole-cell lysates were analyzed by western blotting for cleaved

Caspase-3. (B) Cells were treated with 10 μM CPT, 100 μM etoposide, 2 mM HU for 12 hours, or were irradiated with 30 J/m^2 UV and let to recover for 12 hours. Cells were harvested and analyzed as in (A). (C) Cells were treated with 300 μM Etoposide for 24 hours and Caspase-3/7 activities were measured using the luminogenic Promega Caspase-Glo assay. Statistical significance was determined by student t-test for 3 independent samples. Bars represent SD; **, $P<0.002$. (D) Cells were treated with 10 μM CPT for 12 hours or irradiated with 30 J/m^2 UV and let to recover for 12 hours. Whole-cell lysates were analyzed for the cleavage of Caspase-9, Caspase-7 and Caspase-6 by western blotting.

RPA32 Phosphorylation-Dependent Apoptosis is Dependent on ATM

CPT's ability to generate DSBs depends on replication, making it an important chemotherapy agent because it targets rapid replicating cells. Since RPA is extremely important for replication [64] and because RPA32 phosphorylation after CPT treatment prevents RPA's association with p53 [89], we will primarily use CPT as our apoptosis-inducing agent.

Non-repaired DSBs are a main trigger of apoptosis [108] and generally DSBs evoke the ATM-ATR signaling pathway [109]. To test whether these two members of the PIKK family have any role in the RPA32 phosphorylation-deficiency induced apoptosis, we used caffeine, a widely used radio-sensitizing agent that has been shown to inhibit the kinase activities of ATM and ATR [110-113], the ATM kinase inhibitor Ku55933, and the ATR kinase inhibitor NU6027. Figure 3-4A shows that caffeine and the ATM/ATR inhibitors were able to diminish the cleavage of PARP after CPT treatment in both WT-RPA32 and PD-RPA32; however, when we used the ATM and the ATR inhibitors individually, we found that the inhibition of apoptosis was only dependent on ATM but not on ATR (Figure 3-4B and 3-4C).
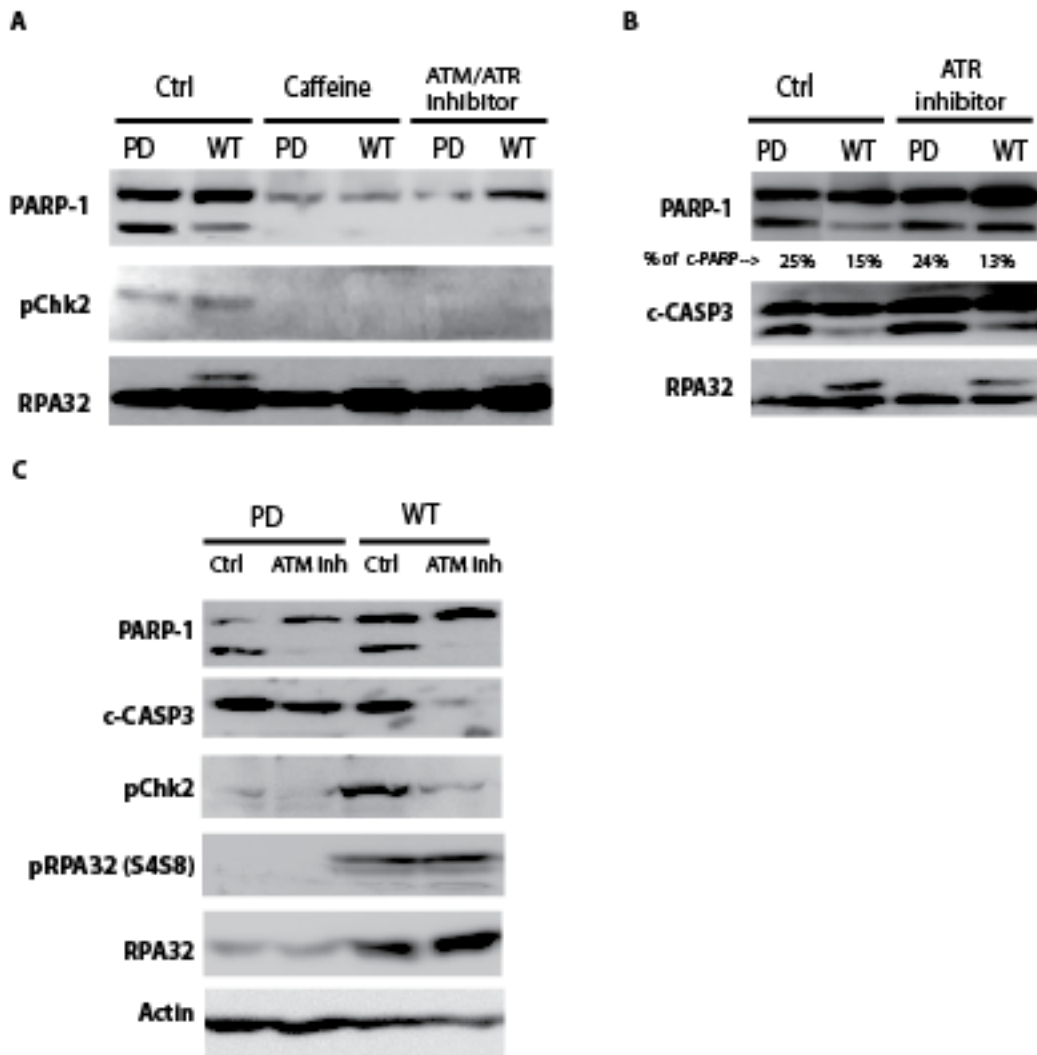
Figure 3-4. RPA32 Phosphorylation-Dependent Apoptosis is Dependent on ATM. (A) Stable U2OS cells expressing WT- or PD- RPA32 were synchronized in S phase, thereafter, caffeine or ATM/ATR inhibitors were added to the culture for 1 hour. Cells were then treated with CPT for 12 hours in the presence of caffeine or the ATM/ATR inhibitors. Cells were harvested and whole cell lysates were loaded in SDS-PAGE. (B,C) Cells were synchronized in S phase and treated with ATR inhibitor (B) or ATM inhibitor (C). 1h after the addition of ATR or ATM inhibitor cells were treated with CPT for 12 hours in the presence of the inhibitors. Whole-cell extracts were then loaded in SDS-PAGE.

