**Post-translational Regulation of RPA32, ATM and Rad17**

**Controls the DNA Damage Response**

by

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Department of Pharmacology and Cancer Biology
Duke University

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Weiguo Zhang

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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2009

ABSTRACT

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# Abstract

The eukaryotic genome integrity is safeguarded by the DNA damage response, which is composed of an elaborate network of signal transduction pathways that can detect DNA lesions, arrest cell cycle progression, motivate DNA repair processes, or induce apoptosis or senescence when cells incur irreparable DNA damage. During this process, DNA damage-induced post-translational modifications, most notably protein phosphorylation, of a variety of DNA damage-responsive proteins has been shown to mediate the initiation, transduction and reception of the DNA damage signals, resulting in alterations of their stability, activities or subcellular localizations, ultimately leading to activation of various downstream effector pathways.

While a lot has been elucidated on the downstream events of the DNA damage response, little is known about how DNA damage is detected. Two still ongoing studies of this dissertation attempt to address this question. Our preliminary work on ATM indicates that serine 2546 is critical for its kinase activity. Substitution of this residue with phosphomimetic aspartate, but not nonphosphorylable alanine, abrogates the kinase activity of ATM and fails to rescue the checkpoint-deficient phenotype exhibited by the ATM-deficient cells, suggesting that removal of an inhibitory phospho group at S2546 might be required for the activation of ATM. In another study, we identified a novel DNA-damage responsive threonine residue (T622) in Rad17, which undergoes ATM/ATR-dependent phosphorylation *in vitro* and *in vivo*. Ectopic expression of a phosphodeficient mutant (T622A) of Rad17, but not its wild-type control, shows a pronounced defect in sustaining Chk1 phosphorylation and the corresponding G2/M checkpoint upon DNA damage, suggesting that phosphorylation at T622 might complement that on the two

previously reported phosphorylation sites, S635 and S645, to mediate G2/M checkpoint activation while the latter is primarily responsible for intra-S phase checkpoint.

Although a large amount of knowledge has been accumulated about the initiation and activation process of the DNA damage response, how cells recover, the equally important flip side of the response, has remained poorly understood. We have found that in cells recovering from replication stress, RPA32 phosphorylation at ATM/ATR-responsive sites T21 and S33, which reportedly suppresses DNA replication and recruiting other checkpoint and repair proteins to the DNA lesions, is reversed by the serine/threonine protein phosphatase 2A (PP2A). Cells with a RPA32 persistent-phosphorylation mimic (T21D/S33D) exhibit normal checkpoint activation and re-enter the cell cycle normally after recovery, but display a pronounced defect in the repair of DNA breaks. These data indicate that PP2A-mediated RPA32 dephosphorylation may be a required event during the repair process in the DNA damage response.

In summary, these studies in this dissertation highlight the importance of reversible phosphorylation and dephosphorylation in the modulation of the DNA damage response. They extend our knowledge and deepen our understanding of this process by revealing that dephosphorylation may positively regulate the activation of cell cycle checkpoints, which is seemingly dominated by protein phosphorylation upon DNA damage, that phosphorylation of certain checkpoint proteins at different sites may result in distinct consequences, and that dephosphorylation of some activated checkpoint/repair proteins may function as an important mechanism for cells to recover from the DNA damage response.

# Dedication

To my parents, Jianming Feng and Guanghua Xie, for being the wind behind my sail that has driven me thus far; to my wife, Shuang Song, for accompanying and safeguarding me through this bumpy yet wonderful adventure; to my son, William S. Feng, for being my compass that has helped me sail out of being lost and will guide me for the next journey.

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**Chapter 1**



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# List of Abbreviations

| | |
|---|---|
| 53BP1 | p53 binding protein |
| A-T | Ataxia telangiectasia |
| ATM | Ataxia telangiectasia mutated |
| ATR | ATM-Rad3-related |
| ATRIP | ATR interacting protein |
| BRCA | Breast cancer susceptibility gene |
| BRCT | BRCA1 carboxy terminal |
| CDK | Cycline-dependent kinase |
| Chk1 | Checkpoint kinase 1 |
| CHX | Cycloheximide |
| DBD | DNA binding domain |
| DNA-PK | DNA-dependent protein kinase |
| DSB | DNA double-stranded break |
| FACS | Flow cytometric analysis |
| HU | Hydroxyurea |
| IF | Immunofluorescence |
| IP | Immunoprecipitation |
| IR | Ionizing radiation |
| MAPK | mitogen-activated protein kinase |
| MDC1 | Mediator of DNA damage checkpoint 1 |
| MRN | Mre11-Rad51-Nbs1 complex |

| | |
|---|---|
| NCS | Neocarzinostatin |
| OA | Okadaic acid |
| PCNA | Proliferating cell nuclear antigen |
| PIKK | Phosphoinositide 3-kinase related protein kinase |
| Plk1 | Polo-like kinase 1 |
| PP1 | Protein phosphatase 1 |
| PP2A | Protein phosphatase 2A |
| PP4 | Protein phosphatase 4 |
| PP5 | Protein phosphatase 5 |
| PP6 | Protein phosphatase 6 |
| RFC | Replication factor C |
| RPA | Replication protein A |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| shRNA | Short Hairpin RNA |
| siRNA | Small interfering RNA |
| ssDNA | Single-stranded DNA |
| TopBP1 | Topoisomerase binding protein 1 |
| UV | Ultraviolet |
| Wip1 | Wild-type p53-induced phosphatase 1 |

# Acknowledgements

I would like to first thank my mentor, Dr. Xiao-Fan Wang, for his constant encouragement, helpful guidance and full support through these graduate school years at Duke. I deeply appreciate his full-hearted trust in me that has allowed me to explore unexplored directions, make mistakes and move forward. His typical open-mindedness, resourcefulness and optimism have exerted invaluably positive influences on me from which I am sure that I will be benefited for my whole life.

I want to also thank my committee members, Drs. Sally Kornbluth, Jeffrey Rathmell, Tso-Pang Yao, and Weiguo Zhang, for their insightful comments and constructive suggestions on my research, without which I would have made bigger mistakes and wasted more time in my graduate study. I feel also grateful to our off-campus collaborators, Drs. Xiaohua Wu, Mark Wold, Xin-Hua Feng and Xuan Liu for providing critical reagents that have made my studies possible and smooth.

It has been a really great honor to work with the Wang lab members, all being talented, patient and willing to help. These wonderful people include our previous lab members Ji Zhang, Yanan Fang, Tony Dang, Ryohei Furumai, Hongyan Jian and Xing Shen, Chaoyu Ma, Xing Guo, Yu Rong, Anthony Cheng, Melanie Jardim, Irwin Liu, and our present lab members Yong Yu, Timothy Wakeman, Steve Schilling, Daniel Radiloff, Sheila Yong, Hui Wang, Valerie Curtis, Qinhong Wang, Tao Sun and Pengyuan Yang. I feel particularly thankful to Ji Zhang, who gave careful instructions and offered invaluable trainings to me when I first waded in the field of DNA damage response, to Timothy Wakeman, who has provided constructive suggestions and wholehearted help

on both the techniques and my paper writing, and to other lab members that had worked or are working in the same field, Yanan Fang, Tony Dang, Ryohei Furumai, Melanie Jardim, Qinhong Wang and Sheila Yong, for their technical assistance and helpful discussions. I also appreciate faculties, colleagues and friends in many labs on this campus for their assistance and encouragement.

I owe a huge thankfulness to my close friends that I was luckily acquainted with in the Duke community, Yong Yu and Meifang Dai, Hongyan Jian, Yanan Fang and Qing Shi, Chaoyu Ma and Nu Zhang, Xing Guo and Zhiping Wang, Penny Luo, Yuhong Wu and Fei Liu, Wei Wu and Lixian Zhong, Fumin Lin, Ran Li, Hui Wang, Sheila Yang and Anthony Cheng, who have given me unconditional help in every aspect of my life here at Duke; without them, it would have become more difficult and less enjoyable.

Lastly I would like to express my deepest love and gratitude to my family. My wife, Shuang Song, has always been there with me not only in times of joy and happiness but at the moments of frustration and despair, accompanying and safeguarding me through this bumpy yet wonderful journey. Her inherent nature of being positive and optimistic has been a healing blessing to me and has quietly yet profoundly reshaped me for ever. My currently twenty-month-old son, William S. Feng, has given me a tremendous mental boost in my difficult times, guiding me out of being lost and through this adventure of self-discovery. Also luckily I have my parents-in-law, Gang Song and Luojing Meng, for their self-sacrifice in sharing our burden of taking care of the everyday life. And last but never least, to my parents, Jianming Feng and Guanghua Xie, for being the wind behind my sail that has driven me this far, for their endless love,

merits of fearlessness and generosity that has always been bringing me comfort, confidence and hope. I love you all.

# 1 Overview

## *1.1 The DNA damage response*

The genomes of all living cells are under constant attack by exogenous DNA-damaging agents (e.g. ionizing radiation, UV light, reactive chemicals) as well as the intracellular byproducts of normal metabolism (e.g. reactive oxygen intermediates, inaccurately replicated DNA) (Abraham, 2004; Sancar et al., 2004). To cope with this challenge and preserve the integrity of the genomic information, eukaryotic cells have evolved an elaborate surveillance, maintenance and execution system termed DNA damage response, which is composed of a set of signal transduction pathways that can detect DNA lesions, arrest cell division, motivate repair processes, or induce apoptosis or senescence when the level of DNA damage is beyond repair (Figure 1-1) (Su, 2006). Deficiencies in these pathways cause accumulation of harmful mutations and aberrant cell division, leading to genomic instability and decreased cell survival. In human, defective DNA damage response underlies the pathogenesis of a variety of diseases including immunodeficiency, cardiovascular diseases, neurodegenerative disorders, and cancer (Kastan and Bartek, 2004; Zhou and Elledge, 2000).

Over the past two decades, significant progress has been made in our understanding of the DNA damage response pathways. A huge number of DNA damage-responsive proteins have been identified and characterized, which are conceptually categorized into three functional modules according to their roles in the whole cellular response to genotoxic insults: cell cycle checkpoints, DNA damage repair, and apoptosis/senescence (Figure 1-1). Mounting evidence has shown that these

modularly organized proteins cooperate in a spatially and temporally tightly controlled manner to safeguard the genome integrity (Su, 2006; Zhou and Elledge, 2000).

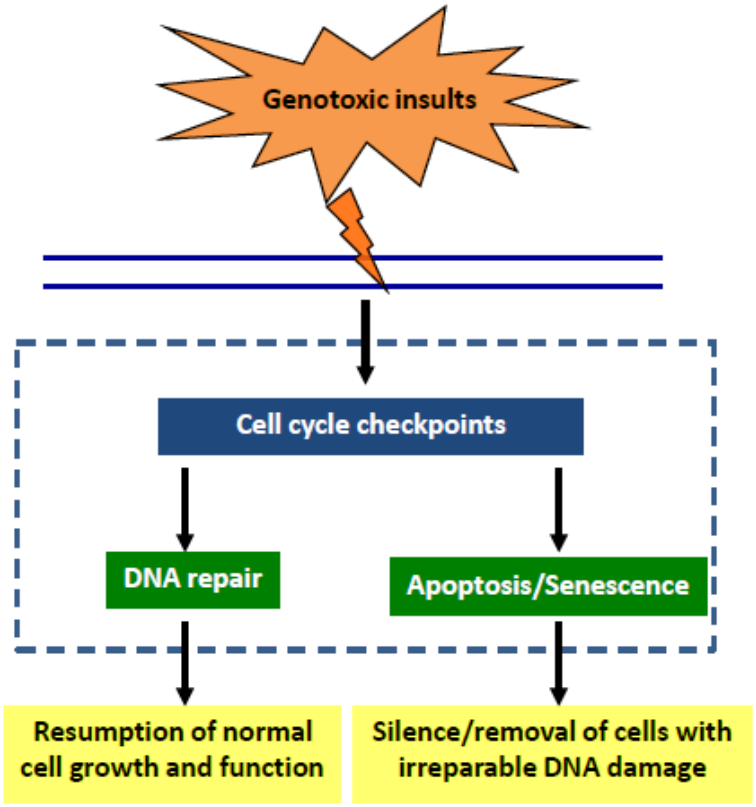


**Figure 1-1: Overview of the eukaryotic DNA damage response.**

In response to genotoxic insults, the checkpoints are activated to arrest the cell cycle progression. Depending on the levels of DNA lesions, checkpoints may motivate DNA repair and allow mitotic re-entry, or induce apoptosis/senescence.

Upon genotoxic stresses, cell cycle checkpoints are activated to delay or arrest cell cycle progression, allowing time for the downstream DNA damage repair pathways to fix the damaged DNA. Cells can survive and resume the normal cell growth and function if the DNA lesions are effectively removed. If however, the level of DNA damage

is beyond the capability to repair, cells may be eliminated by apoptosis or silenced by senescence (Bartek and Lukas, 2007; Harrison and Haber, 2006).

### 1.1.1 The cell cycle checkpoints

In an unperturbed cell division cycle, an eukaryotic cell progresses through four consecutive phases (G1, S, G2 and M) and divides into two identical daughter cells. To ensure the accurate transmission of genetic information during this process, cells employ a series of specialized biochemical pathways, termed checkpoints, to closely monitor the proper progression of cell cycle, which operate mainly at the three major control points in the cell cycle: the transition points G1/S, G2/M, and the intra-S phase (Abraham, 2001). The overall function of this surveillance system is to detect damaged or abnormally structured DNA, and to coordinate cell-cycle progression with DNA repair.

In essence, cell cycle checkpoints are signal transduction pathways which involve a huge network of proteins that fall into four conceptual categories: DNA damage sensors, signal mediators, checkpoint transducers, and effectors (Figure 1-2) (Sancar et al., 2004). Many of these checkpoint-essential proteins are highly conserved across species, whose orthologues have been found from yeasts to humans (Table 1-1).

It is noteworthy that there is no absolute demarcation among the various checkpoint components and some important checkpoint proteins may play multiple roles in the signal transduction pathways. For example, the eukaryotic DNA replication-essential protein, replication protein A (RPA), has been demonstrated to be able to detect DNA lesions (a sensor), as well as mediating suppression of DNA synthesis upon DNA damage (an effector). In addition, many of the sensor, mediator and transducer

proteins are commonly shared among the various checkpoint pathways (G1/S, intra-S and G2/M checkpoints) which nonetheless employ distinct effector components to directly suppress the cell cycle progression at the various control points of the cell cycle (Su, 2006). In this sense, the distinct natures of various checkpoints are defined mainly by these different effectors.

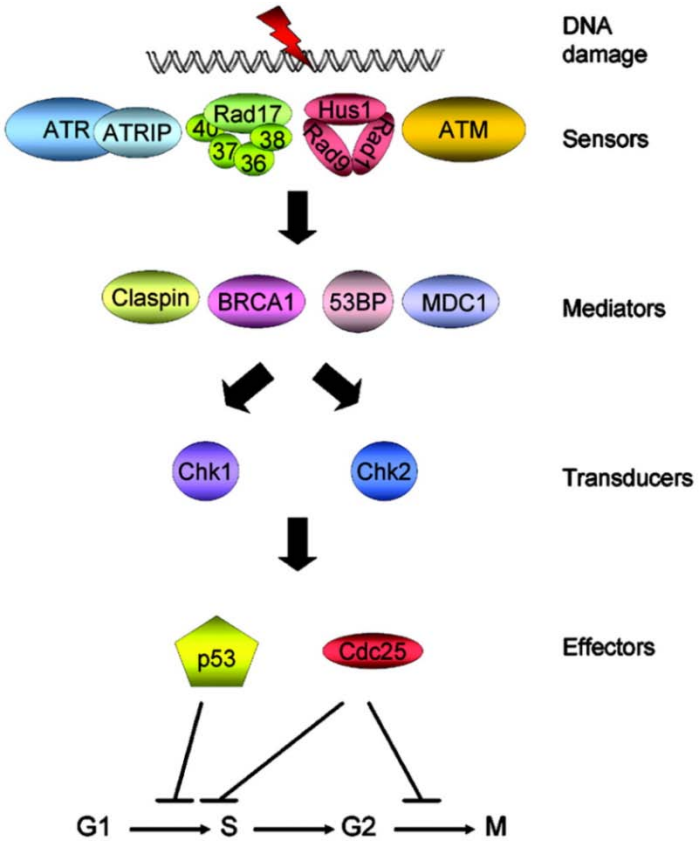


**Figure 1-2: The hierarchy of mammalian cell cycle checkpoint pathways.**

The DNA damage is detected by sensors that, with the aid of mediators, transduce the signal to transducers. The transducers, in turn, activate or inactivate other effector proteins that directly participate in inhibiting the G1/S transition, S-phase progression, or the G2/M transition (from (Sancar et al., 2004)).

**Table 1-1 Highly conserved DNA damage checkpoint proteins (excerpted from Sancar, Lindsey-Boltz et al. 2004)**

| Protein function | Mammals | *S. pombe* | *S. cerevisiae* |
|---|---|---|---|
| Sensors | | | |
| RFC-like | Rad17 | Rad17 | Rad24 |
| PCNA-like | Rad9 | Rad9 | Ddc1 |
| | Rad1 | Rad1 | Rad17 |
| | Hus1 | Hus1 | Mec3 |
| PI3-Kinases (PIKK) | ATM | Tel1 | Tel1 |
| | ATR | Rad3 | Mec1 |
| PIKK binding partner | ATRIP | Rad26 | Ddc2/Lcd1/Pie1 |
| Mediators | | | |
| | MDC1 | | |
| | 53BP1 | | |
| | TopBP1 | Cut5 | Dpb11 |
| | Claspin | Mrc1 | Mrc1 |
| | BRCA1 | Crb2/Rph9 | Rad9 |
| Transducers | | | |
| Kinase | Chk1 | Chk1 | Chk1 |
| | Chk2 | Cds1 | Rad53 |

#### 1.1.1.1 DNA damage sensors

The proper execution of cell cycle checkpoints requires the timely recognition of various chromosomal lesions to initiate downstream effector events, which is conducted by a group of specialized proteins termed DNA damage sensors. Two subsets of proteins have been identified to play critical roles in this process: two Rad groups of checkpoint protein complexes (Rad17-RFC and Rad9-Rad1-Hus1) and two structurally related protein kinases, Ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR).

It is notable that the Rad17-RFC and Rad9-Rad1-Hus1 (9-1-1) complexes bear significant structural and functional homology with replication factor C (RFC) and proliferating cell nuclear antigen (PCNA), and may act in concert with one another in the recognition of DNA damage and activation of downstream checkpoints (Sancar et al., 2004). The current consensual model holds that the Rad17-RFC protein complexes may recognize the DNA lesions and load the PCNA-like 9-1-1 sliding clamp onto sites of DNA damage, in a manner similar to the RFC-dependent recruitment of PCNA onto DNA during unperturbed replication (Nyberg et al., 2002). In humans, the DNA damage-induced interaction between these two protein complexes is reportedly dependent on phosphorylation of hRad17 on Ser635 and Ser645, which is mediated by ATM/ATR (Bao et al., 2001). In addition, the 9-1-1 complex may have an additional role of processing DNA damage to enhance checkpoint signaling to other checkpoint/repair proteins (Lydall and Weinert, 1995).

ATM and ATR belong to the phosphoinositide 3-kinase-like kinase (PIKK) family, which both exhibit significant sequence homology with the archetypal phosphoinositide-3 kinase at the C-terminal kinase domain, but nonetheless lack lipase activity. In response to genotoxic stresses, ATM/ATR phosphorylate a great variety of DNA damage-responsive proteins including Chk1, Chk2, p53, NBS1, BRCA1 and RPA, relaying the DNA damage signal downstream or directly activating or suppressing effector proteins in the checkpoint pathways. They are therefore regarded as apical kinases in the checkpoint signal transduction cascade. Both ATM and ATR target serine and threonine residues of their substrates which is followed by glutamine (SQ/TQ), and they share most, if not all, of the downstream substrate proteins. Nonetheless, ATM and ATR differ

in the way to be activated upon detection of DNA damage and in the types of DNA damage to which they respond. Based on current models, whereas ATM activation requires autophosphorylation-dependent breakdown from inactive oligomeric form to the active monomers that subsequently bind to the ends of DNA termini, ATR stimulation needs direct recruitment onto DNA through its binding partner, ATR interacting protein (ATRIP). In accordance with their different damage sensing and activation mechanisms, ATM responds preferentially to DNA damaging reagents that mainly induce DNA double-stranded breaks (DSBs, e.g. IR, CPT), while ATR is activated when cells are challenged with other types of DNA stressors that involve base damages or replication stalk (e.g. UV, HU).

Recently, it has been demonstrated other proteins may play important yet previously oblivious roles in the detection of DNA damage and activation of ATM/ATR. The Mre11-Rad50-Nbs1 (MRN) complex, originally known as a DNA processing protein involved in various types of DNA metabolism, has been shown to serve as a DSBs sensor to recruit and activate ATM at sites of DNA breaks (Lee and Paull, 2004, 2005). The recruitment of ATR to locations of DNA lesions may depend on the interaction between ATRIP and RPA, which can sense the presence of the exposed single-stranded DNA (ssDNA) intermediate due to its unique ssDNA binding capability (Zou and Elledge, 2003).

#### 1.1.1.2 Checkpoint mediators

Checkpoint mediators facilitate the relay of the DNA damage signals originated by the DNA damage sensors downstream to the checkpoint/repair apparatuses by

simultaneously associating with damage sensors and downstream transducer or effector proteins. Biochemically these proteins function as adaptor proteins by localizing downstream checkpoint/repair proteins to sites of DNA damage and/or presenting them for ATM/ATR-dependent phosphorylation (Li and Zou, 2005). The essentiality of these mediators is explicit by the observations that checkpoints are abrogated in cells with mutated or reduced level of mediator proteins which include MDC1, BRCA1, 53BP1 and Claspin.

In humans, at least four BRCA1 carboxy terminal (BRCT) domain-containing proteins have been implicated in the transduction of checkpoint signals, which include the prototypal breast cancer susceptibility gene 1, BRCA1; the topoisomerase binding protein 1, TopBP1; the mediator of DNA damage checkpoint 1, MDC1; and the p53 binding protein, 53BP1 (Nyberg et al., 2002; Sancar et al., 2004). In response to DNA damage, these BRCT mediators translocate to sites of DNA lesions, which appear to be necessary for the efficient ATM/ATR-dependent phosphorylation of downstream substrates such as RPA32, Chk1, Chk2 and p53, as well as the optimal formation of DNA damage-induced repair foci by checkpoint/repair proteins including MRN, FANCD2 and SMC1 (Canman, 2003). The recent findings that many BRCT-containing proteins have phospho-serine and phospho-threonine specific binding activity suggests that BRCT domain might mediate phosphorylation-dependent protein–protein interactions during checkpoint activation (Manke et al., 2003; Yu et al., 2003). It should be mentioned that all of these mediators known to date are themselves substrates of ATM/ATR, phosphorylation of which might generate docking sites for other mediators or downstream transducers (Chk1 and Chk2) with phospho-protein binding motifs.

Besides these *bona fide* BRCT mediators, other important DNA damage responsive proteins such as Claspin, SMC1, H2AX and MRN complex, do not carry BRCT domains yet play similar roles in the transduction of checkpoint signals and formation of DNA repair foci (Li and Zou, 2005; Sancar et al., 2004); they are also deemed as checkpoint mediators in this sense.

#### 1.1.1.3 Checkpoint transducers

With the assistance of checkpoint mediators, the DNA damage signals originating from sites of DNA lesions are transmitted downstream, in the form of protein phosphorylation, to two major signal-transducing kinases, Chk1 and Chk2. These so-called checkpoint transducers in turn propagate and amplify the checkpoint signals throughout the whole nucleoplasm and subsequently to their downstream effectors, most notably p53 and Cdc25, ultimately leading to cell cycle arrest, DNA damage repair, or apoptosis/senescence (Li and Zou, 2005).

Chk1 and Chk2 are structurally unrelated serine/threonine protein kinases, yet exhibit a surprising level of similarities. Both kinases carry several important ATM/ATR-responsive SQ/TQ sites, S345/Q346 and S317/Q318 in Chk1 and T68/Q69 in Chk2, whose phosphorylation has been demonstrated to be required for the DNA damage-induced stimulation of their respective kinase activities (Melchionna et al., 2000; Zhao and Piwnica-Worms, 2001). Chk1 and Chk2 also have an overlapping spectrum of downstream substrates. Once activated, both kinases have been shown to phosphorylate important checkpoint effectors such as Cdc25A, Cdc25C, Mdm2 and p53,

resulting in delay or arrest of cell cycle at appropriate control points (Bartek and Lukas, 2003; Niida and Nakanishi, 2006).

Despite their seemingly redundant roles in the DNA damage response, Chk1 and Chk2 are differentially activated and may have distinct functions which are still being studied. In mammalian cells, the DSB signal sensed by ATM is transduced primarily by Chk2, while the activation of Chk1 is predominantly ATR-dependent as a response to other types of genotoxic insults such as replication stress and UV irradiation (Sancar et al., 2004). The intimate relationship between Chk1/Chk2 and their respective preferred kinases ATR/ATM, as revealed by a large body of evidence, has prompted an emerging model that regard ATM-Chk2 and ATR-Chk1 as two parallel functional modules pivotal in the DNA damage checkpoint pathways (Bartek et al., 2007). In addition, although Chk1 and Chk2 share most of the substrate effectors, they display some level of substrate specificity. It has been revealed that phosphorylation of DNA damage responsive proteins BRCA1, E2F1, PML and PLK3 is mediated predominantly by Chk2, while Chk1 may exclusively phosphorylate Tlk kinases (Bartek and Lukas, 2003). The most striking difference is observed in the biological requirements for Chk1 and Chk2. Whereas Chk1 knockout results in acute cell death in mammalian cells and embryonic lethality in mice, Chk2 (-/-) mice are viable, fertile, and appear to exhibit near-normal checkpoint responses (Bartek and Lukas, 2003; Sancar et al., 2004).

#### 1.1.1.4 Checkpoint effectors

In eukaryotes, the Cdc25 family of dual-specificity phosphatases has been identified to play a major role in promoting cell cycle progression by dephosphorylating

the inhibitory threonine and tyrosine residues on the cycline-dependent kinases (CDK) (Nyberg et al., 2002). In accordance, Cdc25 is a critical target of DNA damage checkpoints that ensure that cell cycle does not progress until DNA lesions are efficiently fixed or the genomic DNA is faithfully and completely replicated (Kastan, 2001; Sancar et al., 2004). Mammalian cells have three Cdc25 isoforms (Cdc25A, Cdc25B and Cdc25C), all of which can be phosphorylated by Chk1 or Chk2. The genotoxic stress-induced phosphorylation inactivates Cdc25 proteins, by direct inhibition (Blasina et al., 1999; Uto et al., 2004), by initiating proteolytic degradation (Falck et al., 2001), or by excluding the protein from the nucleus through binding with the cytoplasmic 14-3-3 proteins (Lopez-Girona et al., 1999; Peng et al., 1997).

Another important checkpoint effector is the tumor suppressor p53, a transcription factor whose function is to arrest cell cycle at G1/S boundary, or induce apoptosis. In response to DNA damage or replication stress, p53 is phosphorylated at Ser15 by ATM/ATR and Ser20 by Chk1/Chk2 (Chehab et al., 1999; Shieh et al., 1997). Phosphorylation of p53 inhibits Mdm2-dependent proteolysis and nuclear export, causing increased levels of p53, which in turn results in transcriptional upregulation of the CDK inhibitor p21/CIP1/WAF1, or proapoptotic BAX, PUMA, NOXA and BID, leading to G1/S arrest or apoptosis respectively (el-Deiry et al., 1993; Harper et al., 1993; Haupt et al., 2003). It is noteworthy that although Chk1/Chk2-dependent Cdc25A inhibition is also shown to participate in the G1/S checkpoint, it only weakly arrests G1/S transition at the initiation stage, and the subsequent maintenance stage of arrest is attributable mainly to p53 activation, which is more robust and could last for hours (Bartek and Lukas, 2001).

Besides these well-documented DNA damage-responsive proteins, recent studies have identified more and more other proteins as checkpoint effectors as well. Wee1 and Mik1, two tyrosine kinases that inhibit the CDK Cdc2, have been shown to be phosphorylated and positively regulated by Chk1 in *S. pombe*, presumably to corroborate the G2/M checkpoint performed by the previously known Cdc25 downregulation (Baber-Furnari et al., 2000; O'Connell et al., 1997; Raleigh and O'Connell, 2000). In addition, ATM/ATR-dependent phosphorylation of DNA repair proteins SMC1, BRCA1, FANCD2, NBS1 and RPA, likely plays an important role in the activation of the intra-S phase checkpoint and downstream repair processes (Sancar et al., 2004).

### 1.1.2 The DNA damage repair pathways

The canonical model of the eukaryotic DNA damage response holds that as a surveillance system, the cell cycle checkpoints monitor DNA lesions and delay or arrest cell cycle progression in the presence of DNA damage, allowing sufficient time for the downstream maintenance system to repair the damaged DNA, which is essentially a huge collection of DNA repair pathways. However, it should be mentioned that although cell cycle checkpoints are seemingly dedicated for cell cycle arrest, they may also play a direct role in recruiting or participating in DNA repair machinery, as indicated by the observation that checkpoint-deficient cells pulse-arrested by drugs are not as viable as similarly treated wild-type cells after infliction of DNA damage (Nyberg et al., 2002).

Evolution has armed eukaryotic cells with an abundance of DNA repair pathways to cope with the daunting complexity of DNA damages. Based on their natures, the

various DNA lesions are classified into covalent changes in DNA structure and noncovalent anomalous structures. The former class of DNA damage includes cross-links, DNA base and backbone damages, and are primarily processed by the DNA repair and recombination pathways; the latter includes base substitution and insertion-deletion mismatches arising from non-perfect DNA replication and homologous recombination, and is targeted mainly by the mismatch repair pathways (Figure 1-3) (Kunkel and Erie, 2005; Sancar et al., 2004; Schofield and Hsieh, 2003).

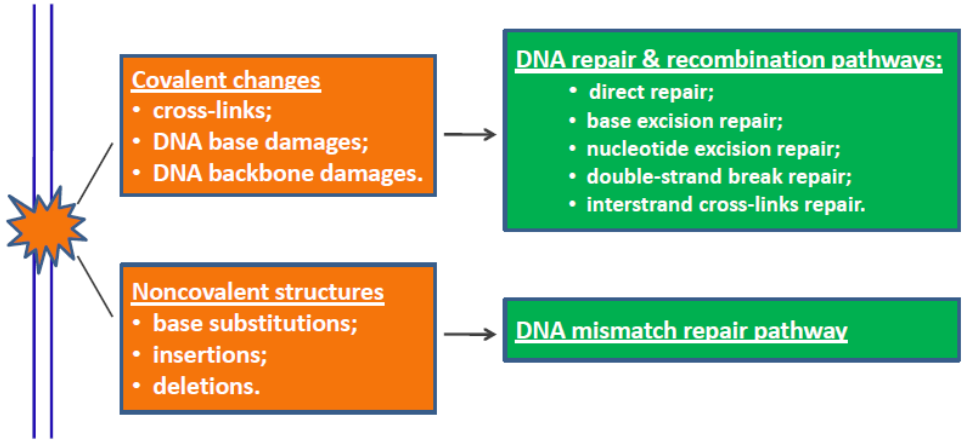


**Figure 1-3: Overview of the eukaryotic DNA repair pathways.**

Eukaryotic cells employ distinct repair pathways to cope with different types of DNA damage. Based on the nature of the DNA damage, various DNA lesions are classified into covalent changes and noncovalent structures, which are repaired primarily by DNA repair and recombination pathways and DNA mismatch repair pathways respectively.

Despite the types of DNA damages and the repair pathways that are activated to fix the lesions, these DNA repair proteins are commonly organized at sites of DNA damage as mega-Dalton protein complexes, termed DNA damage foci, which are essentially the DNA repair centers that do not resolve until the repair process is

completed (Dellaire and Bazett-Jones, 2007; Lisby and Rothstein, 2004). Accumulating evidence also suggests that these DNA damage-induced protein foci are far from static and undergo tight spatiotemporal control, likely reflecting the fact the DNA damage repair is a complicated multistep process which is delicately orchestrated by the various DNA repair proteins (Lisby et al., 2004).

#### 1.1.2.1 DNA repair and recombination pathways

The DNA repair and recombination pathways are mobilized when cells are inflicted with DNA damaging reagents that produce covalent alterations in DNA structure. These changes may include base damages, such as reduced or oxidized bases produced by reactive oxygen species or by IR or UV irradiation; bulky or alkyl base adducts by various DNA damaging chemicals; and cyclobutane pyrimidine dimers and (6–4) photoproducts specifically by UV irradiation. Some DNA damaging reagents may damage DNA backbones, including single- and double-strand DNA breaks, induced primarily by oxidative damage and IR, and abasic sites, caused by spontaneous degeneration of unstable base adducts or by base excision repair. In addition, cross-links may occur interstrandedly or even between DNA and proteins (Sancar et al., 2004).

Depending on the types of DNA damage, different pathways are deployed to repair the various DNA lesions. Five DNA repair pathways have been defined and studied so far, which include direct repair, base excision repair, nucleotide excision repair, double-strand break repair, and repair of interstrand cross-links. Different pathways employ distinct set of specialized repair proteins to resolve corresponding DNA lesions (Table 1-2). Of special note is that DNA double-strand breaks, the most

lethal form of DNA damage, can be repaired by two independent pathways: homologous recombination (HR) or nonhomologous end-joining (NHEJ). However, it should be mentioned that despite this apparent division of labor there are extensive crosstalks among various DNA repair pathways and some critical proteins, such as RPA, may participate in multiple repair pathways.

**Table 1-2 The eukaryotic DNA repair proteins (excerpted from Sancar, Lindsey-Boltz et al. 2004)**

| DNA Repair pathways | DNA repair proteins |
|---|---|
| direct repair | methylguanine methyltransferase, DNA photolyase |
| base excision repair | DNA glycosylases, APE1 endonuclease, XRCC1(Lig3) , Polβ/δ/ε, PCNA, FEN1 and Lig1 |
| nucleotide excision repair | RPA, XPA, XPC, TFIIH, XPB, XPD, XPG, XPF ERCC1, PCNA, RFC and Polδ/ε |
| cross-link repair | XPF, ERCC1, RPA, Rad51, Rad52, XRCC2, XRCC3, MUS81, MMS4, BRCA1, BRCA2, FANC-A, -C, -E, -F and Polη |
| homologous recombination repair | MRN, Rad52, Rad54, Rad55, Rad57, BRCA1, BRCA2, RPA, MUS81 and MMS4 |
| nonhomologous end-joining repair | Ku70, Ku80, DNA-PK, MRN, RPA and XRCC4 |

### 1.1.2.2 DNA mismatch repair pathways

The evolutionarily conserved DNA mismatch repair (MMR) pathways guards against mispaired bases in the genomic DNA that arise from errors in replication and homologous recombination, or as a result of DNA damage. The importance of the MMR in mammals is explicit by the observation that individuals deficient in MMR have an elevated rate of spontaneous mutation, frequently exhibit microsatellite instability and increased predisposition to cancer, most notably the hereditary nonpolyposis colorectal cancer. (Peltomaki, 2001; Schofield and Hsieh, 2003).

