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GENOTOXIN-INDUCED ACETYLATION OF THE WERNER SYNDROME PROTEIN (WRN) AND EFFECT ON ITS DNA METABOLIC FUNCTION

Enerlyn Meliza Lozada Santiago
University of Kentucky, enerlyn@gmail.com

ABSTRACT OF DISSERTATION

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The Graduate School
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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Enerlyn Meliza Lozada Santiago

Lexington, Kentucky

Director: Dr.David Orren, Professor of Toxicology

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Loss of function of the WRN protein causes the genetic disorder Werner Syndrome that is characterized by increased cancer and premature aging. WRN belongs to the RecQ helicase family that plays key roles in preventing genome instability. In response to treatment with genotoxins, WRN is subject to post-translational modification. The relationship of post-translational modification of WRN with its function in DNA metabolism is unknown. There is accumulating evidence suggesting that WRN contributes to the maintenance of genomic integrity through its involvement in DNA replication. Consistent with this notion, WS cells are sensitive to DNA replication inhibitors and DNA damaging agents that tend to block replication fork progression. The cells exhibit an extended S phase, as well as defects in normal bi-directional progression of replication forks diverging from the majority of replication origins. To elucidate the relationship between post-translational modifications of WRN with its function in DNA metabolism, here the acetylation of WRN was studied. In our studies, we provide evidence that WRN acetylation is a dynamic process that strongly correlates to blockage of replication by persistent DNA damage. We also determined the effect of WRN acetylation on its specificity and enzymatic functions. In addition, our studies reveal how agents that block replication regulate the nature of WRN interactions with RPA, a factor known to bind to single-stranded DNA generated at blocked replication forks. Our results demonstrated that WRN and RPA form a stable direct association under normal physiological conditions and treatments that block replication fork progression increase their association, further supporting the idea that WRN is involved in DNA replication through its action at blocked or stalled replication forks. Thus, these studies point to both 1) an important role for acetylation of WRN and 2) its interaction with RPA in the putative function of WRN in response to blocked replication. Overall, our results impact knowledge regarding the relationship between DNA damage, genome instability and the development and progression of aging and cancer.

KEYWORDS: Werner Protein, Acetylation, Genotoxins, DNA damage, Replication

Enerlyn Meliza Lozada Santiago

Students's Signature

9/30/2011

Date

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By

Enerlyn Meliza Lozada Santiago

David K. Orren, Ph.D.

Director of Dissertation

Liya Gu, Ph.D.

Director of Graduate Studies

9/30/2011

Date

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…….....to my family and God because all my accomplishments to them I owe.

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CHAPTER I

INTRODUCTION

WERNER SYNDROME

Werner Syndrome (WS) is a rare autosomal recessive genetic disease caused by a deficiency of a single gene product known as WRN. This syndrome was first identified by Otto Werner, a German scientist who described the syndrome in his doctoral thesis in 1904. WS is characterized by increased cancer and early onset or increased frequency of specific age-related phenotypes, including graying and loss of hair, skin ulceration, atherosclerosis, osteoporosis, cataracts, hypertension, and diabetes mellitus type II [Goto et al., 1997; Chen et al., 2002; Orren 2006]. The syndrome has specific symptoms not found in normal aging, including short stature, hyperkeratosis and soft tissue calcification. WS patients usually develop normally until they reach the second decade of life. In fact, most WS patients are not diagnosed until their 20's or even 30's. The median age of patients who die from WS is 54. Primarily they die from cancer or cardiovascular disease [Huang et al., 2006]. Because of this large overlap with normal aging, the relatively normal early development, and the mildness of aging symptoms, it is believed that WS is an excellent model system for the study of human aging [Martin et al., 1978].

ROLE OF WRN IN GENOME MAINTENANCE

Cells from WS patients show several distinct abnormalities when compared with normal cells, including elevated frequency of spontaneous chromosomal aberrations characterized by deletions, insertions, and translocations as well as an increased rate of telomere shortening [Gebhart et al., 1988; Honma et al., 2002]. In an effort to determine the role of WRN in DNA metabolism, WRN-deficient cells have been subjected to many DNA damaging regimens. They are hypersensitive to DNA damaging agents such as 4-nitroquinolone-1-oxide (4NQO), interstrand crosslinking agents including mitomycin C (MMC) and cisplatin, and topoisomerase inhibitors such as camptothecin [Gebhart et al.,

1988; Ogburn et al., 1997; Poot et al., 1999]. In addition, they are hypersensitive to agents that tend to block progression of replication forks, including hydroxyurea (HU) [Pichierri et al., 2001]. Together, this evidence suggests that WRN functions in cellular responses to maintain genome stability, such as DNA repair, replication and/or recombination pathways. However, the sensitivity of WS cells to damaging agents does not appear to reflect a direct role in an established DNA repair pathway. Instead, sensitivity to certain DNA damaging agents and HU suggest that WRN plays a role in responding to replication blockage by lesions or other obstructions.

Additional evidence supports the role of WRN in DNA replication. WRN expression is upregulated during S phase in highly proliferative transformed cell lines [Kawabe et al., 2000b]. WS cells have dramatically reduced replicative capacity leading to very early cellular senescence; they grow slowly and have been reported to have a longer S phase as well as possibly replication initiation and elongation abnormalities [Martin et al., 1970; Takeuchi et al., 1982b; Salk et al., 1985; Poot et al., 1992; Yan et al., 1998]. WRN-deficient cells are defective in normal bi-directional progression of replication forks diverging from the majority of replication origins [Rodriguez-Lopez et al., 2002]. This suggests a high frequency of replication fork stalling and is consistent with the notion that WRN may play an important but non-essential role in replication [Takeuchi et al., 1982a; Hanaoka et al., 1985]. Thus, it has been suggested that WRN is involved either in preventing the collapse of stalled replication forks or in resolving intermediates present at blocked forks. Consistent with this idea, recent studies have shown that: 1) upon replication arrest, WRN is redistributed to distinct nuclear foci (where it co-localizes with RPA) and is phosphorylated and acetylated [Constantinou et al., 2000; Sakamoto et al., 2001; Blander et al., 2002], and 2) WRN can coordinate its unwinding and pairing activities to regress a model replication fork substrate [Machwe et al., 2006 and 2007]. From these observations (summarized in Table 1.1), it seems likely that WRN plays a role in a DNA metabolic pathway that allows the cell to properly deal with blockage of replication forks. Thus, the nature of genomic instability, increased cancer and premature aging observed in WS may be the result of improper resolution of blocked replication and illegitimate recombination caused by loss of WRN function.

Table 1.1 Evidence indicating a role of WRN in the maintenance of genomic integrity through its involvement in DNA replication

Findings	References
WS cells exhibit a reduced replicative life span.	Martin et al., 1970; Salk et al., 1985
WS cells exhibit an extended S phase.	Takeuchi et al., 1982b; Poot et al., 1992
WS cells are hypersensitive to agents that tend to block progression of replication forks.	Gebhart et al., 1988; Ogburn et al., 1997; Pichierri et al., 2001; Poot et al., 1999, 2001
WRN moves to nuclear foci that correspond to sites of ongoing replication following HU and DNA damaging treatments.	Karmakar et al., 2005; Sakamoto et al., 2001; Constantinou et al, 2000
WRN expression is upregulated during S phase in highly proliferative transformed cell lines.	Kawabe et al., 2000b
WS cells are defective in normal bi-directional progression of replication forks diverging from the majority of replication origins	Rodriguez-Lopez et al., 2002
WRN regress model replication forks *in vitro.*	Machwe et al., 2006 and 2007
Functional and physical interaction with RPA, FEN-1, PCNA, Topoisomerase I, and DNA Polymerase δ	Shen et al., 1998a, 2003; Brosh et al., 1999; Doherty et al., 2005; Sommers et al., 2005 ; Brosh et al., 2001, 2002; Sharma et al., 2004 and 2005 ; Lebel et al., 1999; Rodriguez-Lopez et al., 2003 ; Lebel et al., 1999, Lane et al.,2003 ; Kamath-Loeb et al., 2000, 2001; Szekely et al., 2000

It is well known that WRN associates or interacts directly with factors involved in DNA replication (references in Table 1.1). For example, WRN interacts with proteins required for lagging strand synthesis such as PCNA and FEN-1. The interaction with PCNA is remarkable since PCNA has key roles in important processes, besides DNA replication, such as DNA repair and recombination. WRN also interacts physically and functionally with RPA that protects single-stranded DNA and binds to gaps at blocked replication forks. What is more, Topo I, a protein that facilitates DNA replication by relaxing the tension generated by winding/unwinding of DNA, interacts with WRN. This interaction is not only physical but also functional; since WRN stimulates the ability of Topo I to relax negatively supercoiled DNA and Topo I inhibit the ATPase activity of WRN. However, the direct link of WRN involvement in DNA replication is its interaction with DNA polymerase δ, a major replicative DNA polymerase. Taken together, these observations suggest that WRN is involved in maintaining functional DNA replication forks and absence of these interactions in WS cells might contribute to the inability of those cells to properly respond to blockage of replication.

POST-TRANSLATIONAL MODIFICATIONS OF WRN

WRN is subject to post-translational modifications that modulate the localization and activities of WRN upon DNA damage (Figure 1.1 summarizes the results of the latest studies in WRN modification). Among the most common post-translational modifications are phosphorylation, sumoylation and acetylation, all of which have been reported for WRN.

The first reported post-translational modification on WRN was sumoylation when endogenous and ectopically expressed WRN was shown to be modified by ubiquitin-like SUMO-1 molecules within cells. Conjugation of SUMO to WRN has been shown to be promoted by the nucleolar tumor suppressor p14 and the SUMO-conjugating enzyme Ubc9 [Kawabe et al., 2000a; Woods et al., 2004]. This modification correlates with WRN redistribution within the nucleus, suggesting that sumoylation might affect the availability or localization of WRN.