Effect of DNA-PK on RPA32 Phosphorylation Deficiency-Induced Apoptosis

We, as well as others, have shown that the induction of RPA32 phosphorylation after CPT treatment is attributed to DNA-PK [41,89] and not to any other PIKK family member. If the apoptotic phenotypes that are observed in the PD-RPA32 cells are due to lack of RPA32 phosphorylation, we should be able to mimic this apoptotic response by inhibiting the kinase activity of DNA-PK in the WT-RPA32 cells. Figure 3-5A shows that in the WT-RPA32 cells both the DNA-PK inhibitor and the siRNA to DNA-PK inhibited the phosphorylation of RPA32. The apoptotic response of the WT-RPA32 cells was amplified with this inhibition; the percentage of PARP-1 degradation after CPT treatment in the presence of the DNA-PK inhibitor was significantly increased when compared to control sample that had no DNA-PK inhibitor (Figure 3-5A, compare lanes 2 with 4). Similar results were obtained with the siRNA to DNA-PK (Figure 3-5A, compare lanes 6 and 8). Additionally, the amount of Caspase-3 cleavage and the percentage of PARP-1 degradation were proportional to the dose of DNA-PK inhibitor used (Figure 3-5B). As the concentration of the DNA-PK inhibitor was increased, so was the amount of Caspase-3 cleavage and PARP-1 degradation in the WT-RPA32. Overall, Figure 3-5 shows that the phosphorylation of RPA32 is important for the signal transduction that directs the cell towards apoptosis and that the signaling pathway is dependent on DNA-PK.

Figure 3-5. Effect of DNA-PK on RPA32 Phosphorylation Deficiency-Induced Apoptosis. (A, lanes 1-4) Stable U2OS cells expressing WT- or PD- RPA32 were synchronized in S phase and treated with DNA-PK inhibitor 1 hour before CPT treatment (10 μM, 18 hours). Whole-cell lysates were prepared and analyzed by western blotting. (A, lanes 5-8) Cells were transfected with siRNA to DNA-PK for a total of 48 hours. Before CPT treatment (10 μM, 18 hours) cells were synchronized in S phase as described in Materials and Methods. Prepared whole-cell lysates were analyzed by western blotting. (B) Stable U2OS cells expressing WT- or PD- RPA32 were treated with increasing concentrations of DNA-PK inhibitor 1 hour before CPT treatment (18 hours). Whole-cell lysates were prepared and analyzed by western blotting.

Numbers indicate the ratio of cleaved-Caspase-3 in WT-RPA32 to cleaved-Caspase-3 in PD-RPA32 cells.

Effect of p53 on RPA32 Phosphorylation Deficiency-Induced Apoptosis

We noticed that in S phase synchronous cells treated with CPT the levels of p53 in the PD-RPA32 cells were higher than that of the WT-RPA32 (Figure 3-6A, lanes 1and 2). So we investigated the involvement of p53 in the induction of apoptosis in these cells. Our results revealed (Figure 3-6A) that when p53 was depleted using siRNA, the amount of PARP-1 cleavage in the PD-RPA32 cells decreased to the level seen in WT-RPA32 cells (lanes 3 and 4). Additionally, cleaved Caspase-3 levels were decreased. Similar results were obtained when p53-dependent transactivation was inhibited by pifithrin-α: the amount of cleaved PARP-1 (c-PARP-1) in the PD-RPA32 cells was reduced to the same levels of the WT-RPA32 cells (Figure 3-6b). Furthermore, overexpression of p53 revealed that the amount of PARP-1 degradation in the WT-RPA32 increased when compared to the control WT-RPA32 that had basal p53 levels (Figure 3-6C, lanes 3 and 4), suggesting that abundance of p53 in the cell enhances the induction of apoptosis. Overall, Figure 3-6 indicates that the induction of apoptosis by the lack of RPA32 phosphorylation is mediated by p53 through its transactivation pathways.
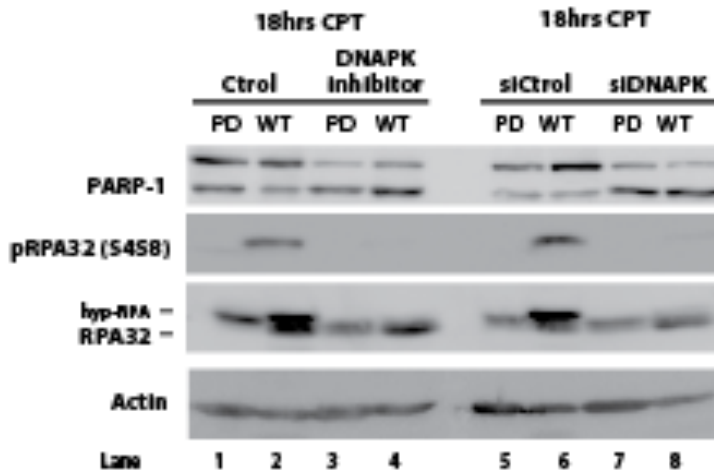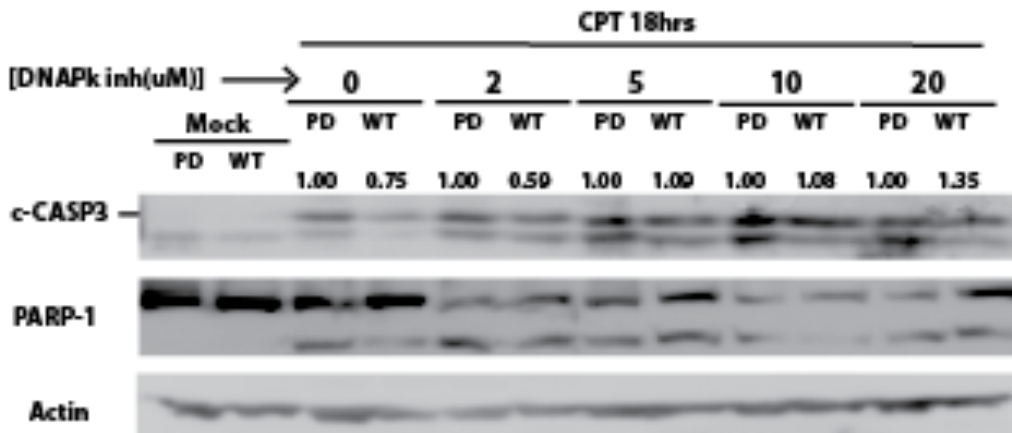
A