In comparison to that in the prototypal *E. coli.*, the eukaryotic MMR performs similarly stepwise repair upon detection of mismatches, but the proteins involved in this process are more specialized and may differ depending on the nature of the mismatch and the substrate for excision. The initiation step involves recognition of a mismatch by MSH2/MSH6 (MutS$\alpha$) and MSH2/MSH3 (MutS$\beta$) complexes, followed by association of a so-termed matchmaker complex MLH1/PMS2 (MutLα), which in turn recruits other important processing/repair factors including EXO (5'→3' exonuclease), PCNA, Pol $\delta$ and $\varepsilon$, and DNA ligase for the subsequent steps of excision, resynthesis and ligation (Figure 1-4). It is worthy of note that this is only a simplified model and the mammalian MMR is more complicated and involves a greater number of more specialized MMR proteins, presumably adapted to dealing with mismatches in the far more complex genomes (Schofield and Hsieh, 2003).

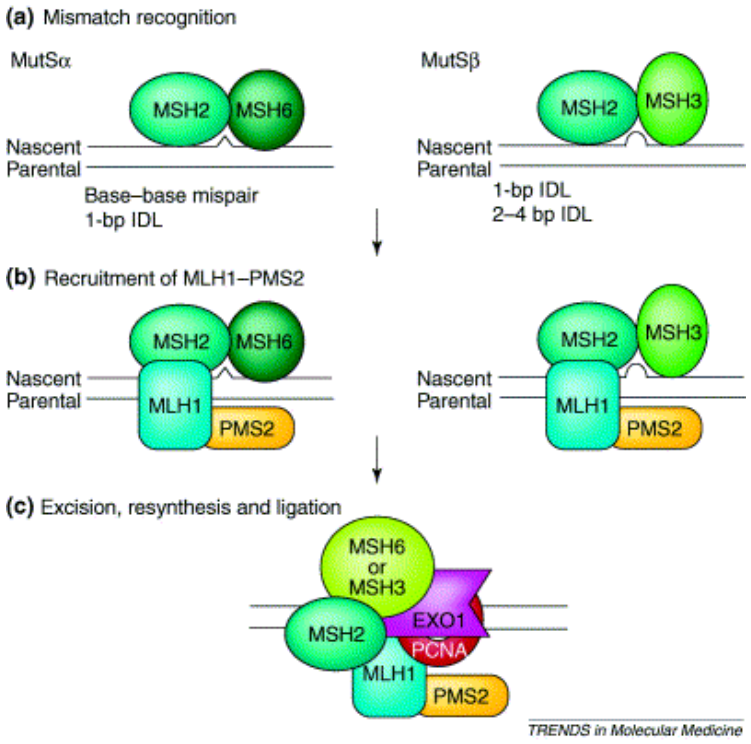


**Figure 1-4: Stepwise model for mammalian DNA mismatch repair.**

(a) Various types of mismatches are recognized by MSH2/MSH6 (MutSα) and MSH2–MSH3 (MutSβ) complexes; (b) The matchmaker protein complex MLH1/PMS2 (MutLα) is subsequently recuited to sites of mismatch to activate downstream repair events; (c) The 5′ to 3′ exonuclease, EXO1, is recruited to process the mismatches, followed by translocation of PCNA to recruit Pol δ and ε for resynthesis, and of ligase to terminate the repair process. (from(Wei et al., 2002)

## *1.2 The recovery process from the DNA damage response*

Cells activate checkpoints to arrest cell cycle, mobilize DNA repair processes when exposed to DNA damages, but after DNA repair has been complete, the checkpoint arrest signal need to be turned off so that cells can re-enter the cell cycle. This is essentially the recovery process from DNA damage response. Until recently little was known about the molecular basis of such a process.

The last five years have witnessed a great advancement in our understanding of the recovery process. It has been shown that in eukaryotic cells, there exist at least two major mechanisms that operate to extinguish the activated DNA damage response following release from genotoxic stresses. One is through dephosphorylation and deactivation of important checkpoint/repair proteins by various protein phosphatases, most notably the serine/threonine families of phosphatases reportedly including PP2A, Wip1/PPM1D, PP1 and PP4; the other is via the ubiquitin/proteasome-mediated degradation of some critical checkpoint proteins (Bartek and Lukas, 2007; Harrison and Haber, 2006).

It is mentionworthy that unicellular yeasts utilize a special mechanism, termed checkpoint adaptation, to survive a long-term exposure to genotoxic stress by re-entering the cell cycle even in the presence of high levels of DNA damage, which shares some, but not all, of the features with the recovery process (Bartek and Lukas, 2007; Harrison and Haber, 2006). This topic will also be discussed in this section.

### 1.2.1 The recovery mechanism involving protein dephosphorylation

Upon genotoxic stresses, a primary response of eukaryotic cells is the stimulation of two parallel functional modules composed of two pairs of kinases: ATM-Chk2 and ATR-Chk1, which in turn phosphorylate and activate a great variety of downstream checkpoint/repair proteins, including Rad17, Nbs1, BRCA1, H2AX, RPA32, Cdc25 and p53, facilitating assembly of DNA repair centers (foci) at the sites of DNA damage or causing alteration of their enzymatic or transcriptional activities, ultimately leading to cell cycle arrest, apoptosis or senescence (Abraham, 2001; Kastan and

Bartek, 2004). Due to the striking prevalence of the genotoxic insults-induced phosphorylation, it appears that ATM/ATR/Chk1/Chk2-dependent phosphorylation of their downstream checkpoint/repair proteins plays a dominant theme in the transduction and execution of the DNA damage response (Figure 1-5).

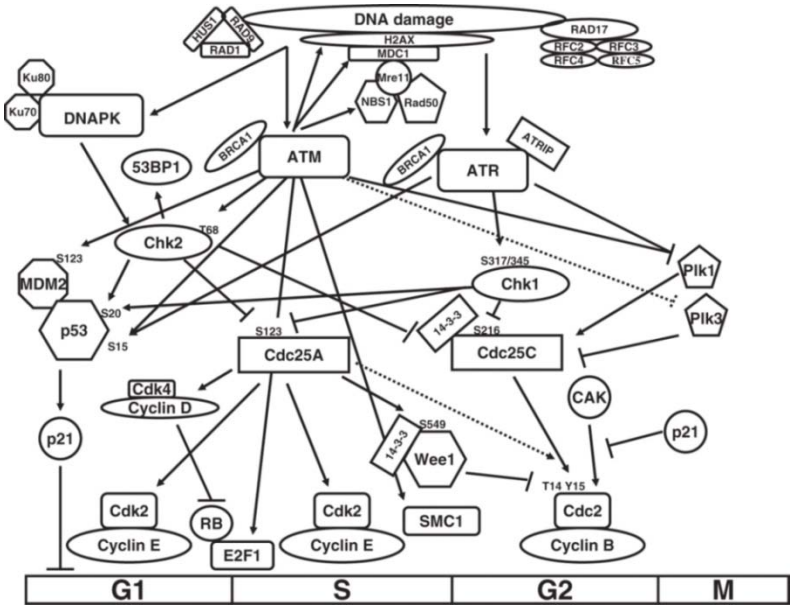


**Figure 1-5: Two kinase pairs dominate DNA damage-induced phosphorylation.**

The ATM-Chk2 and ATR-Chk1 control the DNA damage response by extensive phosphorylation of their downstream checkpoint/repair proteins, which include Rad17, Cdc25A, Cdc25C, p53, MDM2,53BP1, NBS1, MDC1, H2AX, Plk1, BRCA1 and many more. (from (Niida and Nakanishi, 2006))

Given the critical role protein phosphorylation plays in the activation of the DNA damage response, it seems plausible that when cells are recovering, the reversal of this process, protein dephosphorylation, might help to extinguish the DNA damage response. This idea has been supported by several recent findings, which have shown that several PPP (phosphoprotein phosphatase) and PPM (metallo-dependent protein phosphatase;

PP2C) families of serine/threonine (Ser/Thr) protein phosphatases, including PP1, PP2A, PP4 and Wip1/PPM1D, are involved in the recovery process from DNA damage response by dephosphorylating several critical checkpoint/repair proteins such as Chk1, p53, UNG2 and γ-H2AX.

In eukaryotes, removal of the phosphate group from phosphorylated proteins is catalyzed by three families of protein phosphatases. Besides the above mentioned PPP and PPM families of Ser/Thr phosphatases, the PTP family of Tyr phosphatases specifically targets proteins with phosphorylation occurring at tyrosine residues (Barford et al., 1998). Since DNA damage-induced phosphorylation of checkpoint and repair proteins occurs predominantly on serine and threonine residues, only PPP and PPM families of Ser/Thr phosphatases are discussed in this part.

#### 1.2.1.1 PPP family of protein phosphatases

In mammals, the PPP family of Ser/Thr phosphatases include seven members (PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7) (Moorhead et al., 2009). They share a highly conserved catalytic domain but are divergent in the noncatalytic region, which may determine their associated regulatory subunits and define their distinctive activities and functions. Among these, five members (PP1, PP2A, PP4, PP5 and PP6) exhibit a significant level of sensitivity to okadaic acid (OA), and they can be further differentiated by the fact that PP1 is not as OA-sensitive as other four phosphatases, which are collectively known as PP2A-like protein phosphatases (Barford et al., 1998). To date, PP1, PP2A, PP4 and PP5 have been found to participate in the DNA damage response and will be covered here.

PP1 can dephosphorylate a variety of substrate proteins in various cellular processes. The substrate specificity is reportedly determined by the ~ 50 different regulatory/targeting subunits with which the same catalytic subunit (PP1c) is complexed. Association with these distinct regulatory subunits converts PP1c into many different forms, attributing it with distinct substrate specificities, restricted subcellular locations and diverse regulations (Cohen, 2002). It has been demonstrated that a consensus sequence (R-V-x-F), termed RVxF motif, of various regulatory/targeting subunits mediates their binding with PP1c (Cohen, 2002). In some cases, however, PP1 has been shown to bind directly to substrates that share this RVxF motif, which include pRb (Vietri et al., 2006) and Xenopus CDC25 (Margolis et al., 2003).

In contrast with the heterodimeric composition of PP1, PP2A consists of three subunits. In addition to the catalytic subunit (C subunit) and regulatory/targeting subunit (B subunit) which are similar to PP1, PP2A needs another structural subunit (A subunit) to form a holoenzyme (Figure 1-6). Similar with PP1, PP2A also relies on the various regulatory B subunits for its diverse activities and functions. To date, four different subfamilies of B subunits have been identified in mammals, termed B, B', B'' and B''' respectively, which essentially associate with the PP2A core A/C dimer in a mutually exclusive manner (Janssens and Goris, 2001). Further molecular cloning has revealed that multiple isoforms exist for each of the mammalian PP2A subunits. Therefore, given all of the different subfamilies of the B subunit plus the various isoforms found in each subunit (A, C and B-B'''), the possible combinations for the PP2A holoenzymes are dauntingly huge (Figure 1-6). This high level of complexity in its composition underlies

the diverse functions of PP2A in dephosphorylation of myriad substrates in various signal transduction pathways in a wide array of cellular processes.

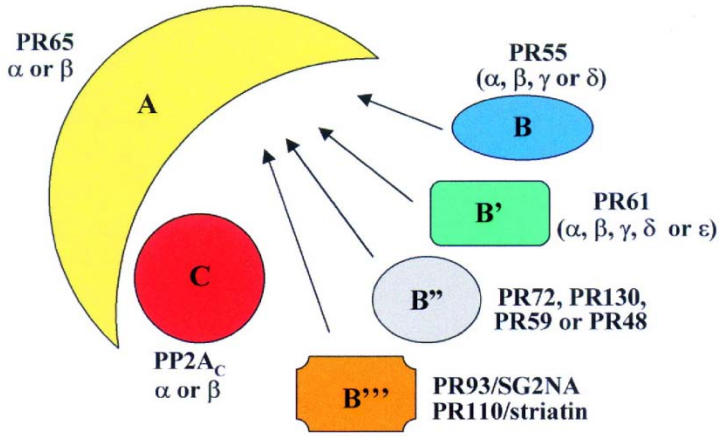


**Figure 1-6: Complex composition of mammalian PP2A.**

The heterotrimeric PP2A is composed of structural A subunit, catalytic C subunit and various regulatory/targeting B subunits, which include four subfamilies (B, B', B'' and B'''). Each subunit contains several isoforms. (from (Janssens and Goris, 2001))

PP4 and PP5 are both expressed ubiquitously in the cell yet in low abundance. Like PP1 and PP2A, the interplay between PP4 catalytic subunit (PP4c) and various regulatory subunits is central to the mode of action of PP4 (Cohen et al., 2005). By contrast, however, PP5 does not seem to have regulatory subunits but is instead modulated by its N-terminal tetratricopeptide repeats (TPR) domain and a short C-terminal segment, which both inhibit PP5 phosphatase activity in normal conditions and may mediate interaction with other proteins, determining the subcellular localization and substrate specificity (Chinkers, 2001).

### 1.2.1.2 PPM family of protein phosphatases

The PPM (PP2C) family phosphatases are $Mn^{2+}$/$Mg^{2+}$-dependent Ser/Thr protein phosphatases. In contrast with most PPP members, PPM phosphatases are resistant to OA and do not have additional subunits to regulate their activities (Barford et al., 1998). To date, eighteen PPM phosphatases have been identified in human, all being monomeric and depending on the various modular domains at the N- and C-terminal extensions to localize the enzymes to specific locations and substrates (Moorhead et al., 2007).

The Wild-type p53-induced phosphatase 1, Wip1 (or PP2Cδ, PPM1D), is an important member of PPM phosphatases that has been demonstrated to play a crucial role in the DNA damage response. Unlike other constitutively expressed Ser/Thr phosphatases such as PP1, PP2A, PP4 and PP5, Wip1/PPM1D is induced in a p53-dependent manner upon exposure to genotoxic stress, possibly operating to reverse the DNA damage-induced phosphorylation cascade as a homeostatic regulator (Lu et al., 2008). Quite a few DNA damage-responsive proteins have been found to be substrates by Wip1/PPM1D, which include the stress-response kinase p38 mitogen-activated protein kinase (MAPK), the base-excision-repair protein UNG2, as well as other important checkpoint regulators Chk1, Chk2, p53 and ATM (Moorhead et al., 2007). It is speculated that Wip1/PPM1D may selectively dephosphorylate these checkpoint/repair proteins by specifically targeting two DNA damage-responsive motifs: pTXpY and pS/pTQ (Lu et al., 2008).

### 1.2.1.3 Phosphatases involved in checkpoint recovery

Accumulating evidence has indicated that protein dephosphorylation by PPP and PPM families of Ser/Thr phosphatases plays a critical role in the recovery process from the DNA damage response.

In *Schizosaccharomyces pombe*, release from G2 checkpoint arrest is reportedly controlled by dephosphorylation (at S345) and inactivation of Chk1, which is mediated by Dis2, a yeast homologue of PP1 (den Elzen and O'Connell, 2004). In *Saccharomyces cerevisiae*, dephosphorylation of Rad53 (Chk2 orthologue) by two protein phosphatases, Pph3‑Psy2 (PP4-R3 homologue) and Ptc2/Ptc3 (two homologues of Wip1/PPM1D), appears to play a non-redundant role in the deactivation of Rad53 and the consequent recovery from checkpoint arrest induced by DNA damaging reagents (Leroy et al., 2003; O'Neill et al., 2007). PP1 may also play an import role in the release from G2 arrest in *Xenopus* oocytes and human cells, possibly through the direct dephosphorylation of Cdc25 on S287 in *Xenopus* and human Cdc25C on S216 (Margolis et al., 2003). In humans, it has been reported that re-entry into the cell cycle after the DNA damage response depends heavily on Wip1/PPM1D, which can reportedly dephosphorylate ATM, Chk1, Chk2, and p53 (Fujimoto et al., 2006; Lu et al., 2005; Shreeram et al., 2006). In other reports, however, dephosphorylation of these important checkpoint proteins may also be mediated by PP1 and/or PP2A (den Elzen and O'Connell, 2004; Dozier et al., 2004; Goodarzi et al., 2004; Li et al., 2006; Liang et al., 2006).

Besides the critical role in checkpoint recovery, protein dephosphorylation is also necessary for the efficient DNA repair in cells recovering from the DNA damage response. For example, completion of the BRCA1-dependent DSBs repair appears to

require dephosphorylation of BRCA1 by PP1 (Hsu, 2007; Yu et al., 2008). Removal of γ-H2AX, the phosphorylated histone H2AX generated at the site of DNA DSBs whose function is to stabilize DNA repair foci, is mediated by PP2A or PP4 and functions to facilitate repair of DNA DSBs and allows subsequent resumption of DNA replication (Chowdhury et al., 2005; Chowdhury et al., 2008). Dephosphorylation of UNG2, a uracil DNA glycosylase essential for the base excision repair, is performed by Wip1/PPM1D, possibly to facilitate its inactivation after completion of DNA repair (Lu et al., 2004).

Based on the above findings, it seems that the concerted actions of multiple Ser/Thr protein phosphatases, especially PP1, PP2A, PP4 and Wip1/PPM1D, are critical in the termination of the eukaryotic DNA damage response. The biological significance of the use of multiple phosphatases over some checkpoint/repair proteins is unclear, yet it is proposed that it may confer the eukaryotic cells with both increased control and extra options for how and when a damaged cell re-enter the cell cycle.

### 1.2.2 The recovery mechanism involving protein degradation

Although protein dephosphorylation plays an essential role in the recovery from DNA damage response, it is arguably a passive process and insufficient to account for the entire recovery process. Recent work has demonstrated the existence of another active mechanism that involves ubiquitin/proteasome-mediated degradation of some of the critical checkpoint regulators, which might complement protein dephosphorylation for the efficient recovery from DNA damage response (Bartek and Lukas, 2007).

### 1.2.2.1 Chk1 degradation in S phase checkpoint recovery

The S-phase DNA replication is under continuous surveillance by checkpoint regulators ATR and Chk1. In response to DNA damaging agents that cause replication stress, ATR phosphorylates Chk1 at S317 and S345, stimulating its kinase activity, and the activated Chk1 in turn delays S phase progression by blocking the firing of DNA replication origins through phosphorylation of the CDK (Cdk2) activator Cdc25A, which primes it for ubiquitination and subsequent proteolytic degradation (Bartek et al., 2004).

In a recent report, however, a novel mechanism was unraveled such that ATR-mediated Chk1 phosphorylation, most notably on S345, not only leads to Chk1 activation, but also marks it for polyubiquitination and proteasome-dependent degradation, a process that is mediated by E3 ligase complexes containing Cul1 or Cul4A (Figure 1-7) (Zhang et al., 2005b). It was speculated that this mechanism may serve as an necessary complement to the Wip1/PPM1D-mediated Chk1 dephosphorylation/deactivation in the process of checkpoint recovery. Although Chk1 dephosphorylation could turn off Chk1 activation and the corresponding checkpoint response it drives, the effect is transient and the kinase activity of Chk1 could readily be turned on again via the ATR-dependent phosphorylation in the presence of unresolved DNA lesions. By active degradation of the stimulated Chk1, however, cells could limit the duration of replication checkpoint signaling, and prevent the active protein kinase from accumulating to levels that would be deleterious to normal S-phase progression. In cases such as prolonged or high-intensity replication stress, however, the active degradation of Chk1 may result in irreversible damage to the stalled replication forks, which could culminate in cell death (Zhang et al., 2006).

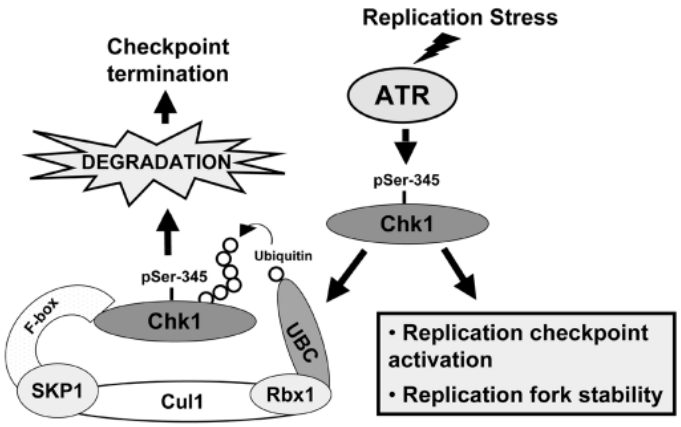


**Figure 1-7: Chk1 activation and degradation induced by replication stress.**

In response to replication stress, ATR phosphorylates Chk1 at S345, leading to activation of this protein kinase and activation of the replication checkpoint. Chk1 phosphorylation at S345 simultaneously marks Chk1 for ubiquitination by Cul1-containing E3 ligases, and ultimately targets Chk1 for degradation by the proteasome, resulting in checkpoint termination. (from (Zhang et al., 2006))

### 1.2.2.2 Claspin/Wee1 degradation in G2 checkpoint recovery

The G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. In the setting that ATR is activated, G2 checkpoint is mediated primarily by Chk1, whose stimulation requires the critical mediator protein-Clapsin. Activated Chk1 inhibits the entry into mitosis by downregulating Cdc25A and upregulating the mitosis-inhibiting kinase Wee1, which together suppress Cdc2/CyclinB activity and block the mitotic entry (Sancar et al., 2004).

Recently, it was shown that recovery from the G2 checkpoint may involve proteolytic degradation of Claspin and Wee1 (Figure 1-8) (Bartek and Lukas, 2007). While the removal of Claspin eliminates the Chk1 coactivator, resulting in the upregulation of the Cdc25A phosphatase, destruction of Wee1 essentially extinguishes a

direct CDK inhibitor. Both processes remove the suppression on the Cdc2/CyclinB complex, allowing the mitotic re-entry (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Degradation of Claspin/Wee1 is mediated by $SCF^{\beta Trcp}$ ubiquitin ligase, which specifically recognizes Claspin and Wee1 following their phosphorylation by the recovery-associated protein kinase-the polo-like kinase 1 (Plk1) (Figure 1-8) (van Vugt et al., 2004).

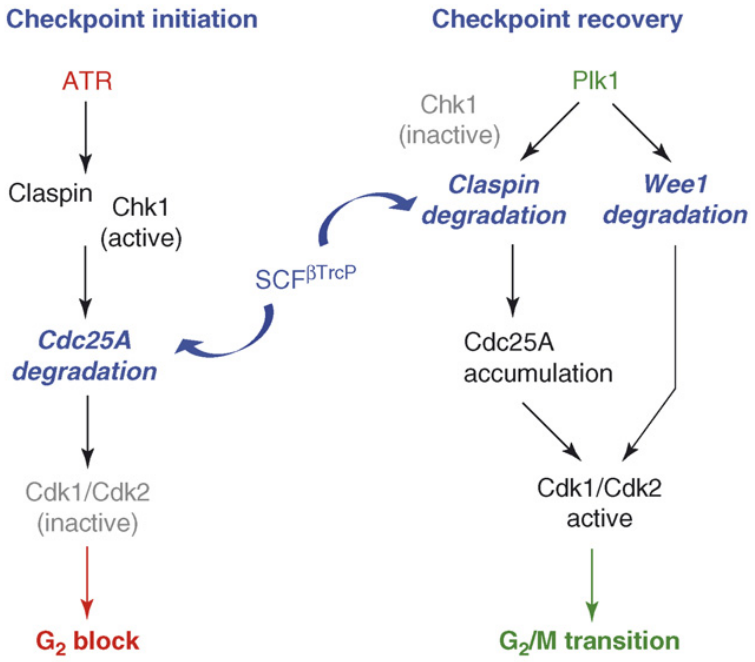


**Figure 1-8: Claspin/Wee1 degradation in G2/M checkpoint recovery.**

Upon DNA damage that induces G2/M checkpoint, Chk1 is activated by ATR and its coactivator Claspin, which in turn earmarks Cdc25A for degradation in an $SCF^{\beta TrcP}$ dependent manner. During checkpoint recovery, the same $SCF^{\beta TrcP}$ enzyme targets Claspin and Wee1 for proteolytic degradation, which is primed by phosphorylation by the kinase Plk1. Destruction of both proteins removes the suppression on the CdKs (Cdk1/Cdk2), allowing the mitotic re-entry. (from (Bartek and Lukas, 2007))

### 1.2.3 Checkpoint adaptation

Checkpoint adaptation, originally identified in the *Saccharomyces cerevisia* (Toczyski et al., 1997), refers to a special checkpoint recovery mechanism by which cells re-enter into cell cycle despite the persistence of unresolved DNA damages. Although this mechanism might confer on the unicellular yeast a growth advantage to survive a sustained cell cycle arrest, it had once been deemed unlikely to exist in cells of higher organisms due to its strong potential for introducing genomic instability. Recent studies, however, have argued otherwise. Adaptation to persistent genotoxic stress has been uncovered in *xenopus* and even in human cells, which were exposed to chronic DNA replication block or persistent ionizing irradiation (Syljuasen et al., 2006; Yoo et al., 2004).

Early studies over yeast adaptation have suggested that several groups of proteins are involved in this process, which include checkpoint regulators Ptc2, Ptc3, Cdc5 (Polo kinase), Ckb1 and Ckb2 (casein kinase II subunits), and HR proteins Ku70, Ku80, Rad51, Srs2 helicase, and Sae2 (Harrison and Haber, 2006). Recently, it was demonstrated that the mitotic re-entry of adapted cells facing persistent genotoxic challenge is facilitated by resumed activity of Plx1/Plk1 and inhibition of the Chk1 kinase, in an process involving Claspin (Syljuasen et al., 2006; Yoo et al., 2004).

So far it is still unclear why adaptation exists in higher organisms, although it was speculated that checkpoint adaptation in higher eukaryotes may facilitate the elimination of cells containing irreparable DNA damage through mitotic catastrophe (Bartek and Lukas, 2007).

# 2 Replication protein A (RPA) Dephosphorylation by PP2A is Required for Efficient DNA Damage Repair

## *2.1 Introduction*

Single-stranded DNA (ssDNA) is a ubiquitous yet important intermediate during all types of eukaryotic DNA metabolism known to date, which include DNA replication, homologous recombination (HR), DNA damage checkpoints and most, if not all, of the major DNA repair pathways (Zou et al., 2006). Thus, it is highly demanded that the intermediate ssDNA structure be protected from unwanted attacks by constantly present environmental and endogenous insults (most notably the nearly omnipresent endonucleases), and maintained at its unwound state to allow for the assembly of various functional apparatuses and the subsequent occurrence of critical DNA metabolic reactions. In both prokaryotes and eukaryotes, there exist several important ssDNA-binding proteins to perform these critical functions.

### 2.1.1 Structure of Replication Protein A (RPA)

The evolutionally conserved RPA complex is the major ssDNA-binding protein in eukaryotes (Wold, 1997). Similar to its orthologues in other organisms, human RPA is a heterotrimeric protein complex composed of the 70kDa, 32kDa and 14kDa subunits (referred to RPA70, RPA32 and RPA14), each consisting of at least one OB-fold (oligo-saccharide/oligonucleotide binding fold) domain commonly found in proteins with ssDNA-binding activities (Figure 2-1) (Binz et al., 2004). The DNA binding activity is mainly attributed to its RPA70 subunit, which contains as many as four OB domain. The RPA32 subunit has one OB domain and only display weak DNA binding activity, but its

N-terminal domain is of special attention, which exists in an extended, flexible conformation, and is demonstrably the functionally important phosphorylation domain and will be addressed in much detail in the following part. Despite the presence of one OB domain, RPA14 does not bind with DNA but is instead necessary for the formation of RPA heterotrimeric complex.

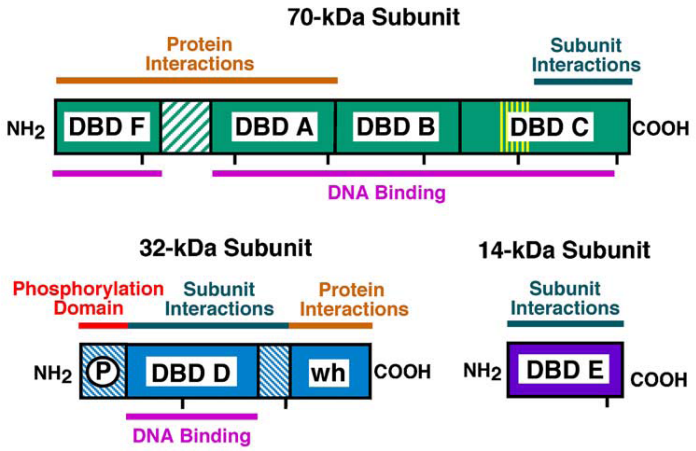


**Figure 2-1: Function domains of human RPA complex.**

The human RPA complex is composed of 70-kDa, 32-kDa and 14-kDa subunits. Each subunit contains 1-4 DNA binding domains (DBD) which may account for DNA binding or protein interaction activities. It is noteworthy that RPA32 subunit carries an important phosphorylation domain at N-terminus and another C-terminal domain involved in interactions with non-RPA proteins. (from (Binz et al., 2004)).

## 2.1.2 Functions of RPA in various types of DNA metabolism

RPA has been demonstrated to play versatile yet crucial roles in all aspects of eukaryotic DNA metabolism known so far, which include DNA replication, homologous recombination, cell cycle checkpoint activation and almost all major types of DNA repair pathways (Zou et al., 2006).

RPA is required for both the initiation and the elongation steps of DNA replication in eukaryotes. RPA associates with the CDC45 origin complex after it has been loaded onto the origins of replication, possibly facilitating the initial DNA opening and the subsequent loading of DNA polymerase $\alpha$ and other replication proteins. Once the replication forks are established, RPA maintains associated as an essential factor during replication elongation (Bell and Dutta, 2002). In accordance with these critical roles in DNA replication, RPA is localized with replication centers in S-phase human cells, which exhibit as punctuate speckles (foci) if observed by epifluorescence microscopy (Dimitrova and Gilbert, 2000).

The importance of RPA in homologous recombination was indicated originally by the observation that mutation of both RPA32 and RPA70 in *S. cerevisiae* causes HR defects (Heyer et al., 1990). Later it was demonstrated that RPA binds with HR proteins Rad51/Rad52/Rad54 (Gasior et al., 1998; Tan et al., 1999), and that RPA may both positively and negatively regulate binding of Rad51 to ssDNA (Sugiyama et al., 1997). In addition, RPA reportedly stimulates the annealing activity of Rad52 and promotes formation of Rad52 (Jackson et al., 2002). Studies over a RPA70 mutant (rfa1-11) in yeast further indicated that RPA plays an essential role in strand invasion step as well as the formation of pre-synapic complex during the whole process of homologous recombination (Wang and Haber, 2004).

Besides these roles in the unperturbed physiological condition, RPA also serves critical functions when cells are challenged with genotoxic stress. Similar to many other DNA damage-responsive proteins, RPA also translocates to nuclear foci which are essentially the DNA repair centers where a large collection of various checkpoint/repair

proteins are concentrated to actively repair the DNA lesions (Golub et al., 1998). RPA has been found to interact with many of these repair-essential proteins such as Rad51 and MRN complex, and is not surprisingly involved in most, if not all, of the major eukaryotic DNA repair pathways identified so far (Binz et al., 2004; Sancar et al., 2004).

Recent evidence has suggested that RPA is beyond being a mere DNA repair protein. It is likely to be an essential yet previously oblivious DNA damage sensor that can specifically detect the presence of ssDNA and cooperate with other sensor proteins, including ATR, to initiate DNA damage signals at locations of DNA damage. RPA is reportedly required for loading of the two established DNA damage sensor protein complexes, Rad17-Rfc2-5 and the Rad9-Rad1-Hus1, to sites of DNA lesions (Ellison and Stillman, 2003; Zou et al., 2003). Most notably, ATR recruitment to DNA lesions and activation towards its downstream transducer kinase Chk1 appears also to rely on RPA, possibly through the direct interaction between RPA70 and ATRIP (Zou and Elledge, 2003). Consistent with these reports, several RPA32 and RPA70 mutants of *S. cerevisiae* display deficient activation of G1/S, intra-S and G2/M checkpoints in response to DNA damage (Lee et al., 1998; Longhese et al., 1996; Pellicioli et al., 2001). Thus RPA is an important player in the activation of various DNA damage checkpoints.

### 2.1.3 Genotoxic stress-induced RPA hyperphosphorylation

The RPA32 N-terminal unstructured segment consisting of ~40 amino acid residues represents a functionally important domain for RPA. Although it is not significantly conserved by the primary sequence, the 40 residue-segment across different species unanimously contains multiple clustered serine and threonine residues,

which have been demonstrated to undergo phosphorylation both during unperturbed cell cycle and following genotoxic stress (Figure 2-2).

The cell cycle-dependent phosphorylation of human RPA32 occurs primarily at the conserved cyclin-CDK phosphorylation sites, serine 23 (S23) and serine 29 (S29), and reportedly happens during G1/S transition and M-phase, and disappears at the end of mitosis (Zou et al., 2006). Despite this strong cell cycle correlation, it is still not clear what biological function the mitotic phosphorylation of RPA32 serves during unperturbed cell cycle, although it has been speculated that RPA32 phosphorylation in mitosis may alter its DNA-binding and protein-protein interactions (Oakley et al., 2003), and may primes RPA32 to undergo additional phosphorylation events in response to genotoxic stress (Anantha et al., 2008).

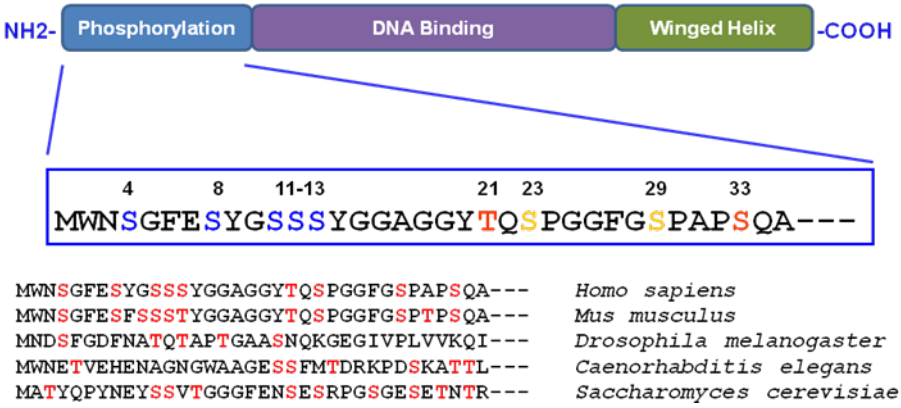


**Figure 2-2: The N-terminal phosphorylation domain of RPA32**

Top: The N-terminal domain is functionally important in human RPA32. Except for S23 and S29 (yellow), which are targeted by Cdk2 and undergo cell cycle-dependent phosphorylation, other residues are phosphorylated in a DNA damage-inducible manner. Among those, T21 and S33 (red) are phosphorylated by PIKKs, while the rest (S4, S8 and S11-13, blue) are putative or proved phosphorylation residues by yet-to-be-identified kinases. Bottom: Cross-species analysis over RPA32 sequence identifies clustered S/T residues (red) that are phosphorylated upon DNA damage in manners similar to those in human RPA32.