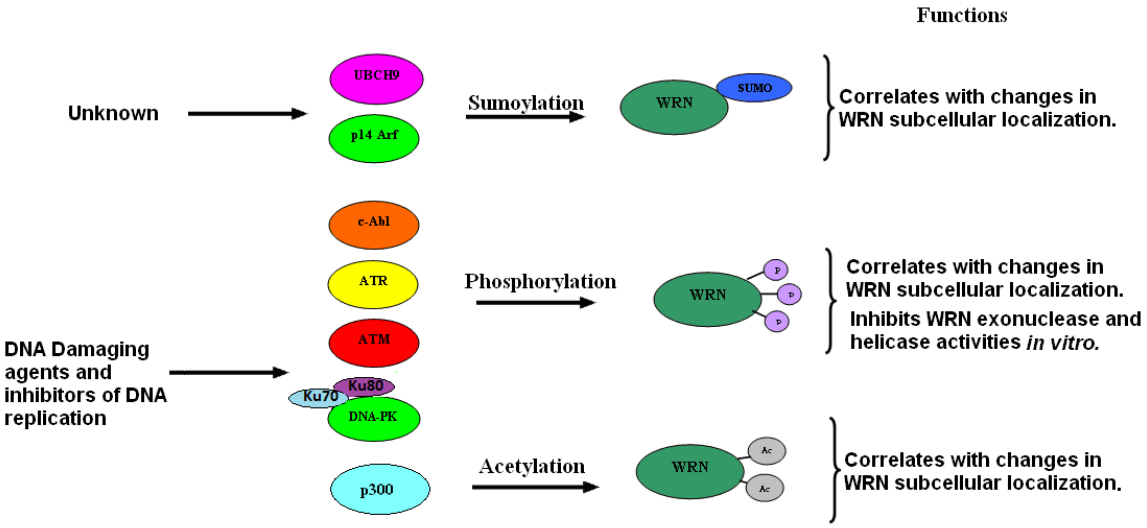

Figure 1.1 Functional consequences of WRN post-translational modifications

In addition, it has been reported that WRN is phosphorylated at multiple ser/thr residues after DNA damaging treatments (including MMC and bleomycin) and other agents that block DNA replication (such as HU) [Pichierri et al., 2003; Karmakar et al., 2002 and 2005; Yannone et al., 2001; Ammazzalorso et al., 2010]. DNA-dependent protein kinase (DNA-PK) was reported to be involved in WRN phosphorylation *in vitro* and is required for WRN phosphorylation *in vivo* [Yannone et al., 2001; Karmakar et al., 2002]. Interestingly, phosphorylation of WRN with DNA-PK inhibits its helicase and exonuclease activities, suggesting that WRN modifications may be a way of regulating WRN catalytic activities. The proteins ATR and ATM, members of the phosphoinositide 3-kinases family (PI3-Ks), as DNA-PK, phosphorylate WRN as well. ATR/ATM-dependent WRN phosphorylation affects WRN translocation and co-localization with its protein partners, including RPA, in nuclear foci after DNA damage [Pichierri et al., 2003; Ammazzalorso et al., 2010]. Additionally, it was found that the nuclear tyrosine kinase c-Abl regulates WRN phosphorylation. c-Abl phosphorylates WRN at tyrosine residues correlating with the translocation of WRN from nucleoli to discrete nuclear foci upon DNA damage [Cheng et al., 2003].

In addition to being sumoylated and phosphorylated, WRN is also acetylated *in vivo* [Blander et al., 2002]. WRN acetylation is promoted by the acetyltransferase p300. Interestingly, deacetylated WRN is localized in the nucleolus and acetylation of WRN correlates with its recruitment to the nucleoplasm. Furthermore, Karmakar and colleagues reported that DNA damaging agents, including MMC, promote WRN acetylation [Karmakar et al., 2005]. Taken together, it is likely that cellular WRN trafficking and activity is regulated by several types of post-translational modifications, which in turn may be related to a specific DNA damage response pathway.

WERNER SYNDROME (WRN) GENE

The gene known to be defective in WS is located on the short arm of chromosome 8 between positions 12 and 11.2. It was initially localized by linkage analysis, and the use of markers that were found to be in linkage disequilibrium in WS patients [Goto et al.,

1992; Schellenberg et al., 1992; Thomas et al., 1993; Ye et al., 1995]. Finally, it was identified in 1996 by positional cloning. The discovery of the gene was viewed as significant, not only because a gene associated with aging was identified, but because it also turned out to be a cancer susceptibility gene [Yu et al., 1996; Nakura et al., 1996]. The WRN gene spans more than 250 kb and consists of 35 exons (from those, only 34 are coding exons) that encode a protein of 1,432 amino acids [Yu et al., 1997]. The gene was cloned by the Martin and Schellenberg groups at the University of Washington, and its coding sequence immediately suggested a role in DNA metabolism. The existence of 7 conserved sequence motifs, typical of proteins with ATPase and/or helicase activity, placed WRN in the RecQ helicase family [Yu et al., 1996].

WRN AS A RECQ MEMBER

In general, helicases use the energy derived from the hydrolysis of ATP to catalyze the unwinding of double-stranded nucleic acids. The RecQ helicases are a subfamily of DNA helicases that are highly conserved through evolution [Bachrati et al., 2003]. Prokaryotes and lower eukaryotic species generally contain a single RecQ family member, including Sgs1 in *Saccharomyces cerevisiae* and RecQ in *Escherichia coli.* However, higher organisms contain multiple RecQ members. The human genome contains at least five RecQ genes (WRN, BLM, RECQ4, RECQ1, and RECQ5) that encode seven distinct proteins including three isoforms of RECQ5 generated by alternative splicing [Bachrati et al., 2008]. All RecQ helicases share a centrally located domain of ~450 residues that contains the seven conserved helicase motifs (see Figure 1.2). Downstream of the helicase domain, some RecQ members have additional regions of homology, known as the RecQ-conserved (RQC) and the Helicase and RNase D C-terminal (HRDC) domains.

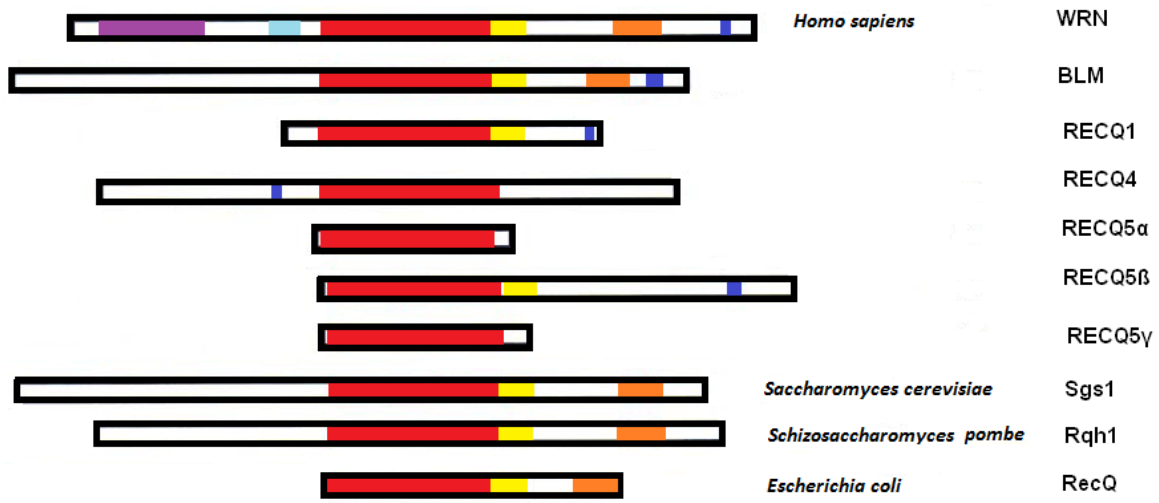

Figure 1.2 Schematic representations of RecQ family members. The name of the organisms and the proteins are listed on the right. The proteins are aligned with respect to their conserved helicase domain (in red). The less conserved RQC and HRDC domains, discussed in the text, are depicted in yellow and orange, respectively. WRN also contains an exonuclease domain (in purple) and a 27 residue direct repeat (in light blue). The NLS sequences are shown in dark blue.

In general, loss of function of a RecQ member results in higher levels of illegitimate recombination, although the resulting types of chromosomal instability can vary somewhat. Thus, RecQ helicase appear to have prominent roles in the maintenance of genome stability, although the precise details of their roles in DNA metabolism are still unknown. Germ-line defects in three of the five human known RecQ helicases are associated with hereditable diseases. Specifically, defects in WRN, BLM or RECQ4 result in Werner, Bloom or Rothmund-Thomson Syndrome, respectively. Individuals with these diseases are highly cancer-prone, but those with Bloom and Rothmund-Thomson have fewer aging characteristics than WS patients [Martin et al., 2000].

PROPERTIES OF THE WRN PROTEIN

WRN has an approximate molecular weight of 162 kilodaltons [Chen et al., 2002]. It has several structural domains (schematically depicted in Figure 1.3) that contribute to its physiological function. Several laboratories, including ours, have overproduced and purified recombinant WRN protein and characterized its domains and the basic catalytic activities associated with them. This section briefly describes each of those domains and the catalytic activities or properties related with them.

Several studies have demonstrated that WRN possesses DNA-binding activity. In fact, it has four distinct DNA-binding regions, including the helicase, RQC and HRDC domains [Liu et al., 1999; von Kobbe et al., 2003; Hu et al., 2005; Lee et al., 2005]. Its exonuclease domain also possesses DNA-binding affinity, albeit much weaker than the aforementioned domains [Machwe et al., 2006a]. Importantly, the DNA binding activity of WRN appears to be dependent upon DNA structure with no apparent nucleotide sequence preference [Orren et al., 1999; Brosh et al., 2002a]. It has higher affinity to single-stranded than for double-stranded DNA [Orren et al., 1999], in a manner influenced by substrate length [Machwe et al., 2006a]. WRN binds most stably, and has higher affinity, to complex DNA structures including substrates containing bubbles and D-loops [Orren et al., 2002; Shen et al., 2000; Brosh et al., 2002a].

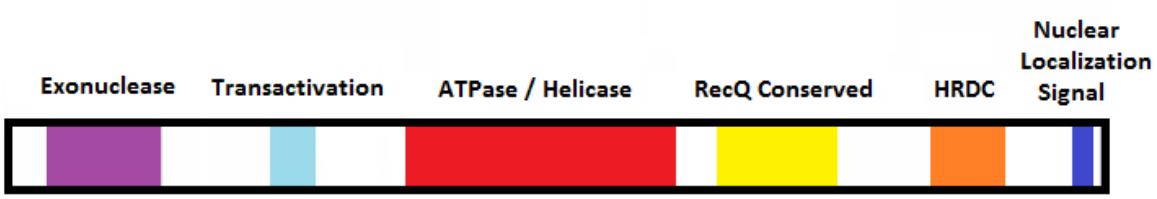

Figure 1.3 Schematic representations of the domains of WRN. The domains of interest are depicted and identified.

Consistent with its strong homology to RecQ helicases, the central region of WRN confers ATPase activity that provides the energy for unwinding DNA with a 3'→5' directionality [Gray et al., 1997; Suzuki et al., 1997; Shen et al., 1998a]. This unwinding activity is highly DNA structure-specific in its action. It prefers special DNA structures, such as those formed during replication and recombination, including forks, bubbles, and Holliday junction intermediates [Constantinou et al., 2000; Brosh et al., 2001a; Mohaghegh et al., 2001; Lebel et al., 1999; Orren et al., 2002; Opresko et al., 2004]. Interestingly, our laboratory has demonstrated that, similar to some recombination proteins, WRN also facilitates the pairing of complementary DNA strands [Machwe et al., 2005]. This annealing activity acts in concert with its helicase activity to perform strand exchange, branch migration, and regression of model replication forks [Constantinou et al., 2000; Machwe et al., 2005, 2006b and 2007]. Thus, these facts suggest that WRN might be involved in recombination and/or replication-related pathways to maintain genome stability.

The existence of an N-terminal RNase D-type domain, not present in other human RecQ members, confers to WRN an intrinsic 3'→5' exonuclease activity [Huang et al., 1998; Shen et al., 1998b; Mian et al., 1997]. Thus, the unique premature aging phenotype of WS may be due to the loss of both helicase and exonuclease functions of WRN in DNA metabolic pathways. Biochemical analysis of WRN exonuclease activity indicates that the enzyme prefers the degradation of DNA duplex with a recessed 3' end and alternate structures such as an internal bubble, D-loops and Holliday junctions [Orren et al., 2002; Shen et al., 2000; Machwe et al., 2002]. Interestingly, a recombinant WRN without the ATPase/helicase domain retains exonuclease activity [Huang et al., 1998], indicating that the exonuclease domain folds into a functional unit that can be uncoupled from WRN helicase activity. Although several biochemical studies have examined possible coordination between the helicase and exonuclease activities of WRN, whether and how they might act together *in vivo* remains unclear.