B

C
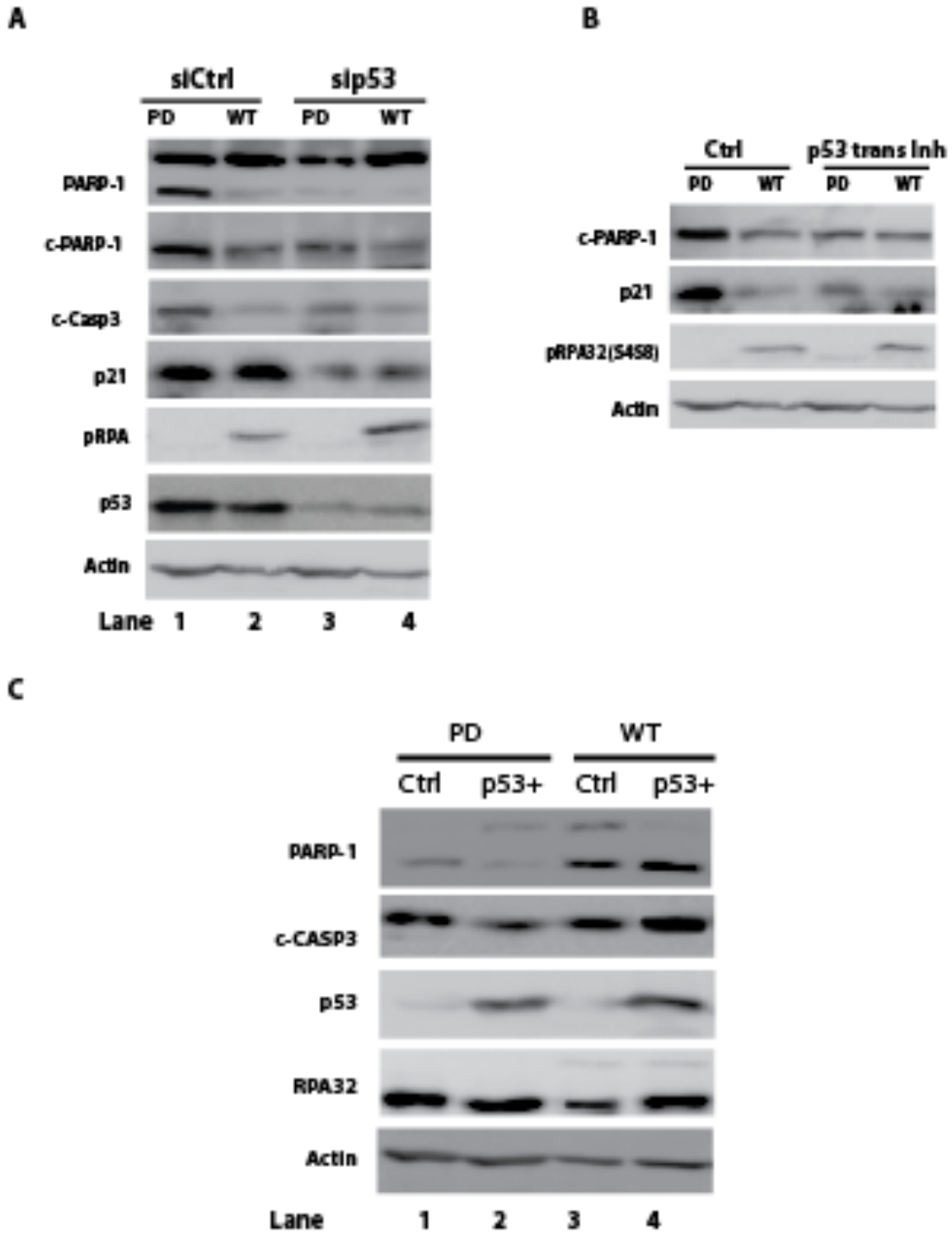
Figure 3-6. Effect of p53 on RPA32 Phosphorylation Deficiency-Induced Apoptosis. (A) Stable U2OS cells expressing WT- or PD- RPA32 were transfected with siRNA to p53 for a total of 48 hours. Before CPT treatment cells were synchronized in S phase as stated in Materials and

Methods. Prepared whole-cell lysates were analyzed by western blotting. (B) Cells were treated with pifithrin-α for 2 hours to inhibit the transcriptional activity of p53. Cells were treated with 10 μM CPT for 18 hours in the presence of pifithrin-α. Whole-cell lysates were analyzed by western blotting. Anti-pRPA32-Ser4/8 was used to detect bands specific to Ser-4/8 phosphorylated in RPA32 (C) U2OS cells were transfected with p53 wild-type for 48 hours, synchronized in S phase and treated with 10 μM CPT for 6 hours. Whole cell lysates were then loaded in SDS-PAGE and were analyzed by western blotting.