Of special note is the genotoxic stress-induced RPA32 hyper-phosphorylation. Upon DNA damage or replication stress, at least seven serine or threonine residues (S4, S8, S11-13, T21 and S33) of the human RPA32 subunit undergo extensive phosphorylation. Among those, the SQ/TQ motif-containing T21 and S33 are shown to be phosphorylated by PIKK kinases ATM, ATR, and DNA-PK. The identities of the kinases responsible for the remaining residues (S4, S8 and S11-13), however, still remain to be determined (Figure 2-2) (Binz et al., 2004; Zou et al., 2006).

It has been demonstrated that RPA32 hyperphosphorylation plays an important role in the DNA damage response. Phosphorylation of RPA32 stimulates genotoxic stress-induced interaction with two critical checkpoint/repair complexes, Mre11-Rad50-Nbs1 and Rad9-Hus1-Rad1, and may also promote the recruitment of the DSBs repair proteins Rad51/Rad52 to sites of DNA damage (Robison et al., 2005; Wu et al., 2005a; Wu et al., 2005b). While competent to translocate to locations of DNA lesions, phosphorylated RPA is unable to associate with replication centers and thus may function to mediate S-phase checkpoint by suppressing DNA replication directly (Olson et al., 2006; Patrick et al., 2005; Vassin et al., 2004). The inability of phosphorylated RPA to support DNA replication is reportedly due to its altered duplex DNA binding/denaturation ability and its decreased interaction with DNA polymerase $\alpha$ (Binz et al., 2003; Oakley et al., 2003; Patrick et al., 2005). It is noteworthy that during this process, ATM/ATR-dependent phosphorylation of RPA at T21 and S33 is critical, whereas phosphorylation at other sites appears to be dispensable, indicating that distinct RPA functions may be differentially regulated by phosphorylation at different sites (Olson et al., 2006).

Given the essential roles that unphosphorylated RPA plays in unperturbed cell growth and division, it is conceivable that DNA damage-induced RPA32 phosphorylation needs to be attenuated when cells are recovering. We found that the ATM/ATR-dependent phosphorylation of RPA32 at T21 and S33 is reversed by PP2A-mediated dephosphorylation. Interference with PP2A activity causes persistent RPA32 phosphorylation and increased sensitivity to a replication stress inducer hydroxyurea (HU). The PP2A catalytic subunit associates with and dephosphorylates RPA32 following HU stress. Through a model that mimics persistent phosphorylation of RPA32, we show that cells substituted with T21/S33-phosphomimetic RPA32 exhibit increased HU/UV sensitivity, but nonetheless possess normal checkpoint activation and indistinguishable resumption of DNA replication after HU release compared with wild type control cells. Further investigation demonstrates that following release from HU stress, cells with mutant RPA32 display persistent DNA damage foci containing RPA and γ-H2AX and exhibit a pronounced defect in the repair of HU-induced DNA breaks, suggesting that PP2A-mediated RPA32 dephosphorylation is required for the efficient repair of DNA lesions.

## *2.2 Materials and Methods*

**Plasmids and Mutagenesis**

The pMKO retroviral RPA32 shRNA expression vector was a generous gift from Dr. Xiaohua Wu (The Scripps Research Institute) and has been described previously (Olson et al., 2006). The expression constructs of the Flag-tagged PP1, PP2A, PP4 and PP6 catalytic subunits were kindly provided by Dr. Xin-Hua Feng (Baylor College of Medicine). The Flag-

PP5 expression construct was described previously (Ali et al., 2004). The C-terminally tagged full-length Flag-RPA32 was generated by subcloning into the pcDNA3 vector (Invitrogen) a PCR product containing the RPA32 coding sequence from p3a-RPA32, a generous gift from Dr. Marc Wold (University of Iowa). The primers used for PCR were: FLAG/C-tagged-F 5'-*GGA TCC ACC ATG TGG AAC AGT GGA TTC GAA*-3' and FLAG/C-tagged-R 5'-*GAA TTC TTA CTT GTC ATC GTC ATC CTT GTA ATC CCC TCC TTC TGC ATC TGT GGA TTT AAA ATG G*-3'. The PCR product was digested with *EcoR*I and *BamH*I and subcloned into pcDNA3 vector. The resulting pcDNA3-RPA32-Flag was further used as template to introduce the T21V/S33A, T21D/S33D substitutions after several rounds of mutagenesis following the QuikChange™ Site-Directed Mutagenesis protocol (Stratagene). The primers used for mutagenesis were: RPA32-T21V/S33A-F 5'-*TAC GGG GGA GCC GGC GGC TAC GTG CAG TCC CCG GGG GGC TTT GGA TCG CCC GCA CCT GCT CAA GCC GAA AAG AAA TCA*-3'; RPA32-T21V/S33A-RC 5'-*TGA TTT CTT TTC GGC TTG AGC AGG TGC GGG CGA TCC AAA GCC CCC CGG GGA CTG CAC GTA GCC GCC GGC TCC CCC GTA*-3'; RPA32-T21D/S33D-F 5'-*TAC GGG GGA GCC GGC GGC TAC GAT CAG TCC CCG GGG GGC TTT GGA TCG CCC GCA CCT GAT CAA GCC GAA AAG AAA TCA*-3' and RPA32-T21D/S33D-RC 5'-*TGA TTT CTT TTC GGC TTG ATC AGG TGC GGG CGA TCC AAA GCC CCC CGG GGA CTG ATC GTA GCC GCC GGC TCC CCC GTA*-3'. The pcDNA3-RPA32-Flag(T21V/S33A) and pcDNA3-RPA32-Flag (T21D/S33D) were used as template to further generate the S4A/S8A/S11-13A/T21V/S33A (phosphodeficient, PD) and S4D/S8D/S11-13D/T21D/S33D (phosphomimetic, PM) sub-stitution mutant. The primers used for these two mutagenesis PCR were: RPA32-5SA-F 5'-*GGA TCC ACC ATG TGG AAC GCT GGA TTC GAA GCC TAT GGC GCT GCA GCA TAC GGG GGA GCC GGC*

*GGC TA*-3'; RPA32-5SA-RC 5'-*TAG CCG CCG GCT CCC CCG TAT GCT GCA GCG CCA TAG GCT TCG AAT CCA GCG TTC CAC ATG GTG GAT CC*-3'; RPA32-5SD-F 5'-*GGA TCC ACC ATG TGG AAC GAT GGA TTC GAA GAC TAT GGC GAT GAC GAT TAC GGG GGA GCC GGC GGC TA*-3' and RPA32-5SD-RC 5'-*TAG CCG CCG GCT CCC CCG TAA TCG TCA TCG CCA TAG TCT TCG AAT CCA TCG TTC CAC ATG GTG GAT CC*-3'. Each o the above Flag-tagged RPA32 variants was subsequently cloned into the *EcoR* I and *BamH*I sites of pQCXIP (Clontech) vector.

**Transfection and Retroviral Infection**

Transient transfection in Hela cells or 293T cells was done using the FuGENE 6 (Roche), or Lipofectamine 2000 (Invitrogen) transfection kits, where appropriate, according to manufacturers' protocols. Retroviruses (pQCXIP, pMKO) were packaged in 293T cells by simultaneous transfection with the desired retroviral constructs and the Gag/Pol and VSV-G helper genes. The medium was changed 24 hours after transfection and the supernatant was then collected 24 and 48 hours later, filtered and mixed with 4μg/ml polybrene (Sigma) before addition to the target Hela cells. After incubation in the retroviral supernatants for 24 hr, cells were under appropriate drug selection.

**Cell Culture**

Hela cells and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. U2OS cells were maintained in McCoy's 5A medium with 10% FBS and antibiotics. The culture of HaCaT and HepG2 cells has been described previously (Waddell et al., 2004). Various Flag-tagged

RPA32 variants (pQCXIP) were introduced into Hela cells by retroviral infection, followed by selection of puromycin-resistant cells. The substitution lineages were further generated by silencing the endogenous RPA32 in these cell lines that ectopically express each of the mutant RPA32, which was accomplished by two rounds of infection with retroviruses (pMKO) expressing the RPA32 shRNA target sequence, followed by G418 selection. All of the above cells were grown at 37°C in 5% $CO_2$. Stable cell populations were maintained under either puromycin (2μg/ml) or G418 (800 μg/ml) selection.

**Antibodies and Reagents**

The antibodies used for immunoblotting were purchased from Abcam (rabbit polyclonal RPA32pT21), Bethyl Laboratories (rabbit polyclonal RPA32pS33, RPA32pS4/8 and Rad17pS645), Calbiochem (mouse monoclonal RPA32 and RPA70), Cell signaling (rabbit polyclonal γ-H2AX and rabbit monoclonal Chk1 pSer345), Upstate (rabbit polyclonal PP1 catalytic subunit, and mouse monoclonal PP2A/C$\alpha$), Santa Cruz (mouse monoclonal p53), and Sigma-Aldrich (mouse monoclonal Flag and γ-tubulin). The reagents used in this study include: hydroxyurea, okadaic acid, and caffeine (all from Sigma-Aldrich) and MG-132 (BioMol).

**Immunoblotting and Immunoprecipitation**

For immunoblotting, cells were lysed in NETN (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) supplemented with protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotonin) and phosphatase inhibitors (20 mM β-

glycerophosphate, 0.5 μM okadaic acid). Cell lysates were kept on ice for 10 min and cleared by full-speed centrifugation for 10 min at 4ºC, followed by quantitative determination of the protein concentrations using the modified Bradford protein assay kit from Bio-Rad. Lysates were then resolved in SDS-PAGE, transferred to Immobilon-P PVDF membrane and blotted with appropriate primary and secondary antibodies. For examination of the interaction between PP2A/C and RPA32, Hela cells were harvested with the previously described PP2A-coIP buffer (Arnold and Sears, 2006) (except that it contained 0.5% Nonidet P-40) containing protease/phosphatase inhibitors. Lysates were precleared by centrifugation, followed by sequential incubation with anti-RPA32 antibody for 4 hr and protein A beads (Calbiochem) for 1 hr. After extensive wash with lysis buffer, the immunoprecipitates were analyzed by immunoblotting with anti-RPA70 and anti-PP2A/Cα antibodies. The control mouse IgG was from Santa Cruz Biotechnology, Inc.

**Immunofluorescence Microscopy**

Cells were sparsely plated onto glass coverslips in six-well plates and incubated for overnight. Cells were then under appropriate treatments before immunofluorescence analysis. In brief, cells were washed once with PBS, and fixed/permeabilized with acetone/methanol (1:1) at -20 °C for 10 min. After rehydration in PBS for 10 min and incubation with blocking buffer (3% BSA in PBS containing 0.1% Triton X-100) for 1 hr, the cells were stained with primary antibodies in blocking buffer for 2 hr at room temperature or overnight at 4 °C, followed by three PBS washes and incubation with the appropriate secondary antibodies (Invitrogen) in blocking buffer for 1 hr at room temperature. After washing with PBS, cells were mounted with the fluorescent mounting medium containing

DAPI (Santa Cruz) and analyzed by fluorescence microscopy. For detection of incorporated BrdU, cells were incubated in medium containing 10 μM BrdU (BD Pharmingen) for 10 min prior to fixing, and the genomic DNA was denatured with 0.1 M HCl according to the manufacturer's protocol. The primary antibodies used for immunofluorescence in this study were RPA32 and RPA70 (mouse monoclonal, Calbiochem), RPA32pS33 (rabbit polyclonal, Bethyl), γ-H2AX (rabbit polyclonal, Cell Signaling, and mouse monoclonal, Upstate), PP2A/C (rabbit polyclonal, Santa Cruz), and anti-BrdU (rat monoclonal, Abcam). Experiments were performed three times and cells detected with five or more discrete foci were regarded as foci-positive.

**Silencing of PP1 or PP2A**

The siRNA oligos against the catalytic subunit of either PP1 or PP2A were purchased from Santa Cruz Biotechnology, Inc. To knockdown PP2A and PP1, approximately $2 \times 10^5$ HeLa cells were seeded per well in six-well plates. The next day, cells were transfected with the PP1 or PP2A siRNA oligos using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Cells were treated with indicated conditions and analyzed 48-72 hr post-transfection. When necessary, cells underwent two rounds of siRNA transfection, and the knockdown effect was examined by immunoblot using anti-PP1 (Upstate) and anti-PP2A (Upstate) antibodies.

***In vitro* Phosphatase Assay**

Hela cells were treated with 10 mM of HU for 6 hr and lysed by the lysis buffer mentioned above except that it lacks phosphatase inhibitors. Cell lysates were incubated on

ice for 10 min and precleared by centrifugation at 4 °C for 10min. Immunoprecipitations were performed using anti-RPA32 antibody and Protein G beads to prepare the phosphorylated RPA32 substrates, which were then incubated with the purified PP2A enzyme (Upstate) in the dephosphorylation buffer (20 mM HEPES, pH 7.0, 1 mM DTT, 1 mM MnCl2, 100 mg/ml BSA and 50 mM leupeptin). The reactions were incubated at 30 °C for 30 minutes in the presence of different concentrations of OA, and terminated by the addition of 4X SDS-PAGE loading buffer and boiling for 5 minutes. Each kinase reaction was separated on an SDS-PAGE gel, and dephosphorylation of RPA32 was analyzed by immunoblot with anti-RPA32pT21 and anti-RPA32pS33 antibodies.

**Cell Proliferation Assay**

The cell proliferation rate of various RPA32 substitution lines was evaluated by the MTS assay using the CellTiter 96 Aqueous Nonradioactive Cell-proliferation Assay Kit from Promega according to the manufacturer's instructions. Specifically, cells were plated at $1\times10^3$/well in 96-well plates and incubated in full medium for various lengths of time (1-5 days). When required, medium was replaced every three days. For the MTS assay, 20 μl of MTS reagent was added to each well and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C for 2-4 hours. The O.D. 490nm of each well was measured by spectrophotometry. The experiment was conducted in triplicates and the readings at various time points were normalized to that at Day 1.

**Clonogenic Survival Assays**

Hela cells were plated at 100 cells per ml in six-well plates or 60 mm dishes. The next day, cells were pulse-exposed to indicated doses of UV or HU (for 24 hours). After treatment, cells were rinsed twice with PBS and incubated in drug-free medium for recovery. The cultures were then incubated for 10-14 days to allow for the colonies to grow, with the medium being changed every three days. Colonies were washed once with PBS, fixed in ice-cold methanol for 10 min, followed by staining with crystal violet (0.5% w/v in methanol, Sigma) at room temperature for 10 min and subsequent washing with water. The colonies at each well or plate were counted, statistically analyzed, and normalized with untreated control. All experiments were performed in triplicates, and only colonies containing 50 or more cells were scored.

**DNA Synthesis Assays**

To evaluate the UV-induced intra-S phase checkpoint and the resumption of DNA synthesis following release from HU block, the DNA synthesis assays were performed as described (Cliby et al., 1998). Specifically, cells were plated onto 12-well plates in triplicates at appropriate densities. Cells were incubated in medium containing 20 nCi/ml of [$^{14}$C] thymidine (NEN) for 24 hr prior to UV treatment or pulse-HU exposure (0.2 mM, 24 hr). At the indicated time points after UV irradiation or release from HU block, cells were pulse-labeled with [$^{3}$H] thymidine (2.5 µCi/ml, 30 min; NEN) and harvested as described (Cliby et al., 1998). The radioactivity was determined by liquid scintillation counting, and the relative DNA synthesis rate was calculated by determining the ratio of $^{3}$H to $^{14}$C and normalization of the treated samples to the appropriate controls.

**Cell Cycle Analysis**

Hela cells were plated into 10 cm plates and incubated for 24-48 hours until the cell density reached ~70%. Cells were then pulse-exposed to indicated dose of HU for 24 hr, washed twice in PBS, and allowed to recover in drug-free medium. Cell were then harvested at various recovery time points by trypsnization and collection in 15 ml conical tubes in PBS. After fixation in 70% ethanol, cells were incubated with RNase A (100 µg/ml, Invitrogen) and propidium iodide (50 µg/ml, Sigma) for 30 min at 37°C. The DNA content for each sample was then determined with a FACScan flow cytometer (BD Biosciences) and the cell cycle distributions were analyzed by CellQuest software.

**Mitotic Index Assays**

The mitotic index assays were carried out to examine the UV-induced G2/M checkpoint and the mitosis progression following release from HU block. Specifically, cells were plated onto 10 cm plates and irradiated with UV or pulse-treated with HU the next day. At the indicated time points following treatment, cells were harvested by trypsinization and fixed in 70% ethanol at -20°C. After permeabilization in 0.25% Triton X-100 in PBS, cells were incubated with anti-phospho-histone H3 antibody (pSer10, Upstate) for 4 hr at room temperature, followed by another incubation with FITC-conjugated secondary antibody for 1 hr (Jackson ImmunoResearch). Cells were subsequently counterstained with propidium iodide, and the phospho-histone H3 fluorescence and the DNA content were determined by flow cytometry. All experiments were performed in triplicates, and the percentage of mitotic cells was calculated as the mitotic index. For examination of the G2/M checkpoint, the mitotic index was normalized to that in the unperturbed controls.

**Single-cell Gel Electrophoresis Assay (Comet Assay)**

The repair kinetics of HU-induced DNA breaks was evaluated by the CometAssay™ Kit (Trevigen) according to the manufacturer's protocol. Briefly, cells plated onto sixe-well plates were pulse-exposed to HU (0.2 mM, 24 hr), washed twice with PBS and allowed to recover in drug-free medium for various length of time (0-12 hr). At each recovery time point, cells were harvested by trypsinization, combined at an appropriate density with the pre-molten low melting agarose (at 37°C), and plated onto CometSlide™. After gelling in dark for 10 min, slides were lysed in pre-chilled Lysis Solution for 60 min, followed by incubation in Alkaline Solution for 30min. Slides then underwent single-cell gel electrophoresis in the alkaline electrophoresis buffer (200 mM NaOH, 1 mM EDTA). After washing twice in dH2O, cells were fixed in 70% ethanol, air-dried and stained with SYBR® Green I. Comets were visualized by epifluorescence on a Zeiss microscope, and the images were analyzed using the public domain software ImageJ. The repair of DNA damages at each recovery point were evaluated by quantifying the tail moment of 75 cells using the comet scoring software, CometScore (http://www.tritekcorp.com/products_cometscore.php).

## *2.3 Results*

### 2.3.1 RPA32 undergoes dephosphorylation in cells recovering from replication stress or UV irradiation.

To investigate if genotoxic insults-induced RPA32 phosphorylation is attenuated during the recovery process, we examined the kinetics of RPA32 phosphorylation in Hela cervical carcinoma cells released from replication stress induced by HU. A potent inhibitor of the ribonucleotide reductase, HU causes ribonucleotide depletion and leads to inhibition of DNA replication and subsequent accumulation of DSBs as a result of

collapse of replication forks (Grallert and Boye, 2008). As shown in Figure 2-3A, Hela cells accumulated a high level of phosphorylated RPA32 immediately before release from HU block (0.2 mM, 24 hr), as indicated by the mobility upshift of RPA32 protein as well as increase in the intensity of bands detected by the phosphospecific antibodies against RPA32 (pT21 and pS33) in comparison with unperturbed cells. Following release from HU treatment, phosphorylated RPA32 reduced gradually and dropped to near basal levels at 12 hr post-recovery, suggesting that RPA32 might undergo dephosphorylation in this recovery process (Figure 2-3A). However, this could occur

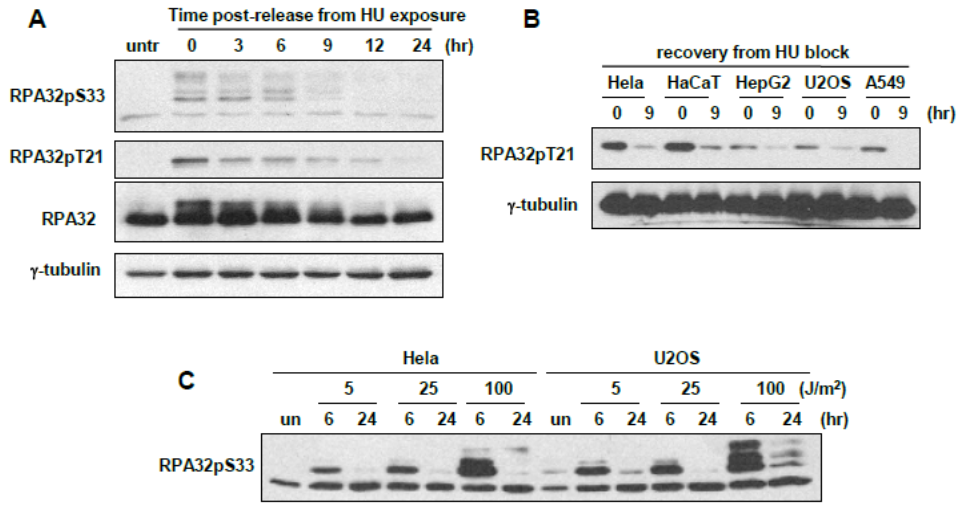


**Figure 2-3: RPA32 phosphorylation is attenuated during recovery from HU/UV.**

(A) Hela cells were pulse-treated with HU (0.2 mM) for 24 hr, washed twice with PBS, and allowed to recover in drug-free medium. The levels of phosphorylated RPA32 were detected at the indicated recovery time points by immunoblot using RPA32 total and phosphospecific antibodies(anti-RPA32pT21 and anti-RPA32pS33). (B) A panel of five different cells lines, including Hela, HaCaT, HepG2, U2OS, A549, were released from HU block (0.2 mM, 24 hr), washed and allowed to recover in drug-free medium. Levels of RPA32 phosphorylation at T21 was analyzed by immunoblot at the indicated times after recovery. (C) Hela and U2OS cells were exposed with 0-100 $J/m^2$ UV irradiation. At 6 hr or 24 hr post-irradiation, RPA32 dephosphorylation at S33 was examined by immunoblot.

specifically in Hela cells only. For this reason, we examined four other cell lines (HaCat, HepG2, U2OS and A549), which were pulse-treated with HU (0.2 mM) for 24 hr, along with Hela cells. After release, all cell lines exhibited apparent reduction of RPA32 phosphorylation at 12 hr recovery time point, suggesting that this observation is not cell line-specific (Figure 2-3B). To explore if RPA32 dephosphorylation could occur in cells recovering from genotoxic insult other than HU, Hela and U2OS cells were both irradiated with UV light. Apparent RPA32 dephosphorylation at S33 was observed 24 hr post UV exposure, indicating that this is a general phenomenon (Figure 2-3C).

It is possible that the observed decrease in the level of phosphorylated RPA32 was not due to RPA32 dephosphorylation, but instead the result of ubiquitin-dependent proteasomal degradation of the phosphorylated RPA32. To investigate this possibility, the proteosome inhibitor MG132 was applied to Hela cells pulse-exposed to HU. As shown in Figure 2-4A, while the presence of MG132 effectively abrogated degradation of p53, a protein which is continuously targeted for proteasomal degradation in Hela cells due to the presence of human papillomavirus (HPV) viral E6 protein (Scheffner et al., 1990), addition of MG132 did not block the decrease in the phosphorylated form of RPA32 or had any noticeable effect on total RPA32 protein levels, suggesting that phospho-RPA32 is not targeted for proteosomal degradation. Thus RPA32 might likely undergo dephosphorylation during the recovery process. To investigate this idea, we tested if the reduction of RPA32 phosphorylation at T21 and S33 observed in recovering cells could be inhibited by okadaic acid (OA), a wide-spectrum inhibitor of serine/threonine protein phosphatases (Janssens and Goris, 2001). As indicated in Figure 2-4B, in cells releasing from HU block, OA suppressed the attenuation of RPA32

phosphorylation in a dose dependent manner, and presence of as low as 50 nM of OA could display a notable inhibition effect, suggesting that dephosphorylation by an OA-sensitive protein phosphatase may account for the declining RPA32 phosphorylation. However, it is possible that OA exerted its effect not by inhibiting the RPA32 phosphatase, but by stimulating the kinases that phosphorylate RPA32 at T21/S33, which may include all of the three DNA damage responsive PIKK kinases ATM, ATR and DNA-PK. We ruled out this possibility by demonstrating that OA still prevented disappearance of phosphorylated RPA32 at T21 and S33 in the presence of 2 mM of

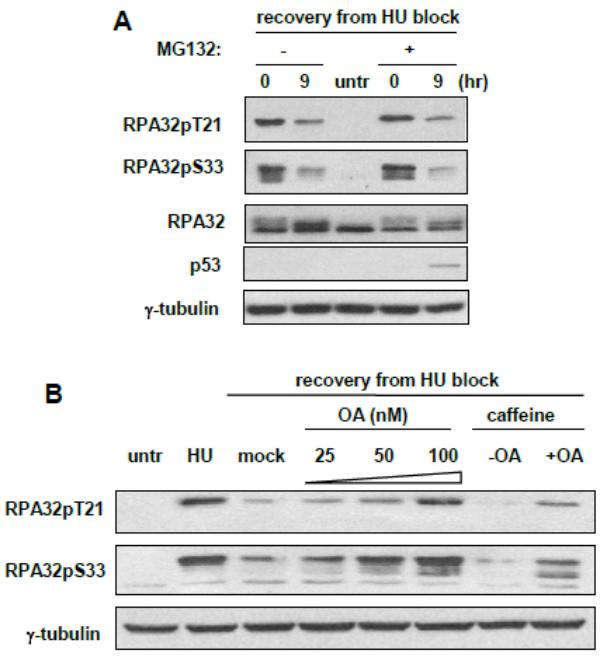


**Figure 2-4: Attenuation of RPA32 phosphorylation is due to dephosphorylation.**

(A) Hela cells were released from HU block (0.2 mM, 24 hr) and the RPA32 phosphorylation levels (at T21 and S33) were analyzed at 0 hr or 9 hr post-recovery in the absence or presence of MG132 (20 μM). (B) Hela cells recovering from HU block (0.2 mM, 24 hr) were examined for the attenuation of RPA32 phosphorylation in the presence of 0-100 nM of OA or OA (50 nM) combined with caffeine (2 mM).

caffeine, a well-known inhibitor of ATM/ATR (Figure 2-4B). It should be mentioned that although DNA-PK was previously thought to be less caffeine-sensitive, it was reported that 2 mM of caffeine could still efficiently inhibit DNA-PK-dependent phosphorylation of RPA32 at T21 (Block et al., 2004).

Taken these data together, in cells recovering from replication stress or UV irradiation, RPA32 phosphorylation is attenuated through dephosphorylation by an okadaic acid-sensitive protein phosphatase.

### 2.3.2 PP2A mediates RPA32 dephosphorylation at T21 and S33

Although widely held as a general inhibitor of PPP subfamily of serine/threonine protein phosphatases, OA has been demonstrated to inhibit some phosphatases, which includes PP1, PP2A, PP4, PP5 and PP6, more potently than others (Janssens and Goris, 2001). In addition, among all of the OA-sensitive PPP phosphatases, PP1 has been shown to be 100-fold less sensitive to OA ($IC_{50}$=~50 nM) than others, which all exhibit similar level of OA sensitivity as PP2A ($IC_{50}$=~0.5 nM), and are therefore collectively termed PP2A-like protein phosphatases (Bialojan and Takai, 1988).

In order to identify which OA-sensitive protein phosphatase mediates RPA32 dephosphorylation, a screen was performed by overexpressing the five known OA-sensitive protein phosphatases in Hela cells prior to HU treatment. Levels of T21 and S33 phosphorylation were notably reduced in cells overexpressing PP1 and PP2A compared with cells transfected with empty vector or other phosphatases (Figure 2-5A), suggesting that RPA32 might be dephosphorylated by these two phosphatases. However, since RPA32 dephosphorylation could be efficiently inhibited by as low as 50

nm of OA *in vivo* (Figure 2-4B), PP2A seems to be more likely the phosphatase responsible for RPA32 dephosphorylation, based on the fact that PP1 has $IC_{50}$=~50 nM *in vitro*. Consistent with this idea, there was a report that PP2A could be effectively inhibited by 100 nM of OA in culturing MCF7 cells, whereas the inhibition on PP1 was not observed (Favre et al., 1997). To further confirm that PP2A, but not PP1, mediates the dephosphorylation of RPA32 at T21 and S33, Hela cells were transfected with siRNA oligos against either PP1 or PP2A catalytic subunits prior to HU pulse-exposure. Knockdown of PP2A but not PP1 significantly attenuated RPA32 dephosphorylation at T21 and S33 in cells recovering from HU stress, suggesting that PP2A is likely the phosphatase that targets RPA32 (Figure 2-5B).

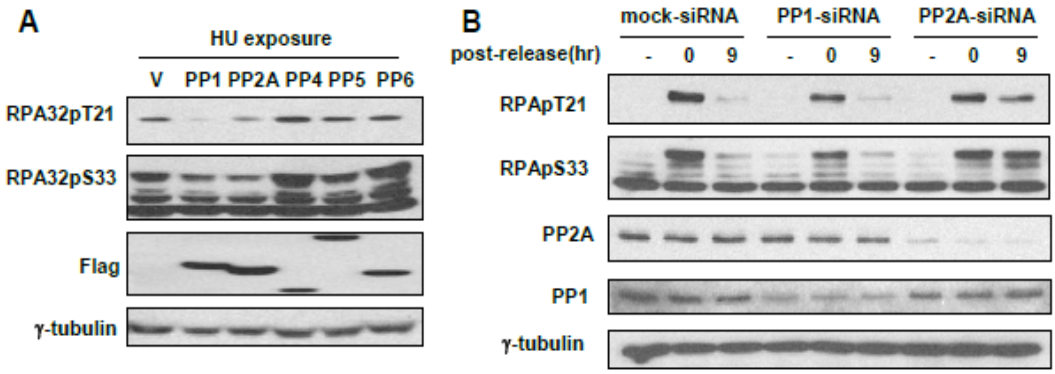


**Figure 2-5: PP2A mediates RPA32 dephosphorylation.**

(A) Hela cells were transfected with empty vector, or constructs that overexpress PP1, PP2A, PP4, PP5 or PP6. Twenty four hours post-transfection, cells were treated with HU (0.2 mM) for 24 hr before analysis of phosphorylated RPA32 by immunoblot. (B) Hela cells were transfected with a control siRNA oligo, or oligos against PP1 or PP2A catalytic subunit. Cells were pulse-treated with HU (0.2 mM, 24 hr) 24 hr post-transfection, and RPA32 dephosphorylation at T21/S33 were compared at the indicated time points.

In consistency with this result, phosphorylated RPA32 foci persisted significantly longer in the PP2A/C-silenced cells, with 53.21% of cells being foci-positive 12 hr post-release from HU block (Figure 2-6). This was in sharp contrast with the mock control cells that showed a significantly reduced number of foci and had only 18.53% of foci-

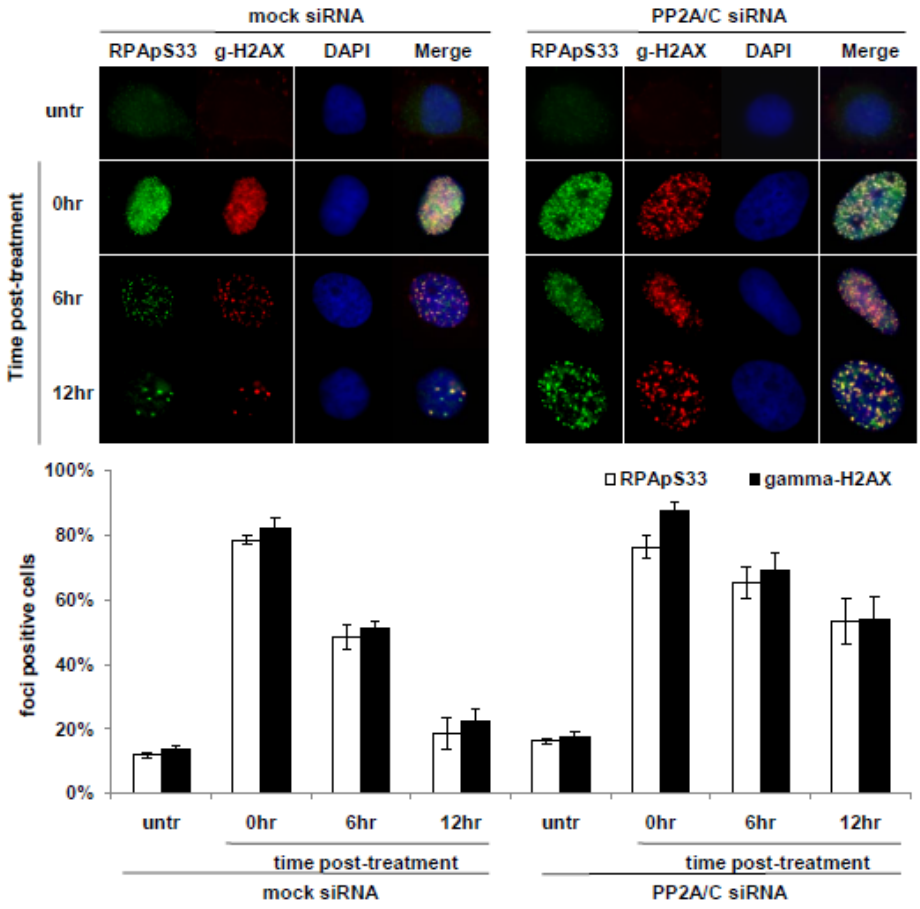


**Figure 2-6: PP2A dephosphorylates RPA32 and γ-H2AX simultaneously.**

Hela cells transfected with control siRNA or siRNA against PP2A/C were pulse-treated with HU (0.2 mM, 24 hr). At 0-12 hr post-release, cells were fixed and co-stained with DAPI and anti-RPA32pS33 and anti-γ-H2AX antibodies. DNA damage foci were visualized by immuno-fluorescence and the percentage of phospho-RPA32 and γ-H2AX foci-positive cells was quantified, normalized and plotted. The error bars represent the standard 2 deviation (SD) from three independent experiments.

positive cells at the 12 hr time point. It is also noteworthy that phosphorylated RPA32 foci colocalized with γ-H2AX foci, which displayed a similar kinetics to disappear during unperturbed recovery and also exhibited longer persistency in the PP2A-silenced cells (Figure 2-6). This data is consistent with a previous report that PP2A mediates γ-H2AX dephosphorylation following pulse-exposure to camptothecin, a topoisomerase I inhibitor (Chowdhury et al., 2005).