WRN has a direct repeat of a highly acidic 27-amino acid sequence localized between the region containing the exonuclease and helicase activities. This acid repeat has been

shown to be involved in protein-protein interaction such as the WRN and RPA interaction [Doherty et al., 2005]. The C terminus contains the nuclear localization signal (NLS). Individuals with WS have WRN gene mutations that truncate the gene product prior to the NLS. This has lead to the theory that the observed aging phenotypes result from the total absence of WRN's nuclear functions.

In summary, WS is a well-established model system for investigating the relationships between chromosomal instability and development of cancer and other age-related diseases. At the cellular level, WRN deficiency results in replication abnormalities, extended S-phase, and hypersensitivity to certain DNA-damaging and replication blocking agents. In normal cells, subsequent to DNA-damaging treatments or HU, WRN is recruited to distinct nuclear foci and co-localizes with replication factors. WRN preferentially acts on complex DNA structures, such as those formed during replication and recombination. Collectively, this evidence indicates that WRN may play an important role in response to replication blockage.

RESEARCH OBJECTIVES

Accumulating evidence indicates that WRN has a critical function for maintaining genomic stability. Consistent with its putative role in DNA metabolism, WRN is localized in the nucleus. However, upon DNA damage and blockage of replication, WRN migrates into discrete nuclear foci. These subnuclear foci are sites of ongoing or arrested DNA replication, supporting WRN function in restoration of blocked replication. In support of a replication-related role for WRN, it colocalizes with the replication factor RPA in replication foci. The latter suggests that WRN and RPA might interact at stalled replication forks, influencing WRN role in accurate resolution of replication blockage. Importantly, this WRN redistribution correlates with WRN acetylation, suggesting that this post-translational modification may regulate WRN function at blocked replication forks. The fact that WRN catalytic activities have preference in resolving unusual DNA structures and regressing model replication forks are also consistent with a function of WRN in remodeling or resolving blocked replication forks.

The goal of this study is to further clarify the role of WRN in response to agents that damage DNA and/or block replication, with a particular emphasis on the relationship of acetylation of WRN with its function in DNA metabolism. The specific hypothesis is that blockage of replication by DNA damage in the template or other circumstances (nucleotide depletion, in the case of HU) induces translocation and acetylation of WRN to perform a key function in proper resolution of these obstructions to replication. In the absence of WRN, when replication undergoes pausing as in the case of nucleotide depletion or arrest at the site of DNA damage, the cell cannot properly resolve the resulting structures. Subsequently, collapse of the replication fork causes double-strand break formation and increased illegitimate recombination. This is highly consistent with the increased genomic instability observed in WRN-deficient cells. Thus, to investigate the events surrounding the potential function of WRN in response to DNA damage and replication blockage, our studies have been focused on WRN acetylation to determine its impact on the role of WRN in DNA damage response. The specific aims are: 1) to study the dynamics of WRN acetylation and its relationship to DNA damage, 2) to investigate the effect of WRN acetylation on its biochemical functions, and 3) to study if the nature of WRN interaction with RPA is altered in response to agents that block replication. Our findings should help to determine an important role for WRN in maintaining genome integrity and whether and to what extent acetylation of WRN contributes to genomic integrity surveillance.

This study has addressed the following issues in three chapters:

Chapter 2 – Genotoxin-induced WRN dynamic acetylation and its correlation to blockage of replication by persistent DNA damage

Chapter 3 – WRN acetylation regulates its enzymatic activities

Chapter 4 –WRN interaction with RPA in response to agents that block replication

CHAPTER II

GENOTOXIN-INDUCED WRN DYNAMIC ACETYLATION AND ITS CORRELATION TO BLOCKAGE OF REPLICATION BY PERSISTENT DNA DAMAGE

INTRODUCTION

DNA damage may induce post-translational modification of proteins to allow proteins to be regulated in a temporal and spatial manner, in most cases to ensure efficient regulation of cellular processes in response to genotoxic stress. [Appella et al., 2001; Huen et al., 2008; Polo et al., 2011]. The most common and well-studied protein modifications are acetylation, sumolyation, ubiquitylation, methylation and phosphorylation. The importance of post-translational modification is highlighted by the results of several recent studies that demonstrate interplay between multiple protein modifications that combine to propagate the DNA damage signal to elicit cell cycle arrest, DNA repair, apoptosis and senescence [Huen et al., 2008]. Recent studies have shown that WRN is subject to post-translational modifications in response to several DNA damaging agents. Specifically, WRN is subject to acetylation, sumoylation and phosphorylation [Blander et al., 2002; Karmakar et al., 2002 and 2005; Pichierri et al., 2003; Yannone et al., 2001]. The work presented here has focused on WRN acetylation to investigate the impact of this modification on the function of WRN in DNA metabolism.

First identified for histones over 40 years ago, acetylation of lysine residues of proteins is now known to occur in more than 80 transcription factors, many nuclear regulators, and various cytoplasmic proteins. It is emerging as a key mechanism by which proteins are regulated in several physiological processes such as migration, metabolism and aging [reviewed on Close et al., 2010 and Choudhary et al., 2009]. In humans, the acetylation state of proteins is determined by two main groups of enzymes [Figure 2.1]. The first one is the group of histone aceyltransferases (HATs), also called acetylases,

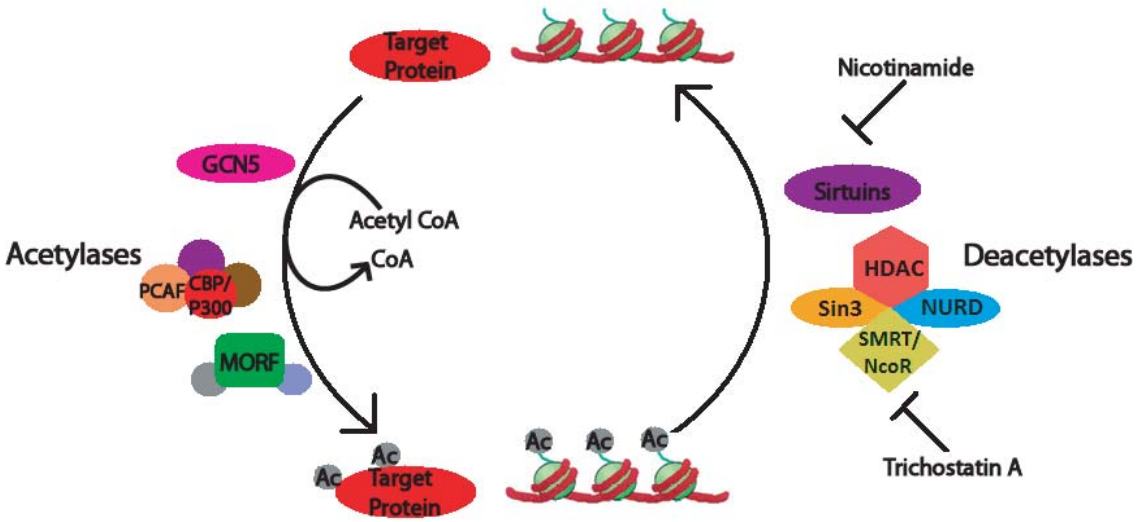

Figure 2.1 Protein Acetylation. The acetylation process involves two main groups of proteins: 1) Acetylases (including GNC5, CBP/p300, PCAF and the MORF complex) that are responsible for adding the acetyl groups onto lysines in proteins, and 2) Deacetylases that remove acetyl groups from lysine amino acids. The main ones are the histone deacetylases (HDACs) and the sirtuins (SIRTs). HDACs are usually found as multisubunit complexes with proteins containing histone deacetylase and remodeling activities, such as NURD, and corepressors such as SMRT/NcoR and Sin3.

that are responsible for adding the acetyl groups onto lysine of proteins. Of these enzymes, the p300/CBP family is the most characterized and continues to receive the most attention. The second group of enzymes is known as deacetylases that remove acetyl groups from lysine amino acids in proteins. The main ones are the histone deacetylases (HDACs) and the sirtuins that include seven members (SIRT1-7). The importance of these enzymes is highlighted by the fact that aberrant protein acetylation and deacetylation activity is associated with various diseases, including solid tumors and leukemias [Marks et al., 2010; Cress et al., 2000]. Given their association with cancer formation, novel compounds endowed with a deacetylase inhibitory activity, such as nicotinamide and Trichostatin A, have gained interest as both cancer chemopreventive and therapeutic agents [Federico et al., 2011].

It has been demonstrated that acetylation may affect the function of a protein, the nature of the interaction with its protein partners, and the sub-cellular localization. For example, acetylation of the genomic guardian and tumor suppressor p53 has been shown to be induced by DNA damaging agents resulting in alteration of its ubiquitination, stability and nuclear localization [Liu et al., 1999; Zhao et al., 2006; Chao et al., 2006]. In addition, p53 acetylation regulates the interaction of p53 with its protein partners (including Mdm2) and its ability to increase transcription of p21 and suppress cell growth [Tang et al., 2008; Zhao et al., 2006]. Together, these findings indicate that acetylation can play a key role exerting multifaceted effects to control various cellular and biological processes *in vivo*.

Recent studies have shown that the cellular function of WRN appears to be regulated by acetylation. Specifically, Blander and colleagues reported that WRN acetylation *in vivo* is promoted by the acetyltransferase p300 [Blander et al., 2002]. Moreover, it has been shown that deacetylated WRN is localized in the nucleolus and that acetylation of WRN correlates with its translocation to nuclear foci (where WRN colocalizes with PML nuclear bodies that are known to contain protein acetyltransferases); these discrete subnuclear regions correspond to sites of ongoing and/or blocked DNA synthesis in which WRN co-localizes with replication factors (such as RPA) in response to DNA

damaging agents and the replication inhibitor HU [Constantinou et al., 2000; Blander et al., 2002; Karmakar et al., 2005]. Taken together, it is likely that regulation of WRN within cells is altered by protein acetylation, and perhaps is related to its function in a specific DNA metabolic pathway.

In order to further understand the regulation of WRN by protein modification, we studied the relationship between DNA damage, inhibition of DNA replication and WRN acetylation. Although some studies indicate potential roles for acetylation in WRN regulation, it remains unclear the circumstances in which WRN becomes acetylated and how is WRN specifically regulated by acetylation. In this chapter, we report that WRN is detectably acetylated under normal conditions and that acetylation of WRN significantly increases after treatment with DNA damaging agents and inhibitors of DNA replication. Importantly, we provide evidence that it is not simply the induction of damage but its persistence that enhances WRN acetylation. The kinetics of WRN acetylation, its cell cycle relationship and the roles of HDACs and sirtuins in regulation of WRN acetylation were also investigated. Together, these studies advance our understanding of the dynamics of WRN modification in response to DNA damage.