Lack of RPA32 Phosphorylation Stimulates the Expression of PUMA

PUMA is a BH-3 only family member originally identified as a p53-inducible gene. The BH-3-only family members also include Bad, Bid, Bik, Hrk, Bim and Noxa; which promote apoptosis by antagonizing anti-apoptotic proteins through their BH3 domain interactions [101]. Specifically, PUMA is thought to bind Bcl-2 and Bcl-XL and promote cytochrome c release and apoptosis [102,103]. Figure 3-7A shows whole cell lysates analyzed by western blotting of both PD-RPA32 and WT-RPA32 cells treated with CPT after S phase synchronization in which the total levels of PUMA in the PD-RPA32 cells were elevated when compared to the WT-RPA32. The elevated levels of PUMA correlate with the higher levels of PARP-1 degradation. In addition, cellular fractionation confirmed the abundance of PUMA in the cytosolic fraction of PD-RPA32 cells and not in the WT-RPA32 cells (Figure 3-7B).

A
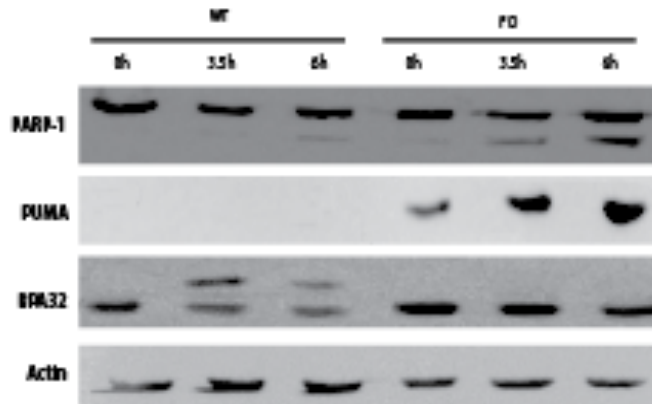
B
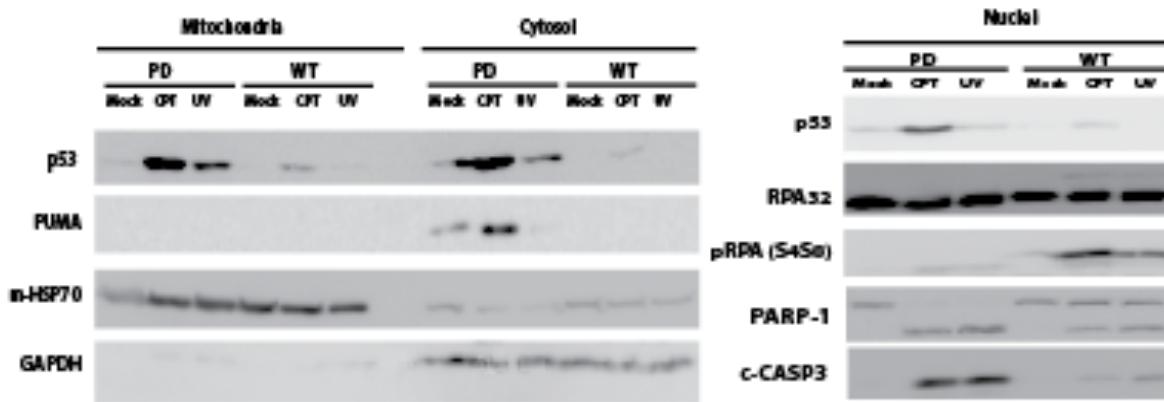
Figure 3-7. Lack of RPA32 Phosphorylation Stimulates the Expression of PUMA.
(A) Stable U2OS cells expressing WT- or PD- RPA32 were synchronized in S phase as indicated
in Material and Methods and treated with 10 μM CPT for the indicated times. Whole cell lysates
were analyzed by western blotting. (B) Mitochondria, cytosol and nuclei were isolated at 8 hours
after CPT (10 μM) or UV (30 J/m^2) treatments. Samples were then analyzed by western blotting.

Discussion

The mechanism that dictate whether a cell should stop dividing and enter the self-death

pathway remain largely obscure and correspond to a major challenge in cancer research.

Defective or inefficient apoptosis is an acquired hallmark of cancer; thus, understanding the

mechanism used to avoid apoptosis will expose efficient treatments for the eradication of such cells. In this project, we reveal a novel role of RPA32 phosphorylation in the DNA damage response that links lack of RPA32 phosphorylation to the induction of apoptosis.

Although we observed the enhanced activation of apoptosis in PD-RPA32 cells after the use of various genotoxic agents such as etoposide and UV light (Figure 3-1 and 3-3B), we decided to focus on dissecting the signaling cascade after CPT treatment only. CPT-treated PD-RPA32 cells showed enhanced PARP-1 degradation and Caspase-3 cleavage (Figure 3-1 and Figure 3-3), in addition to increased of sub-G1 phase population (Figure 3-1C and 3-1D), augmented loss of mitochondrial membrane potential (Figure 3-2A and 3-2B), and significantly more cytochrome c release from mitochondria into cytosol (Figure 3-2C) when compared to the WT-RPA32.

We show that the signaling cascade that leads to apoptosis in CPT-treated cells is dependent on the kinase activity of DNA-PK. Results in Figure 3-5 show that DNA-PK inhibition and not ATM or ATR, results in increased apoptosis in the WT-RPA32 cells. Moreover, such increase of apoptosis in the DNA-PK-inhibited WT-RPA32 mimics the high amounts of apoptosis seen originally in PD-RPA32 cells without the DNA-PK inhibition. In agreement with previous publications exhibiting DNA-PK as the kinase responsible for phosphorylating RPA32 after CPT treatment [41,89], the results in Figure 3-5 show that by using an alternative mechanism for impeding the phosphorylation of RPA32, in this case by limiting the kinase activity of DNA-PK, we are able to enhance the induction of apoptosis.