It is possible that PP2A does not mediate RPA32 dephosphorylation directly but is somehow involved in this process indirectly. To assess this possibility, a co-immunoprecipitation assay was performed to examine if PP2A could interact with PRA32 at endogenous levels. As shown in Figure 2-7A, the PP2A catalytic subunit did not associate with RPA32 in unperturbed cells, but a strong binding was observed when cells were exposed to HU stress. This binding decreased significantly after 9 hrs of

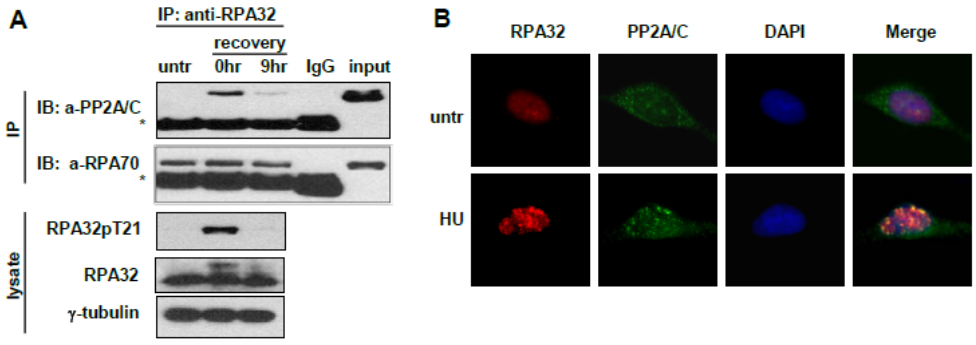


**Figure 2-7: PP2A and RPA32 interacts in an HU-inducible manner.**

(A) Immunoprecipitation assays were performed in unperturbed cells, or cells recovering from pulse-HU exposure (0.2 mM, 24 hr) at the indicated times after release using an anti-RPA32 antibody. The association of RPA32 with PP2A was detected by immunoblot using an antibody against PP2A/C. Binding of RPA70 was also examined as a positive control. The stars indicate the immunoglobulin light and heavy chains detected in the reaction. (B) Hela cells were mock-treated, or exposed to HU (0.2 mM) for 24 hr. DNA damage-inducible foci of RPA32 and PP2A were examined by immunofluorescence.

recovery. Thus, PP2A likely plays a direct role in the process of RPA32 dephosphorylation due to its induced association with RPA32 following genotoxic exposure. In support of this notion, immunofluorescence analysis also showed that the catalytic subunit of PP2A colocalized with RPA32 foci in an HU stress-inducible manner (Figure 2-7B). To examine if PP2A could dephosphorylate RPA32 directly, phosphorylated RPA32 immunoprecipitated from HU-stressed cells was incubated with recombinant PP2A catalytic subunit. PP2A readily dephosphorylated RPA32 *in vitro*, reducing levels of phosphorylated RPA32 within 30 min, and this process was markedly inhibited by addition of as low as 5 nm of OA (Figure 2-8), a concentration known to block PP2A activity under the assay condition. Taken together, these data indicate that PP2A mediates RPA32 dephosphorylation at T21 and S33 in cells recovering from HU-induced replication stress.

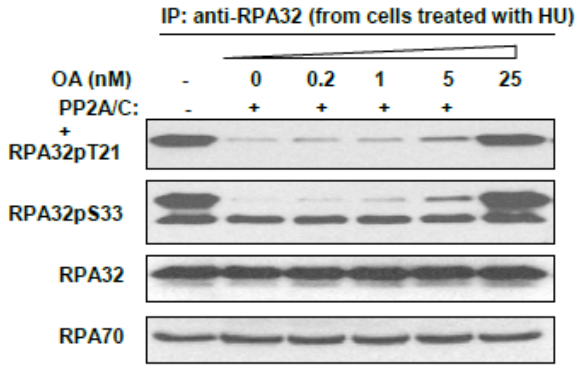


**Figure 2-8: PP2A can dephosphorylate RPA32 *in vitro*.**

RPA32 was immunoprecipitated from Hela cells exposed to HU (10 mM) for 6 hr and used as substrate for the *in vitro* phosphatase assay in the absence or presence of purified PP2A catalytic subunit and different doses of OA (0-25 nM). Western analysis was performed 30 min later to determine levels of phosphorylated RPA32.

### 2.3.3 RPA32 dephosphorylation at T21/S33 is dispensable for the checkpoint activation but is required for efficient recovery

As previously mentioned, the unphosphorylated form of RPA plays an essential role in supporting DNA replication and other types of DNA metabolism under normal cellular conditions. It is therefore plausible that when cells are recovering from DNA damage or replication stress, PP2A-dependent RPA32 dephosphorylation may be required to reverse genotoxic stress-induced RPA32 phosphorylation, consequently restoring RPA32 to its normal condition. In support of this hypothesis, PP2A-silenced Hela cells exhibited deficient RPA32 dephosphorylation at T21 and S33 after HU pulse-exposure (Figure 2-5B) and more importantly displayed significantly reduced cell viability following pulse-exposure to HU stress (24 hr) compared to cells treated with mock siRNA oligos (Figure 2-9A).

Since PP2A is a versatile protein phosphatase targeting myriad substrates in numerous cellular processes, it is possible that the increased sensitivity to HU induced by PP2A knockdown is caused by PP2A-dependent processes other than attenuated RPA32 dephosphorylation. To better address if RPA32 dephosphorylation is required for this process, we created a RPA32-T21D/S33D mutant (DD hereafter) to mimic the persistent phosphorylation state of RPA32 at T21 and S33 (Figure 2-9B). The serine/threonine-to-aspartate conversion has been extensively utilized as phospho-mimetic to study protein functions, and in many cases these phosphorylation-mimicking proteins have identical structure and activity with the actual phospho-proteins (Huang and Erikson, 1994; Vassin et al., 2004; Wittekind et al., 1989). In order to avoid potential interference by the presence of endogenous wild type RPA32, Hela substitution cells

were created where the endogenous RPA32 was stably replaced with a non-targetable wild type (WT), the RPA32-DD mutant, or phospho-deficient T21V/S33A (VA) mutant (Figure 2-9B). As shown in Figure 2-9C, endogenous RPA32 expression was effectively silenced by retroviral-mediated siRNA, and the levels of the exogenously expressed RPA32 variants were comparable to the endogenous RPA in the vector control cells. Cells expressing the DD mutant of RPA32 displayed normal morphology and a slightly slower growth rate compared to control cells (data not shown). The ability of RPA32-DD to complex with other RPA subunits was also indistinguishable from other RPA variants (data not shown), consistent with a previous report on a RPA32 phopho-mimetic which contained as many as eight substitutions (Vassin et al., 2004). Differing from this report (Vassin et al., 2004), which showed incompetent association of the extensively substituted RPA32 mutant with the DNA replication centers, the RPA32-DD described here displayed no apparent deficiency in its colocalization with the DNA replication centers in the unperturbed cells (Figure 2-9D), indicating that the RPA32-DD is sufficient to support DNA replication, a result that is not surprising given the relatively normal cytology exhibited by the DD cells. No other discernable phenotypes were found after fifty passages.

Since RPA translocation to sites of DNA damage has been demonstrated to be an early event in the DNA damage response and plays an important role in activating ATR-dependent checkpoint pathways, we examined if cells with the RPA32-DD phosphomimetic mutant exhibited defective upstream checkpoint activation following genotoxic stress. After HU treatment, RPA32-DD formed punctuate foci in the nucleus that increased over time at a similar rate to the RPA32-WT, indicating that the RPA32-

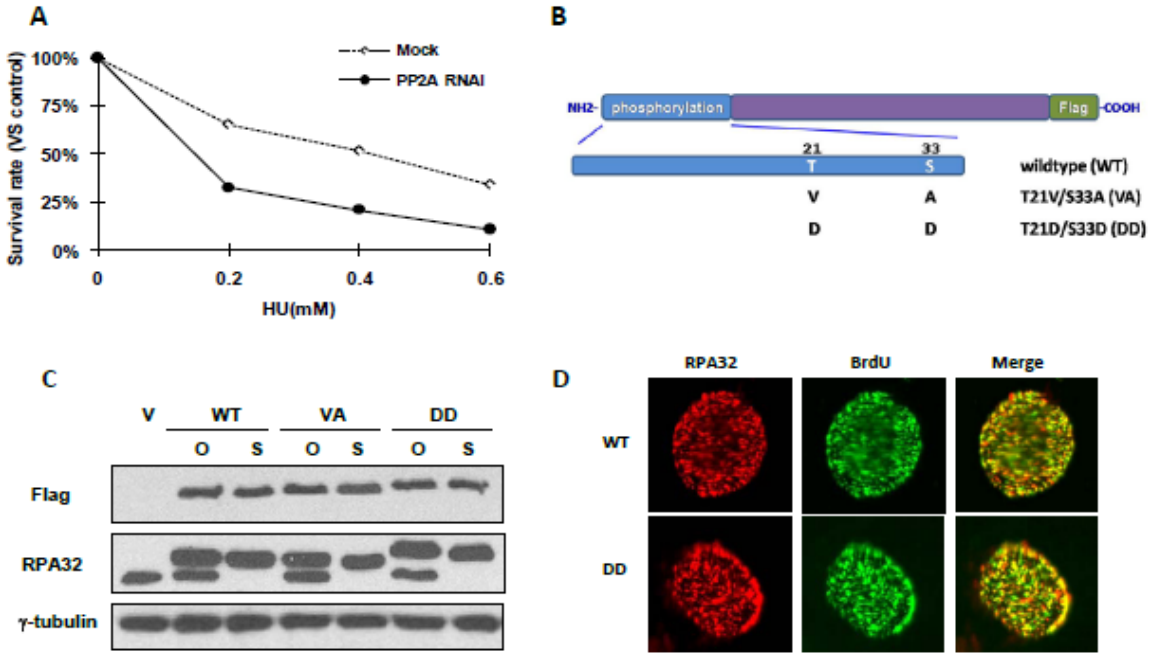


**Figure 2-9: Creation/characterization of RPA32 persistent-phosphorylation mimic.**

(A) Hela cells were transfected with control siRNA, or siRNA specific for the PP2A catalytic subunit. Twenty-four hours after transfection, cells were pulse-treated with 0-0.6 mM HU for 24 hr, washed twice with PBS and incubated in drug-free medium for 10-14 days to allow the growth of colonies for the clonogenic survival assay. (B) Schematic presentation of the wildtype (WT), phosphodeficient (T21V/S33A, VA) and phosphomimetic (T21D/S33D, DD) mutant of RPA32, all being Flag-tagged. (C) The various RPA32 substitution cell lines (S) were created by stably expressing in Hela cells the empty vector (V) or Flag-tagged RPA32 variants (WT, VA and DD) followed by retrovirally silencing endogenous RPA32 in these overexpression cells (O). RPA32 levels in these cell lines were examined by western blot analysis. (D) WT and DD cells were pulse-labeled with BrdU for 10 min and then analyzed by immunofluorescence using anti-BrdU and anti-RPA32 antibodies.

-DD mutant translocates to chromatin normally upon DNA damage (Figure 2-10A). In addition, ATR-dependent phosphorylation of two important checkpoint regulators, Chk1 and Rad17, displayed no distinguishable difference in the DD cells compared with other cells, suggesting that the RPA32-DD substitution does not affect ATR activation (Figure 2-10B). The DD cells have an intact intra-S checkpoint as evidenced by a reduction in

DNA synthesis following UV irradiation to an extent comparable to the wildtype and vector control lines, but in contrast with the phospho-deficient AA cells which maintained a relatively higher level of DNA synthesis (Figure 2-10C). This observation confirms a previous report that RPA32 phosphorylation at T21/S33 is required for the intra-S checkpoint (Olson et al., 2006). In addition, the mitotic index of the DD cells, as measured by phospho-histone H3 staining, was reduced significantly upon UV exposure, to a level similar to other controls cells (Figure 2-10D), indicative of a normal G2/M checkpoint in the DD cells. These data suggest that substitution of endogenous RPA32 with the RPA32-T21D/S33D phosphomimetic mutant does not affect the early checkpoint activation in the DNA damage response. Therefore, for studies of the recovery process from the DNA damage response where RPA32 is dephosphorylated at the T21 and S33, the RPA32-DD substitution cells provide us with an opportunity to investigate the events influenced by RPA32 dephosphorylation by serving as a persistent-phosphorylation mimic model.

To examine whether RPA32 dephosphorylation is required for the efficient recovery from replication stress, the substitution cells were pulse-treated to a range of doses of HU for 24 hrs, and their viability after recovery was analyzed by the clonogenic survival assay. As shown in Figure 2-11A, DD cells displayed a significantly reduced survival rate compared to WT or vector control cells; only 30.91% of DD cells survived and formed colonies after recovery from pulse-exposure to 0.2 mM of HU, which was in sharp contrast with WT (72.39%) and the vector control cells (70.78%). Similarly, DD cells were also less capable to survive UV irradiation compared to control cells, albeit to a lesser extent than with HU treatment (Figure 2-11B).

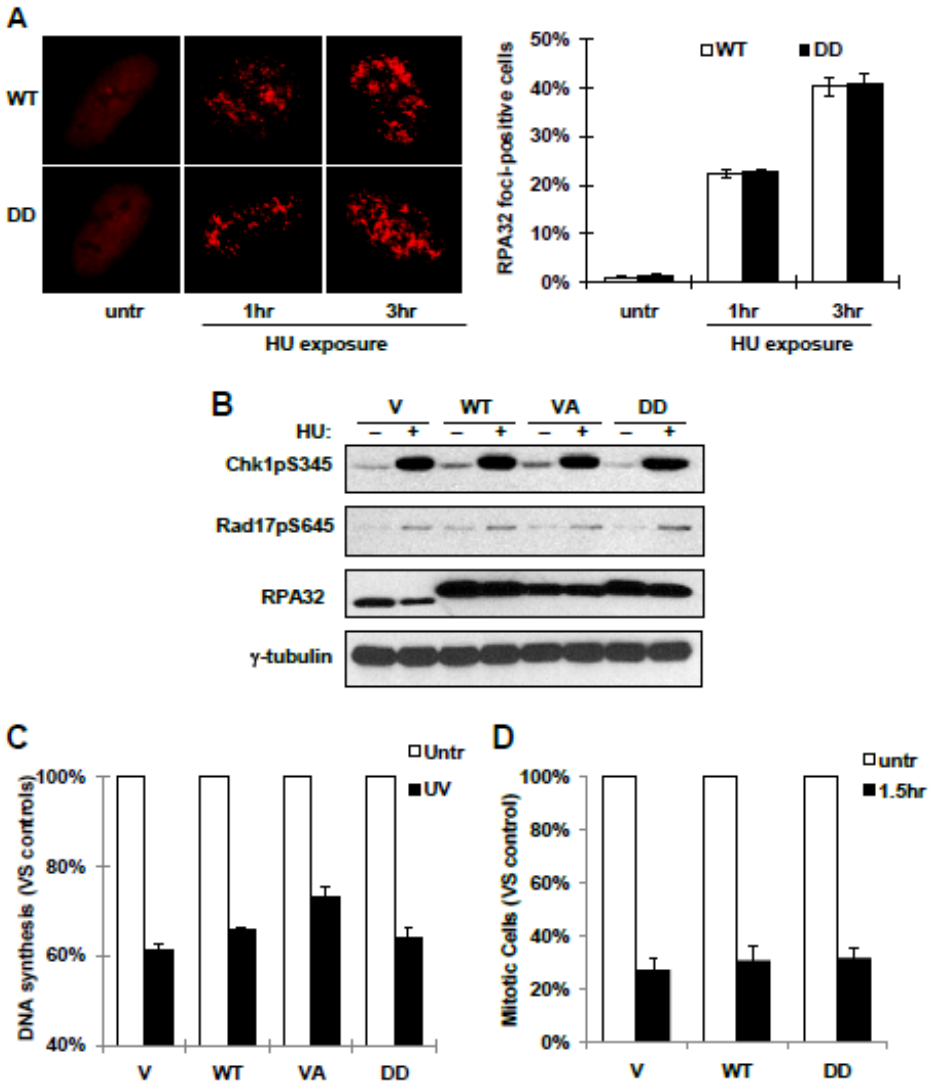


**Figure 2-10: RPA32 dephosphorylation is not required for checkpoint activation.**

(A) WT and DD cells were treated with HU (5 mM). At 0-3 hr post-exposure, cells were fixed and stained with anti-RPA32 antibody. The RPA32 foci were visualized by immunofluorescence and the percentage of foci-positive cells were quantified, normalized and plotted. Error bars represent the SD from three independent experiments. (B) The substitution cells were mock-treated or exposed to HU (5 mM), and phosphorylation of Chk1 at S345 and Rad17 at S645 was examined by western blot 3 hr later. (C) Various cells were irradiated with 25 J/m$^2$ UV, and 3 hrs later the DNA synthesis rates were determined and normalized to that in untreated cells. (D) The substitution cells were left untreated or irradiated with 25 J/m$^2$ UV. 1.5 hrs later, cells were co-stained with PI and anti-phospho-H3 antibodies, and the mitotic fractions were determined by FACS analysis and normalized to that of unperturbed cells.

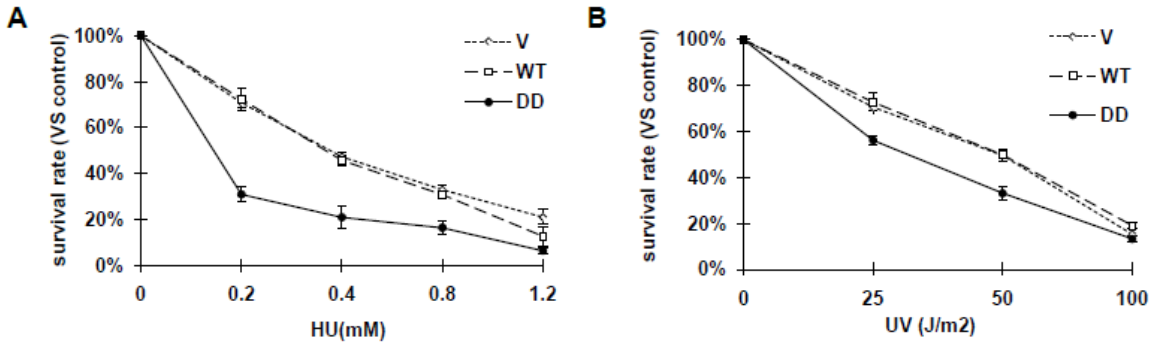


**Figure 2-11: RPA32 dephosphorylation is required for recovery from HU/UV stress.**

(A) The RPA32 substitution lines were pulse-exposed to 0-1.2 mM HU for 24 hr, and then allowed to recover in drug-free medium for 10-14 days. The cell viability of various lineages was analyzed by the clonogenic survival assay. (B) The substitution lines were irradiated with 0-100 J/m$^2$of UV, cultured for 10-14 days, and analyzed for their viability by the clonogenic survival assay. All the data points in (A) and (B) represent the mean SD of triplicate determinates of three independent experiments.

Taken all the data together, Hela cells containing the RPA32 persistent phosphorylation mimetic (RPA32-DD) mutant are functional in their checkpoint activation, but are deficient to recover effectively from replication stress or UV irradiation. These results suggest that RPA32 dephosphorylation at T21 and S33 might be a required event in this process.

## 2.3.4 RPA32 dephosphorylation is not required for the cell cycle re-entry

It has been reported that genotoxic stress-induced RPA32 phosphorylation prevents its association with replication centers and thus may suppress DNA synthesis following DNA damage (Vassin et al., 2004). Recent evidence indicates that phosphorylation at the ATM/ATR-responsive T21/S33 sites is critical for this function (Olson et al., 2006). Given these findings it is plausible that RPA32 dephosphorylation at

T21/S33 might be required for the resumption of DNA replication following recovery from HU block. However, as the thymidine incorporation assay indicated, the DNA synthesis activity of the RPA32-DD substitution cells increased at a comparable rate to that of WT and vector control lines within three hours post-release from HU pulse-exposure (0.2 mM, 24 hr, Figure 2-12A), implying that RPA32 dephosphorylation is not required to resume the arrested DNA replication following release from HU block.

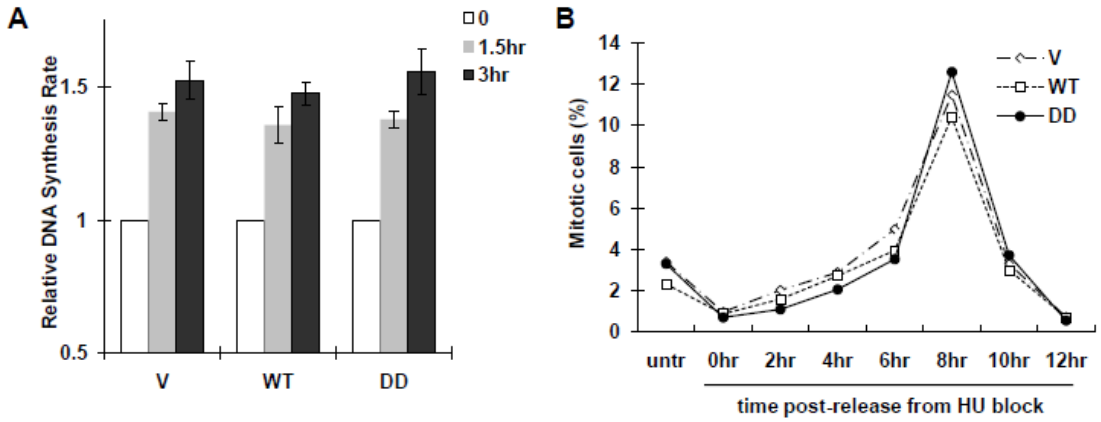


**Figure 2-12: RPA32-DD is normal to resume DNA replication and initiate mitosis.**

(A) The substitution cells were released from pulse HU exposure (0.2 mM, 24 hr). At 0-3 hr post-release, the DNA synthesis rates were determined and normalized to the rate at 0 hr time point. All samples were tested in triplicates, and consistent results were obtained among three independent experiments. (B) Various cell lineages were allowed to recover from pulse-exposure to HU (0.2 mM, 24 hr). Cells at the indicated time points were fixed and co-stained with PI and anti-phospho-H3 antibody, and the mitotic fractions were further determined by FACS analysis.

HU is a reversible inhibitor of DNA replication, and cells synchronized by HU exposure re-enter the cell cycle if released from HU block. Since the resumption of DNA replication seems to be unaffected, it is possible that progression through the downstream G2/M boundary requires RPA32 dephosphorylation at T21/S33. To test this

possibility, the various substitution lines were released from pulse-exposure to HU (0.2 mM, 24 hr) and their progression through mitosis was evaluated by phospho-histone-H3 staining at different time points. As indicated in Figure 2-12B, the mitotic profile of the DD cells showed no noticeable difference compared with that of other two lines (V and WT); the mitotic indices of all three lines remained low until 4 hr post-release when cells started to divide, and the percentage of the mitotic cells peaked at 8 hr and dropped to a level similar to 0 hr time point at 10 hr. Thus, RPA32 dephosphorylation at T21/S33 might be dispensable for mitotic progression in cells recovering from HU block.

The above results were supported by an independent experiment performed on Hela cells pulse-exposed to two different levels of HU for 24 hours. In sharp contrast with cells pulse-treated with a sublethal dose (0.2 mM) of HU, where RPA32 was apparently dephosphorylated gradually during recovery, cells treated with a lethal dose (2 mM) exhibit persistent RPA32 phosphorylation up to 24 hours post-release (Figure 2-13A). The lethally treated cells could resume DNA replication in S-phase and re-enter the cell cycle with no apparent defects except that they displayed approximately a 3 hour delay in their recovery compared with sublethally treated cells (Figure 2-13B), indicating a dispensable role of RPA32 dephosphorylation in the resumption of DNA replication and the subsequent mitosis following release from replication block.

There is a possibility that DNA replication resumes only in cells where RPA32 is dephosphorylated, or alternatively at sites where the bound RPA32 is dephosphorylated and not at sites where RPA32 remains phosphorylated. To test these possibilities, Hela cells were pulse-labeled with BrdU for 10 min six hours post-release from HU block (2

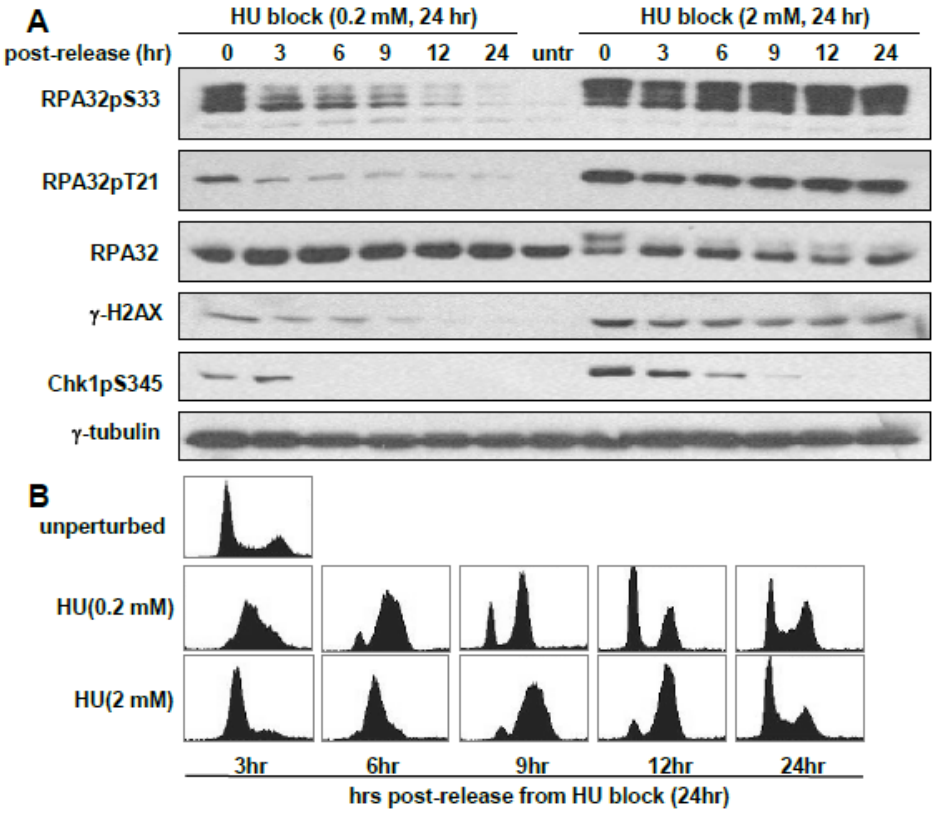


**Figure 2-13: RPA32 dephosphorylation is not required for cell cycle re-entry.**

(A & B) Hela cells were treated with sublethal (0.2 mM) or lethal dose (2 mM) of HU for 24 hr, and then allowed to recover. At 0-24 hr after release, cells were harvested and analyzed by immunoblot using appropriate antibodies (A). Remaining cells underwent cell cycle profiling analysis (B).

mM, 24 hr) and then tested for colocalization between phosphorylated RPA32 and incorporated BrdU, which usually indicates where DNA replication is occurring. As shown in Figure 2-14A, phosphorylated RPA32 and BrdU strongly co-localized, confirming that resumption of DNA replication is independent of RPA32 dephosphorylation. To confirm that RPA32 dephosphorylation is not required for resumption of mitosis following recovery from HU block, mitotic index analysis was

carried out. As revealed in Figure 2-14B, the lethally-treated Hela cells exhibit a lagging but nonetheless apparent progression through mitosis in comparison with the cells pulse-exposed to a sublethal dose of HU, indicating that RPA32 dephosphorylation at T21/S33 is not required for this process. Taken together, these data indicate that RPA32 dephosphorylation is not required to resume DNA replication or progress through the mitotic phase during recovery from HU-induced genotoxic stress.

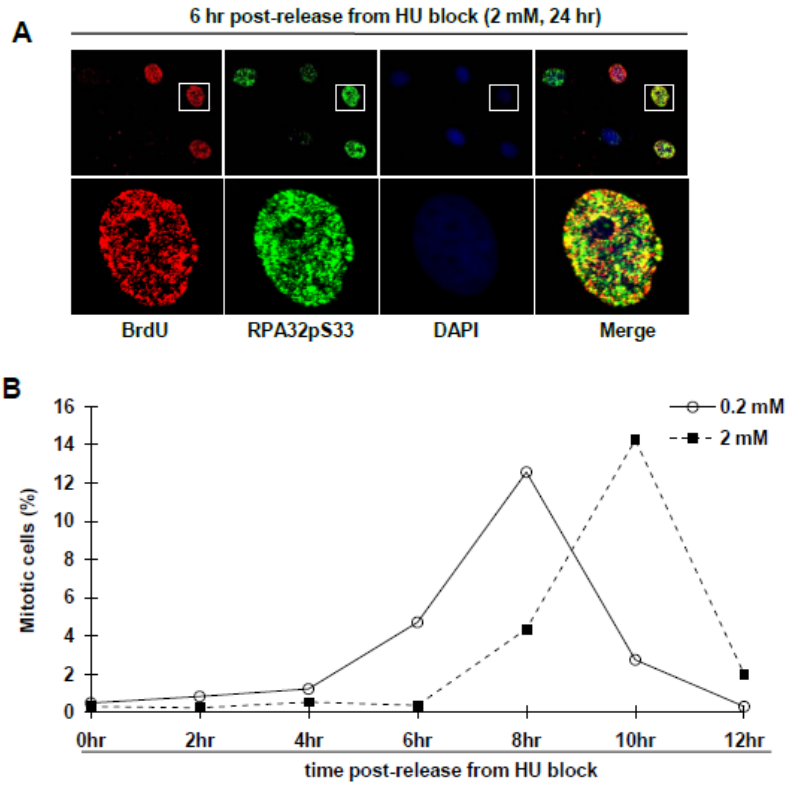


**Figure 2-14: RPA32 dephosphorylation is dispensable for replication resumption or mitosis progression.**

(A) Hela cells were pulse-exposed to lethal dose of HU (2 mM, 24 hr). At 6 hr post-recovery, cells were pulse-labeled with BrdU for 10 min followed by immunofluorescence analysis using anti-BrdU and anti-RPA32pS33 antibodies. The cell positive for both types of staining was boxed and magnified for clearer visualization. (B) Hela cells were allowed to recover from pulse-HU treatment (0.2 or 2 mM, 24 hr) and mitotic index analysis was performed by FACS at the indicated time points after release.

It is noteworthy that γ-H2AX dephosphorylation showed similar kinetics to RPA32 dephosphorylation in cells pulse-treated with sublethal dose of HU, and similar to RPA32, its dephosphorylation was significantly abrogated in lethally treated cells (Figure 2-13A). In contrast, dephosphorylation of Chk1 at S345, a site whose phosphorylation has been shown to be critical for Chk1 activation (Chen and Sanchez, 2004), occurred under both conditions, albeit with a 3 hr delay in lethally treated cells (Figure 2-13A). It should also be noted that Chk1 dephosphorylation showed a strong temporal correlation with the release from replication arrest in both cases (Figure 2-13A and 2-13B). These results are not surprising since γ-H2AX colocalizes very well with phosphorylated RPA32 (Figure 2-15), which are both dephosphorylated by the common phosphatase PP2A (Chowdhury et al., 2005), whereas Chk1 dephosphorylation is reportedly mediated by a different protein phosphatase Wip1/PPM1D (Lu et al., 2005). Given the pivotal role Chk1

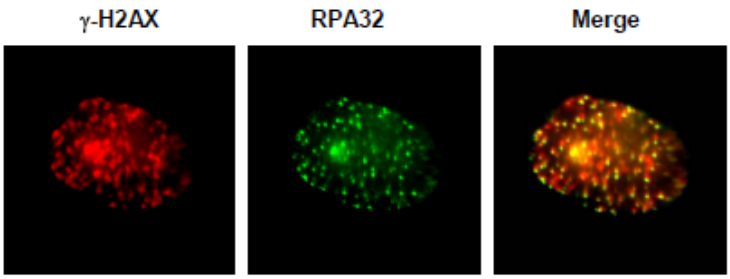


**Figure 2-15: RPA32 colocalizes with γ-H2AX after DNA damage.**

Hela cells were treated with HU (0.2 mM) for 24 hr before analysis of colocalization between RPA32 and γ-H2AX by immunofluorescence.

plays in checkpoint activation, it seems that Chk1 deactivation through dephosphorylation may also dictate checkpoint recovery from replication stress. This

idea is supported by a recent report which showed that the reversal of intra-S and G2/M checkpoint activation requires Wip1/PPM1D-dependent dephosphorylation of Chk1 (Lu et al., 2005).

### 2.3.5 RPA32 dephosphorylation at T21/S33 is critical for repair of DNA breaks induced by replication stress.

Because RPA has been shown to be involved in various DNA repair pathways (Sancar et al., 2004; Wold, 1997; Zou et al., 2006), it is possible that the deficient recovery from HU stress and UV irradiation displayed by cells containing the RPA32 phosphomimetic mutant might be due to defective DNA damage repair. To investigate this possibility, the substitution cells were pulse-exposed to HU (0.2 mM, 24 hr) and then analyzed for the level of DNA breaks by the alkaline single-cell gel electrophoresis (alkaline comet assay) at different recovery time points. Even though DD cells accumulated slightly lower levels of DNA breaks before HU release (data not shown), repair of the DNA breaks, as indicated by the decline of the relative comet tail moments, was significantly less efficient compared with WT cells (Figure 2-16A and B). At 12 hr post-release, the DD cells still contained a substantially higher level of unresolved DNA breaks (22.4%) compared to the WT cells (3.3%) (Figure 2-16B). These data indicate that cells expressing the RPA32 phosphomimetic mutant are less efficient in the repair of DNA breaks induced by HU, suggesting that RPA32 dephosphorylation at T21 and S33 is required for this process.

Consistent with this result, PP2A/C-silenced Hela cells exhibit less RPA32 dephosphorylation (Figure 2-17A) and a significantly higher level of unresolved DNA breaks 12 hr after recovery from HU stress (36.7% relative to 0 hr point, calculated by

the ratio between comet tail moments, Figure 2-17B and 2-17C). This is in sharp contrast to the mock control cells where RPA32 was fully dephosphorylated and DNA repair was essentially complete (1.4%, Figure 2-17A and 2-17C).

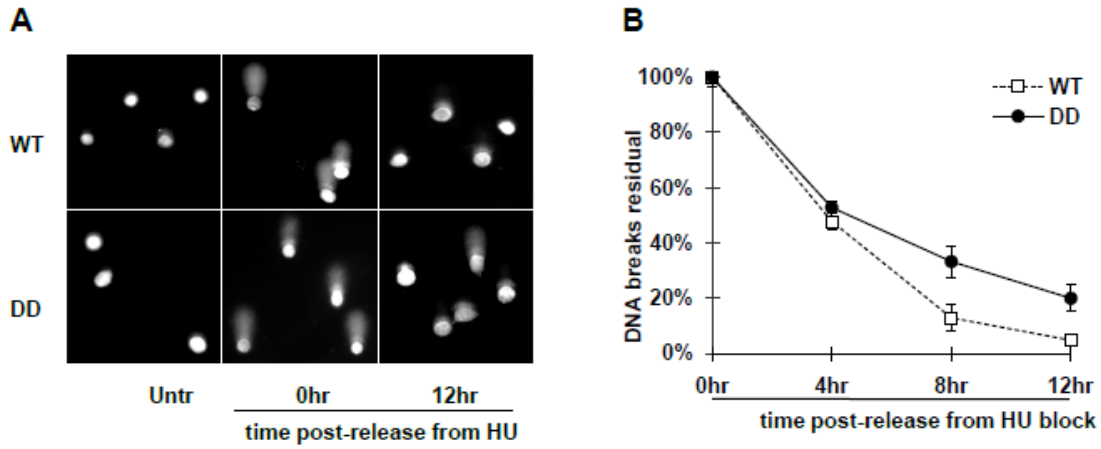


**Figure 2-16: RPA32-DD is defective in repair of DNA breaks induced by HU.**

(A & B) WT and DD cells were allowed to recover from pulse-HU exposure (0.2 mM, 24 hr). At 0-12 hr post-recovery, cells were harvested and analyzed by the alkaline comet assay. The pictures shown in (A) were taken from three data points, and were for demonstration purpose only. (B) Repair of DNA breaks was evaluated by the level of the residual DNA breaks which is calculated by the comet tail moments at various recovery time points relative to that at the 0 hr time point. All data points represent the mean ± SD of three independent experiments.