RESEARCH DESIGN

SPECIFIC AIM: To study the dynamics of WRN acetylation and its relationship to DNA damage.

Aim a. To establish methods to measure endogenous WRN acetylation

Aim b. To investigate the relationship between DNA damage and/or replication blockage and WRN acetylation

Aim b1. To determine the influence of DNA damaging agents and/or replication inhibitors on WRN acetylation levels

Aim b2. To examine whether WRN acetylation is directly related to induction of DNA damage and/or its persistence

Aim b3. To establish the kinetics and cell cycle relationship of WRN acetylation

Aim c. To study the roles of HDAC and sirtuins deacetylases in regulation of WRN acetylation

RATIONALE

The cellular function of WRN appears to be regulated by posttranslational modification [Kusumoto et al., 2007], including acetylation. Consistent with this idea, WRN modification correlates with its translocation to nuclear foci in response to DNA damage and replication blockers [Blander et al., 2002; Karmakar et al., 2005]. It is well established that DNA damage and blockage of replication may induce post-translational modifications to allow temporal and spatial control over the modified protein relocalization, interactions and function [Huen et al., 2008; Appella et al., 2001; Polo et al., 2011]. Collectively, these facts support the notion of an association between WRN modification and DNA damage and blockage of replication. To further elucidate the function of WRN in response to DNA damage, we wanted to investigate WRN acetylation in greater depth. We hypothesized in this study that DNA damaging agents and replication blocking agents induce WRN acetylation. First, we established a method to measure acetylation of endogenous WRN (Aim a). Then, we used this method to analyze if endogenous WRN acetylation is influenced in response to DNA damaging agents and replication inhibitors (Aim b1). If our hypothesis is true, increasing the frequency of damage should lead to increased levels of WRN acetylation. Experimentally, this can be accomplished by preventing removal of damage by standard repair pathways to determine if WRN acetylation is amplified after appropriate DNA damage when the DNA repair pathway involved in removing the induced damage is absent and/or defective (Aim b2). Since lysine acetylation is a reversible posttranslational process that could be related to detection and propagation of specific cellular responses [Huen et al., 2008], we analyzed the kinetics and potential relationship to cell cycle (Aim b3). Given that lysine acetylation is governed by the opposing actions of acetyltransferases and deacetylases, we analyzed the role of deacetylases in regulation of WRN acetylation (Aim c). Specifically, we studied whether WRN is a substrate for the activity of HDAC and sirtuin classes of deacetylase enzymes.

Aim a. To establish methods to measure endogenous WRN acetylation. Although expression systems have been use to examine acetylation of proteins, including WRN, the detection of endogenous acetylated WRN requires additional challenges and optimization of protocols to achieve high sensitivity. Thus, we established our own protocol to obtain better sensitivity and provide quantitative data. This was accomplished by analyzing the specificity of different commercially available antibodies against WRN and acetylated lysine residues in proteins during immunoprecipitation and immunoblotting techniques. The utility and validation of the approach was assessed by using purified wild-type and acetylated WRN.

Aim b. To investigate the relationship between DNA damage and/or replication blockage and WRN acetylation. WRN-deficient cells are hypersensitive to several DNA damaging agents, including methylmethanesulfonate (MMS), mitomycin C (MMC) and cisplatin, and the replication inhibitor hydroxyurea (HU) [Poot et al., 2001; Imamura et al., 2002; Harrigan et al., 2006]. Since WRN function is needed for resistance to these agents, then it might be regulated in response to these treatments, including by post-translational modification. As support for this idea, in normal cells WRN relocalizes from (primarily) the nucleolus to punctuate nuclear foci (also containing replicator factors) following treatment with MMS and DNA replication inhibitors [Karmakar et al., 2005; Constantinou et al., 2000]. This movement of WRN to nuclear foci correlates with WRN modification, specifically with WRN phosphorylation and acetylation [Pichierri et al., 2003; Blander et al., 2002].

Since WRN cells are hypersensitive to MMS, MMC, cisplatin and HU, we used these agents to produce DNA damage or maximally block replication for the subsequent examination of WRN acetylation **(aim b1)**. To determine if WRN acetylation is indeed related to DNA damage, we studied whether WRN modification is increased in cells with deficient or inhibited repair pathways after appropriate DNA damaging treatments **(aim b2)**. Since cells with deficient or inhibited repair pathways cannot repair the lesions caused by specific DNA damaging agents, the damage will persist. If DNA damage results in WRN acetylation, then disabling specific DNA repair pathways should amplify

WRN acetylation. Thus, this strategy should help to establish whether the effect of the agent is specifically mediated by the DNA damage that it generates and to examine whether the response (acetylation) is related to initial damage induction or to the persistence of the DNA damage. If the latter is the case, this may imply a downstream effect of the damage on DNA metabolism.

Acetylation is a reversible posttranslational modification [Yang et al., 2004]. Thus, to establish the kinetics of WRN acetylation **(aim b3),** we studied the rate at which WRN is acetylated and the duration of this modification on WRN. To this end, WRN acetylation was monitored at several time points after MMS treatment. Importantly, such experiments should help to determine the kinetics of WRN acetylation as a function of time. It has been shown that protein acetylation status could be associated with (events occurring during) specific cell cycle phases. For example, PCNA acetylation has been shown to be related to S phase progression [Naryzhny et al., 2004]. Thus, as part of this study, we also sought to identify if WRN acetylation is associated with a specific phase of the cell cycle. This relationship was analyzed first by comparing the cell cycle profile of population of cells untreated or treated with a DNA damaging agent that induced WRN acetylation. The efficacy of the experiment was verified by flow cytometry following Hoechst staining of permeabilized cells. Then, to analyze if WRN acetylation has a relationship to DNA replication (or the lack thereof), DNA synthesis in the presence and absence of DNA damaging agents or replication inhibitors was directly measured by bromodeoxyuridine (BrdU) incorporation, a synthetic thymidine analog that gets incorporated into a cell's DNA during replication.

Aim c. To study the roles of HDAC and Sirtuin deacetylases in regulation of WRN acetylation. The acetylation state of a protein is determined by the opposing action of acetylases and deacetylases. In this aim, we investigate the role of deacetylases in regulating WRN acetylation. To date, eighteen genes encoding proven or putuative deacetylases have been identified in humans [Johnstone et al., 2002; Witt et al., 2009]. These have been divided into four distinct classes based on based on their size, cellular localization, number of catalytic active sites, and homology to yeast HDAC proteins.

Class I, II, and IV are called the ‘‘classical” HDACs and Class III is known as sirtuins [Witt et al., 2009]. Therefore, we used drugs to inhibit the different classes of deacetylases: trichostatin A (TSA) selectively inhibits the Class I, II and IV of histone deacetylase enzymes and nicotinamide has been shown to inhibit the Class III that are the sirtuins [Moradei et al., 2005; Bieliauskas et al., 2008; Walkinshaw et al., 2008; Cen et al., 2010; Witt et al., 2009]. These inhibitors were used alone and in combination, as well as with DNA damaging agents, to investigate the dynamics of WRN acetylation and the roles of each class of deacetylases.

METHODS

Culture medium and reagents. The SV40-transformed fibroblast cell lines, 1-O and 8-D, used in my experiments were obtained from J. Christopher States, University of Louisville. Methylmethanesulfonate (MMS), phenylmethylsulfonylfluoride (PMSF), TSA, nicotinamide, HU, MMC, cisplatin, O^6-benzylguanine and protease inhibitor cocktail were purchased from Sigma-Aldrich. Olaparib was purchased from ChemieTek. Cell culture media and reagents were purchased from Invitrogen. Cells were grown in MEM-α Glutamax medium supplemented with 10% FBS, 1% HEPES, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For DNA damaging treatments, we chose MMS, MMC, and cisplatin. We also used the replication inhibitor HU. After we chose these drugs for our experiments, we initially treated actively growing, subconfluents populations of cells with doses reported from other studies representing physiological conditions. Then, we monitored toxicity by counting viable cells to establish the proper dose range to work. Cells were incubated in growth medium containing 1 mM MMS for 4 h, 2 mM HU for 16 h (or 10 h when indicated), 7 μM MMC for 16 h or 25 μM cisplatin for 19 h before harvesting. For inhibition of deacetylases, cells were incubated in growth medium containing 1 μM TSA and/or 5 mM nicotinamide for 4 h or 10 h (as indicated in figures) in the presence or absence of MMS or HU. For inhibition of MGMT, cells were pre-incubated in growth medium containing 40 μM O^6-benzylguanine for 4 h followed by incubation with 1 mM MMS for an additional 4 h. For treatment with O^6-benzylguanine alone, cells were treated with 40 μM

O^6-benzylguanine for 8 h. To study the inhibition of PARP, cells were treated with 5 nM olaparib for 38 h followed by incubation with 1 mM MMS for an additional 4 h. For treatment with olaparib alone, cells were treated with 5 nM olaparib for 42 h.

Expression and purification of unmodified and acetylated WRN. Purified WRN proteins (unmodified and acetylated) were used for optimization of immunoprecipitation methods as well as for enzymatic studies (conducted as described in Chapter III). Recombinant unmodified WRN was expressed using the baculovirus-insect cell system as described [Orren et al., 1999]. FLAG-tagged WRN and FLAG-tagged acetylated WRN were overexpressed in HEK293 cells using a transient transfection assay (developed by Dr. Jianyuan Luo, University of Maryland Medical School). To obtain unmodified FLAG-WRN, cells were transfected with vector specifying production of FLAG-WRN. To obtain acetylated WRN, cells were co-transfected with individual vectors specifying production of FLAG-WRN and CMV-p300 or CMV-CBP (p300 and CBP are two acetyltransferases that acetylate WRN *in vivo*) [Blander et al., 2002]. To maximally acetylate WRN protein, cells were treated with TSA and nicotinamide to inhibit cellular deacetylase activity 6 h before harvest. To obtain deacetylated FLAG-WRN, cells were co-transfected with FLAG-WRN, CBP and SIRT1 (a histone deacetylase) vectors. Cells were harvested 36 h after transfection and were lysed in a FLAG-lysis buffer (50 mM Tris-HCl pH 7.8, 137 mM NaCl, 1 mM NaF, 1 mM $NaVO_3$, 1% Triton X-100, 0.2% Sarkosyl, 1 mM DTT, 10% glycerol) containing fresh protease inhibitors, PMSF, 10 μM TSA and 5 mM nicotinamide. After anti-FLAG M2 immunoprecipitation, the immobilized FLAG-WRN proteins were released using FLAG peptide (Sigma) and purified unmodified or acetylated FLAG-WRN was collected. To determine relative protein concentration, the eluted protein products were resolved by 8% SDS-PAGE gels and analyzed by western blot with anti-WRN antibody (Santa Cruz Biotechnology). To determine the level of acetylation, the products were analyzed by western blot with anti-acetylated lysine antibody (Cell Signaling).