Additionally, when the kinase activity of ATM is inhibited, not that of DNA-PK or ATR, the signal that triggers apoptosis diminishes in both cell lines. This piece of evidence puts ATM as an important checkpoint activator for the CPT response that leads to apoptosis in these cells,

but it also shows that ATM is not a modulator of the response. In other words, because ATM inhibition cause the same effect in both WT- and PD- RPA32 cells, ATM does not account for the enhanced apoptotic response seen in the cells incapable of undergoing RPA32 phosphorylation.

Thereafter, Figure 3-6 shows that the p53-dependent pathway modulates the activation of apoptosis in PD-RPA32. PD-RPA32 cells induce higher levels of p53 when treated with CPT and the inhibition of p53 by siRNA results in the reduction of the apoptotic response in this cell line (Figure 3-6A and 3-6B). Furthermore, the overexpression of p53 was able to increase apoptosis in WT-RPA32 cells while the PD-RPA32 cells did not show a significant change (Figure 3-6C). In addition, the p53 transcriptional activity plays a role in the activation of apoptosis as pifithrin-α, a p53 transcriptional inhibitor, decreases the apoptotic signaling in PD-RPA32 cells (Figure 3-6B). Finally, CPT-treated PD-RPA32 cells activate the canonical p53 target PUMA which is a direct inducer of apoptosis (Figure 3-7) [103].

During this study, we discovered that CPT treatment is a good vehicle for analyzing the behavior of RPA phosphorylation in the induction of apoptosis. CPT is a topoisomerase I inhibitor which generates DSBs when the replication machinery crashes into the topoisomerase I-nicked DNA intermediate complex. For this reason, CPT is a more potent chemotherapy drug in actively replicating cells because it can generate high amounts of DSBs. Together with previous data stating that the replication machinery can discriminate the hyperphosphorylated state of RPA32 and prevents hyperphosphorylated RPA from stable association with pre-replication origins (Figure S3-1) and replication centers [64] leads us to infer that, upon CPT treatment, the PD-RPA32 cells are unable to slow down replication; thus, generating more DSBs.

Meanwhile, WT-RPA32 cells are able to dissociate from replication centers and slow down replication because of the phosphorylated stated of RPA32, generating fewer DSBs.

Additionally, our lab and others have shown that the hyperphosphorylated state of RPA32 is important for efficient repair of DSBs [62,89]. The generation of more abundant DSBs by the PD-RPA32 cells and the fact that prompt repair of the DNA lesions requires RPA32 phosphorylation [62,89] points us to believe that the augmented apoptotic response is due to these two factors: inefficient repair and augmented generation of DSBs. Moreover, the results in Figure 3-5 show that the inhibition of DNA-PK increases the apoptotic response of the WT-RPA32 cells, revealing that the apoptotic signal from PD-RPA32 cells is, in part, a cause of inefficient repair considering that the major role of DNA-PK is in the initiation of DSB repair by NHEJ. Additionally, HU-treated cells did not show enhanced apoptosis in PD-RPA32 cells (Figure 3-1B and Figure 3-4B). HU is a chemotherapy drug that reduces the production of deoxyribonucleotides (dNTPs) via the inhibition of ribonucleotide reductase. The treatment of HU initially results in stalled replication forks that, after prolong treatment, collapse into DSB's. The lack of apoptosis seen after HU treatment could be attributed to the innate characteristic of HU taking extended periods time to generate DSB's. In addition, in the PD-RPA32 background specifically, the lack of DSBs generation in HU-treated cells could be credited to HU itself inhibiting replication by the depletion of dNTPs. When replication is inhibited by other means, in this case by HU, the generation of DSBs is not enhanced by the lack RPA32 phosphorylation. In other words, HU-treated cells do not use the phosphorylation of RPA32 as the main sensor for signaling the stoppage of replication. Other drugs used in this study (etoposide, CPT and UV) that show up-regulated apoptotic response do not slow down replication directly.

Taken together, our results show that the inhibition of RPA32 phosphorylation enhances the apoptotic response of CPT-treated cells by the complementary contribution of two cellular machineries: DNA replication and DNA repair. By using CPT and disabling RPA32 phosphorylation, the generation of DNA damage is increased by pressing the cells to continue replicating while topoisomerase I-nicked intermediates generate; thus, creating higher levels of DSBs. Meanwhile, the DSB repair machinery is hampered by the lack of phosphorylation of RPA32 [62,89]. The combination of both augmented DSB production and inefficient DSB repair by CPT-treated PD-RPA32 cells could be of importance for generating more efficient chemotherapies.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Human health is challenged every day by the constant exposure to a variety of genotoxic agents which cause DNA damage, mutations and potentially cancers. The major biological defense system against DNA damage is the DNA repair machinery that recognizes and removes DNA lesions in cells. It is believed that efficient DNA repair occurs in cells through the coupling of the DNA repair machineries with DNA damage checkpoints. RPA is a eukaryote single-stranded DNA-binding protein shown to be directly involved in a variety of DNA metabolic pathways. Importantly, the RPA32 subunit undergoes N-terminal hyperphosphorylation (hyp-RPA32N) by DNA damage checkpoint kinases and this hyperphosphorylation is crucial for the cellular responses to DNA damage. Therefore, in this project we addressed the notion that the recognition of the hyp-RPA32N motif would represent a newly stimulated interaction between the new form of RPA and the recognizing protein. This recognition may also disrupt native RPA interaction with other proteins. Previously, our lab has shown that, upon hyperphosphorylation, RPA undergoes a structural reorganization featured by the binding of hyp-RPA32N to DBD-B in RPA70 [60]. Because DBD-B is involved in many RPA-protein interactions, we hypothesized that a competitive binding on hyp-RPA32N to DBD-B may inhibit these RPA-protein interactions. The change in equilibrium marked by this type of dynamic competition is believed to be the basis for the regulation that modulates the different biochemical pathways in cells. This hands-off/hands-on mechanism may allow RPA to coordinate different components of the DNA damage response mechanisms.