In response to genotoxic stress, a large number of checkpoint and repair proteins including the Mre11/Rad50/Nbs1 complex, RPA, 53BP1 and γ-H2AX accumulate at sites of DNA damage and form huge protein complexes termed DNA damage foci, which are essentially the DNA repair centers that do not resolve until the repair process is completed (Dellaire and Bazett-Jones, 2007; Lisby and Rothstein, 2004). For this reason, these protein foci are regarded as markers for DNA damage. To confirm the

above results through the comet assays, we examined the kinetics of RPA foci and γ-H2AX foci, two markers broadly used to indicate DNA damage, in the substitution cells

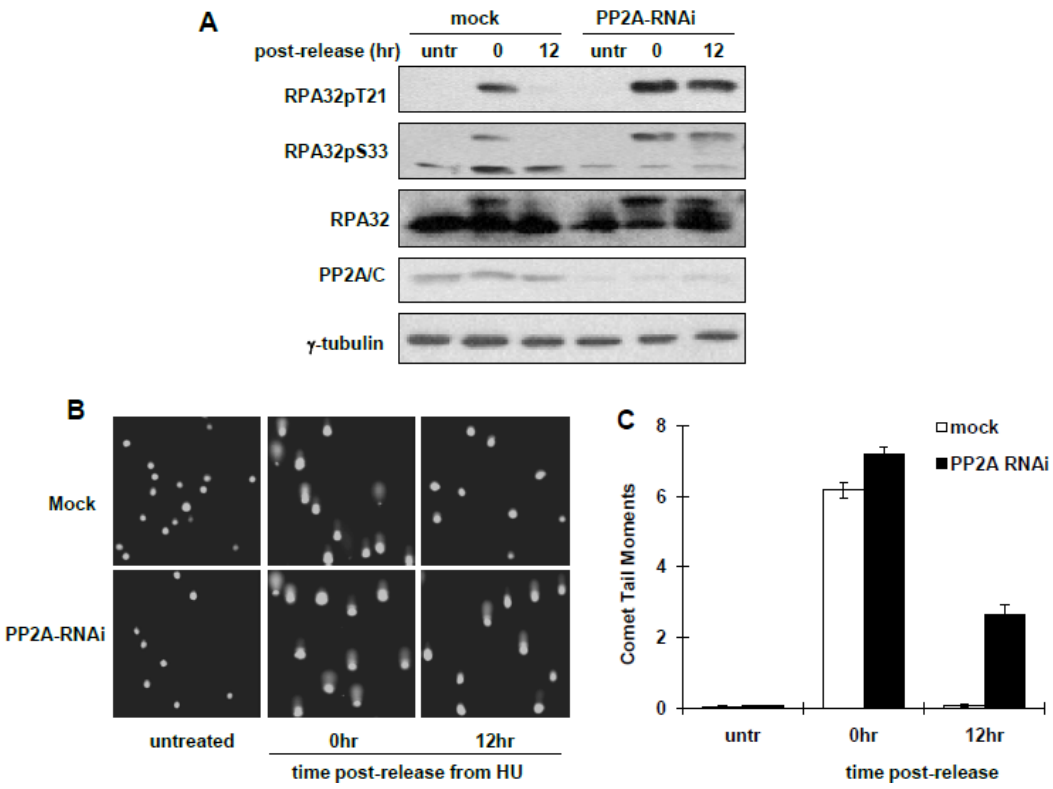


**Figure 2-17: RPA32 dephosphorylation is required for efficient DNA repair.**

Hela cells were transfected with mock siRNA or siRNA against the PP2A catalytic subunit. Twenty-four hours post-transfection, cells were pulse-exposed to HU (0.2 mM, 24 hr) and then allowed to recover. RPA32 dephosphorylation was analyzed by immunoblot (B) and repair of DNA breaks was evaluated by alkaline comet assay (B). Quantitative analysis of the comet tail moments at various time points was performed and plotted as (C). All the data points represent the mean ± SD of three independent experiments.

recovering from HU block (0.2 mM, 24 hr). As shown in Figure 2-18, both RPA70 and γ-H2AX exhibited HU stress-induced punctate staining in WT and DD cells, which also displayed a significant level of colocalization. In both substitution cells, the number of the speckles formed by these two proteins as well as the percentage of the foci-positive cells

declined over time following recovery from HU pulse-exposure. However, the rate of foci reduction in DD cells was significantly slower than that in WT cells; at 12 hr post-recovery, only 18.72% of WT cells but as many as 46.58% of DD cells were γ-H2AX foci-positive, and the percentage of DD cells that contained unresolved RPA70 foci was almost three times that of WT cells (29.19% VS 10.17%), confirming again that DNA damage repair is defective in the DD cells (Figure 2-18).

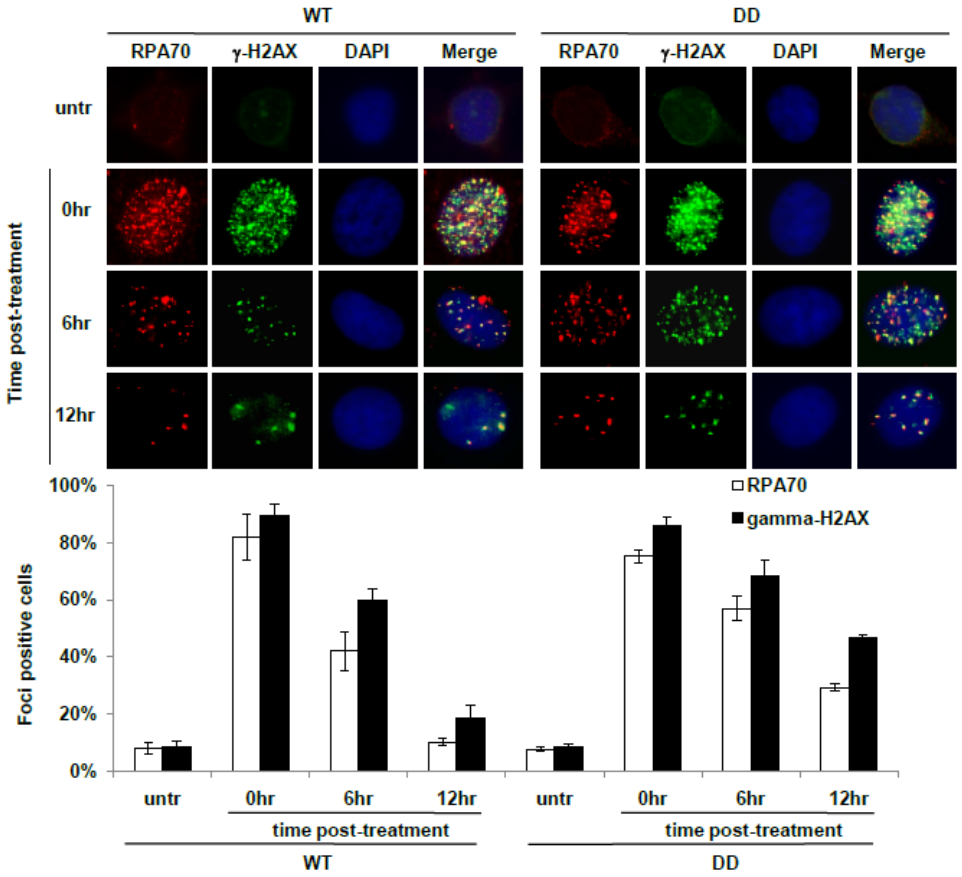


**Figure 2-18: DNA damage foci resolved slower in RPA32-DD cells.**

WT and DD cells were allowed to recover from pulse-HU treatment (0.2 mM, 24 hr). At 0-12 hr post-release, cells were fixed and stained with DAPI, anti-RPA70 and anti-γ-H2AX antibodies. Immunofluorescence was carried out to visualize the DNA damage foci formed by RPA70 and γ-H2AX and the percentages of the foci-positive cells were calculated, normalized and plotted. All the data points in represent the mean ± SD of three independent experiments.

Taken together, these results indicate that the phosphomimetic DD mutant cells are deficient in the repair of DNA breaks induced by replication stress, demonstrating that PP2A-dependent RPA32 dephosphorylation at T21 and S33 is necessary for efficient DNA damage repair.

## *2.4 Discussion*

In summary, our results have identified a novel mechanism whereby dephosphorylation of human RPA32, which is mediated by the important Ser/Thr protein phosphatase 2A, plays a crucial role for cells to survive genotoxic insults. Specifically, we show that in cells recovering from the replication stress induced by HU, RPA32 undergoes PP2A-dependent dephosphorylation at T21 and S33, a process dispensable for checkpoint activation or cell cycle re-entry but critical for the efficient repair of DNA breaks.

### 2.4.1 Sequential RPA32 phosphorylation and dephosphorylation controls proper progression of DNA damage response

An essential ssDNA binding protein complex, RPA has been shown to be crucial in all major types of DNA metabolisms known to date: replication, recombination, checkpoint activation and DNA repair (Iftode et al., 1999; Wold, 1997). Even in the last two processes that are collectively termed DNA damage response, RPA also demonstrably plays versatile yet essential roles in almost all aspects of this complicated pathway, which include recognizing and stabilizing DNA breaks, activating/mediating checkpoint pathways, and assisting with the downstream repair of diverse types of DNA lesions (Zou et al., 2006). However, little is known about how the versatile functions of

RPA are regulated. Based on our study and others, it seems that the sequential phosphorylation and dephosphorylation of RPA32 at T21/S33 may provide a mechanism controlling the switch between checkpoint activation and DNA damage repair, the proper execution of which guarantees the efficient progression of the entire DNA damage response from initiation to recovery.

Upon DNA damage or replication stress, RPA32 is phosphorylated by ATM/ATR at sites of DNA damage or stalled replication forks, and phosphorylated RPA suppresses ongoing DNA replication and helps recruit other checkpoint/repair proteins. Later, during recovery from DNA damage-induced checkpoint arrest, phosphorylated RPA32 is removed through PP2A-dependent dephosphorylation, an event that is essential for efficient DNA repair (Figure 2-19). For this entire process, initial phosphorylation and subsequent dephosphorylation of RPA32 need to be tightly regulated in order for a proper DNA damage response to occur. Suppression or failure of initial RPA32 phosphorylation causes a defective S-phase checkpoint leading to DNA damage-resistant DNA synthesis, and delay or dysfunction of subsequent RPA32 dephosphorylation results in inefficient repair of DNA lesions.

It is noteworthy that in addition to the ATM/ATR-responsive T21 and S33, several other serine residues (S4, S8, S11, S12 and S13) on the N-terminus of RPA32 also undergo DNA damage-inducible phosphorylation. Therefore, it is possible that dephosphorylation of these sites is also regulated by PP2A and required for the DNA damage response. Based on our observations, PP2A seems to be responsible for dephosphorylation of some, if not all, of these residues as well, since western blots using an anti-RPA32 antibody showed an apparent PP2A-dependent reduction of multiple

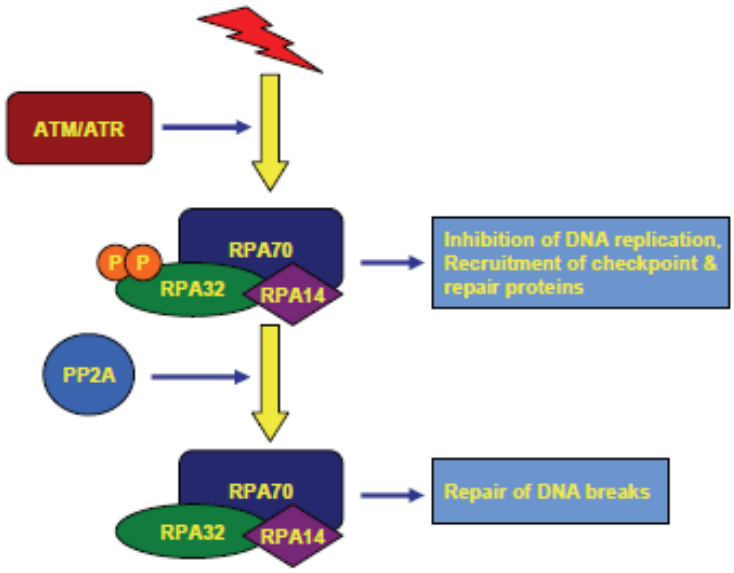


**Figure 2-19: Sequential phosphorylation & dephosphorylation at T21/S33 controls proper progression of the DNA damage response.**

In response to genotoxic stress, activated ATM/ATR phosphorylate RPA32 on T21 and S33 at sites of DNA damage, where the phosphorylated RPA mediates intra-S checkpoint by directly suppressing DNA replication, and facilitates the recruitment of downstream checkpoint/repair proteins. Following recovery, PP2A translocates to DNA repair foci, associating with and dephosphorylating RPA32. This process is required for efficient repair of DNA breaks.

slower migrating bands in cells recovering from HU stress, which can be indicative of loss of protein phosphorylation. In support of this, western blot using an antibody that recognizes doubly phosphorylated RPA32 on both S4 and S8 suggested at least one of these two sites may also be dephosphorylated in a PP2A-dependent manner (data not shown). Due to lack of appropriate phosphospecific antibodies against all of these seven individual sites we did not address this question further. Regardless of whether PP2A is involved, given the high level of cytotoxicity induced by overexpression of RPA32 hyperphosphomimic mutant that has substitution of all seven serine/threonine site (data not shown) and the essential role unphosphorylated RPA32 plays in supporting

unperturbed DNA replication, it is conceivable that cells would not survive persistent RPA32 hyperphosphorylation following genotoxic stress and that dephosphorylation of these DNA damage-inducible sites is likely a required event during recovery.

### 2.4.2 Phosphorylated RPA suppresses DNA replication during checkpoint activation yet supports resumed DNA replication during recovery

Genotoxic stress-induced phosphorylation of RPA32 has been shown to be critical in activation of intra-S phase checkpoint by suppressing DNA replication directly, due to its reduced capability to bind with DNA polymerase $\alpha$ and to denature duplex DNA (Binz et al., 2003; Patrick et al., 2005; Vassin et al., 2004). Surprisingly, however, our data reveals that in cell recovering from HU stress, DNA replication resumes irrespective of RPA32 phosphorylation status, as cells released from a lethal dose of HU resumed DNA replication normally regardless of persistent RPA32 phosphorylation (Figure 2-12).

One possible explanation is that DNA synthesis during unperturbed S-phase replication and in the recovery from replication stress may utilize different DNA polymerases and involve distinct mechanisms. While the former process requires Pol $\alpha$ primase and unphosphorylated RPA-dependent initiation at replication origins and relies heavily on two other processive, high-fidelity polymerases (Pol $\delta$ and Pol $\varepsilon$) at replication elongation, DNA synthesis in the recovering cells mainly resumes at the arrested replication forks or starts as part of a repair process and therefore may not need the initiation step and may be mediated by non-replicative but repair-specific polymerases (Hubscher et al., 2002; Shcherbakova et al., 2003; Weill and Reynaud, 2008). In support

of this idea, several non-replicative DNA polymerases including X family polymerases λ, μ, TdT, Y family polymerase η and B family polymerase ζ and Rev1 have been found to be involved in the none-homologous end joining (NHEJ) and homologous recombination (HR) repair synthesis of DSBs, the major type of DNA breaks induced by collapse of replication forks following HU exposure (de Feraudy et al., 2007; Hirano et al., 2008; McIlwraith et al., 2005; Shcherbakova et al., 2003; Weill and Reynaud, 2008). In addition, it has also been shown that the Pol α-primase complex is not required for DSBs-induced HR during the mating type switch in budding yeast (Wang et al., 2004). However, it is possible that resumption of DNA replication may also involve initiation from replication origins, but it should be pointed out that even in cells arrested with lethal dose of HU, a detectable level of RPA32 remains unphosphorylated in the nucleoplasm which should suffice for supporting replication initiation (data not shown).

### 2.4.3 Role of PP2A-dependent RPA32 dephosphorylation in the repair of DNA breaks.

This study has established a causal relationship between RPA32 dephosphorylation and repair of DNA breaks induced by replication stress, however the question remains open about what specific role RPA32 dephosphorylation plays in this process. Presumably, RPA32 dephosphorylation may function to recruit certain repair-essential proteins to or removing other suppressive ones from the locations of DNA breaks where the DNA repair process is occurring. Accumulating evidence has shown that the repair of DNA breaks is a complicated multi-step process orchestrated by a variety of DNA repair proteins that form dynamic yet tightly regulated repair foci at sites of DNA damage (Houtsmuller et al., 1999; Lisby et al., 2004; Lukas et al., 2003;

Solomon et al., 2004). Alternatively, phosphorylated RPA32 may negatively regulate certain downstream steps of DNA repair pathways that do not involve translocation of any repair proteins, and activation of these steps may therefore necessitate prior PP2A-dependent dephosphorylation of RPA32.

To understand what specific role RPA32 dephosphorylation plays in the DNA repair process, it would be inviting to temporally dissect the entire process, including recruitment/removal of the repair proteins and activation of the stepwise DNA repair pathways. Since prolonged replication stress mainly cause accumulation of DSBs as a result of collapse of replication forks, we would like to examine if RPA32 dephosphorylation is necessary for the repair of DSBs, which is mediated by two major pathways: homologous recombination (HR) and the non-homologous end joining (NHEJ). As a matter of fact, it has been shown that recruitment to DSB sites of one HR-essential repair protein Rad51, which is also an ssDNA-binding protein, requires dislodging of RPA from DSBs. Under this scenario, it is possible that RPA32 dephosphorylation may be needed for the displacement of RPA complex from or replacement of the Rad51 to the sites of DSBs. Thus this would be one of our immediate future direction to pursue. It is, however, also possible that RPA32 dephosphorylation is required for the NHEJ repair pathway, which is mainly mediated by proteins such as Ku70, Ku80 and DNA-PK. Thus translocation of these NHEJ-essential proteins would be another potentially important possibility to test.

### 2.4.4 Checkpoint adaptation also exists in human cells.

Although resumption of DNA replication does not require RPA32 dephosphorylation, it showed a good temporal correlation with Chk1 dephosphorylation at S345 regardless of the level of HU exposure (Figure 2-12). Based on a previous report that PPM1D-dependent dephosphorylation of Chk1 abrogates checkpoints, we believe that Chk1 dephosphorylation-dependent deactivation may dictate resumption of DNA replication as part of a checkpoint recovery process (Lu et al., 2005).

Interestingly, while cells pulse-treated with lethal dose (2 mM) of HU underwent apparent checkpoint recovery by resuming cell cycle following release, these cells contained persistently high levels of phosphorylated RPA32 and γ-H2AX, suggesting that a large number of DNA breaks were yet to be resolved. This is reminiscent of checkpoint adaptation, an intriguing mechanism originally discovered in yeast. In this mechanism, cells re-enter the cell cycle despite the persistence of unrepaired DNA breaks, an advantage presumably conferred on unicellular yeast cells to allow them to survive a long-term arrest when facing persistent genotoxic stresses (Toczyski et al., 1997). Checkpoint adaptation had been believed to be absent in metazoans because of the possibility that it could promote genomic instability, but recent studies have shown that such a phenomenon may exist in cells of higher organisms as well (Harrison and Haber, 2006; Syljuasen et al., 2006; Yoo et al., 2004). These data in our study evidently indicate that checkpoint adaptation also occurs in human cells.

It is unclear what role checkpoint adaptation plays in mammalian cells when confronted with persistent genotoxic stress like HU. Based on our data that these adapted cells died out ultimately, we speculate that checkpoint adaptation might serve

as a mechanism to eliminate cells which carry irreparable DNA damage, possibly through mitotic catastrophe. However, this speculation needs further experimental evidence.

# 3 Define the role of serine 2546 in regulation of ATM activity

## *3.1 Introduction*

DNA double-strand breaks (DSBs) may represent the most lethal form of DNA lesions and can be generated intracellularly by the metabolic byproducts of cellular respiration (e.g. reactive oxygen species, ROS), or be induced exogenously by various DNA damaging agents, including ionizing radiation (IR) and chemicals that mimic radiation (e.g. neocarzinostatin, NCS) or inhibit DNA topoisomerases (e.g. camptothecin and etoposide) (Khanna and Jackson, 2001). If repaired inefficiently, DSBs could pose a serious threat to cell viability and genome stability by increasing the likelihood of chromosome breakage and rearrangement, mutagenesis and loss of crucial genetic information (Riches et al., 2008; Shrivastav et al., 2008).

Despite the overall genotoxicity, it is worthy of note that DSBs can be beneficial under certain physiological conditions. For example, in meiosis and V(D)J recombination that is specific to mammals, cells can take advantage of DSB-induced recombination to generate genetic diversity (Kleckner, 1996; Roeder, 1990; Smith and Jackson, 1999). In these cases, DSBs are purposely produced by certain endogenous nucleases, then the same repair machinery used to repair unwanted DSBs is mobilized to remove the DNA breaks, introducing the recombination of genetic information as a consequence (Daboussi et al., 2002; Khanna and Jackson, 2001).

### 3.1.1 Structure and function of ATM

In eukaryotes, DSBs are responded primarily by the PIKK kinase Ataxia-telangiectasia Mutated (ATM). Upon detection of DSBs, ATM initiates and transduces the damage signals to the downstream checkpoint/repair apparatuses, leading to arrest of cell cycle and mobilization of repair proteins that can specifically repair DSBs (Abraham, 2004; Khanna and Jackson, 2001; Koundrioukoff et al., 2004; Westphal, 1997). Two parallel pathways may be activated by ATM to repair DSBs, which include the homologous recombination (HR) repair pathway and the non-homologous end joining (NHEJ) repair pathway (Karran, 2000).

ATM is a highly conserved gene, with its orthologs being found in all eukaryotic cells examined to date (Smith et al., 1999). In accordance with its role in checkpoint activation and repair of DSBs, mutations of human *atm* gene have been causally associated with ataxia-telangiectasia (A-T), an autosomal recessive human genetic disease characterized by cerebellar ataxia, immune deficiencies and increased risk of cancers, particularly lymphomas (Boultwood, 2001; Dasika et al., 1999; Meyn, 1999). Cells from A-T patients exhibit abnormal telomere morphology, increased chromosomal breakage, hypersensitivity to radiation, indicative of defective DNA damage checkpoints (Meyn, 1995; Pandita, 2002). In consistency with the above observations, mice with defective *atm* gene exhibit a similar phenotype as human A-T and their cells display dysfunctional checkpoint activation and are hypersensitive to DNA damaging agents (Shiloh, 1997).

Human *atm* encodes a huge protein kinase which contains 3056 residues and has a molecular weight of ~370 kDa. Sequence analysis over hATM protein reveals that its kinase domain (PI-3 kinase related kinase domain, PIKK) is at the near end of its

carboxyl terminus and is flanked by two loosely conserved domains known as FAT (FRAP/ATM/TRRAP) and FATC, which have been suggested to be involved in intra- or intermolecular interactions (Figure 3-1) (Bosotti et al., 2000).

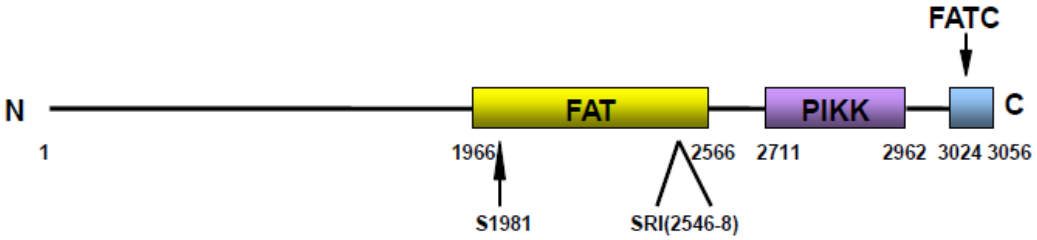


**Figure 3-1: The schematic domain-structure of human ATM.**

Human ATM contains 3056 amino acid residues, consisting of FAT domain, the kinase domain (PIKK) and the c-terminal FATC domain. The S1981 represents the autophosphorylation site, and the SRI (2546-2548) region is located at the C-terminus of FAT domain and is critical for ATM kinase activity. The vast N-terminal region remains unknown.

### 3.1.2 ATM activation mechanism

Probably due to its large molecular weight and low abundance, investigation over how the kinase activity of ATM is stimulated by DSBs has proven to be challenging until recently, when significant advances have been made towards an understanding about how it is activated.

It had been suggested that ATM may require certain covalent or sterical modifications to become activated because IR treatment could stimulate the kinase activity of immunoprecipitated ATM (Banin et al., 1998; Canman et al., 1998). Consistent with this idea, it was reported that IR induces ATM autophosphorylation at S367, S1893, and S1981, an event that may stimulate the kinase activity of ATM by triggering disassociation of inactive ATM dimers or oligomers into catalytically active monomers (Bakkenist and Kastan, 2003; Kozlov et al., 2006). The autophosphorylation at S1981,

and possibly at the other two sites (S367 and S1893) as well, might be negatively regulated by PP2A, which in undamaged cells associates with ATM and keeps it inactive, but dissociates from ATM upon IR treatment, allowing its subsequent activation (Goodarzi et al., 2004). PP5, on the other hand, may serve as a positive regulator. It binds to ATM in a genotoxic stress-inducible manner, and interference with the expression or the activity of PP5 attenuated DNA-damage-induced activation of ATM, leading to defects of the IR-induced intra-S-phase checkpoint (Ali et al., 2004). Plausibly PP5 may be required to remove an inhibitory phospho group on ATM. Besides phosphorylation, ATM has also been demonstrated to undergo rapid acetylation by the Tip60 histone acetyltransferase (HAT) in response to DNA damage, which is also crucial for the kinase activation (Sun et al., 2005). In a recent study, Aven, a previously reported apoptotic inhibitor, was identified to associate with and potently activate ATM upon DNA damage (Guo et al., 2008), suggesting that some regulators might control ATM activation through their genotoxic stress-induced change of association with ATM.

Intriguingly, the activation of ATM kinase activity does not seem to require direct contact with DSBs, as one study shows that ATM autophosphorylation and ATM-dependent p53 phosphorylation has no correlation with the number of DSBs (Kitagawa et al., 2004). However, the opposite is seen with other DNA damage foci-forming substrates such as Nbs1, H2AX, and SMC1, whose phosphorylation appears to depend on the recruitment of ATM to sites of DNA damage (Horejsi et al., 2004; Kitagawa et al., 2004; Mochan et al., 2003).

As discussed above in Chapter 1, a special group of DNA damage-responsive proteins termed checkpoint mediators may facilitate association of ATM with particular

substrates, which essentially add another layer of complexity to ATM activation. It was shown that the ability of recombinant Mre11-Rad50-Nbs1 complex to stimulate the *in vitro* kinase activity of ATM requires functional Nbs1 for increased phosphorylation of Chk2, but not nucleoplasmic p53 (Lee and Paull, 2004). Cells lacking functional Nbs1 or three other BRCT proteins (BRCA1, 53BP1 or MDC1) displayed relative normal p53 responses yet striking defects in the translocation of phosphorylated ATM to sites of DNA damage and the subsequent phosphorylation of its foci-forming substrates such as Chk2, BRCA2, as well as these mediator proteins themselves (Horejsi et al., 2004; Kitagawa et al., 2004; Lee and Paull, 2004; Mochan et al., 2003).

Taken these discoveries together, an ATM activation model arises which describes IR-induced ATM activation as a multistep process which includes stimulation of its kinase activity, autophosphorylation and dimer dissociation, recruitment by the adaptors to the nuclear foci at the breaks and presentation by the mediator proteins to its downstream substrates (Figure 3-2) (Bakkenist and Kastan, 2004).

It should be mentioned, however, that despite this current prevalent model that regards ATM autophosphorylation as a key step for kinase activation (Bakkenist and Kastan, 2003), two recent reports, which were published in the same group, argued against this widely accepted causal role of ATM autophosphorylation in its kinase activation. By use of a transgenic mouse model in which all three conserved ATM serine autophosphorylation sites (S367/1899/1987), which correspond to S367/1893/1981 in human ATM, have been replaced with non-phosphorylable alanine, they found that the mutant murine ATM could be normally activated without causing any detectable defects in the DNA damage response, demonstrating that ATM autophosphorylation is merely

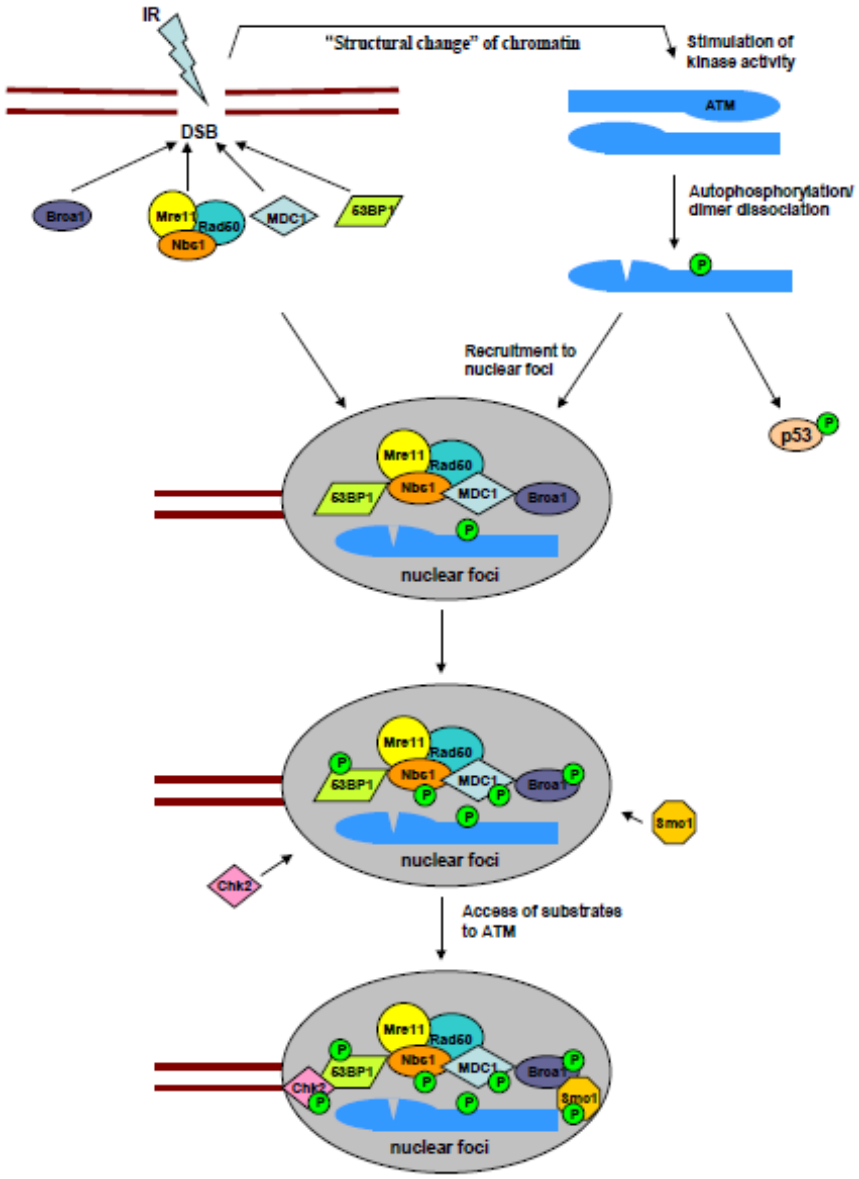


**Figure 3-2: Model of ATM activation towards phosphorylation of its substrates.**

DSBs cause structural change of chromatin which in turn stimulates the kinase activity of ATM. ATM then undergoes autophosphorylation at S1981 and dimer dissociation. The active, monomeric ATM either directly phosphorylates nucleoplasmic p53 or is recruited to sites of DNA breaks by adaptor proteins such as MDC1, 53BP1, BRCA1 and Mre11-Rad50-Nbs1 complex, which independently translocate to the DSBs. In the nuclear foci, ATM can phosphorylate these checkpoint proteins. Phosphorylation of other originally nucleoplasmic proteins like Chk2 and SMC1 by ATM may also require mediators. Some adaptor proteins like 53BP1 and Brca1 may also serve as mediators.

a consequence, rather than a cause of ATM activation. (Daniel et al., 2008; Pellegrini et al., 2006).

### 3.1.3 The SRI (2546-2548) region

One of the most common deletion mutations found in A-T patients, the *Atm* 7636del9 mutation causes deletion of a short fragment containing three amino acid residues (SRI:2546-2548). This segment is located at the near C-terminal of the above mentioned FAT domain and is ~150aa upstream of the kinase domain (Figure 3-1) (Savitsky et al., 1995; Watters et al., 1997).

Functional studies revealed that ATM-ΔSRI has no kinase activity *in vitro*, and when transfected, does not rescue the radiosensitive phenotype in ATM-deficient cells. Furthermore, overexpression of ATM-ΔSRI in ATM-sufficient cells dominantly interferes with IR-induced ATM kinase activity and results in enhanced chromosomal aberrations and reduced viability following IR exposure (Scott et al., 2002). Consistent with these observations, ATM-ΔSRI homozygous mice exhibited similar level of radiosensitivity as $ATM^{-/-}$ mice and the ATM-ΔSRI heterozygous mice displayed increased susceptibility to developing tumors whereas no tumors are observed in $ATM^{+/-}$ mice (Spring et al., 2002; Spring et al., 2001).

### 3.1.4 The study rationale

Based on the above reports, it is obvious that this short deletion mutation, despite being outside the kinase domain, has significant functional effects on the ATM activity, but the reason for the abolished kinase activity remains unclear. Several possible scenarios could potentially explain this intriguing question. Among them, one

possibility is that this segment may harbor a critical post-translational modification site such that the deletion may abrogate the modification that is indispensable for ATM activation. Starting from this idea, we hypothesize that phosphorylation and dephosphorylation at serine 2546 (S2546) might be critical for ATM activation. Although S2546 is not in the conserved PI3-kinase domain, it is immediately adjacent and may provide an essential regulatory element for the whole kinase activity.

In this study, we examined this hypothesis by generating the S2546 phosphodeficient and phosphomimetic mutants (ATM-S2546A and ATM-S2546D) and then performing a series of comparative analyses over these ATM mutants, along with the wildtype ATM. In contrast with ATM-S2546A, which showed no detectable defects compared with WT, ATM-S2546D had a dramatically reduced kinase activity towards the p53 substrate *in vitro*, and when expressed in an ATM-deficient cell line, failed to phosphorylate Nbs1 and Rad17 following NCS treatment and did not rescue the radio-resistant DNA synthesis (RDS) phenotype displayed by the A-T cells, suggesting that S2546 might represent an important regulatory site for ATM activation. Based on these data, we propose that phosphorylation on S2546 may inhibit ATM kinase activity in normal conditions, and that its dephosphorylation may be a required event for ATM activation in response to DNA DSBs.