Immunoprecipitation and detection of WRN acetylation. For immunoprecipitation experiments, cells were lysed by sonication in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA) supplemented with protease inhibitor cocktail, 1 mM PMSF and 10 units/ml of DNase I (New England Biolabs). After centrifugation at 21,000 × g for 12 min at 4°C, the supernatants/clarified lysates were isolated and their protein concentrations measured. Aliquots of the clarified lysates (~600 ug of protein each) were then pre-cleared with Protein G Plus/Protein A agarose beads (Calbiochem) and 1 ug of normal mouse IgG (Santa Cruz) for 1 h, then incubated with anti-acetylated lysine antibody (Cell Signaling) and 30 µl of Protein G Plus/Protein A bead suspension for 18 h at 4°C. After collection by centrifugation and removal of supernatant, the beads were then washed three times with RIPA buffer supplemented with protease cocktail inhibitors, 1 mM PMSF and 200 ug/ml ethidium bromide. After removal of the final wash, equal portions of RIPA and 2X SDS sample buffer were added to the beads and immunoprecipitated proteins were released by heating at 95°C for 5 min. Equal volumes of each sample were resolved by SDS-PAGE (8%). For the loading control, 30-50 ug of each sample (as specified in figures) were also resolved by SDS-PAGE (8%). Proteins were transferred to PVDF membranes (Bio-Rad) by electroblotting. The membranes were blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris, pH 7.4, 140 mM NaCl and 0.1% Tween-20) and analyzed by western blotting with mouse monoclonal anti-WRN (Abcam) antibody for 18 h at 4°C followed by incubation with peroxidase-labeled secondary anti-mouse (GE Healthcare) for 1 h at room temperature. Signal was visualized by chemiluminescent analysis using ECL Plus (GE Healthcare).

Micronucleus Assay. 8-D and 1-O cells were incubated in growth medium without or with 25 µM Cisplatin for 19 h. After 19 h incubation, the media was removed and cells were washed twice with growth medium. New media was added to the cells and cytochalasin B (3 ug/mL, from Sigma-Aldrich) was added to the culture for 24 h. Cells were harvested, fixed (methanol:acetic acid 3:1) at 4°C and seeded onto microscope slides (75 x 25 mm, 1 mm thick from Fisher Scientific). Slides were air dried, then stained with a 10% Giemsa (Sigma-Aldrich) solution and examined at 1000×

magnification (oil) using a bright field microscope. One thousand (1000) binucleated cells per treatment were examined and the number of micronuclei was recorded.

Flow Cytometry Analysis. 1-O cells were incubated in growth medium containing +/- 1 mM MMS for 2 h. After incubation, cells were harvested and counted using a hemocytometer. Cells were suspended at a density of 5×10^6 cells/ml in sorting buffer [1× PBS (Ca/Mg^{++} free), 1 mM EDTA, 25 mM HEPES pH 7.0, 1% FBS (heat inactivated)]. Hoechst 33342 stain (10 μM, from Sigma-Aldrich) was added and cells were incubated at 37°C for 45 min (with mixing every 5 min). Cells were filtered thru a 40 micron cell strainer (from Fisher Scientific) to remove clumps and debris. Cells were analyzed by flow cytometry and subsequently subjected to immunoprecipitation and immunoblotting as specified above.

BrdU incorporation Assay. The assay was performed using a kit following the manufacturer instructions (Exalpha Biologicals BrdU cell proliferation assay). A suspension of 1-O cells (1.5×10^5 cells/ml) was prepared using culture media. 100 ul was added to each well (using 96-well tissue culture plates). After 8 h, cells were untreated, treated with 2 mM HU for 16 h, or treated with 1 mM MMS for 1-4 h (as indicated in figures). BrdU was added 1 h prior to the end of the treatments. Media was aspirated from the cell wells and cells were fixed (using a solution provided in the kit) at room temperature for 30 min. Cells were washed three times using the washing buffer provided by the manufacturer. An anti-BrdU monoclonal antibody was added and cells were incubated for 1 h at room temperature. Cells were washed three times and incubated for 30 min at room temperature with peroxidase goat anti-mouse IgG conjugate. After three washes, TMB (3,3', 5,5"-tetramethylbenzidine) peroxidase substrate was added and cells were incubated for 30 min at room temperature in the dark. A stop solution was added to every well and the absorbance was measured at 450 nM using a spectrophotometric microtiter plate reader (SpectraMax Plus 384, Molecular Devices).

Statistical analysis. The data was analyzed using one-way ANOVA followed by Newman Keuls post-test (GraphPad Prism-4). A p value of less than 0.05 was considered a significance difference.

RESULTS

Protocol for detection of endogenous acetylated WRN. Previous studies show WRN modification and relocalization to nuclear foci after treatment with DNA damaging agents and blockers of replication. In light of these findings, we reasoned that it should be possible to detect WRN modification, specifically WRN acetylation, particularly as a response to DNA damage and replication stress. Although experimental approaches exist to tease out the role of post-translational modifications on WRN, most of them have been based on ectopic expression of WRN. Because of a lack of methods and information on endogenous WRN modifications, we set out to establish our own protocol to measure endogenous WRN acetylation. First, we tested the specificity of different commercially available antibodies against WRN protein to detect unacetylated and acetylated WRN protein, produced as described in the Methods section. Then, we tested the ability of those antibodies to pull WRN from cell lysates. The specificity of different commercially available antibodies against acetylated lysine proteins (including an antibody against acetylated WRN) was also tested to determine if they can recognize purified acetylated FLAG-WRN protein. The capability of those antibodies to pull down acetylated WRN and their specificity was also established by adding purified acetylated WRN into cell lysates of WRN-deficient and -proficient cell lines. Finally, we tried different combinations of the antibodies to perform IP and immunoblotting techniques. From all the combinations tested, we decided to use an antibody against acetylated lysine for the IP reaction and an antibody against WRN to probe for acetylated WRN since that combination of antibodies gave us clean, consistent and highly sensitive western blots. Importantly, the selected antibody against WRN was able to identify both states (unacetylated and acetylated) of WRN (Figure 2.2A) and the antibody against acetylated lysine specifically recognizes the acetylated form of WRN (Figure 2.2B). In addition, the acetylated lysine antibody specifically pulled down the acetylated form of WRN (Figure

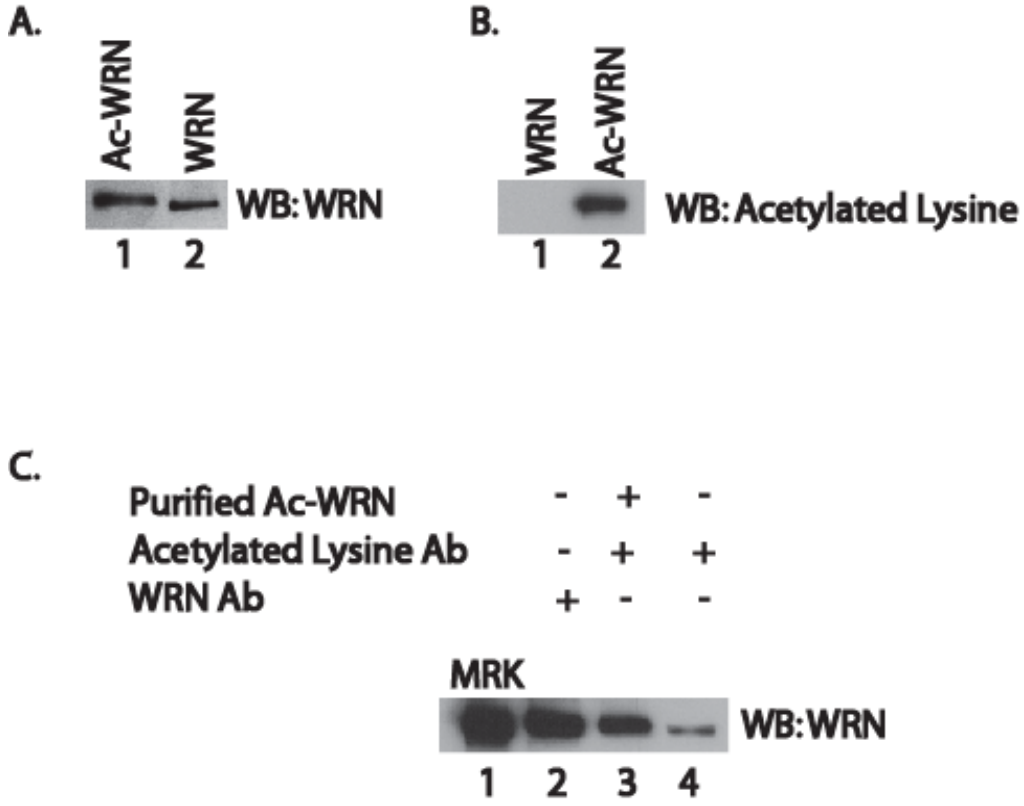

Figure 2.2 Specificity of antibodies selected for our studies. A) Unacetylated and acetylated recombinant WRN proteins were subjected to SDS-PAGE and Western blotting with anti-WRN antibody. **B)** Unacetylated and acetylated recombinant WRN proteins were subjected to SDS-PAGE and Western blotting with anti-acetylated lysine antibody. **C)** Cell lysates were subjected to immunoprecipitation by incubating with the specified antibodies (Ab) in the presence or absence of purified acetylated WRN. The IP products were subjected to SDS-PAGE and analyzed by Western blotting with anti-WRN antibody.

2.2C, compare lanes 3 and 4). The diagram in figure 2.3 shows the established protocol. Briefly, after drug treatments, cell lysates were prepared and the protein concentration of each cell lysate was measured to use comparable amounts of each sample. Lysates were subject to a pre-clearing step to remove proteins that interacted non-specifically with normal IgG and Protein G Plus/Protein A beads. The pre-cleared lysates were incubated with a mouse monoclonal antibody against acetylated lysine to immunoprecipitate the pool of acetylated proteins. After release from the beads, immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western Blot using an antibody specific for WRN protein. The results were quantified using a fluorimager.