Of particular interest is the RPA-p53 interaction. p53 is a tumor suppressor and plays a critical role in cellular DNA damage responses, particularly DNA damage checkpoints. Importantly, the RPA-p53 interaction inhibits homologous recombination [52]. In other words, the disruption of RPA-p53 binding increases HR activity in cells. Since HR is required for repair of DSBs and hyperphosphorylation of RPA enhances RPA interaction with Rad51 and Rad52 [62], two pivotal proteins for HR repair, we decided to conduct studies to address the regulation of the switch-on and –off mechanism of the RPA-p53 complex. Conclusively, we found in Chapter 2 of this dissertation that the RPA-p53 interaction is modulated via site-specific post-translational modifications on both p53 and RPA (Figure 2-7e). Specifically, after DNA damage, RPA is hyperphosphorylated by DNA-PK at multiple sites in RPA32N while p53 is phosphorylated by ATM and ATR at Ser-37 and Ser-46, respectively (Figure 2-5). Such molecular alterations interfere with RPA binding to the N-terminus of p53, therefore, freeing RPA and p53. Figure 2-6 and Figure 2-7 show that a deficiency in RPA phosphorylation and release from the p53–RPA complex significantly reduces repair efficiency of DSBs induced by CPT. In parallel, the released hyp-RPA binds much more efficiently to Rad51 than does native RPA (Figure 2-6d) suggesting that the phosphorylation of RPA and p53 not only releases RPA during the DNA damage response, but also allows RPA to more efficiently recruit Rad51 to the DSB sites during an early step of HR, thus promoting the repair process. In addition, the study in Chapter 2 addresses a lingering hypothesis in the DNA repair field on the cross-communication of the two independent DSB repair pathways: HR and NHEJ [91]. Before this study, no detailed mechanism had been offered for the collaboration of HR and NHEJ in DSB repair. The results in Chapter 2 provide evidence that DNA-PK, the hallmark protein of NHEJ, together with ATR and ATM have a regulatory role in HR repair efficiency of CPT-induced DSBs. Our findings also show, for

the first time, that all three major PIKK family members together are involved in the DNA damage response for DSB repair. Upon DNA damage DNA-PK, ATM or ATR phosphorylates specific sites of p53 or RPA to make a synergistic contribution to inducing p53–RPA dissociation for effective HR repair. Taking together, the switch-on and –off interaction between RPA and p53 constitute an important mechanism for the regulation of DSB by HR with the concomitant contribution of DNA-PK.

The exact mechanisms that command the switch from stoppage of cell division to initiation of cell death remain obscure and still correspond to one of the major challenges in cancer research. Major research has been conducted in understanding the mechanisms that lead to apoptosis for improving therapeutic efficacy and selectivity in killing cancer cells [23]. Because RPA has been reported to be involved in many pathways of the DNA damage responses and the fact that, upon phosphorylation, RPA modulates many cellular processes, we hypothesize that the inhibition of RPA phosphorylation could become an important target for the sensitization of cancer cell killing. One key property of all cancer cells is accelerated growth. This uncontrolled growth is due to a series of breaches in the checkpoint machinery that leads to inefficient stoppage of replication. Some chemotherapy drugs already take advantage of this accelerated growth; prominent examples are the analogues of topoisomerase I inhibitor camptothecin (CPT), which act by generating DSBs when the replication machinery crashes into the topoisomerase I-nicked DNA intermediate complex. CPT is able to generate DSB's only in actively replicating cells, so the killing effects of CPT are potent in cancer cells. Additionally, RPA32 phosphorylation prevents RPA association with replication centers [64] and suppresses the activation of origin firing (Figure S3-1, unpublished data). Both pieces of evidence suggest that the replication machinery can discriminate the hyperphosphorylated state of RPA32 and impede

the initiation of replication. This discrimination has been also shown to lead to stoppage of replication by activation of cell cycle checkpoints [61]. Taken together, the inhibition of RPA phosphorylation after DNA damage can cause failure in arresting the cell cycle.

Because of both, CPT's killing effects being dependent on replication and lack of RPA phosphorylation leading to failure of cell cycle arrest, we hypothesized that this combination should enhance cell death in cancer cells. Conclusively, in Chapter 3 we found that lack of RPA32 phosphorylation in CPT-treated cells leads to enhanced apoptosis. Specifically CPT-treated PD-RPA32 cells showed augmented PARP-1 degradation (Figure 3-1A), Caspase-3 cleavage (Figure 3-3), sub-G1 cell accumulation (Figure 3-1C and 3-1D), loss of mitochondrial membrane potential (Figure 3-2A and 3-2B), and cytochrome c release into the cytosol (Figure 3-2C) when compared to the WT-RPA32. Additionally, we found that the signaling cascade that leads to apoptosis in the PD-RPA32 cells is affected by the kinase activity of DNA-PK and is achieved by the augmentation of p53 levels in the cell. Higher levels of p53 are caused by the generation of persistent DSBs (Figure 2-6A and 2-6B) and activation of the sensor kinase ATM. Augmented p53 levels, in turn, induce higher amounts of apoptosis modulator PUMA. Our results suggest that the lack of RPA32 phosphorylation enhances apoptosis, illuminating a novel target that lies on the crossroads of DNA repair and cell death which could be of importance to the sensitization of cancer cells to chemotherapy.