## *3.2 Materials and Methods*

**Plasmids, Mutagenesis and GST fusion proteins**

The Flag tagged ATM construct has been described previously (Cortez et al., 1999), and was used as template to introduce the S2546A and S2546D substitution

mutations using the QuikChange™ Site-Directed Mutagenesis Kit following the manufacturer's protocol (Stratagene). The Flag-tagged kinase dead mutant of ATM (ATM-KD, D2870A) was described previously (Li et al., 2000b). GST-p53 (1-70) was described elsewhere (Tibbetts et al., 1999).

**Cell Culture and Transfection**

The 293T cells were cultured in DMEM containing 10% FBS (Invitrogen) plus antibiotics. The AT4BI cells were grown in DMEM/F12 supplemented with 20% FBS and antibiotics. For generation of various ATM mutants-overexpression A-T cell lines, the empty vector and the various ATM mutant constructs were tranfected into the AT4BI cell line using the FuGENE 6 transfection kit according to manufacturers' protocol (Roche).

**Reagents and Antibodies.**

The antibodies used for immunoblotting were purchased from Abcam (rabbit polyclonal ATMpS1981) Bethyl Laboratories (rabbit polyclonal Rad17pS645), Calbiochem (rabbit polyclonal ATM), Cell signaling (rabbit polyclonal Nbs1 and Nbs1pSer343), Santa Cruz (rabbit polyclonal hRad17, p53pSer15, and mouse monoclonal p53), and Sigma-Aldrich (mouse monoclonal Flag and γ-tubulin). The neocarzinostatin (NCS) was purchased from Sigma-Aldrich.

**Immunoblotting**

Cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol) supplemented with protease inhibitors (20 μg/ml leupeptin, 10

μg/ml pepstatin A, 10 μg/ml aprotonin) and phosphatase inhibitors (20 mM β-glycero-phosphate, 0.5 μM okadaic acid). Cell lysates were kept on ice for 10 min and cleared by full-speed centrifugation for 10 min at 4ºC, followed by quantitative determination of the protein concentrations using the modified Bradford protein assay kit from Bio-Rad. Lysates were then resolved in SDS-PAGE, transferred to Immobilon-P PVDF membrane and blotted with appropriate primary and secondary antibodies.

**Radioresistant DNA synthesis assay**

Various mutant ATM expression constructs were transfected into the AT4BI cells. The next day, cells were plated onto 12-well plates in triplicates at appropriate densities and incubated in medium containing 20 nCi/ml of [$^{14}$C] thymidine (NEN) for another 24 hr prior to NCS treatment (300 ng/ml). Four hours after NCS treatment, cells were pulse-labeled with [$^{3}$H] thymidine (2.5 μCi/ml, 30 min; NEN) and harvested as described (Cliby et al., 1998). The radioactivity was determined by liquid scintillation counting, and the relative DNA synthesis rate was calculated by determining the ratio of $^{3}$H to $^{14}$C and normalization of the treated samples to the appropriate controls.

***In vitro* kinase assay**

GST-p53 (1-70) fusion protein was bacterially expressed, purified, and eluted as previously described (Tibbetts et al., 1999). AT4BI cells were grown to approximately 50% confluency, transiently transfected with various mutant ATM expression constructs. Two days post-transfection cells were mock treated or treated with NCS (300 ng/ml) for 30 min before harvest and cell lysis. The recipe for the lysis buffer was as follows (50 mM

Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol) supplemented with protease inhibitors (20 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml aprotonin) and phosphatase inhibitors (20 mM β-glycero-phosphate, 0.5 µM okadaic acid). The ATM variants were then immunoprecipitated using anti-Flag antibody. Immune complexes were washed twice with kinase buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 4 mM $MnCl_2$, 6 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM dithiothreitol, and 100 mM $NaVO_4$). Substrate (200 ng to 1 mg), 10 mCi of $\gamma[^{32}P]$adenosine triphosphate (ATP), and ATP to a final concentration of 5 mM were added, and the reaction mixtures were incubated at 30°C for 15 min. After electrophoresis in SDS-polyacrylamide gels, the reaction products were visualized by autoradiography.

## *3.3 Results*

### 3.3.1 ATM-S2546D failed to rescue the RDS phenotype displayed by ATM deficient cells

ATM is a crucial checkpoint sensor kinase involved in the initiation of multiple checkpoint pathways in response to DSBs. Thus cells deficient in ATM (A-T cells) are defective in their cell cycle checkpoints, resulting in a variety of abnormal phenotypes including increased chromosomal breakage and hypersensitivity to radiation (Meyn, 1995; Pandita, 2002). Among the multiple DNA damage-responsive checkpoint pathways, the intra-S phase checkpoint closely monitors the ongoing DNA replication and suppresses DNA synthesis when cells are inflicted with replication stress or DNA damage (Sancar et al., 2004). Due to the deficiency in the *atm* gene, the A-T cells are defective in this checkpoint and not surprisingly exhibit a radioresistent DNA synthesis (RDS) phenotype.

To investigate if S2546 substitutions affect ATM functions, we performed a rescue experiment where all of the ATM variants were overexpressed in the ATM deficient AT4BI cells. Cells were then treated with the radiomimetic NCS to induce DSBs and then assayed for their capabilities to suppress DNA synthesis. As shown in Figure 3-3, the DNA synthesis in the A-T cells transfected with empty vector was not efficiently suppressed by NCS treatment (68% relative to that in untreated cells), indicative of a RDS phenotype. Ectopical overexpression of ATM-WT and ATM-S2546A, however, rescued this phenotype efficiently by restoring the NCS-induced suppression of DNA synthesis significantly, as judged by their lower relative DNA synthesis rates (52% and 48%). In contrast, no rescue is observed in the A-T cells overexpressing ATM-S2546D, which still exhibited a high level of NCS-resistant DNA synthesis rate (65%).

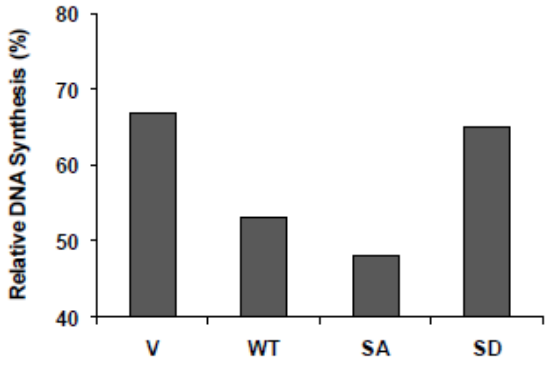


**Figure 3-3: ATM-S2546D failed to rescue the RDS phenotype exhibited by AT cells.**

Various ATM constructs, as well as the empty vector, were transfected into the ATM-deficient AT4BI cells. Forty-eight hours post-transfection, cells were treated with NCS to induce DSBs. The DNA synthesis rates of various NCS-treated cells were measured by double thymidine labeling, and compared with those in unperturbed condition. The relative DNA synthesis rates of various cells were then plotted for comparison of their capability to suppress DNA synthesis upon DSBs.

Therefore, while the phosphodeficient ATM-S2546A is fully functional, ATM containing the S2546D phosphomimetic substitution failed to rescue the phenotype caused by ATM-deficiency, suggesting that removal of an inhibitory phospho-group on the S2546 residue might be required for ATM activation.

### 3.3.2 ATM-S2546D exhibited significantly reduced kinase activity *in vitro*.

Failure of ATM-S2546D to rescue the RDS phenotype in A-T cells might be due to its defective kinase activity *per se*. To investigate this possibility, we conducted an *in vitro* kinase assay, which is meant to examine the ability of various ATM mutants to phosphorylate their substrate in a cell-free system.

The ATM-S2546A and ATM-S2546D were overexpressed in AT4BI cells, along with the wild-type positive control (ATM-WT) and the kinase dead negative control (ATM-KD). Cells were exposed to NCS for 30 min to induce DSBs. ATMs were then immunoprecipitated from the cell lysates and incubated with substrate GST-p53 (1-70) to allow for the kinase reaction. The kinase activity of various ATM mutants was then evaluated by phosphorylation of the substrate p53, as well as their autophosphorylation levels. Compared with ATM-S2546A, which showed similar level of kinase activity as the wildtype ATM, ATM-S2546D exhibited significantly reduced kinase activity towards p53 (Figure 3-4). In consistency with this result, autophosphorylation of ATM-S2546D also displayed notable level of reduction, if the phosphorylation levels were normalized with total ATM protein level and compared with ATM-WT and ATM-S2546A (Figure 3-4). Based on this result, we conclude that S2546 is an important residue for ATM activation. Given the distinct effect on the ATM activity by the phosphodeficient S2546A and

phosphomimetic S2546D substitutions, it appears that ATM kinase activity might be negatively regulated by the phosphorylation on S2546, whose dephosphorylation thus may be a necessary event for the ATM activation.

However, it should be noted that S2546D substitution, which is unlike the kinase dead D2870A mutation, significantly reduces but does not completely abrogate the kinase activity of ATM. In addition, NCS-induced stimulation of the ATM kinase activity seems to be also normal in the S2546D mutant, which showed about two fold-increase after NCS treatment (Figure 3-4), a level comparable to the WT and S2546A mutant. Thus other regulatory mechanisms must exist to stimulate ATM kinase activity besides S2546 dephosphorylation, which presumably may include DSBs-induced covalent or sterical modifications.

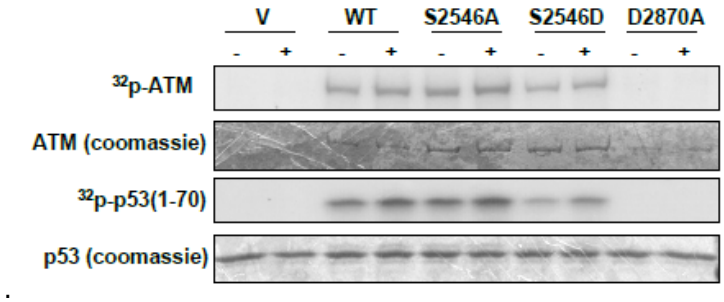


**Figure 3-4: ATM-S2546D has significantly reduced kinase activity.**

AT4BI cells were transfected with empty vector and ATM variants: WT, S2546A, S2546D and D2870A. 48 hr post-transfection, cells were treated with NCS to induce DSBs and ATMs were pulled down by immunoprecipitation and their kinase activities were evaluated by *in vitro* kinase assays. Both ATM autophosphorylation and p53 phosphorylation was analyzed and compared across various ATM mutants.

### 3.3.3 Phosphorylation of Nbs1 and Rad17 was defective in ATM-S2546D overexpressed A-T cells.

To further investigate if the ATM-S2546D mutant also exhibits defective kinase activity *in vivo*, we examined the DSBs-induced phosphorylation of various known ATM substrates in the A-T cells, which were transiently transfected with empty vector, wildtype ATM, ATM-S2546A or ATM-S2546D respectively. As shown in Figure 3-5, thirty minutes after IR or NCS treatment, phosphorylation of Nbs1 and Rad17 exhibited defects in ATM-S2546D transfected A-T cells, but not in cells overexpression ATM-S2546A, suggesting that ATM-S2546D might be a defective kinase.

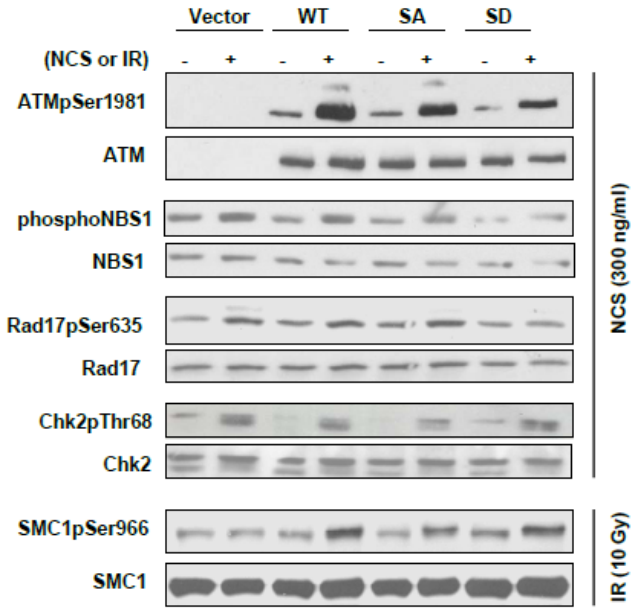


**Figure 3-5: ATM-S2546D shows some substrate preference.**

AT4BI cells were transfected with empty vector and ATM variants: WT, S2546A, S2546D and D2870A. 48 hr post-transfection, cells were treated with NCS (300 ng/ml) or IR (10 Gy) to induce DSBs. Phosphorylation of various ATM substrates, including .Nbs1, Rad17, Chk2 and SMC1, as well as it autophosphorylation at S1981, were examined by immunoblot.

Surprisingly, however, phosphorylation of Chk2 and SMC1, two other important ATM substrates, displayed no discernable differences between these two mutants. In addition, ATM autophosphorylation at S1981 also seemed to show a rather slight reduction in the ATM-S2546D cells (Figure 3-5). This result, if combined with the data shown above, may suggest that ATM-S2546D may show some level of substrate preference *in vivo*, which in response to DSBs phosphorylates SMC1 and Chk2 with no detectable defects yet fails to phosphorylate others efficiently including Rad17 and Nbs1.

Nonetheless it should be mentioned that the parental AT4BI cells surprisingly exhibited some level of NCS-induced phosphorylation of substrates Nbs1, Rad17 and Chk2, regardless of the fact that these cells are ATM-deficient (Figure 3-5). We speculate that this might be due to the limitation of NCS, which might produce secondary DNA lesions that may stimulate the partially redundant kinase ATR. This idea appears to be supported by the observation that treatment with IR, a better DSBs-inducer, did not cause SMC1 phosphorylation (Figure 3-5). Alternatively, these might be specific to the AT4BI cell line because of its aberrant genetic background. Thus, although it appears that ATM-S2546D is defective in its activity towards some substrates *in vivo*, more convincing data are needed to make a more definitive conclusion.

## *3.4 Discussion*

### 3.4.1 S2546 and the SRI region is critical for ATM kinase activity.

Although no definitive conclusion could be achieved at this moment due to the limitations of the experiments performed on this still ongoing study, it is clear that the S2546 residue, and the SRI region it resides in as well, represent a critical yet intriguing

site for ATM activity. Its essentiality for ATM is explicit by the observations that an in-frame deletion of SRI segment, or the S2546D substitution mutation, could cause deleterious consequences to the ATM kinase, resulting in a total loss (in ATM-ΔSRI) or significant reduction (in ATM-S2546D) of the kinase activity. Because such a critical site is not in the conserved PIKK kinase domain but is notably ~150 amino acid residues upstream of it (Figure 3-1), it is unlikely to play a structural role in supporting the ATM kinase activity, but instead may represent a crucial regulation locus to modulate ATM activity. Though uncommon, this type of regulation is not without precedents. The mammalian target of rapamycin ( mTOR), another PIKK family kinase, contains a serine residue (S2035) upstream of its kinase domain, whose substitution to aspartic acid residue reportedly abolished the binding of mTOR with FKBP12, pointing to a similarly negative effect on its kinase activity (Chen et al., 1995).

It is also noteworthy that the SRI sequence is in the relatively conserved but functionally poorly understood FAT domain. In fact, a conserved SRI segment (2147-2149) can also be found in another closely related PIKK kinase ATR (Figure 3-6A). It is of special attention that the protein sequences flanking the SRI in ATR display a notable level of homology with that in ATM, suggesting the essentiality of both SRI sites in their respective kinase proteins. Since S2546 in ATM is crucial for its kinase activity, it is inviting to test if S2147 in the conserved SRI sequence in ATR is also important for ATR kinase activity. This speculation has in fact been confirmed by a similar experiment performed on ATR. We found that substitution of serine 2147 to aspartic acid, but not alanine, abolished ATR kinase activity in an *in vitro* kinase assay (Figure 3-6B), in a manner similar to S2546 in ATM.

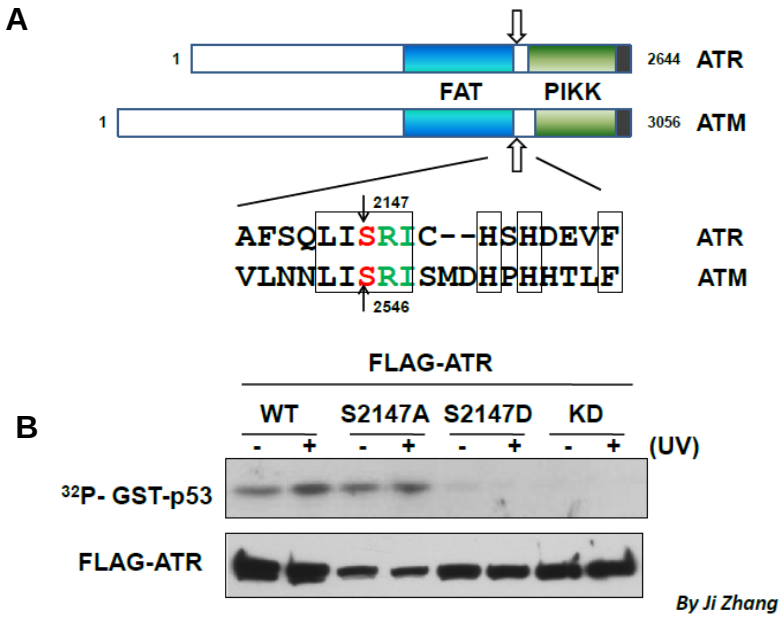


**Figure 3-6: The SRI-residing S2147 is critical for the kinase activity of ATR.**

(A) The SRI sequence is conserved among ATM and ATR. The schematic protein structures of human ATM and ATR. Both kinases contain a conserved SRI sequence (2546-2548 in ATM & 2147-2149 in ATR) which is at C-terminal of the common FAT domain but upstream of their respective kinase domains (PIKK). The SRI sequences are aligned below, with the conserved residues being boxed. The SRI residues are highlighted by colors and indicated by arrows. (B) The ATR mutants containing phosphodeficient S2147A and phosphomimetic S2147D substitutions were transfected into 293T cells, along with the wildtype (WT) and kinase-dead (KD) controls. The *in vitro* kinase assays were then performed across different lines in response to UV irradiation (by Ji Zhang).

Therefore, though distinctive in many ways of their activities, ATM and ATR may share some common mechanism in their respective activation processes, wherein the SRI-residing serine residues (S2546 in ATM, S2147 in ATR) may play a critical role in the activation of both kinases.

### 3.4.2 S2546 may harbor an inhibitory phospho group for ATM

Our still ongoing study, though far from being conclusive, points to a likely role of phosphorylation and dephosphorylation at S2546 to control ATM activation. Given that the phosphomimetic S2546D substitution, but not the phosphodeficient (S2546A) one, significantly reduces ATM kinase activity and fails to rescue the RDS phenotype of the A-T cells, it appears that S2546 may harbor an inhibitory phospho group that suppresses ATM activity in normal conditions yet needs to be removed in response to DSBs to allow for the full activation of ATM. If this scenario holds true, dephosphorylation of ATM at S2546 may represent another layer of regulation on the ATM kinase activity, adding to the already daunting level of complexity beneath the activation mechanism whereby the ATM is stimulated upon DSBs (Figure 3-2).

It should be mentioned that the presence of one or multiple inhibitory phospho groups on the serine or threonine residues of ATM has been suggested by our previous study, which showed that the Ser/Thre protein phosphatase PP5 associates with ATM in an IR-inducible manner, possibly serving as a positive regulator for the stimulation of the ATM kinase activity (Ali et al., 2004). Therefore, it is a tempting hypothesis that PP5 may precedingly dephosphorylate S2546 to promote ATM activation in response to DSBs. In addition, since S2147D in ATR behaved similarly as S2546D in ATM, it is also worth a close investigation if PP5 could also dephosphorylate S2147 to activate ATR, as our previous data showed that PP5 binds with ATR in a DNA damage-inducible manner as well (Zhang et al., 2005a).

Surprisingly, however, although ATM-S2546D showed reduced kinase activity *in vitro* and failed to rescue the RDS phenotype of A-T cells *in vivo*, phosphorylation of a

subgroup of ATM-responsive checkpoint/repair proteins (SMC1 and Chk2) seemed to be normal in contrast with others (Nbs1 and Rad17), whose ATM-dependent phosphorylation is abolished (Figure 3-5). Although this result still needs to be confirmed, it may suggest that S2546 dephosphorylation may not simply activate ATM kinase activity *per se* in the initial step, but may be involved in the subsequent steps of the complicated activation process as well, which include the arguable step of autophosphorylation and dimer dissociation, followed by its recruitment by the adaptors to the nuclear foci at the DSBs and presentation by the mediator proteins to its downstream substrates (Figure 3-2) (Bakkenist and Kastan, 2004). At each step, phosphorylation of a subgroup of substrates may rely on a different set of the adaptor/mediator proteins that serve to bring ATM kinase and substrates together, allowing the phosphorylation reaction to occur. The S2546D mutation might affect the binding of ATM with certain adaptor/mediator proteins but not others, leading to seemingly distinct substrate preferences. Regardless, whether this reflects the complicated nature of ATM activation or the limitations of the experimentation conditions remains to be determined.

Despite these discussions, it should be pointed out that they are based on the hypothesis that S2546 undergoes reversible phosphorylation, which nonetheless has not been tested yet and thus calls for solid evidences. We may examine this hypothesis by direct sequencing through mass spectrometry or by immunoblot using a phospho-specific antibody.

### 3.4.3 Alternative explanations.

Although our current data have suggested that the reversible phosphorylation at S2546 might regulate ATM activity, it is still a yet-to-be-examined hypothesis. Therefore, it justifies consideration of alternative explanations before this hypothesis is proved real and true.

One possibility is that the SRI(2546-2548) sequence might be crucial for the binding of a positive regulator of the ATM kinase. Thus the SRI deletion mutation, and possibly the S2546D as well, ablates the association of this regulator which in turn lead to reduction or complete loss of the ATM kinase activity. In another possible scenario, the SRI region may contain an alternative type of post-translational modification (e.g. methylation/ demethylation at R2547) that is critical for ATM activity but is lost in the SRI deletion and abrogated by S2546D mutation. Both mutations thus could potentially abolish the activation of ATM in response to DSBs. Examination of these two possibilities may need identification of the SRI-binding regulator through promeomic and/or genetic approaches (yeast two hybrid) and require the generation of an antibody that can specifically recognize this particular modification (methylated R2547) in addition to a mass spectrometry-based direct sequencing. However, although unlikely, it is in theory possible that deletion of the SRI region or the S2546D substitution may cause a conformational change in the ATM protein that dramatically reduces or completely inactivates the kinase activity.

It should be pointed out that the above discussions apply to ATR as well. Considering the homology of the SRI regions local to SRIs in both ATM and ATR, the phenotypical resemblance between the phosphomimetic mutants at their respective

serine residues (S2546 in ATM and S2147 in ATR), as well as the similar requirement of PP5 for their kinase activation, it is reasonable to believe that ATM and ATR may share some common features in their individual activation processes which may involve a similar dephosphorylation at the key serine residues, methylation/demethylation at the following arginine residues (R2547 in ATM and R2148 in ATR), or a common regulator that binds at the SRI regions and positively modulates the activation of both kinases. Thus, a parallel study over these two kinases is warranted.

# 4 Define the role of a novel phosphorylation site (T622) of Rad17 in checkpoint activation

## *4.1 Introduction*

The radiation-sensitive (Rad) group of checkpoint proteins have long been identified to function at an early step in the eukaryotic DNA damage checkpoints to arrest cell cycle progression in response to DNA damage (Hartwell et al., 1994). They have now been determined to be responsible for the detection of various DNA lesions, thus are termed DNA damage sensors (Sancar et al., 2004; Zhou and Elledge, 2000). As a critical member of Rad protein, Rad17 acts in concert with the Rad9-Rad1-Hus1 complex to detect various type of DNA lesions and facilitate the initiation of DNA damage signals from two apical checkpoint kinases, ATM and ATR (Abraham, 2001; Sancar et al., 2004).

### 4.1.1 Structure and function of Rad17

In accordance with its crucial role in the surveillance of cell cycle progression, Rad17 orthologues have been found across the entire eukaryotic kingdom (Melo and Toczyski, 2002). The human *rad17* gene encodes a basic hydrophilic protein that contains 670aa residues and exhibits DNA binding activity. It is notable that Rad17 bears significant sequence homology with all five subunits of the replication factor C (RFC) (Figure 4-1), which functions to open the PCNA complex and load it onto DNA during unperturbed DNA replication (Griffiths et al., 1995; Majka and Burgers, 2004). Sequence analysis over these RFC-related proteins reveals that homology is concentrated mainly in a region containing seven conserved sequence motifs, which

include a Walker A-type nucleotide-binding motif and a metal binding catalytic site termed Walker B motif (Bluyssen et al., 1999; Cullmann et al., 1995). These features link Rad17 with all other RFC subunit proteins into a highly abundant and functionally diverse group of proteins referred to as the AAA+ class of ATPases (Neuwald et al., 1999; Venclovas et al., 2002).


**Figure 4-1: Schematic protein structure of hRad17.**

The human *rad17* gene encodes a 80 kDa protein containing 670aa residues. hRad17 protein has seven segments that share significant sequence homology with RFC proteins, which are indicated by the shaded boxes. The critical nucleotide binding motif and two phosphorylation sites, Ser635 and Ser645, are shown by arrows.

It has been shown that the ATPase activity is critical for Rad17 in checkpoint activation. A single mutation (K118E) in the nucleotide-binding motif of the fission yeast Rad17 abrogates the intrinsic ATP binding capacity and dramatically reduces its DNA binding ability (Kai et al., 2001), leading to a checkpoint defect mimicking that caused by a Rad17 null mutation (Griffiths et al., 1995). Consistent with these findings, several mammalian Rad17 variants with mutation in the nucleotide-binding motif associate poorly with chromatin, abolishing the checkpoint activation upon DNA damage when overexpressed (Bao et al., 1998; Rauen et al., 2000; Zou et al., 2002).

In sequence, Rad17 is most similar to the large subunit of RFC (p140, RFC1), and in response to DNA damage, replaces RFC1 to form a chromatin-bound complex

with the other four RFC subunits (RFC2-5) (Nyberg et al., 2002). The resulting Rad17-RFC complex functions to facilitate the translocation of another essential DNA damage sensor protein complex, Rad9-Rad1-Hus1, to the sites of DNA damage, in a manner mimicking the p140-RFC-dependent recruitment of PCNA during DNA replication (Figure 4-2) (Melo and Toczyski, 2002; Parrilla-Castellar et al., 2004). Though low in sequence homology with PCNA, the Rad9-Rad1-Hus1 complex forms a PCNA-like trimeric protein complex, whose Rad17-dependent recruitment to DNA lesions tethers downstream checkpoint/repair proteins for ATM/ATR-dependent activation and the subsequent DNA repair (Parrilla-Castellar et al., 2004; Sancar et al., 2004).

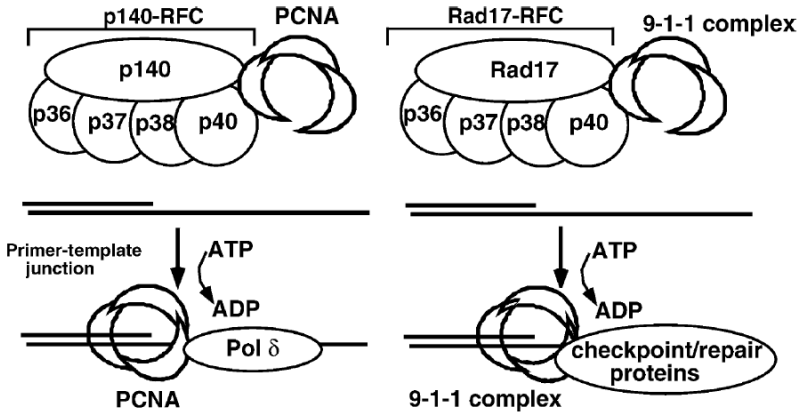


**Figure 4-2: Rad17–RFC recruits 9-1-1 complex in a way mimicking RFC-dependent loading of PCNA sliding clamp.**

During unperturbed DNA replication, p140–RFC loads the homotrimeric PCNA clamp around DNA at sites of replication in a reaction that hydrolyzes ATP to ADP. DNA-bound PCNA tethers DNA-metabolizing proteins such as DNA polymerase δ to the site of replication. In the Rad17–RFC complex, Rad17 replaces the large p140 subunit and complexes with the other four small RFC subunits. The Rad17–RFC clamp loader loads the heterotrimeric Rad9-Rad1-Hus1 (9–1–1) complex onto DNA at sites of DNA damage in an ATP-dependent manner. The 9–1–1 complex then tethers checkpoint and/or DNA repair proteins to the DNA lesion. (from (Parrilla-Castellar et al., 2004))

### 4.1.2 DNA damage-dependent and independent phosphorylation of Rad17

Similar to many checkpoint/repair proteins, Rad17 also undergoes DNA damage-responsive phosphorylation. Previously, our lab has demonstrated that two SQ/TQ motif-containing serine residues, S635 and S645, which are at the far C-terminus of hRad17, are phosphorylated in an ATM/ATR-dependent manner following genotoxic stresses (Figure 4-1) (Bao et al., 2001). Phosphorylation of Rad17 on these two sites appears to be critical for DNA damage-induced checkpoint activation in that human fibroblasts overexpressing an hRad17 phosphodeficient mutant on both sites (hRad17-S635A/S645A) displayed deficient activation of the G2/M checkpoint and reduced survival following genotoxic stress. Further analysis indicates that the defective G2/M checkpoint activation might be due to inefficient association between mutant Rad17 with Rad1, a component of the Rad9-Rad1-Hus1 complex (Bao et al., 2001).

A recent study, however, has argued against this claim. By use of a substitution system wherein the endogenous wildtype Rad17 was replaced with an S635A/S645A doubly phosphodeficient mutant, this report revealed that Rad17 phosphorylation at these two sites is not essential for G2/M checkpoint activation, but is instead required for the intra-S phase checkpoint induced by replication stress (Wang et al., 2006). Detailed investigation over the underlying mechanism further demonstrates that the defective response to replication stress caused by loss of Rad17 phosphorylation at S635/S645 might be due to the abolished association of mutant Rad17 with Claspin, an important checkpoint mediator protein required for ATR-dependent phosphorylation of Chk1. Although the initial Chk1 phosphorylation upon replication stress was not affected, it

failed to be maintained in the later stage (Wang et al., 2006). Therefore, this study established that Rad17 phosphorylation at S635 and S645 is essential for the intra-S phase checkpoint.

Although the above reports have demonstrated a critical role of Rad17 phosphorylation in response to genotoxic stresses, Rad17 may also undergo cell cycle-dependent phosphorylation. It was reported that both human and mouse Rad17 are phosphorylated during unperturbed S phase in replicating tissues, in a process that relies on ATR but is independent of DNA damage and ATM, and that the phosphorylated Rad17 preferentially localizes to DNA replication sites, possibly to monitor the progress of DNA replication through its interaction with DNA polymerase ε (Post et al., 2001; Post et al., 2003).

### 4.1.3 The study rationale

Although the use of a substitution system as described in the 2006 report (Wang et al., 2006) may have an advantage over our overexpression system (Bao et al., 2001) and give a better depiction of what the role of Rad17 phosphorylation (at S635/S645) plays in the process of checkpoint activation, it alone does not sufficiently explain the whole picture. As demonstrated elsewhere, Rad17 is linked to activation of various checkpoint pathways including intra-S phase checkpoint and G2/M checkpoint (Griffiths et al., 1995; Lydall and Weinert, 1995). Whereas the ATM/ATR-dependent phosphorylation of hRad17 at S635 and S645 is required for the former process (Wang et al., 2006), it is dispensable for the latter, suggesting that DNA-damage induced regulations of Rad17 other than ATM/ATR-dependent phosphorylation at S635/S645

might exist to account for the activation of G2/M checkpoint. The study to be covered in this chapter attempts to address this question by examining if Rad17 contains other ATM/ATR phosphorylation site(s) that is responsible for the activation of the G2/M checkpoint.

### 4.1.4 Rad17 may contain other ATM/ATR responsive site(s)

In a sequence analysis over human Rad17, we find that besides S635 and S645 there are four more serine/threonine residues (T88, S180, T548 and T622) that are followed by glutamine (Q) residues (Figure 4-3). The co-called SQ/TQ motif has long been established as the consensus ATM/ATR recognition site (Sancar et al., 2004). Among them, it is notable that T548 and T622 are within 100 residues from S635 and S645, a fact that makes Rad17 a putative SQ/TQ cluster domain (SCD)-containing protein. The term SCD was coined by Elledge and co-workers when they identified that the majority of the ten SQ/TQ motifs in a 246-residue segment of BRCA1 and similarly seven within just 51 residues of Chk2 are extensively phosphorylated by ATM (Cortez et al., 1999; Matsuoka et al., 1998). To date, several dozen DNA damage checkpoint/repair


**Figure 4-3: Rad17 carries multiple SQ/TQ sites.**

hRad17 has four more SQ/TQ sites besides the well-studied Ser635 and Ser645 (indicated by stars), which are Thr88, Ser180, Thr548 and Thr622. Among those, Thr548 and Thr622 are clustered with Ser635 and Ser645, which together make a putative SQ/TQ cluster domain (SCD) that may undergo extensive phosphorylation by ATM/ATR upon DNA damage.

proteins, including Rad9, Nbs1, 53BP1, MDC1, Chk1 and Chk2, have been found to contain SCDs and many of the SCD-residing Ser/Thr residues have been proved to be *bona fide* ATM/ATR phosphorylation sites in response to genotoxic stresses (Table 4-1). The presence of SCDs is now considered another structural hallmark of DNA damage response proteins, in addition to the well-established BRCT and FHA domains that display phospho-serine and phospho-threonine binding activity (Traven and Heierhorst, 2005).