DNA damaging agents/replication inhibitors enhance WRN acetylation. WS cells are hypersensitive to certain DNA damaging agents, including MMS, MMC and cisplatin. In addition, WS cells are hypersensitive to the replication inhibitor HU. Collectively, these findings suggest that WRN might be regulated in response to those treatments, including by post-translational modifications. Thus, we used these agents as possible candidates for the induction of WRN acetylation. As a negative control, cells were analyzed after UV-C irradiation, a treatment that does not cause enhanced cell death in WS cells compared to normal cells. MMS is a DNA-alkylating agent that methylates DNA bases, producing 7-methylguanine, 3-methyladenine and O^6-methylguanine [Wyatt et al., 2006]. MMC is a crosslinking agent that induces interstrand crosslinks [Dusre et al., 1989]. Cisplatin is a platinum-based chemotherapy drug that acts by generating mostly intrastrand crosslinks, repaired by nucleotide excision repair (NER), as well as some interstrand crosslinks [Jamieson et al., 1999]. HU depletes nucleotide pools by inhibition of the enzyme ribonucleotide reductase, resulting in blocking progression of all replication forks [Skog et al., 1992]. WS cells are hypersensitive to HU, apparently by apoptosis of cells with stalled replication forks [Pichierri et al., 2001]. UV-C causes direct photochemical damage to DNA producing covalent linkages between adjacent cytosine and thymidine bases creating cyclobutane pyrimidine dimers and 6-4 photoproducts [Sinha et al., 2002; Markovitsi et al., 2010]. Experiments were performed with human fibroblasts treated either with or without 1 mM MMS for 4 h, 2 mM HU for 16 h, 7 μm MMC for 16 h, 25 μM cisplatin for 19 h or irradiated with 20 J/m^2 or 40 J/m^2 UV-C. Interestingly, a low

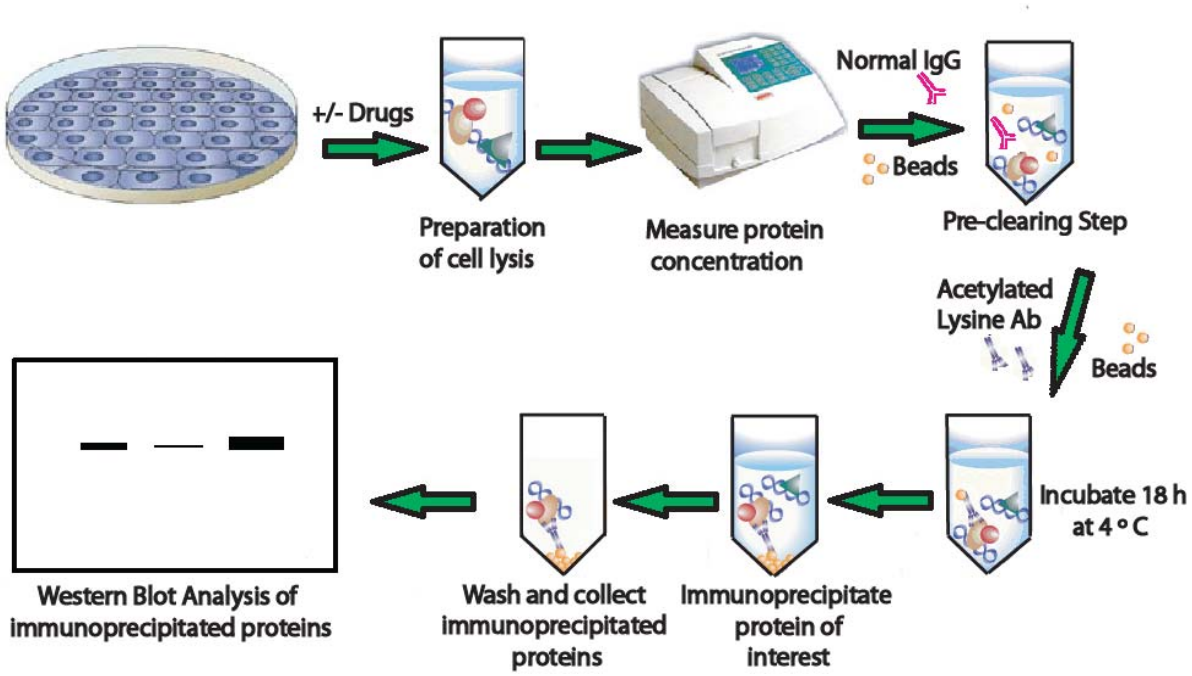

Figure 2.3 Schematic representation of the protocol used.

level of acetylation of WRN is detectable even in untreated cells (Figure 2.4A, lane 2). More importantly, the amount of acetylated WRN is significantly increased in cells treated with MMS, HU, MMC, and cisplatin (Figure 2.4A, upper panel, compare lines 2 to 3-6). Quantitation of data from multiple independents experiments indicates that treatment with MMS results in 3.3 fold increase and treatment with HU in 2.1 fold increase in the levels of WRN acetylation. The crosslinking agents also increase the levels of acetylated WRN, since treatment with MMC and cisplatin resulted in 2.7- and 2.5-fold increase (quantitation in Figure 2.4B), respectively. This effect was not due to an increase in WRN expression or abundance caused by any of the treatments, since a parallel Western analysis of lysates with anti-WRN antibody indicated that the same amount of total WRN is present following each treatment (Figure 2.4A lower panel). In contrast, irradiation of cells with 20 J/m^2 or 40 J/m^2 UV-C does not increase significantly the levels of acetylated WRN (Figure 2.4C, compare line 2 vs. lines 3 and 4, quantitation on figure 2.4D). Collectively, this data indicates that WRN acetylation is upregulated in response to certain DNA damaging treatments (but not UV) and replication inhibitors.

Correlation between persistent DNA damage and WRN acetylation. Although the experiments above suggest an association between DNA damage and WRN acetylation *in vivo,* we wanted to confirm this relationship and investigate it further. Our strategy was to inhibit removal of particular types of DNA damage to increase the frequency of those lesions in the DNA template. If WRN acetylation is related to DNA damage, inhibition of repair pathways that remove the relevant type of damage should amplify the effect of the damaging agent on WRN acetylation. Thus, we monitored WRN acetylation in cells with normal and compromised repair pathways after appropriate DNA damaging treatments.

Initially, we focused on MMS because this agent gave us the highest increase in WRN acetylation. MMS methylates DNA at the N^7-deoxyguanine, N^3-deoxyadenine and O^6-deoxyguanine positions. Thus, we examined the effect of persistence of these adducts on WRN acetylation. The 7-methylguanine and 3-methyladenine adducts are repaired by base excision repair (BER) [Wyatt et al., 2006]. BER is involved in repair of DNA damage arising from spontaneous base loss or genotoxic agents that modify bases, such

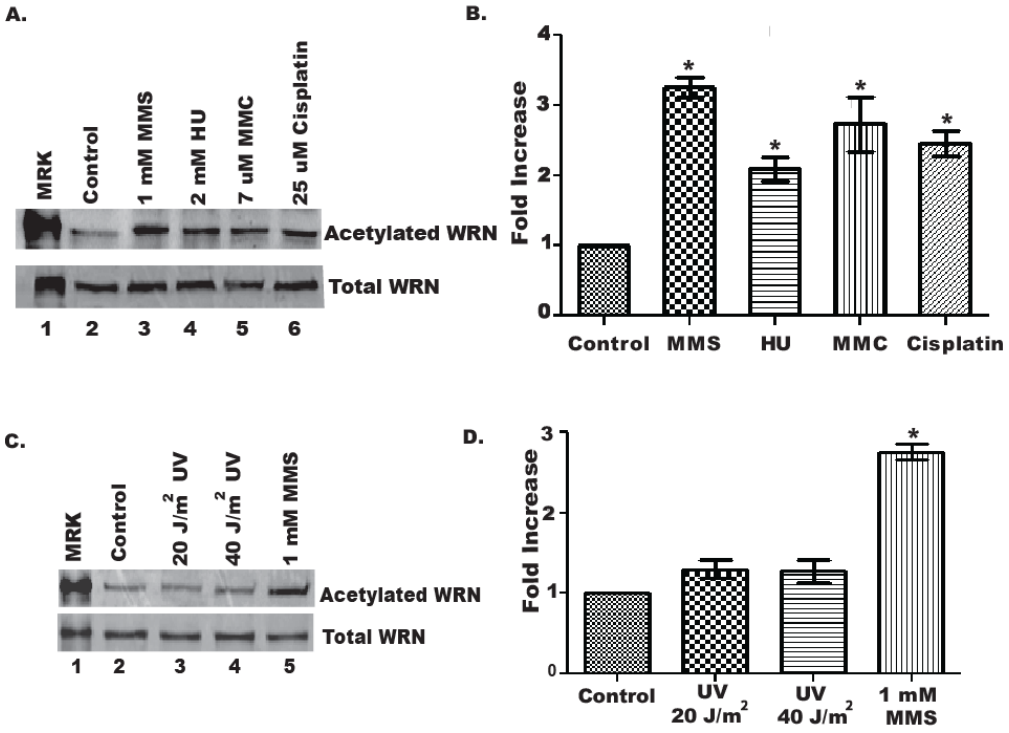

Figure 2.4 WRN is acetylated in response to DNA damaging agents/replication inhibitors. A) 8-D cells were incubated in growth medium with or without 1 mM MMS for 4 h, 2 mM HU for 16 h, 7 uM MMC for 16 h or 25 uM Cisplatin for 19 h (before harvest) and clarified cell lysates were processed for IP with anti-acetylated lysine antibody. The IP products were subjected to SDS-PAGE and western blotting with anti-WRN antibody (upper panel). 50 ug of each cell lysate were subjected to SDS-PAGE and western blotting with anti-WRN antibody (lower panel) to ensure that equal amount of total WRN was present in the IP reactions as control. **B)** Quantitative bar graph for WRN acetylation (mean ± SEM of 3 independent experiments. * = $P < 0.05$ when compared with control untreated cells). **C)** Cells were irradiated (20 J/m^2 or 40 J/m^2 UV-C) or treated with 1 mM MMS for 4 h (before harvest) for IP with anti-acetylated lysine antibody. The IP products were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (upper panel). 40 ug of each cell lysate were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (lower panel). **D)** Quantitative bar graph for WRN acetylation (mean ± SEM of 4 independent experiments. * = $P < 0.05$ when compared with control untreated cells). Lanes 1 in figure A and C are purified acetylated WRN (upper panel) or purified unmodified WRN (lower panel) that we used as marker (MKR).

as MMS [Wyatt et al., 1999 and 2006; Horton et al., 2005]. The O^6-methylguanine adducts are acted on by the enzyme O^6-methylguanine methyltransferase (MGMT) that repairs O^6-methylguanine by direct transfer of the alkyl group on guanine to a cysteineresidue in its active site [Gerson et al., 2002; Kaina et al., 2007]. Thus, we used the drugs O^6-benzylguanine and olaparib to inhibit the repair of the DNA lesions produced by MMS. The agent O^6-benzylguanine is a potent inactivator of MGMT by acting as a substrate for the protein. Specifically, MGMT transfers the benzyl group in O^6-benzylguanine to the cysteine residue in the active site of the protein resulting in MGMT inactivation and, therefore, lesions present at the O^6 position of DNA induced by MMS remain unrepaired [Dolan et al., 1990 and 1997; Murakami et al., 2007]. First, cells were pre-treated with O^6-benzylguanine for 4 h before the addition of MMS for an additional 4 h. Interestingly, co-treatment of cells with MMS and O^6-benzylguanine did not appear to increase WRN acetylation (Figure 2.5A, upper panel, compare lines 3 and 4). Quantitation of data from multiple independent experiments is shown in Figure 2.5B. This result suggests that O^6-methylguanine lesions are not responsible for triggering WRN acetylation.