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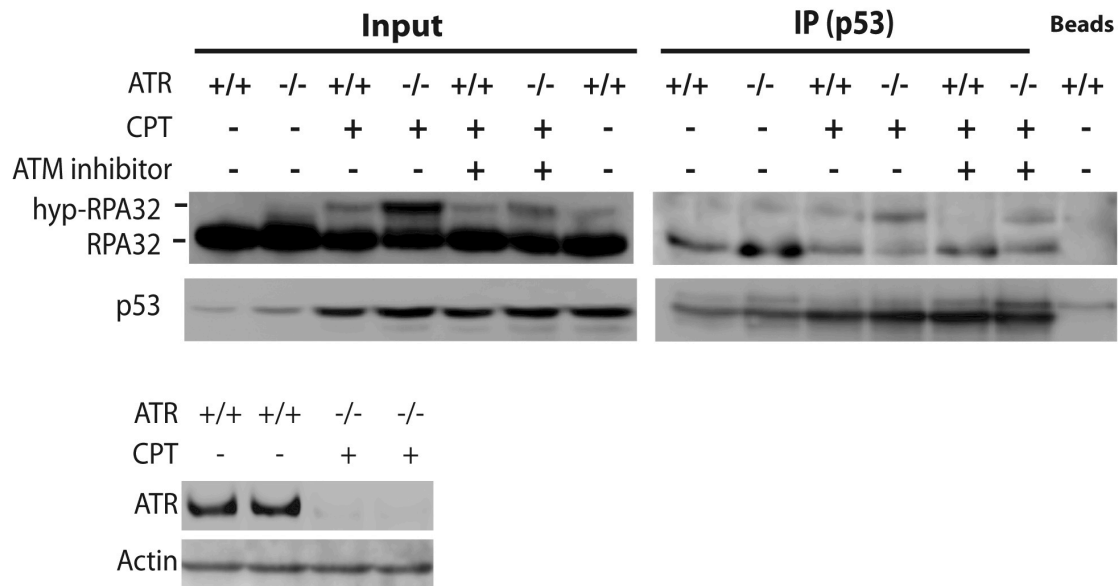
APPENDIX A

SUPPLEMENTARY FIGURES

Figure S2-1. Modulation of p53-RPA Binding is Dependent on ATM and ATR. HCT-116 ATR^{-/-} cells were synchronized in S phase as stated in Materials and Methods Chapter 2 and treated with 10 μM ATM inhibitor (Ku55933) for 1 hour prior to CPT treatment (10 μM for 2.5 hours). Whole cell lysates were subjected to DNase I digestion and 5% of the sample was loaded as input. IP was subsequently performed using anti-p53 antibody and co-immunoprecipitated proteins were analyzed by western blotting with the indicated antibodies. Bottom figure shows a western blot of whole cell lysates of HCT-116 ATR^{+/+} and HCT-116 ATR [flox/-] cells used in this figure.
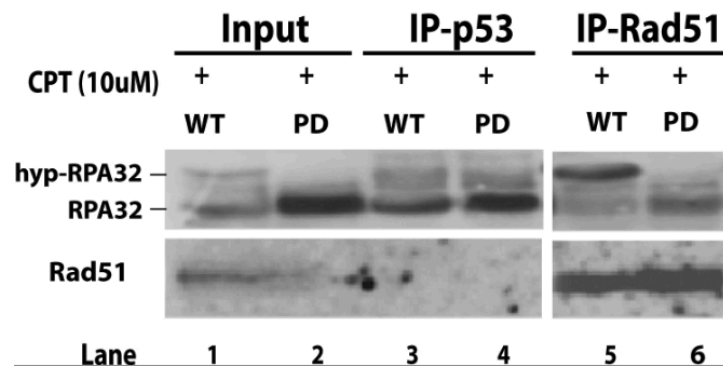
Figure S2-2. Hyperphosphorylation of RPA Promotes RPA-Rad51 Interaction. Stable U2OS WT or PD cells were synchronized in S phase with APH, and then treated with 10 μM CPT for 3 hrs. Chromatin was isolated and incubated with DNase I. Subsequently, samples were incubated with anti-p53 antibody and co-immunoprecipitated proteins were analyzed by western blotting (lanes 3-4). The supernatant after IP was immunoprecipitated again using Rad51 antibodies and analyzed (lanes 5-6).
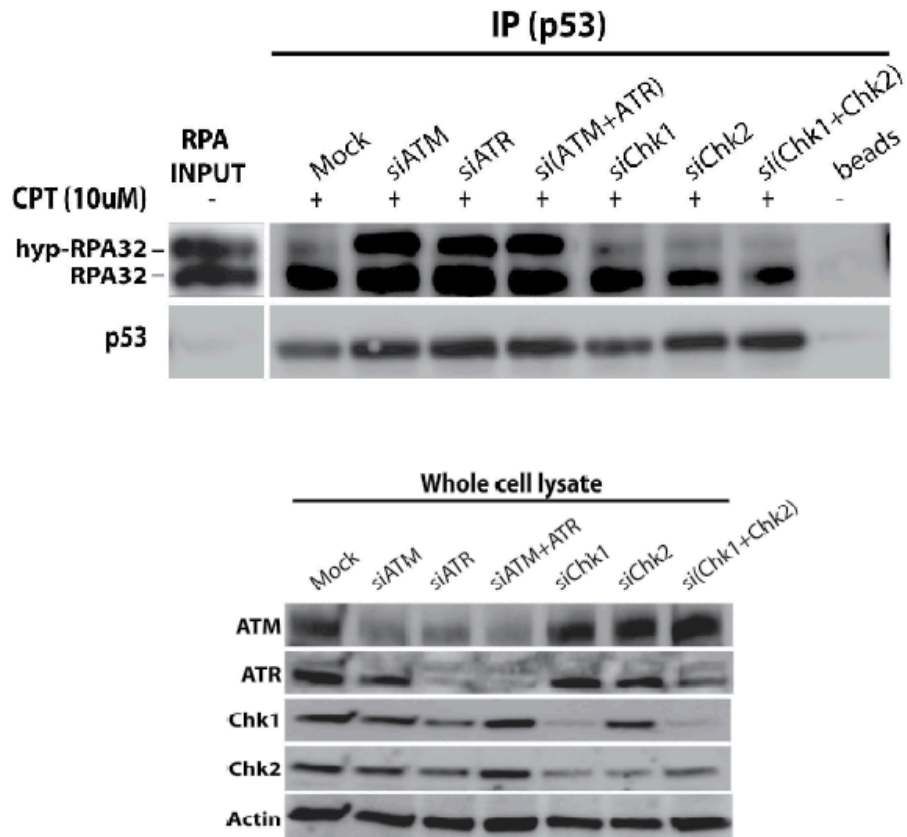
Figure S2-3. Effects of ATR, ATM, Chk2 and Chk1 on p53-RPA Interaction. A549 cells were transfected with siRNAs targeting ATM, ATR, Chk1, Chk2 or combinations for 48 hours. Cells were treated with 10 μM CPT for 3 hrs to induce RPA phosphorylation. Whole cell lysates were prepared and analyzed by western blotting to confirm silencing (bottom). Nuclear lysates were isolated and treated with DNase I. Samples were incubated with anti-p53 antibodies and co-immunoprecipitated proteins were analyzed by western blotting.
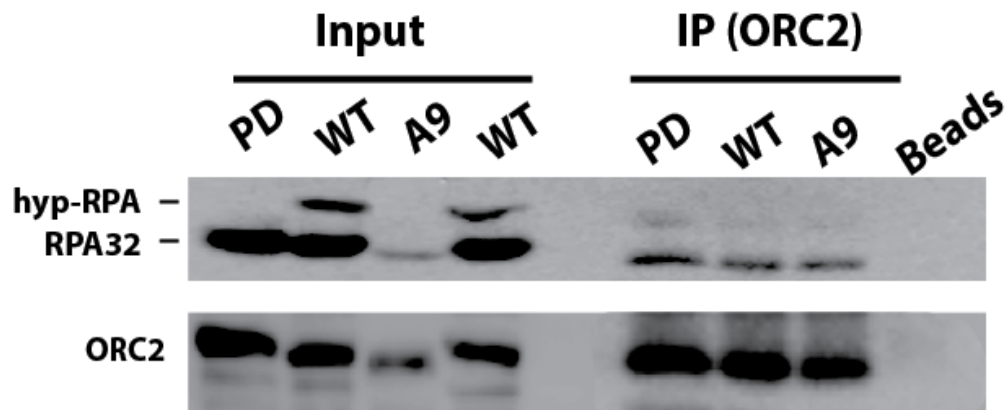
Figure S3-1. Hyperphosphorylated RPA is Unable to Associate with Replication Origins. Stable U2OS WT or PD cells were synchronized in S phase with APH, followed by treatment with 10 μM CPT for 3 hrs. Chromatin Immunoprecipitation (ChIP) assay was performed following a previously published procedure [114]. After treatment, cells were incubated with formaldehyde to crosslink interacting protein-DNA and protein-protein complexes. The crosslinking reaction was terminated with 250 mM glycine in PBS. Nuclei were prepared by fractionation. The chromatin was sheared to 200-1500 bp fragments by sonication. The sheared chromatin was then incubated with anti-ORC2 (a subunit of ORC) antibody, followed by precipitation with Protein G Sepharose beads. The immunoprecipitates were boiled for 30 minutes to reverse the crosslinks. Proteins that co-precipitate with replication origin-containing chromatin were detected by western blotting. Top panel: western blotting with anti-RPA32 antibody. The arrow indicates the hyperphosphorylated forms of RPA32. Bottom panel: western Blotting with anti-ORC2. It has been well documented that one of the prerequisites for initiation of DNA replication is the binding of RPA to the short ssDNA region of the DNA bubble and its interaction with ORC (origin recognition complex) at replication origins [115]. This data suggest that the hyperphosphorylation of RPA32 inhibits its interaction with the replication origins thus inhibiting origin firing or the start of replication.