**Table 4-1: DNA damage-responsive proteins that contain the SCD domain (excerpted from (Traven and Heierhorst, 2005)).**

| Protein | Organism | Position of SCD (amino acids) | Number of SQ/TQ residues/ length of SCD (1st-last SQ/TQ) |
|---|---|---|---|
| KINASES | | | |
| CHK1* | *Homo sapiens* | 317–367 | 4/51 |
| CHK2** | *Homo sapiens* | 19–69 | 7/51 |
| DNA PK | *Homo sapiens* | 2609–2648 | 4/40 |
| MEDIATORS | | | |
| BRCA1 | *Homo sapiens* | 1143–1525 | 12/383 |
| 53BP1 | *Homo sapiens* | 6–179 (SCD1) | 8/174 |
| | | 437–893 (SCD2) | 13/457 |
| | | 1068–1369 (SCD3) | 8/302 |
| MDC1 | *Homo sapiens* | 292–766 (SCD1) | 13/475 |
| | | 869–1226 (SCD2) | 10/358 |
| | | 1716–1834 (SCD3) | 5/119 |
| Rad9*** | *S. cerevisiae* | 390–458 | 6/69 |
| NBS1 | *Homo sapiens* | 278–494 | 5/217 |
| Claspin | *Homo sapiens* | 839–951 | 5/113 |
| Mrc1**** | *S. cerevisiae* | 90–273 | 12/184 |
| OTHER SCD PROTEINS | | | |
| Rad26 | *S. pombe* | 32–73 | 4/42 |
| RAD17 | *Homo sapiens* | 548–646 | 4/99 |
| SMC1 | *Homo sapiens* | 951–967 | 3/17 |
| FANCD2 | *Homo sapiens* | 596–718 (SCD1) | 5/123 |
| | | 1079–1113 (SCD2) | 3/35 |
| | | 1257–1419 (SCD3) | 5/163 |
| WRN | *Homo sapiens* | 991–1153 | 4/163 |
| CtIP | *Homo sapiens* | 664–746 | 3/83 |
| p53 | *Homo sapiens* | 15–167 | 4/153 |
| MDM2 | *Homo sapiens* | 351–420 | 7/70 |
| UPF1 | *Homo sapiens* | 10–98 (SCD1) | 5/89 |
| | | 982–1068 (SCD2) | 11/87 |
| Esc4 | *S. cerevisiae* | 743–807 | 4/65 |
| Mdt1 | *S. cerevisiae* | 310–597 | 14/288 |
| ASCIZ | *Homo sapiens* | 265–656 | 17/392 |

In this regard, the fact that Rad17 might contain a putative SCD at the far C-terminus might warrant T548 and T622 as two likely residues for ATM/ATR-dependent phosphorylation besides the previously-studied S635 and S645. We investigated this hypothesis and identified that T622 is a *bona fide* phosphorylation site which can be phosphorylated *in vitro* and *in vivo* and may be involved to maintain Chk1 phosphorylation and sustain G2/M checkpoint.

## *4.2 Materials and Methods*

### Cell Culture and Transfection

293T cells were cultured in DMEM containing 10% FBS (Invitrogen) plus antibiotics. U2OS cells were grown in MaCoy5A complemented with 10% FBS and antibiotics. Transfection of appropriated constructs was all performed with the FuGENE 6 transfection kit according to manufacturers' protocol (Roche).

### Plasmids and shRNAs

The constructs of GST-Rad17(486-670) and V5-Rad17 has been described previously (Bao et al., 2001), and used as templates to generate various GST-Rad17 mutants using the QuikChange™ Site-Directed Mutagenesis Kit following the manufacturer's protocol (Stratagene). The Flag-tagged ATR was described previously (Tibbetts et al., 1999).

### Antibodies and Reagents

The phospo-Rad17 (T622) antibody was generated by immunizing the rabbit with a phospho-peptide that corresponds to the T622 region of Rad17. The Rad17 polyclonal antibody was purchased from Santa Cruz Biotechnology. The γ-tubulin antibody was from Sigma-Aldrich and V5 from Invitrogen. The hydroxyurea was from Sigma-Aldrich.

**Immunoblotting**

Cells underwent appropriate treatment before being harvested in the lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol) supplemented with protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotonin) and phosphatase inhibitors (20 mM β-glycero-phosphate, 0.5 μM okadaic acid). Cell lysates were kept on ice for 10 min and cleared by full-speed centrifugation for 10 min at 4ºC, followed by quantitative determination of the protein concentrations using the modified Bradford protein assay kit from Bio-Rad. Lysates were then resolved in SDS-PAGE, transferred to Immobilon-P PVDF membrane and blotted with appropriate primary and secondary antibodies.

***In Vitro* Kinase Assay**

GST fusion proteins of various Rad17 mutants (486-670) were bacterially expressed, purified, and eluted as previously described (Bao et al., 2001). 293T cells were grown to approximately 50% confluency, transiently transfected with Flag tagged ATR expression constructs. Two days post-transfection cells were stimulated with UV (100 $J/m^2$) and harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol) which was supplemented with protease inhibitors (20 μg/ml leupeptin, 10

μg/ml pepstatin A, 10 μg/ml aprotonin) and phosphatase inhibitors (20 mM β-glycero-phosphate, 0.5 μM okadaic acid). The ATR kinase protein was then immunoprecipitated using anti-Flag antibody. Immune complexes were washed twice with kinase buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 4 mM $MnCl_2$, 6 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM dithiothreitol, and 100 mM $NaVO_4$). Various GST-Rad17 mutant protein substrates (200 ng to 1 mg), 10 mCi of $\gamma[^{32}P]$adenosine triphosphate (ATP), and ATP to a final concentration of 5 mM were added, and the reaction mixtures were incubated at 30°C for 15 min. After electrophoresis in SDS-polyacrylamide gels, the reaction products were visualized by autoradiography.

**Mitotic Index Assays**

The mitotic index assays were carried out to examine the IR-induced G2/M checkpoint. Specifically, cells were plated onto 10 cm plates and irradiated with IR. At the indicated time points following treatment, cells were harvested by trypsinization and fixed in 70% ethanol at -20°C. After permeabilization in 0.25% Triton X-100 in PBS, cells were incubated with anti-phospho-histone H3 antibody (pSer10, Upstate) for 4 hr at room temperature, followed by another incubation with FITC-conjugated secondary antibody for 1 hr (Jackson ImmunoResearch). Cells were subsequently counterstained with propidium iodide, and the phospho-histone H3 fluorescence and the DNA content were determined by flow cytometry. All experiments were performed in triplicates, and the percentage of mitotic cells was calculated as the mitotic index. For examination of the G2/M checkpoint, the mitotic index was normalized to that in the unperturbed controls.

## *4.3 Results*

### 4.3.1 T622 is a novel ATM/ATR-responsive phosphorylation site.

Since ATR-mediated phosphorylation of human Rad17 at S635 and S645 is critical for the intra-S phase checkpoint activation but unessential for the G2/M checkpoint pathway (Wang et al., 2006), we hypothesized that Rad17 may carry other yet-to-be-identified ATM/ATR responsive residues whose phosphorylation is required for the G2/M checkpoint activation upon DNA damage.

We performed a sequence analysis and identified four more residues (T88, S180, T548 and T622) that could potentially be targeted by ATM/ATR. Among them, T548 and T622 are in the putative SCD along with S635/S645, thus are more likely to undergo ATM/ATR-dependent phosphorylation upon DNA damage. To test this idea, we generated a variety of phosphodeficient mutant proteins of GST-Rad17 (486-670), in which one or multiple serine/threonine residues (T548, T622, S635 and S645) were substituted with non-phosphorylable alanine residue. An kinase assay was carried out to evaluate the potential of these mutant proteins to be phosphorylated by ATR *in vitro*. As shown in Figure 4-4, the wildtype GST-Rad17 protein displayed maximum phosphorylation after incubation with the ATR kinase in the presence of [$^{32}$P]-labeled ATP, whereas the S635/S645 double mutant and T548/S635/S645 triple mutant of GST-Rad17 exhibited a similarly reduced level of phosphorylation, suggesting that T622 but not T548 is phosphorylated by ATR. In consistency with this idea, phosphorylation of both the T622/S635/S645 triple mutant and the non-phosphorylable T548/T622/S635/S645 mutant was totally abrogated. Thus T622 may represent another ATR-responsive site in addition to the well-established S635 and S645.

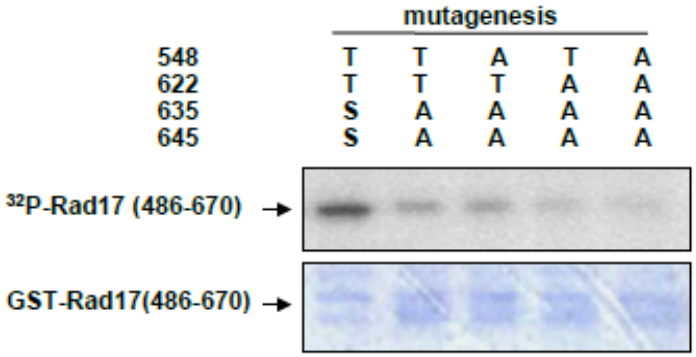


**Figure 4-4: T622 but not T548 can be phosphorylated *in vitro* by ATR.**

Various GST-Rad17 mutant proteins containing one or multiple Ser/Thr-to-Ala substitutions were generated and incubated with stimulated ATR kinase protein *in vitro*. The level of mutant proteins was examined by coomassie staining and ATR-dependent phosphorylation of these proteins was evaluated and compared by electrophoresis and autoradiography.

To confirm the above *in vitro* result, we generated a rabbit polyclonal antibody that could specifically recognize the phosphorylated T622. Cells were treated with various DNA damaging reagents including IR, UV and HU, and an immunoblot was conducted to examine if the endogenous hRad17 undergoes DNA damage-inducible phosphorylation at T622 using this phosphospecific antibody. As shown in Figure 4-5, a significant increase in the level of phosphorylated Rad17 was observed in genotoxically stressed cells compared with that in unperturbed cells, indicating that T622 is a *bona fide* phosphorylation site of hRad17 that responds to DNA damage. Since UV and HU treatment primarily activate ATR, this data confirmed the above *in vitro* result that ATR phosphorylates Rad17 at T622 *in vivo*. It is also noteworthy that T622 may be targeted by ATM as well, in that IR exposure, which preferentially stimulates ATM, also caused an increase of Rad17 phosphorylation at T622, albeit to a less extent than UV/HU treatment (Figure 4-5).

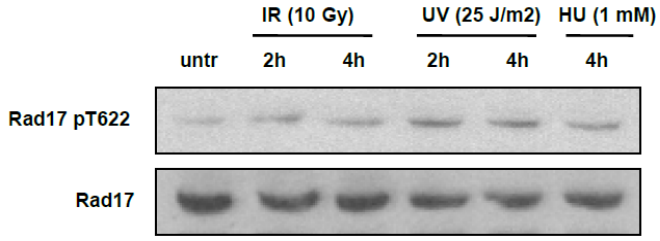


**Figure 4-5: T622 is phosphorylated in a DNA damage-inducible manner *in vivo*.**

Cells were exposed to indicated doses of DNA damaging reagents including IR (10 Gy), UV (25 J/m$^2$) and HU (1 mM) The level of phosphorylated Rad17 was examined by immunoblot using a phosphospecific antibody that recognizes phospho-T622.

Taken together, these data indicate that the SQ/TQ consensus motif-residing threonine 622 represents a novel DNA damage-responsive site that is phosphorylated by ATM/ATR following DNA damage.

### 4.3.2 Overexpression of a T622A mutant Rad17 fails to sustain Chk1 phosphorylation and G2/M checkpoint.

Previously it has been established that the ATM/ATR-dependent phosphorylation of Rad17 at S635 and S645 is essential to sustain Chk1 phosphorylation and maintain the subsequent intra-S phase checkpoint upon replication stress, but is surprisingly dispensable for the G2/M checkpoint (Wang et al., 2006), although Rad17 has long been shown to be critical for DNA damage-induced activation of various checkpoints (Griffiths et al., 1995; Lydall and Weinert, 1995). We hypothesize that ATM/ATR-mediated phosphorylation at T622 might complement that on S635 and S645 to mediate G2/M checkpoint activation.

To investigate this hypothesis, we generated a construct that can express in mammalian cells a mutant Rad17 where the T622 residue was substituted to the non-phosphorylable alanine. The resulted phosphodeficient Rad17-T622A was transiently transfected in U2OS cells, along with the wildtype (WT) and several other mutant forms of Rad17. As shown in Figure 4-6, both WT and Rad17-T622A could be efficiently expressed. We next examined if overexpression of this Rad17 mutant could cause a deficient G2/M checkpoint through a mitotic index analysis. Although cells overexpressing this T622-phosphodeficient mutant Rad17 could still initiate G2/M checkpoint with no detectable defects compared with those expressing an empty vector or the wildtype Rad17, as indicated by the similar level of reduction of the mitotic index within two hours post-IR exposure, they failed to sustain G2/M checkpoint and cells started to re-enter into mitosis 4 hr post-IR treatment (Figure 4-7). Thus Rad17 phosphorylation at T622 may play a crucial role in G2/M checkpoint; although it is unessential for the initiation step, it is necessary for the checkpoint to be maintained at the later stage.

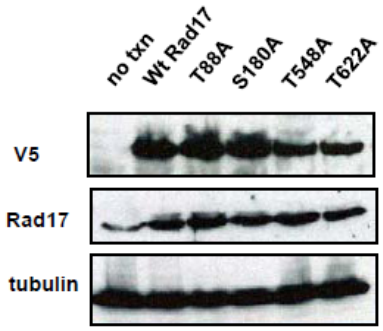


**Figure 4-6: Overexpression of Rad17-T622A, along with other substitution mutants.**

Various V5-tagged Rad17 mutant constructs were transfected into U2OS cells, which underwent immunoblot analysis forty-eight hours post-transfection using appropriate antibodies.

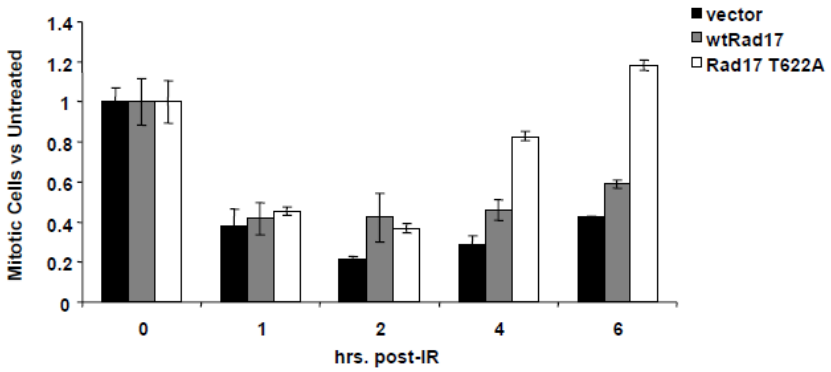


**Figure 4-7: Overexpression of Rad17-T622A failed to maintain G2/M checkpoint.**

The empty vector, wildtype (Wt) Rad17 and T622A substitution mutant Rad17 were transfected in U2OS cells, which were irradiated with IR forty-eight hours post-transfection. At the indicated time points, the mitotic indices were measured, normalized with untreated control cells and compared across different cells.

Because the current consensus holds that the genotoxic stress-induced Chk1 phosphorylation is the key event that mediates both checkpoint initiation and maintenance, we investigated whether the failure to sustain G2/M checkpoint in cells overexpressing Rad17-T622A is a consequence of dysfunctional Chk1 phosphorylation. In consistency with the above functional assay, Chk1 phosphorylation in Rad17-T622A overexpressing cells was initiated with no defect within two hours post-IR exposure, but started to taper off after four hours, in sharp contrast with cells transfected with the empty vector or wild-type Rad17, which displayed persistent phosphorylation of Chk1 in the 6 hr time window after IR irradiation (Figure 4-8). Thus overexpression of the T622 phosphodeficient mutant of Rad17 causes Chk1 phosphorylation unable to sustain, which in turn leads to failure of the host cells to maintain G2/M checkpoint.

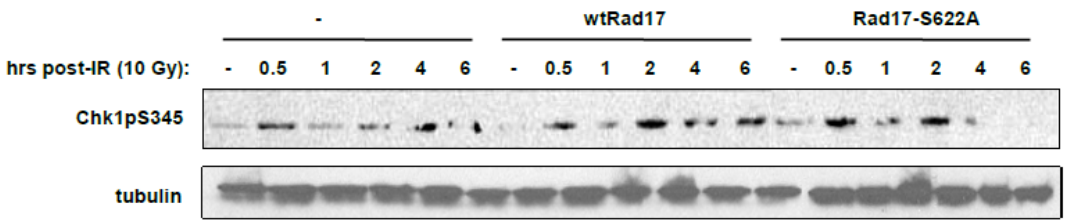


**Figure 4-8: Overexpression of Rad17-T622A failed to sustain Chk1 phosphorylation.**

The empty vector, wildtype (Wt) Rad17 and T622A substitution mutant Rad17 were transfected in U2OS cells, which were irradiated with IR forty-eight hours post-transfection. Chk1 phosphorylation at S345 was examined at the indicated time points after IR irradiation.

Taken together, these data suggest that DNA damage-inducible phosphorylation of hRad17 at threonine 622 is a critical event in the G2/M checkpoint activation in response to genotoxic stress. It is nonessential for the initiation activation of G2/M checkpoint, but is required to maintain Chk1 phosphorylation and sustain the G2/M checkpoint.

## *4.4 Discussion*

Although our current data are still preliminary, it is clear that T622 represents a novel site in human Rad17 that upon DNA damage undergoes ATM/ATR-dependent phosphorylation in addition to the two previously-documented serine residues (S635 and S645).

Based on the functional studies, we postulate that ATM/ATR-dependent phosphorylation at T622 might complement that on S635 and S645 to mediate G2/M checkpoint activation while the latter is primarily responsible for intra-S phase checkpoint. If this yet-to-be-confirmed hypothesis holds true, it may demonstrate an intriguing phenomenon that ATM/ATR-dependent phosphorylation of Rad17 at different

yet notably close sites may differentially regulate activation of distinct checkpoints. It is also worthy of note that these two functionally distinct phosphorylation events seem to involve a common mechanism, whereby the maintenance of different checkpoints is correlated with and very likely dependent on the duration of Chk1 phosphorylation. How the common Chk1 phosphorylation pathway is differentially sustained at various checkpoints by these two distinct phosphorylation events on Rad17 would be an interesting question that deserves an in-depth investigation. Presumably, phosphorylation of Rad17 at different sites (T622 V.S. S635/S645) may induce association of different checkpoint regulators that are specific to distinct checkpoints, leading to sustained Chk1 phosphorylation and the consequent maintenance of corresponding checkpoints.

It should be mentioned that all of the above discussions are based on our preliminary conclusion that T622 phosphorylation is responsible mainly for G2/M checkpoints, which nonetheless needs to be confirmed. To overcome the potential pit of causing artifacts by use of the overexpression system, we are currently replacing in U2OS cells the endogenous wildtype Rad17 with several mutant forms of Rad17, including the T622A substitution mutant and several other mutants with various combinations of substitution at the three phosphorylation residues. Use of this system should provide a more convincing data leading to more reliable conclusion whether T622 phosphorylation complements S635 and S645 phosphorylation in G2/M checkpoint. In addition, although Rad17 phosphorylation at T622 may differentiate from that on S635/ S645 in controlling G2/M checkpoint, theoretically it may be involved in regulating intra-S checkpoint as well, possibly to corroborate the role of Rad17 phosphorylation at

S635/S645. This possibility is also worth further examination by using this same substitution system.

# 5 Conclusions and Perspectives

The last two decades have witnessed great advances in our understanding of how eukaryotic cells respond to the nearly omnipresent genotoxic insults to preserve the integrity of their genetic information, thanks to the extensive and concerted efforts by numerous genetists, biochemists and cell biologists all around the world. It has been established that the DNA damage-induced extensive phosphorylation and activation of a great variety of checkpoint/repair proteins by two pivotal kinase pairs, ATM-Chk2 and ATR-Chk1, plays a seemingly dominant theme in the activation of the DNA damage response. As a consequence, cell cycle is delayed or arrested, repair proteins are mobilized to sites of DNA lesions, and in cases that DNA damage is beyond repair, cells are removed or silenced by apoptosis/senescence.

While a lot has been elucidated on the downstream events that mediate various effector pathways, little is known about how a specialized group of proteins termed DNA damage sensors, which include ATM, ATR, Rad17-RFC and Rad9-Rad1-Hus1, cooperate to detect DNA lesions and initiate the DNA damage signal upon genotoxic stress. Two studies of this dissertation attempt to address this question and may potentially further our understanding of the complicated DNA damage sensing process. Our preliminary work on ATM has suggested a critical role of serine 2546 in controlling the kinase activity of ATM which, if confirmed, could lead to a novel discovery that ATM activation may require removal of an inhibitory phospho group on this residue. In another still ongoing study, we have identified a novel ATM/ATR-responsive threonine residue in Rad17, whose phosphorylation might divert the role of Rad17 in G2/M checkpoint

activation from its previously reported function in intra-S phase checkpoint mediated by phosphorylation at two other residues, S635 and S645.

Although a large amount of knowledge has been accumulated about the initiation process of DNA damage response, how cells recover, the equally important flip side of the response, has remained less studied and poorly understood. Recently mounting evidence has suggested that dephosphorylation of some activated checkpoint/repair proteins may juxtapose another just emerging proteolytic degradation pathway to help conclude the DNA damage response, resulting in completion of DNA repair and extinguishment of checkpoints, ultimately allowing genotoxically stressed cells to re-enter into cell cycle (Figure 5-1). One study in this dissertation supports this view. We

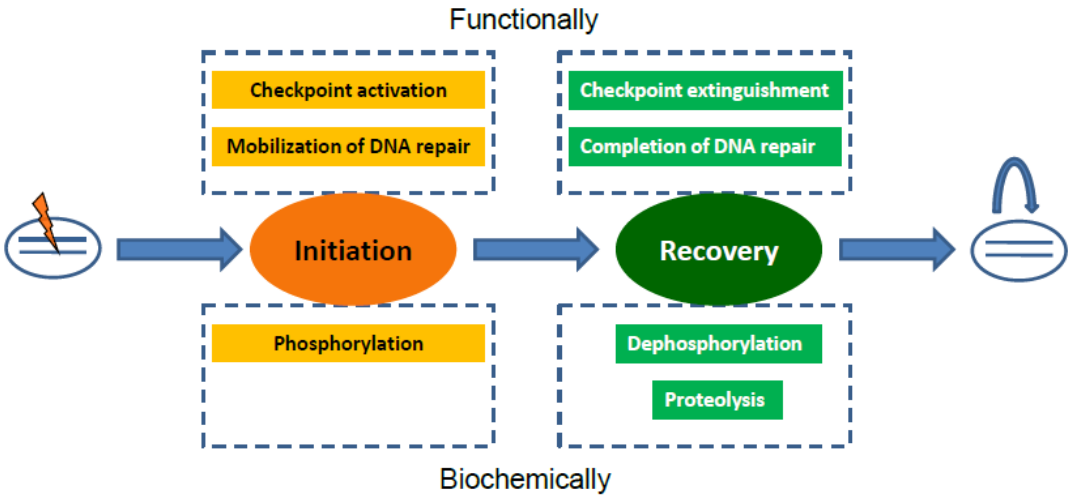


**Figure 5-1: Recovery is an equally important flip side of DNA damage response.**

In response to genotoxic insults, checkpoint and repair proteins are activated to arrest the cell cycle and mobilized at sites of DNA damage to prepare for DNA repair, in a process that depends on ATM/ATR/Chk1/Chk2-mediated phosphorylation. When cells are recovering, activated proteins undergo PP2A/Wip1-dependent dephosphorylation, or in some cases ubiquitin-dependent proteolysis, which help to extinguish checkpoints and complete DNA repair, ultimately leading to cell cycle re-entry.

have identified a critical role for the Ser/Thr protein phosphatase PP2A in the recovery process, which mediates dephosphorylation of RPA32 and γ-H2AX at the sites of DNA breaks, likely to facilitate DNA repair in cells recovering from replication stress. Work from this study also suggested that the successful recovery may require a proper coordination between checkpoint extinguishing and DNA repair completion, which might primarily be mediated by Wip1/PPM1D and PP2A respectively. Aside from deepening our understanding of the recovery process from DNA damage response, our study also has implication for novel cancer therapy.

## *5.1 RPA dephosphorylation is essential for efficient recovery.*

As has been revealed in Chapter 2, PP2A-dependent dephosphorylation of RPA32 at T21 and S33 is required for human cells to recover efficiently from HU-induced replication stress, possibly by participating in the DNA repair process. Despite a lack of direct evidence, it appears that PP2A may also mediate dephosphorylation of some, if not all, of the remaining serine residues (S4, S8, S11, S12 and S13), which also undergo DNA damage-inducible phosphorylation (Binz et al., 2004). Based on the observation that overexpression of a RPA32 hyperphosphomimic mutant induces high level of cytotoxicity to the parental cells (data not shown), we believe that dephosphorylation of these understudied serine residues is also a required event during recovery from replication stress, which may corroborate the role of T21/S33 dephosphorylation in the process of DNA breaks repair.

This conclusion may likely be extrapolated to more general cases. Besides replication stress, RPA32 hyperphosphorylation can be induced by many other types of genotoxic stress as well, which include IR, NCS, UV and various DNA alkylating agents such as methyl methanesulfonate and adozelesin (Liu et al., 2003; Morgan and Kastan, 1997; Wang et al., 2001). Although recovery-related RPA32 dephosphorylation under these conditions remains to be confirmed, given the essential role unphosphorylated RPA32 plays in supporting unperturbed DNA replication, it is conceivable that cells would not survive persistent RPA32 hyperphosphorylation following genotoxic stress and that dephosphorylation of these DNA damage-inducible sites is likely a necessary event in the recovery process. Based on our study, we speculate that RPA32 dephosphorylation may be involved in the DNA repair process as well.

However, as has been well-established, distinct DNA repair pathways involving different sets of repair proteins are mobilized in accordance to various types of DNA damage that cells are exposed to (Sancar et al., 2004). Thus although our study demonstrates that RPA32 dephosphorylation is required for the repair of HU-induced DSBs, it may be differentially involved among the distinct DNA repair pathways. Nonetheless, just as these distinct DNA repair mechanisms may still utilize some common factors, including RPA itself, it is not impossible that RPA32 dephosphorylation plays a more general role across these different repair pathways. Definitive answers to these intriguing questions may require identification of what particular role PP2A-mediated RPA32 dephosphorylation plays in the process of DNA repair under each stress condition. Studies over these questions not only help us understand the role of RPA32 dephosphorylation, they also expand our knowledge and deepen our

understanding over the still elusive processes of DNA repair, which have long been speculated to be stepwise and under tight spatiotemporal regulations (Sancar et al., 2004).

## *5.2 PP2A and Wip1/PPM1D may represent two master regulators driving the recovery process.*

Our study unequivocally demonstrates that PP2A serves as a crucial player in cellular recovery from DNA damage response. Besides RPA32, PP2A has also been shown to dephosphorylate γ-H2AX, another critical DNA damage responsive protein involved in formation and maintenance of DNA repair foci, possibly to promote the repair of DNA double-stranded breaks (Chowdhury et al., 2005). Since both RPA and γ-H2AX form DNA damage-inducible foci which also colocalize with PP2A at DNA lesions, as has been revealed in our study, it appears plausible that PP2A may be generally involved to facilitate DNA damage repair, which in response to DNA damage translocates to the DNA repair foci, actively dephosphorylating RPA and γ-H2AX to allow for the efficient repair of DNA lesions. It is thus far from being a merely passive process counteracting the seemingly dominant phosphorylation events in response to DNA damage. It should be noted that there are a great variety of DNA damage-responsive proteins that could also be phosphorylated following genotoxic stress and concentrate at the same DNA repair foci as RPA and γ-H2AX, which include Rad17, Rad9-Rad1-Hus1 complex, BRCA1, BRCA2, 53BP1 Mre11-Rad50-Nbs1 complex, MDC1 and SMC1 (Bekker-Jensen et al., 2006; Sancar et al., 2004). It remains an interesting open

question whether PP2A could target and dephosphorylate these repair foci-forming proteins as well, serving as a necessary event for the subsequent DNA repair.

Related to the above discussions, since PP2A usually relies on the regulatory B subunit for the substrate specificity and subcellular localization, it is also inviting to identify which particular B subunit targets the PP2A catalytic subunit to sites of DNA damage and mediates recovery-related dephosphorylation of RPA, γ-H2AX and possibly many others. However, given the presence of a large number of B subunits currently identified in mammals, which include four subfamily (B, B', B'' and B''') each further comprising up to a dozen of isoforms (Janssens and Goris, 2001), it is a dauntingly uneasy job for the determination of the responsible B subunit. Nonetheless it is crucial for a thorough understanding of the critical role PP2A plays in the recovery process from the DNA damage response.

The p53-induced protein phosphatase, Wip1/PPM1D, may represent another critical Ser/Thr phosphatase in cells recovering from DNA damage response (Moorhead et al., 2007). According to several recent studies, Wip1/PPM1D may function as a homeostatic regulator to actively turn off the DNA damage checkpoint signals, by dephosphorylating and deactivating important checkpoint proteins, Chk1, Chk2, p53 and p38 stress-response kinase (Fujimoto et al., 2006; Lu et al., 2004; Lu et al., 2005; Moorhead et al., 2007). All these Wip1/PPM1D substrates are phosphorylated by ATM/ATR at the original sites of DNA lesions yet become diffusive in the nucleoplasm afterwards. This interesting phenomenon, if not caused by coincidence, could lead to a bold yet generalized view that Wip1/PPM1D may be responsible for dephosphorylation/deactivation of nucleoplasmic checkpoint proteins to extinguish

activated checkpoints, as opposed to PP2A, which functions mainly at the sites of DNA damage to facilitate DNA repair (Figure 5-2).

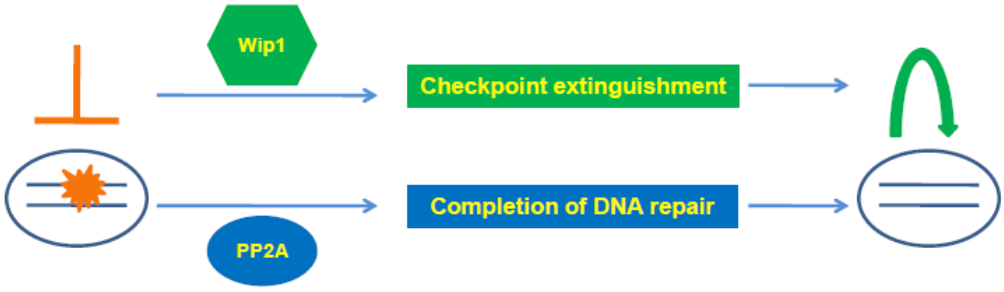


**Figure 5-2: PP2A and Wip1/PPM1D are two master regulators driving the recovery process.**

Activation of the DNA damage response arrests the cell cycle and mobilizes repair proteins at DNA lesions. The recovery process involves turning off checkpoints and completion of DNA repair, which are primarily mediated and driven by Wip1/PPM1D and PP2A respectively.

It should be mentioned that this may only represent a simplified model and that other Ser/Thr protein phosphatases, reportedly PP1 and PP4, may also be involved to dephosphorylate and deactivate checkpoint/repair proteins, as has been reported elsewhere (Chowdhury et al., 2008; Hsu, 2007). Whether these phosphatases serve as a necessary complement or are merely functionally redundant to PP2A and Wip1/PPM1D remain to be determined. Yet given their broader implications and substrate spectrums, it is plausible that PP2A and Wip1/PPM1D represent two master regulators controlling the eukaryotic recovery from DNA damage response.

## *5.3 Repair of DNA lesions and checkpoint recovery need to be well coordinated.*

Under a normal situation, cells do not re-enter into cell cycle until all the DNA lesions are fixed, purposely to avoid harmful accumulation of mutations, deleterious

genomic instability and even deadly mitotic catastrophe. Thus it is imaginable that the successful recovery from DNA damage response would demand that the DNA repair process and the checkpoint recovery process be well coordinated which, if the simplified model we propose in the above part holds true, are primarily mediated by PP2A and Wip1/PPM1D respectively (Figure 5-2).

This division of labor among these two recovery-associated protein phosphatases, though mainly speculative, may partly explain the non-coordinated dephosphorylation of Chk1 versus that of RPA32 and γ-H2AX and the consequent uncoupling between cell cycle re-entry and repair of DNA breaks observed in our study (Figure 2-13 and 2-14). In this experiment, recovering Hela cells that had been pulse-exposed to lethal dose of HU exhibited apparent dephosphorylation of Chk1 and concomitant re-entry into cell cycle but nonetheless displayed persistent phosphorylation of RPA32 and γ-H2AX, which is indicative of high level of DNA breaks (Figure 2-13). Despite the cell cycle reentry, cells did not survive the HU stress and ultimately died out, as indicated by the clonogenic assay (Figure 2-11). Although this mechanism of so-termed checkpoint adaptation may attribute unicellular yeasts with an advantage to survive a long-term arrest when facing persistent genotoxic stresses (Toczyski et al., 1997), it is overall detrimental to the multicellular metazoans because of its high potentials for genomic instability and mitotic catastrophe (Harrison and Haber, 2006; Syljuasen et al., 2006; Yoo et al., 2004).

This discovery exemplifies and highlights the importance of the tight coordination between these two protein phosphatases and the respective processes that they control. If the coordination goes awry, cells could be arrested infinitely, leading to a possible

consequence of senescence, or incur deadly mitotic catastrophe, culminating in cell death, either way being unfavorable for the well-being of the organisms. It is unclear, however, about how these two processes are coordinated. Presumably, this could be achieved by some cross-talks between these two processes that require future identification and characterization.

## *5.4 A novel regulation mechanism of ATM/ATR kinase activity*

Our preliminary data has demonstrated that two comparable serine residues, S2546 in ATM and S2147 in ATR, which are both notably ~ 100 residues upstream of their respective kinase domains, surprisingly affect the activities of both kinases. Although substitution of the serine residue to phosphodeficient alanine has no effect, replacement with phosphomimetic aspartic acid residue significantly reduces the kinase activities in both cases. Based on these observations, we put forward a bold hypothesis that there may exist a common activation mechanism between ATM and ATR, which requires DNA damage-induced removal of an inhibitory phospho group on the key serine residues upon genotoxic stress.

Assuming that this model holds true, given the high level of homology of the sequences flanking the key serine residues between ATM and ATR, it seems likely that dephosphorylation at these comparable sites of both kinases may be mediated by a common protein phosphatase that essentially plays a positive role in their respective activation processes. PP5 is a plausible candidate, as our previous studies have reported that PP5 associates with ATM and ATR in a DNA damage-inducible manner, and more importantly is required for the activation of both kinases (Ali et al., 2004;

Zhang et al., 2005a). Other protein phosphatases, such as PP2A, also exhibit a change in its interaction with ATM in response to DNA damage, thus deserve further investigation (Goodarzi et al., 2004). Regardless of what phosphatase mediates activation of ATM and/or ATR, demonstration of this model will shed light on the still elusive regulation mechanisms that control DNA damage-induced activation of both kinases. However, it should be pointed out that this hypothesis needs material evidences, which would hinge on either a direct sequencing through mass spectrometry or the generation of phosphospecific antibodies that can recognize these two comparable serine residues in ATM or ATR.

As has been discussed in Chapter 3, although our current data point to the above model that reversible phosphorylation at the key serine residues (S2546 in ATM and S2147 in ATR) regulates the activities of both kinases, alternative models exist that may involve methylation/demethylation at the following ariginine residues (R2547 in ATM and R2148 in ATR), non-covalent binding of a positive regulator, or even a mere conformational/functional essentiality of this 3-residue segment *per se* for their kinase activities. These possibilities are worth careful examination when necessary.

No matter which model is real, our findings unequivocally identify in ATM and ATR two comparably critical serine residues, and the local regions they resides in as well, which are outside of the PIKK kinase domains but nonetheless essential for the activity of both kinases. Studies over these interesting observations could lead to discovery of a novel regulation mechanism for the still poorly understood DNA damage-inducible activation of ATM and ATR.