Then, we followed a similar strategy as above but using olaparib that inhibits the enzyme poly (ADP-ribose) polymerase (PARP) and consequently BER. Olaparib is an analog of the substrate NAD+ that binds to the catalytic site of PARP [Plummer et al., 2006; Lord et al., 2008]. PARP is involved in BER by binding gaps and nicks in DNA and helping to open up the damaged DNA to allow access to other components of the repair process [Petrucco et al., 2003]. Specifically, olaparib inhibits PARP by preventing its automodification that is necessary for its release from DNA and the recruitment of proteins involved in BER [Horton et al., 2005]. Importantly, olaparib is not cytotoxic at concentrations necessary to achieve PARP inhibition [Cepeda et al., 2006]. Interestingly, co-treatment of cells with MMS and olaparib further increased WRN acetylation (Figure 2.5C, upper panel, compare lines 3 and 4). The increase in WRN acetylation was not due to olaparib treatment since the cells treated with olaparib alone had comparable amounts of acetylated WRN as control untreated cells (Figure 2.5C, upper panel, compare lines 2

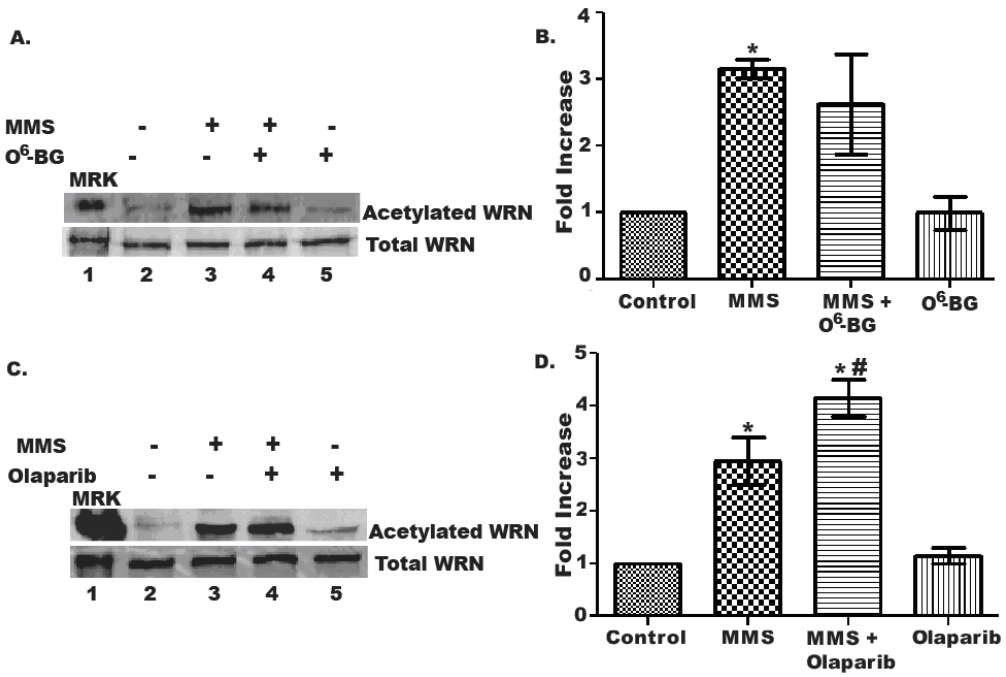

Figure 2.5 Correlation between DNA damage and WRN acetylation. **A)** 8-D cells were incubated in growth medium with or without 40 μM O^6-Benzylguanine (O^6-BG) for 4 h followed by incubation with 1 mM MMS for an additional 4 h. For treatment with O^6-BG alone, cells were treated with 40 μM O^6-BG for 8 h. Cells were harvested and subjected to IP with anti-acetylated lysine antibody and immunobloting with anti-WRN antibody (upper panel). 40 ug of each cell lysate were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (lower panel). **B)** Quantitative chart for WRN acetylation (mean ± SEM of 3 independent experiments. * = $P < 0.05$ when compared with control untreated cells). **C)** 8-D cells were incubated in growth medium with or without 5 nM Olaparib for 38 h followed by incubation with 1 mM MMS for an additional 4 h. For treatment with Olaparib alone cells were treated with 5 nM Olaparib for 42 h. Cells were harvested and subjected to IP with anti-acetylated lysine antibody. IP products were analyzed by immunoblotting with anti-WRN antibody (upper panel). 60 ug of each cell lysate were analyzed by Western blotting with anti-WRN antibody (lower panel). **D)** Quantitative chart for WRN acetylation (mean ± SEM for two independent experiments. * = $P < 0.05$ when compared with control, and # = $P < 0.05$ when compared with cells treated with MMS alone). Lanes 1 in figure A and C are purified acetylated WRN (upper panel) or purified unmodified WRN (lower panel) that we used as marker (MKR).

and 5). Quantitation of data from multiple independent experiments indicates that co-treatment with MMS and the PARP inhibitor increases WRN acetylation by 4.1 fold, compared to a 3.1 fold increase for MMS treatment alone (Figure 2.5D). These results suggest that persistence of 7-methylguanine and 3-methyladenine lesions induce a further amplification of WRN acetylation.

Our previous results show that WRN is acetylated after cisplatin treatment. Cisplatin primarily produces bulky DNA adducts repaired by nucleotide excision repair (NER) [Sancar et al., 1995; Wang et al., 2001; Zheng et al., 2003]. Therefore, NER-deficient cell lines provide opportunities to examine the effects of persistent DNA damage generated by cisplatin on WRN acetylation. Experiments were performed by comparing WRN acetylation after 19 h cisplatin treatment in NER-proficient (8-D cells, normal NER+) and NER-deficient (1-O cells, NER- since they are XPA-deficient) human fibroblasts. As shown in Figure 2.6A, WRN acetylation is further amplified in NER-deficient cells after cisplatin treatment when compared with NER-proficient cells (upper panel, compare lines 3 and 5). This increase is not due to a difference in WRN expression levels between the cell lines, since a parallel Western analysis of samples using an anti-WRN antibody indicated that approximately the same amount of total WRN is present (Figure 2.6A, lower panel). In addition, the levels of acetylated WRN in the untreated 1-O and 8-D cell lines are comparable (Figure 2.6A, upper panel, compare lines 2 and 4). Quantitation of data from multiple independent experiments indicates that WRN acetylation in NER-proficient cells was higher (2.2 fold) when they are treated with cisplatin as compared to their control untreated. However, this difference increases when NER is defective; i.e., when NER-deficient cells are treated with cisplatin, the difference was 3.8 fold when compared to their untreated control (Figure 2.6B). Therefore, these experiments indicate that WRN acetylation corresponds to DNA damage generated by cisplatin and is amplified by the persistence of lesions subject to NER.

To ensure that indeed NER-deficient cells lines have lower efficiency in their DNA repair system, and be confident in the correlation that WRN acetylation increases with

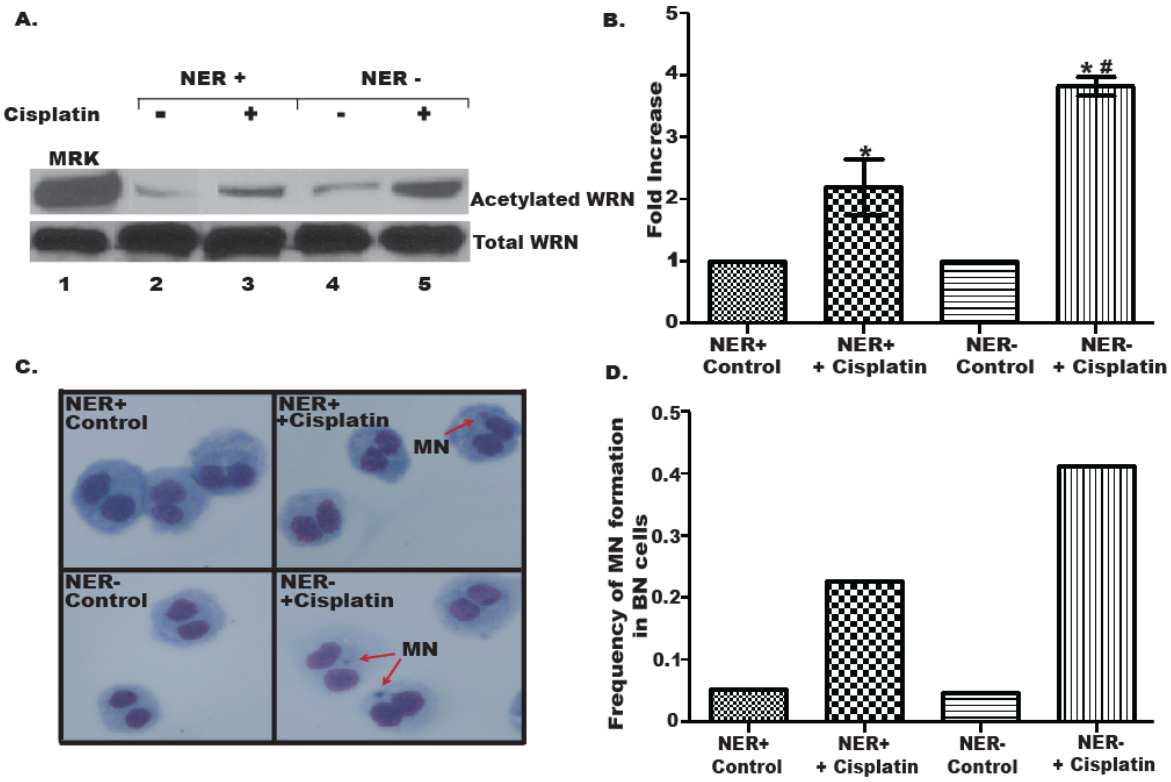

Figure 2.6 WRN acetylation is amplified as a result of persistent DNA damage. A) 8-D (NER+) and 1-O (NER-) cells were incubated in growth medium with or without 25 uM cisplatin for 19 h before harvest for IP with anti-acetylated lysine antibody. The IP products were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (upper panel). 60 ug of each cell lysate were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (lower panel). Lanes 1 is purified acetylated WRN (upper panel) or purified unmodified WRN (lower panel) that we used as marker (MKR). **B)** Quantitative bar graph for WRN acetylation (mean ± SEM. of 4 independent experiments. * = $P < 0.05$ when compared with control untreated cells, and # = $P < 0.05$ when compared with NER Proficient cells treated with Cisplatin). **C)** To measure micronucleus (MN) formation, 8-D cells and 1-O were incubated in growth medium with or without 25 uM Cisplatin for 19 h. Cells were washed twice with growth medium and cytochalasin B (3 ug/mL) was added to the culture for 24 h. Cells were harvested, fixed (methanol:acetic acid 3:1) and stained with a 10% giemsa solution. **D)** Quantitative bar graph for frequency of MN formation in binucleated (BN) cells.

persistence of DNA damage generated by cisplatin, we employed the cytokinesis-block micronucleus assay that measures genome instability that is a consequence of DNA damage. In this technique, genomic instability is scored specifically by counting micronuclei that are biomarkers of chromosome breakage and/or chromosome loss, in binucleated cells [Fenech et al., 2007 and 2008]. The binucleated appearance of cells is a result of blocking cytokinesis with cytochalasin-B, an inhibitor of microfilament ring assembly required for the completion of cytokinesis. Trapping cells at this stage maximizes detection of chromosome breakage. Higher number of micronuclei is indicative of higher chromosome damage. Briefly, NER-proficient and NER-deficient cells were incubated with or without cisplatin for 19 h, following incubation with cytochalasin-B for 24 h. Cells were harvested, fixed and stained and the number of micronuclei formed was scored. The results indicate that, indeed, the NER-deficient cell line had higher numbers of micronuclei formed (scoring in Table 2.1) after the cisplatin treatment when compared with the NER-proficient cell line (see Figure 2.6C). Calculation of the frequency of micronuclei formed in NER-proficient and deficient cell lines indicate that cisplatin treatment induces higher levels of micronuclei formation in the NER-deficient cells when compared to NER-proficient cells (0.412 vs. 0.227 micronuclei/binucleated cells, respectively). Quantitation is shown in Figure 2.6D. Thus, the increased frequency of micronuclei in NER-deficient cells following cisplatin treatment indicates these cells maintain a larger burden of DNA damage and support the notion that they are deficient in repair of cisplatin-induced lesions. Collectively, our results support the notion that WRN acetylation is amplified as a result of inhibition of repair of cisplatin lesions by NER and of 3-methyladeninde and/or 7-methylguanine lesions by BER.