APPENDIX B

ABBREVIATIONS

DN, dominant negative

LOH, loss of heterozygosity

GOF, gain of function

9-1-1 complex, Rad9-Rad1-Hus11 complex

ATM, Ataxia telangiectasia mutated

ATR, Ataxia telangiectasia and Rad3 related

ATRIP, ATR interacting protein

BER, base excision repair

ChIP, chromatin immunoprecipitation assay

CPT, camptothecin

DBD, DNA-binding domain

DDR, DNA damaged response

DMEM, Dulbecco's modified Eagle medium

DNA-PK, DNA-dependent protein kinase

DNA, deoxyribonucleic acid

DSB, double-strand break

DSBR, double-strand break repair

dNTPs, deoxyribonucleotides

FBS, fetal bovine serum

HR, Homologous recombination

HtH, helix-turn-helix

hyp-RPA32, hyperphosphorylated RPA32

hyp-RPA32N, hyperphosphorylated N-terminus of RPA32

IDL, insertion of a deletion loop

IR, ionizing radiation

kDa, kilo Dalton

LFS, Li-Fraumeni syndrome

MMR, mis-match repair

MRN, Mre11/Rad50/NBSI complex

NER, nucleotide excision repair

NHEJ, nonhomologous end joining

OB-fold, oligonucleotide/oligosaccharide binding fold.

PARP-1, Poly-ADP ribose polymerase-1

PD-RPA32, phospho-deficient replication protein A 32 kDa subunit

PI 3-kinase, phophoinositide 3-kinase

RFC, replication factor C

RPA, replication protein A

RPA14, replication protein A 14 kDa subunit

RPA32, replication protein A 32 kDa subunit

RPA32N, N-terminus of RPA32

RPA70, replication protein A 70 kDa subunit

SDS-PAGE, sodium docecyl sulfate polyacrylamide gel electrophoresis

siRNA, small interfering ribonucleic acid

SSBR, single-strand break repair

ssDNA, single-stranded DNA

SV40, Simian vacuolating virus

WT-RPA32, wild-type replication protein A 32 kDa subunit

XRCC4, X-ray repair cross-complementin protein 4

APPENDIX C

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