## *5.5 Phosphorylation of Rad17 at different sites may activate distinct checkpoints.*

It has been well-established that the DNA damage sensor protein Rad17 undergoes DNA damage-inducible phosphorylation as a crucial early event in the activation of various checkpoints including G1, intra-S and G2/M checkpoints (Abraham, 2001; Sancar et al., 2004). Previously two C-terminal serine residues, S635 and S645, have been identified to be phosphorylated by ATM/ATR upon DNA damage (Bao et al., 2001), whose phosphorylation has been pinpointed to be nonessential for the G2/M checkpoint but nonetheless necessary for sustaining Chk1 phosphorylation and the consequent intra-S checkpoint (Wang et al., 2006).

How Rad17 activates G2/M checkpoint has since then become an interesting open question. The preliminary data from our still ongoing study might provide a possible answer to this question. We have shown that T622, an SQ/TQ motif-residing residue, is a novel ATM/ATR-responsive site that also undergoes DNA damage inducible phosphorylation in addition to the well-studied S635 and S645. More importantly, phosphorylation at T622 is likely to mediate G2/M checkpoint in that cells overexpressing a Rad17 mutant with T622A substitution failed to maintain Chk1 phosphorylation and exhibited a defect to sustain G2/M checkpoint. We are currently applying a cleaner substitution system to confirm this result. If substantiated, our findings may demonstrate distinct roles of two phosphorylation events, one on T622 and the other on S635/S645, in activating different checkpoints in response to DNA damage. However, how cells achieve this differentiation is unclear. Presumably, phosphorylation

of Rad17 at distinct sites may recruit different checkpoint mediators to the DNA damage foci leading to activation of distinct checkpoints.

It is noteworthy that the novelly identified phosphorylation residue T622 is very close to S635 and S645, which together form an SQ/TQ cluster domain (SCD) that is featured in quite a few important checkpoint proteins such as Rad9, Nbs1, 53BP1, MDC1, Chk1 and Chk2 (Traven and Heierhorst, 2005). Since ATM/ATR-dependent phosphorylation on the SCDs of these well-studied proteins may affect their activities and/or regulate the interaction with other proteins, we speculate that phosphorylation of Rad17 on these SCD-residing residues may play similar roles as well. However, since our study points to different roles of various phosphorylation events in activation of distinct checkpoints, it seems plausible that phosphorylation at T622 may affect its activity or more likely induce its interaction with other checkpoint regulators in a way distinguishing from that at S635/S645. It would be inviting to address the underlying mechanisms that determine the distinct consequences caused by phosphorylation at different sites. My speculation is that either T622 phosphorylation creates a novel interacting site for another checkpoint protein, plausibly the phospho-threonine preferred FHA-containing protein such as Nbs1 and Chk2 (Li et al., 2000a), or alternatively its phosphorylation, if combined with that at S635 and S645, merely achieves a certain threshold level of phosphorylation that is required for association of certain downstream checkpoint protein that would remain otherwise unbound. An example for the latter situation can be found from a study on the *Saccharomyces cerevisiae* Rad9, which showed that a threshold level of phosphorylation in the Rad9 SCD has to be reached for efficient Rad53 activation and subsequent cell survival in response to DNA damage

rather than phosphorylation of a particular site (Schwartz et al., 2002). Regardless of which hypothesis holds true, this study would help deepen our understanding of the complicated roles Rad17 phosphorylation plays in the activation of various checkpoints in response to DNA damage.

# References


Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev *15*, 2177-2196.

Abraham, R.T. (2004). PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. DNA Repair (Amst) *3*, 883-887.

Ali, A., Zhang, J., Bao, S., Liu, I., Otterness, D., Dean, N.M., Abraham, R.T., and Wang, X.F. (2004). Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. Genes Dev *18*, 249-254.

Anantha, R.W., Sokolova, E., and Borowiec, J.A. (2008). RPA phosphorylation facilitates mitotic exit in response to mitotic DNA damage. Proc Natl Acad Sci U S A *105*, 12903-12908.

Arnold, H.K., and Sears, R.C. (2006). Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. Mol Cell Biol *26*, 2832-2844.

Baber-Furnari, B.A., Rhind, N., Boddy, M.N., Shanahan, P., Lopez-Girona, A., and Russell, P. (2000). Regulation of mitotic inhibitor Mik1 helps to enforce the DNA damage checkpoint. Mol Biol Cell *11*, 1-11.

Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

Bakkenist, C.J., and Kastan, M.B. (2004). Initiating cellular stress responses. Cell *118*, 9-17.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-1677.

Bao, S., Shen, X., Shen, K., Liu, Y., and Wang, X.F. (1998). The mammalian Rad24 homologous to yeast Saccharomyces cerevisiae Rad24 and Schizosaccharomyces pombe Rad17 is involved in DNA damage checkpoint. Cell Growth Differ *9*, 961-967.

Bao, S., Tibbetts, R.S., Brumbaugh, K.M., Fang, Y., Richardson, D.A., Ali, A., Chen, S.M., Abraham, R.T., and Wang, X.F. (2001). ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. Nature *411*, 969-974.

Barford, D., Das, A.K., and Egloff, M.P. (1998). The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu Rev Biophys Biomol Struct *27*, 133-164.

Bartek, J., Bartkova, J., and Lukas, J. (2007). DNA damage signalling guards against activated oncogenes and tumour progression. Oncogene *26*, 7773-7779.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol *5*, 792-804.

Bartek, J., and Lukas, J. (2001). Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol *13*, 738-747.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell *3*, 421-429.

Bartek, J., and Lukas, J. (2007). DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol *19*, 238-245.

Bekker-Jensen, S., Lukas, C., Kitagawa, R., Melander, F., Kastan, M.B., Bartek, J., and Lukas, J. (2006). Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol *173*, 195-206.

Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem *71*, 333-374.

Bialojan, C., and Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J *256*, 283-290.

Binz, S.K., Lao, Y., Lowry, D.F., and Wold, M.S. (2003). The phosphorylation domain of the 32-kDa subunit of replication protein A (RPA) modulates RPA-DNA interactions. Evidence for an intersubunit interaction. J Biol Chem *278*, 35584-35591.

Binz, S.K., Sheehan, A.M., and Wold, M.S. (2004). Replication protein A phosphorylation and the cellular response to DNA damage. DNA Repair (Amst) *3*, 1015-1024.

Blasina, A., de Weyer, I.V., Laus, M.C., Luyten, W.H., Parker, A.E., and McGowan, C.H. (1999). A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr Biol *9*, 1-10.

Block, W.D., Merkle, D., Meek, K., and Lees-Miller, S.P. (2004). Selective inhibition of the DNA-dependent protein kinase (DNA-PK) by the radiosensitizing agent caffeine. Nucleic Acids Res *32*, 1967-1972.

Bluyssen, H.A., Naus, N.C., van Os, R.I., Jaspers, I., Hoeijmakers, J.H., and de Klein, A. (1999). Human and mouse homologs of the Schizosaccharomyces pombe rad17+ cell cycle checkpoint control gene. Genomics *55*, 219-228.

Bosotti, R., Isacchi, A., and Sonnhammer, E.L. (2000). FAT: a novel domain in PIK-related kinases. Trends Biochem Sci *25*, 225-227.

Boultwood, J. (2001). Ataxia telangiectasia gene mutations in leukaemia and lymphoma. J Clin Pathol *54*, 512-516.

Canman, C.E. (2003). Checkpoint mediators: relaying signals from DNA strand breaks. Curr Biol *13*, R488-490.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Chehab, N.H., Malikzay, A., Stavridi, E.S., and Halazonetis, T.D. (1999). Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci U S A *96*, 13777-13782.

Chen, J., Zheng, X.F., Brown, E.J., and Schreiber, S.L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc Natl Acad Sci U S A *92*, 4947-4951.

Chen, Y., and Sanchez, Y. (2004). Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA Repair (Amst) *3*, 1025-1032.

Chinkers, M. (2001). Protein phosphatase 5 in signal transduction. Trends Endocrinol Metab *12*, 28-32.

Chowdhury, D., Keogh, M.C., Ishii, H., Peterson, C.L., Buratowski, S., and Lieberman, J. (2005). gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. Mol Cell *20*, 801-809.

Chowdhury, D., Xu, X., Zhong, X., Ahmed, F., Zhong, J., Liao, J., Dykxhoorn, D.M., Weinstock, D.M., Pfeifer, G.P., and Lieberman, J. (2008). A PP4-phosphatase complex dephosphorylates gamma-H2AX generated during DNA replication. Mol Cell *31*, 33-46.

Cliby, W.A., Roberts, C.J., Cimprich, K.A., Stringer, C.M., Lamb, J.R., Schreiber, S.L., and Friend, S.H. (1998). Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J *17*, 159-169.

Cohen, P.T. (2002). Protein phosphatase 1--targeted in many directions. J Cell Sci *115*, 241-256.

Cohen, P.T., Philp, A., and Vazquez-Martin, C. (2005). Protein phosphatase 4--from obscurity to vital functions. FEBS Lett *579*, 3278-3286.

Cortez, D., Wang, Y., Qin, J., and Elledge, S.J. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science *286*, 1162-1166.

Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. (1995). Characterization of the five replication factor C genes of Saccharomyces cerevisiae. Mol Cell Biol *15*, 4661-4671.

Daboussi, F., Dumay, A., Delacote, F., and Lopez, B.S. (2002). DNA double-strand break repair signalling: the case of RAD51 post-translational regulation. Cell Signal *14*, 969-975.

Daniel, J.A., Pellegrini, M., Lee, J.H., Paull, T.T., Feigenbaum, L., and Nussenzweig, A. (2008). Multiple autophosphorylation sites are dispensable for murine ATM activation in vivo. J Cell Biol *183*, 777-783.

Dasika, G.K., Lin, S.C., Zhao, S., Sung, P., Tomkinson, A., and Lee, E.Y. (1999). DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. Oncogene *18*, 7883-7899.

de Feraudy, S., Limoli, C.L., Giedzinski, E., Karentz, D., Marti, T.M., Feeney, L., and Cleaver, J.E. (2007). Pol eta is required for DNA replication during nucleotide deprivation by hydroxyurea. Oncogene *26*, 5713-5721.

Dellaire, G., and Bazett-Jones, D.P. (2007). Beyond repair foci: subnuclear domains and the cellular response to DNA damage. Cell Cycle *6*, 1864-1872.

den Elzen, N.R., and O'Connell, M.J. (2004). Recovery from DNA damage checkpoint arrest by PP1-mediated inhibition of Chk1. EMBO J *23*, 908-918.

Dimitrova, D.S., and Gilbert, D.M. (2000). Stability and nuclear distribution of mammalian replication protein A heterotrimeric complex. Exp Cell Res *254*, 321-327.

Dozier, C., Bonyadi, M., Baricault, L., Tonasso, L., and Darbon, J.M. (2004). Regulation of Chk2 phosphorylation by interaction with protein phosphatase 2A via its B' regulatory subunit. Biol Cell *96*, 509-517.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Ellison, V., and Stillman, B. (2003). Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA. PLoS Biol *1*, E33.

Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature *410*, 842-847.

Favre, B., Turowski, P., and Hemmings, B.A. (1997). Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. J Biol Chem *272*, 13856-13863.

Fujimoto, H., Onishi, N., Kato, N., Takekawa, M., Xu, X.Z., Kosugi, A., Kondo, T., Imamura, M., Oishi, I., Yoda, A., and Minami, Y. (2006). Regulation of the antioncogenic Chk2 kinase by the oncogenic Wip1 phosphatase. Cell Death Differ *13*, 1170-1180.

Gasior, S.L., Wong, A.K., Kora, Y., Shinohara, A., and Bishop, D.K. (1998). Rad52 associates with RPA and functions with rad55 and rad57 to assemble meiotic recombination complexes. Genes Dev *12*, 2208-2221.

Golub, E.I., Gupta, R.C., Haaf, T., Wold, M.S., and Radding, C.M. (1998). Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. Nucleic Acids Res *26*, 5388-5393.

Goodarzi, A.A., Jonnalagadda, J.C., Douglas, P., Young, D., Ye, R., Moorhead, G.B., Lees-Miller, S.P., and Khanna, K.K. (2004). Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. EMBO J *23*, 4451-4461.

Grallert, B., and Boye, E. (2008). The multiple facets of the intra-S checkpoint. Cell Cycle *7*, 2315-2320.

Griffiths, D.J., Barbet, N.C., McCready, S., Lehmann, A.R., and Carr, A.M. (1995). Fission yeast rad17: a homologue of budding yeast RAD24 that shares regions of sequence similarity with DNA polymerase accessory proteins. EMBO J *14*, 5812-5823.

Guo, J.Y., Yamada, A., Kajino, T., Wu, J.Q., Tang, W., Freel, C.D., Feng, J., Chau, B.N., Wang, M.Z., Margolis, S.S., *et al.* (2008). Aven-dependent activation of ATM following DNA damage. Curr Biol *18*, 933-942.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

Harrison, J.C., and Haber, J.E. (2006). Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet *40*, 209-235.

Hartwell, L., Weinert, T., Kadyk, L., and Garvik, B. (1994). Cell cycle checkpoints, genomic integrity, and cancer. Cold Spring Harb Symp Quant Biol *59*, 259-263.

Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003). Apoptosis - the p53 network. J Cell Sci *116*, 4077-4085.

Heyer, W.D., Rao, M.R., Erdile, L.F., Kelly, T.J., and Kolodner, R.D. (1990). An essential Saccharomyces cerevisiae single-stranded DNA binding protein is homologous to the large subunit of human RP-A. EMBO J *9*, 2321-2329.

Hirano, Y., Reddy, J., and Sugimoto, K. (2008). Role of budding yeast Rad18 in repair of HO-induced double-strand breaks. DNA Repair (Amst).

Horejsi, Z., Falck, J., Bakkenist, C.J., Kastan, M.B., Lukas, J., and Bartek, J. (2004). Distinct functional domains of Nbs1 modulate the timing and magnitude of ATM activation after low doses of ionizing radiation. Oncogene *23*, 3122-3127.

Houtsmuller, A.B., Rademakers, S., Nigg, A.L., Hoogstraten, D., Hoeijmakers, J.H., and Vermeulen, W. (1999). Action of DNA repair endonuclease ERCC1/XPF in living cells. Science *284*, 958-961.

Hsu, L.C. (2007). Identification and functional characterization of a PP1-binding site in BRCA1. Biochem Biophys Res Commun *360*, 507-512.

Huang, W., and Erikson, R.L. (1994). Constitutive activation of Mek1 by mutation of serine phosphorylation sites. Proc Natl Acad Sci U S A *91*, 8960-8963.

Hubscher, U., Maga, G., and Spadari, S. (2002). Eukaryotic DNA polymerases. Annu Rev Biochem *71*, 133-163.

Iftode, C., Daniely, Y., and Borowiec, J.A. (1999). Replication protein A (RPA): the eukaryotic SSB. Crit Rev Biochem Mol Biol *34*, 141-180.

Jackson, D., Dhar, K., Wahl, J.K., Wold, M.S., and Borgstahl, G.E. (2002). Analysis of the human replication protein A:Rad52 complex: evidence for crosstalk between RPA32, RPA70, Rad52 and DNA. J Mol Biol *321*, 133-148.

Janssens, V., and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J *353*, 417-439.

Kai, M., Tanaka, H., and Wang, T.S. (2001). Fission yeast Rad17 associates with chromatin in response to aberrant genomic structures. Mol Cell Biol *21*, 3289-3301.

Karran, P. (2000). DNA double strand break repair in mammalian cells. Curr Opin Genet Dev *10*, 144-150.

Kastan, M.B. (2001). Cell cycle. Checking two steps. Nature *410*, 766-767.

Kastan, M.B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Khanna, K.K., and Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet *27*, 247-254.

Kitagawa, R., Bakkenist, C.J., McKinnon, P.J., and Kastan, M.B. (2004). Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. Genes Dev *18*, 1423-1438.

Kleckner, N. (1996). Meiosis: how could it work? Proc Natl Acad Sci U S A *93*, 8167-8174.

Koundrioukoff, S., Polo, S., and Almouzni, G. (2004). Interplay between chromatin and cell cycle checkpoints in the context of ATR/ATM-dependent checkpoints. DNA Repair (Amst) *3*, 969-978.

Kozlov, S.V., Graham, M.E., Peng, C., Chen, P., Robinson, P.J., and Lavin, M.F. (2006). Involvement of novel autophosphorylation sites in ATM activation. EMBO J *25*, 3504-3514.

Kunkel, T.A., and Erie, D.A. (2005). DNA mismatch repair. Annu Rev Biochem *74*, 681-710.

Lee, J.H., and Paull, T.T. (2004). Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. Science *304*, 93-96.

Lee, J.H., and Paull, T.T. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science *308*, 551-554.

Lee, S.E., Moore, J.K., Holmes, A., Umezu, K., Kolodner, R.D., and Haber, J.E. (1998). Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell *94*, 399-409.

Leroy, C., Lee, S.E., Vaze, M.B., Ochsenbien, F., Guerois, R., Haber, J.E., and Marsolier-Kergoat, M.C. (2003). PP2C phosphatases Ptc2 and Ptc3 are required for DNA checkpoint inactivation after a double-strand break. Mol Cell *11*, 827-835.

Li, D.W., Liu, J.P., Schmid, P.C., Schlosser, R., Feng, H., Liu, W.B., Yan, Q., Gong, L., Sun, S.M., Deng, M., and Liu, Y. (2006). Protein serine/threonine phosphatase-1 dephosphorylates p53 at Ser-15 and Ser-37 to modulate its transcriptional and apoptotic activities. Oncogene *25*, 3006-3022.

Li, J., Lee, G.I., Van Doren, S.R., and Walker, J.C. (2000a). The FHA domain mediates phosphoprotein interactions. J Cell Sci *113 Pt 23*, 4143-4149.

Li, L., and Zou, L. (2005). Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells. J Cell Biochem *94*, 298-306.

Li, S., Ting, N.S., Zheng, L., Chen, P.L., Ziv, Y., Shiloh, Y., Lee, E.Y., and Lee, W.H. (2000b). Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. Nature *406*, 210-215.

Liang, X., Reed, E., and Yu, J.J. (2006). Protein phosphatase 2A interacts with Chk2 and regulates phosphorylation at Thr-68 after cisplatin treatment of human ovarian cancer cells. Int J Mol Med *17*, 703-708.

Lisby, M., Barlow, J.H., Burgess, R.C., and Rothstein, R. (2004). Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. Cell *118*, 699-713.

Lisby, M., and Rothstein, R. (2004). DNA damage checkpoint and repair centers. Curr Opin Cell Biol *16*, 328-334.

Liu, J.S., Kuo, S.R., and Melendy, T. (2003). Comparison of checkpoint responses triggered by DNA polymerase inhibition versus DNA damaging agents. Mutat Res *532*, 215-226.

Longhese, M.P., Neecke, H., Paciotti, V., Lucchini, G., and Plevani, P. (1996). The 70 kDa subunit of replication protein A is required for the G1/S and intra-S DNA damage checkpoints in budding yeast. Nucleic Acids Res *24*, 3533-3537.

Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999). Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature *397*, 172-175.

Lu, X., Bocangel, D., Nannenga, B., Yamaguchi, H., Appella, E., and Donehower, L.A. (2004). The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair. Mol Cell *15*, 621-634.

Lu, X., Nannenga, B., and Donehower, L.A. (2005). PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. Genes Dev *19*, 1162-1174.

Lu, X., Nguyen, T.A., Moon, S.H., Darlington, Y., Sommer, M., and Donehower, L.A. (2008). The type 2C phosphatase Wip1: an oncogenic regulator of tumor suppressor and DNA damage response pathways. Cancer Metastasis Rev *27*, 123-135.

Lukas, C., Falck, J., Bartkova, J., Bartek, J., and Lukas, J. (2003). Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. Nat Cell Biol *5*, 255-260.

Lydall, D., and Weinert, T. (1995). Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science *270*, 1488-1491.

Mailand, N., Bekker-Jensen, S., Bartek, J., and Lukas, J. (2006). Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. Mol Cell *23*, 307-318.

Majka, J., and Burgers, P.M. (2004). The PCNA-RFC families of DNA clamps and clamp loaders. Prog Nucleic Acid Res Mol Biol *78*, 227-260.

Mamely, I., van Vugt, M.A., Smits, V.A., Semple, J.I., Lemmens, B., Perrakis, A., Medema, R.H., and Freire, R. (2006). Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery. Curr Biol *16*, 1950-1955.

Manke, I.A., Lowery, D.M., Nguyen, A., and Yaffe, M.B. (2003). BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science *302*, 636-639.

Margolis, S.S., Walsh, S., Weiser, D.C., Yoshida, M., Shenolikar, S., and Kornbluth, S. (2003). PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. EMBO J *22*, 5734-5745.

Matsuoka, S., Huang, M., and Elledge, S.J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

McIlwraith, M.J., Vaisman, A., Liu, Y., Fanning, E., Woodgate, R., and West, S.C. (2005). Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination. Mol Cell *20*, 783-792.

Melchionna, R., Chen, X.B., Blasina, A., and McGowan, C.H. (2000). Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat Cell Biol *2*, 762-765.

Melo, J., and Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. Curr Opin Cell Biol *14*, 237-245.

Meyn, M.S. (1995). Ataxia-telangiectasia and cellular responses to DNA damage. Cancer Res *55*, 5991-6001.

Meyn, M.S. (1999). Ataxia-telangiectasia, cancer and the pathobiology of the ATM gene. Clin Genet *55*, 289-304.

Mochan, T.A., Venere, M., DiTullio, R.A., Jr., and Halazonetis, T.D. (2003). 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. Cancer Res *63*, 8586-8591.

Moorhead, G.B., De Wever, V., Templeton, G., and Kerk, D. (2009). Evolution of protein phosphatases in plants and animals. Biochem J *417*, 401-409.

Moorhead, G.B., Trinkle-Mulcahy, L., and Ulke-Lemee, A. (2007). Emerging roles of nuclear protein phosphatases. Nat Rev Mol Cell Biol *8*, 234-244.

Morgan, S.E., and Kastan, M.B. (1997). Dissociation of radiation-induced phosphorylation of replication protein A from the S-phase checkpoint. Cancer Res *57*, 3386-3389.

Neuwald, A.F., Aravind, L., Spouge, J.L., and Koonin, E.V. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res *9*, 27-43.

Niida, H., and Nakanishi, M. (2006). DNA damage checkpoints in mammals. Mutagenesis *21*, 3-9.

Nyberg, K.A., Michelson, R.J., Putnam, C.W., and Weinert, T.A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet *36*, 617-656.

O'Connell, M.J., Raleigh, J.M., Verkade, H.M., and Nurse, P. (1997). Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. EMBO J *16*, 545-554.

O'Neill, B.M., Szyjka, S.J., Lis, E.T., Bailey, A.O., Yates, J.R., 3rd, Aparicio, O.M., and Romesberg, F.E. (2007). Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage. Proc Natl Acad Sci U S A *104*, 9290-9295.

Oakley, G.G., Patrick, S.M., Yao, J., Carty, M.P., Turchi, J.J., and Dixon, K. (2003). RPA phosphorylation in mitosis alters DNA binding and protein-protein interactions. Biochemistry *42*, 3255-3264.

Olson, E., Nievera, C.J., Klimovich, V., Fanning, E., and Wu, X. (2006). RPA2 is a direct downstream target for ATR to regulate the S-phase checkpoint. J Biol Chem *281*, 39517-39533.

Pandita, T.K. (2002). ATM function and telomere stability. Oncogene *21*, 611-618.

Parrilla-Castellar, E.R., Arlander, S.J., and Karnitz, L. (2004). Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. DNA Repair (Amst) *3*, 1009-1014.

Patrick, S.M., Oakley, G.G., Dixon, K., and Turchi, J.J. (2005). DNA damage induced hyperphosphorylation of replication protein A. 2. Characterization of DNA binding activity, protein interactions, and activity in DNA replication and repair. Biochemistry *44*, 8438-8448.

Pellegrini, M., Celeste, A., Difilippantonio, S., Guo, R., Wang, W., Feigenbaum, L., and Nussenzweig, A. (2006). Autophosphorylation at serine 1987 is dispensable for murine Atm activation in vivo. Nature *443*, 222-225.

Pellicioli, A., Lee, S.E., Lucca, C., Foiani, M., and Haber, J.E. (2001). Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. Mol Cell *7*, 293-300.

Peltomaki, P. (2001). DNA mismatch repair and cancer. Mutat Res *488*, 77-85.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-1505.

Peschiaroli, A., Dorrello, N.V., Guardavaccaro, D., Venere, M., Halazonetis, T., Sherman, N.E., and Pagano, M. (2006). SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. Mol Cell *23*, 319-329.

Post, S., Weng, Y.C., Cimprich, K., Chen, L.B., Xu, Y., and Lee, E.Y. (2001). Phosphorylation of serines 635 and 645 of human Rad17 is cell cycle regulated and is required for G(1)/S checkpoint activation in response to DNA damage. Proc Natl Acad Sci U S A *98*, 13102-13107.

Post, S.M., Tomkinson, A.E., and Lee, E.Y. (2003). The human checkpoint Rad protein Rad17 is chromatin-associated throughout the cell cycle, localizes to DNA replication sites, and interacts with DNA polymerase epsilon. Nucleic Acids Res *31*, 5568-5575.

Raleigh, J.M., and O'Connell, M.J. (2000). The G(2) DNA damage checkpoint targets both Wee1 and Cdc25. J Cell Sci *113 ( Pt 10)*, 1727-1736.

Rauen, M., Burtelow, M.A., Dufault, V.M., and Karnitz, L.M. (2000). The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9. J Biol Chem *275*, 29767-29771.

Riches, L.C., Lynch, A.M., and Gooderham, N.J. (2008). Early events in the mammalian response to DNA double-strand breaks. Mutagenesis *23*, 331-339.

Robison, J.G., Lu, L., Dixon, K., and Bissler, J.J. (2005). DNA lesion-specific co-localization of the Mre11/Rad50/Nbs1 (MRN) complex and replication protein A (RPA) to repair foci. J Biol Chem *280*, 12927-12934.

Roeder, G.S. (1990). Chromosome synapsis and genetic recombination: their roles in meiotic chromosome segregation. Trends Genet *6*, 385-389.

Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem *73*, 39-85.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., and et al. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science *268*, 1749-1753.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell *63*, 1129-1136.

Schofield, M.J., and Hsieh, P. (2003). DNA mismatch repair: molecular mechanisms and biological function. Annu Rev Microbiol *57*, 579-608.

Schwartz, M.F., Duong, J.K., Sun, Z., Morrow, J.S., Pradhan, D., and Stern, D.F. (2002). Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint. Mol Cell *9*, 1055-1065.

Scott, S.P., Bendix, R., Chen, P., Clark, R., Dork, T., and Lavin, M.F. (2002). Missense mutations but not allelic variants alter the function of ATM by dominant interference in patients with breast cancer. Proc Natl Acad Sci U S A *99*, 925-930.

Shcherbakova, P.V., Bebenek, K., and Kunkel, T.A. (2003). Functions of eukaryotic DNA polymerases. Sci Aging Knowledge Environ *2003*, RE3.

Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-334.

Shiloh, Y. (1997). Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. Annu Rev Genet *31*, 635-662.

Shreeram, S., Demidov, O.N., Hee, W.K., Yamaguchi, H., Onishi, N., Kek, C., Timofeev, O.N., Dudgeon, C., Fornace, A.J., Anderson, C.W., *et al.* (2006). Wip1 phosphatase modulates ATM-dependent signaling pathways. Mol Cell *23*, 757-764.

Shrivastav, M., De Haro, L.P., and Nickoloff, J.A. (2008). Regulation of DNA double-strand break repair pathway choice. Cell Res *18*, 134-147.

Smith, G.C., Divecha, N., Lakin, N.D., and Jackson, S.P. (1999). DNA-dependent protein kinase and related proteins. Biochem Soc Symp *64*, 91-104.

Smith, G.C., and Jackson, S.P. (1999). The DNA-dependent protein kinase. Genes Dev *13*, 916-934.

Solomon, D.A., Cardoso, M.C., and Knudsen, E.S. (2004). Dynamic targeting of the replication machinery to sites of DNA damage. J Cell Biol *166*, 455-463.

Spring, K., Ahangari, F., Scott, S.P., Waring, P., Purdie, D.M., Chen, P.C., Hourigan, K., Ramsay, J., McKinnon, P.J., Swift, M., and Lavin, M.F. (2002). Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. Nat Genet *32*, 185-190.

Spring, K., Cross, S., Li, C., Watters, D., Ben-Senior, L., Waring, P., Ahangari, F., Lu, S.L., Chen, P., Misko, I., *et al.* (2001). Atm knock-in mice harboring an in-frame deletion corresponding to the human ATM 7636del9 common mutation exhibit a variant phenotype. Cancer Res *61*, 4561-4568.

Su, T.T. (2006). Cellular responses to DNA damage: one signal, multiple choices. Annu Rev Genet *40*, 187-208.

Sugiyama, T., Zaitseva, E.M., and Kowalczykowski, S.C. (1997). A single-stranded DNA-binding protein is needed for efficient presynaptic complex formation by the Saccharomyces cerevisiae Rad51 protein. J Biol Chem *272*, 7940-7945.

Sun, Y., Jiang, X., Chen, S., Fernandes, N., and Price, B.D. (2005). A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci U S A *102*, 13182-13187.

Syljuasen, R.G., Jensen, S., Bartek, J., and Lukas, J. (2006). Adaptation to the ionizing radiation-induced G2 checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases. Cancer Res *66*, 10253-10257.

Tan, T.L., Essers, J., Citterio, E., Swagemakers, S.M., de Wit, J., Benson, F.E., Hoeijmakers, J.H., and Kanaar, R. (1999). Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation. Curr Biol *9*, 325-328.

Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C., and Abraham, R.T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev *13*, 152-157.

Toczyski, D.P., Galgoczy, D.J., and Hartwell, L.H. (1997). CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. Cell *90*, 1097-1106.

Traven, A., and Heierhorst, J. (2005). SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. Bioessays *27*, 397-407.

Uto, K., Inoue, D., Shimuta, K., Nakajo, N., and Sagata, N. (2004). Chk1, but not Chk2, inhibits Cdc25 phosphatases by a novel common mechanism. EMBO J *23*, 3386-3396.

van Vugt, M.A., van de Weerdt, B.C., Vader, G., Janssen, H., Calafat, J., Klompmaker, R., Wolthuis, R.M., and Medema, R.H. (2004). Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. J Biol Chem *279*, 36841-36854.

Vassin, V.M., Wold, M.S., and Borowiec, J.A. (2004). Replication protein A (RPA) phosphorylation prevents RPA association with replication centers. Mol Cell Biol *24*, 1930-1943.

Venclovas, C., Colvin, M.E., and Thelen, M.P. (2002). Molecular modeling-based analysis of interactions in the RFC-dependent clamp-loading process. Protein Sci *11*, 2403-2416.

Vietri, M., Bianchi, M., Ludlow, J.W., Mittnacht, S., and Villa-Moruzzi, E. (2006). Direct interaction between the catalytic subunit of Protein Phosphatase 1 and pRb. Cancer Cell Int *6*, 3.

Waddell, D.S., Liberati, N.T., Guo, X., Frederick, J.P., and Wang, X.F. (2004). Casein kinase Iepsilon plays a functional role in the transforming growth factor-beta signaling pathway. J Biol Chem *279*, 29236-29246.

Wang, H., Guan, J., Perrault, A.R., Wang, Y., and Iliakis, G. (2001). Replication protein A2 phosphorylation after DNA damage by the coordinated action of ataxia telangiectasia-mutated and DNA-dependent protein kinase. Cancer Res *61*, 8554-8563.

Wang, X., and Haber, J.E. (2004). Role of Saccharomyces single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair. PLoS Biol *2*, E21.

Wang, X., Ira, G., Tercero, J.A., Holmes, A.M., Diffley, J.F., and Haber, J.E. (2004). Role of DNA replication proteins in double-strand break-induced recombination in Saccharomyces cerevisiae. Mol Cell Biol *24*, 6891-6899.

Wang, X., Zou, L., Lu, T., Bao, S., Hurov, K.E., Hittelman, W.N., Elledge, S.J., and Li, L. (2006). Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. Mol Cell *23*, 331-341.

Watters, D., Khanna, K.K., Beamish, H., Birrell, G., Spring, K., Kedar, P., Gatei, M., Stenzel, D., Hobson, K., Kozlov, S., *et al.* (1997). Cellular localisation of the ataxia-telangiectasia (ATM) gene product and discrimination between mutated and normal forms. Oncogene *14*, 1911-1921.

Wei, K., Kucherlapati, R., and Edelmann, W. (2002). Mouse models for human DNA mismatch-repair gene defects. Trends Mol Med *8*, 346-353.

Weill, J.C., and Reynaud, C.A. (2008). DNA polymerases in adaptive immunity. Nat Rev Immunol *8*, 302-312.

Westphal, C.H. (1997). Cell-cycle signaling: Atm displays its many talents. Curr Biol *7*, R789-792.

Wittekind, M., Reizer, J., Deutscher, J., Saier, M.H., and Klevit, R.E. (1989). Common structural changes accompany the functional inactivation of HPr by seryl phosphorylation or by serine to aspartate substitution. Biochemistry *28*, 9908-9912.

Wold, M.S. (1997). Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Biochem *66*, 61-92.

Wu, X., Shell, S.M., and Zou, Y. (2005a). Interaction and colocalization of Rad9/Rad1/Hus1 checkpoint complex with replication protein A in human cells. Oncogene *24*, 4728-4735.

Wu, X., Yang, Z., Liu, Y., and Zou, Y. (2005b). Preferential localization of hyperphosphorylated replication protein A to double-strand break repair and checkpoint complexes upon DNA damage. Biochem J *391*, 473-480.

Yoo, H.Y., Kumagai, A., Shevchenko, A., and Dunphy, W.G. (2004). Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. Cell *117*, 575-588.

Yu, X., Chini, C.C., He, M., Mer, G., and Chen, J. (2003). The BRCT domain is a phospho-protein binding domain. Science *302*, 639-642.

Yu, Y.M., Pace, S.M., Allen, S.R., Deng, C.X., and Hsu, L.C. (2008). A PP1-binding motif present in BRCA1 plays a role in its DNA repair function. Int J Biol Sci *4*, 352-361.

Zhang, J., Bao, S., Furumai, R., Kucera, K.S., Ali, A., Dean, N.M., and Wang, X.F. (2005a). Protein phosphatase 5 is required for ATR-mediated checkpoint activation. Mol Cell Biol *25*, 9910-9919.

Zhang, Y.W., Hunter, T., and Abraham, R.T. (2006). Turning the replication checkpoint on and off. Cell Cycle *5*, 125-128.

Zhang, Y.W., Otterness, D.M., Chiang, G.G., Xie, W., Liu, Y.C., Mercurio, F., and Abraham, R.T. (2005b). Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. Mol Cell *19*, 607-618.

Zhao, H., and Piwnica-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol *21*, 4129-4139.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.

Zou, L., Cortez, D., and Elledge, S.J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev *16*, 198-208.

Zou, L., and Elledge, S.J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.

Zou, L., Liu, D., and Elledge, S.J. (2003). Replication protein A-mediated recruitment and activation of Rad17 complexes. Proc Natl Acad Sci U S A *100*, 13827-13832.

Zou, Y., Liu, Y., Wu, X., and Shell, S.M. (2006). Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. J Cell Physiol *208*, 267-273.

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