Kinetics and cell cycle relationship of WRN acetylation. Lysine acetylation could be related to specific cellular responses during the individual cell cycle phases [Huen et al., 2008]. Therefore, we examined whether WRN acetylation is associated with a particular cell cycle phase. Initially, we tried cell cycle synchronization protocols by serum starvation and contact inhibition to verify if the levels of WRN acetylation are enriched in any specific phase of the cell cycle. However, the purity and amount of cells obtained in

CELL LINE	TREATMENT	BN CELLS COUNTED	# MN FOUND	FREQUENCY
8-D Cells (NER Proficient)	-	1000	52	0.052
8-D Cells (NER Proficient)	25 uM Cisplatin	1000	227	0.227
1-0 Cells (NER Deficient)	-	1000	48	0.048
1-0 Cells (NER Deficient)	25 uM Cisplatin	1000	412	0.412

Table 2.1 Frequency of MN formation in NER proficient and deficient cell lines.

the synchronized populations were not suitable for our purposes. To obtain enriched population of cells, we also tried to sort the cells in the different cell cycle phases. However, the percentage of recovered cells (especially in G2) was not enough for our purposes. Thus, we analyzed and compared the cell cycle profile of a population of untreated cells with a population of cells treated with MMS, the treatment that induces the highest levels of WRN acetylation. As seen in Figure 2.7, untreated cells showed a relatively normal asynchronous profile. However, in MMS treated cells, we can see an increase in the percentage of cells at S-phase and a loss of G2/M phase cells. In parallel with flow cytometry experiments, acetylated WRN was immunoprecipitated from cells using an acetylated lysine antibody and the IP products were subsequently analyzed by immunoblotting with an anti-WRN antibody. Again, WRN acetylation increases after the MMS treatment, suggesting that WRN acetylation correlated with the increase in S-phase cells (Figure 2.7).

Although the experiments above suggest a correlation between S-phase progression and WRN acetylation *in vivo*, we wanted to corroborate this relationship and investigate it further. Thus, to determine if indeed WRN acetylation correlates with S-phase or replication blockage, the effect of our treatments on DNA replication was established. To this end, the incorporation of bromodeoxyuridine (BrdU) was measured. BrdU is a synthetic thymidine analog that is incorporated during DNA synthesis. Experiments were performed with cells treated either with or without 1 mM MMS for 4 h or 2 mM HU for 16 h. BrdU incorporation was measured using the protocol described in the Methods section. Interestingly, the incorporation of BrdU was abolished (non-detectable) in HU- and MMS-treated cells when compared with untreated cells, indicating that these HU and MMS treatments dramatically inhibit DNA replication. Taken together, this evidence suggests that acetylation of WRN observed after HU and MMS correlates with blockage of replication during S-phase.

To gain further insight on WRN acetylation in response to induced DNA damage, we investigated the kinetics of WRN acetylation to determine the timing of the onset of acetylation and the duration of this modification. For these experiments, cells were

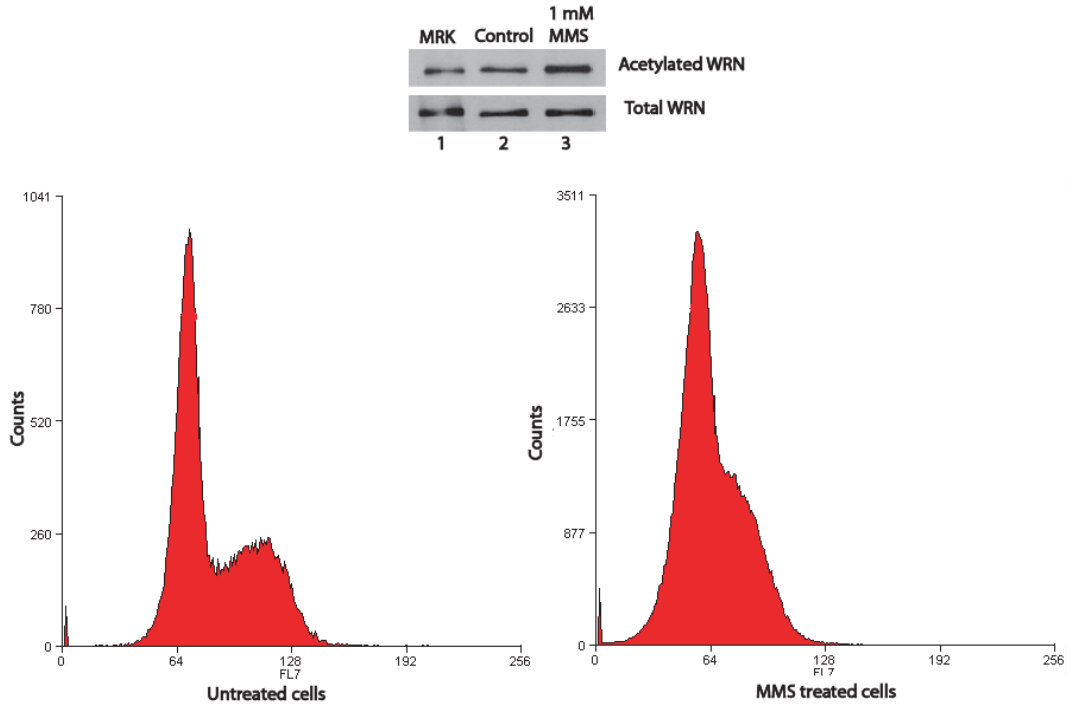

Figure 2.7 WRN acetylation is associated with S-phase progression. A) Cells were incubated in growth medium with or without 1 mM MMS for 2 h before harvest for Hoechst staining. Cells were analyzed by flow cytometry and subsequently subjected to immunoprecipitation with anti-acetylated lysine antibody. The IP products were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (upper panel). 30 ug of each cell lysate were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (lower panel) to ensure that equal amount of total WRN was present in the IP reactions as control. Lanes 1 is purified acetylated WRN (upper panel) or purified unmodified WRN (lower panel) that we used as marker (MKR).

incubated with MMS (since this treatment produced the highest level of acetylated WRN) and harvested at different time points (1 h, 2 h, 4 h). We also analyzed cells 2 h and 4 h after MMS treatment (by removing medium containing MMS and replacing it with fresh medium). After lysis, acetylated WRN was immunoprecipitated as previously and resolved by SDS-PAGE to compare the levels of WRN acetylation at the different time points. As seen in Figure 2.8A, WRN acetylation starts to increase gradually after 1 h, reaching its maximum level of acetylation after 4 h. However, at a later time, specifically 4 h after removal of MMS, the levels of acetylated WRN decrease to normal levels (quantitation on Figure 2.8B). Thus, the increase in WRN acetylation levels seems to be a transient regulatory process for WRN. We also measured the incorporation of BrdU at the different time points. Upon treatment with 1 mM MMS, BrdU incorporation dropped to 29% of the untreated control by 1 h, and to undetectable levels thereafter. Thus, the timing of the increase in WRN acetylation after MMS roughly corresponds to its inhibitory effect on DNA replication.

Role of HDAC and sirtuins in regulation of WRN acetylation. Modulation of protein acetylation depends upon the opposing activities of acetylases and deacetylases. As the name implies, deacetylases remove acetyl groups from the side chain of specific lysine residues on proteins. Eighteen isoforms of mammalian deactylases have been described so far [Walkinshaw et al., 2008]. They have been grouped into two families and four classes. The HDAC family, also known as the classical family, is composed of Class I, II and IV. The sirtuin family is composed of Class III. The two families have entirely different catalytic mechanisms of action and are thus not targeted by the same inhibitor molecules [Bieliauskas et al.; 2008; Federico et al., 2011]. Therefore, to establish the role of deacetylases in regulation of WRN acetylation, we used inhibitors specific to different classes. TSA specifically inhibits Classes I, II and IV by targeting the zinc molecule found in the active site of HDACs. Nicotinamide inhibits Class III by binding to the conserved pocket of sirtuins that participates in NAD^+ cofactor binding and catalysis. First, we treated cells with both inhibitors to hinder deacetylases of the different classes at the same time and gain knowledge about the dynamics of WRN acetylation. In these

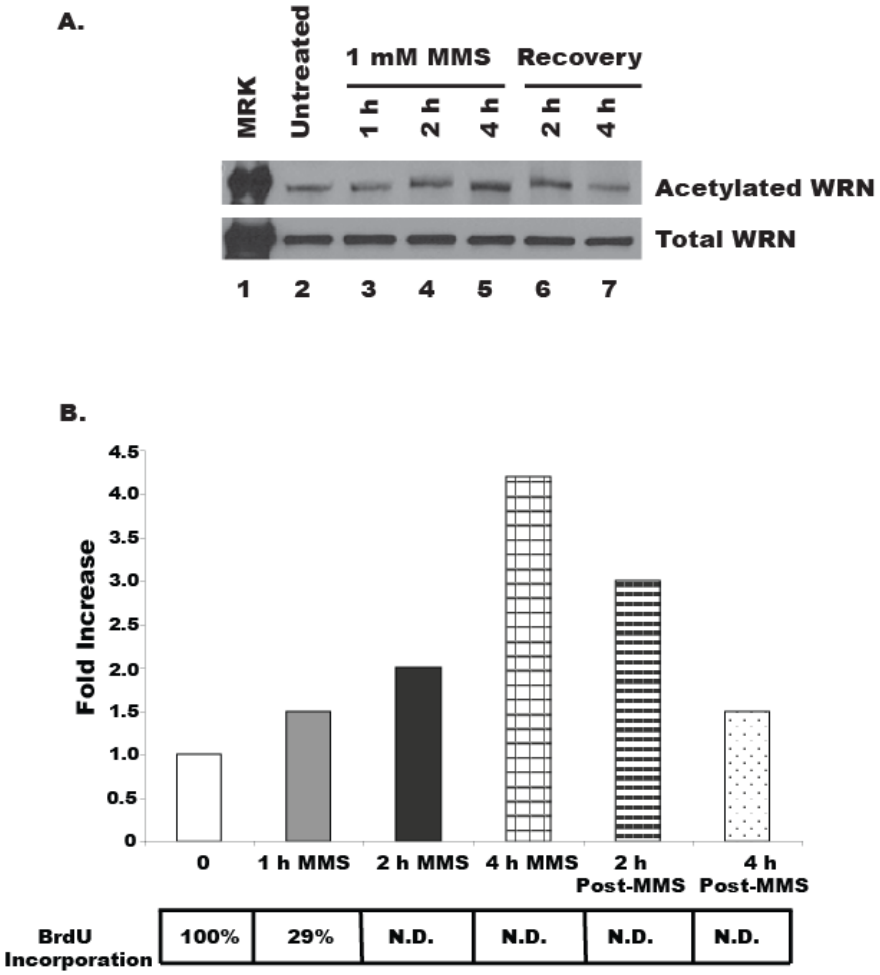

Figure 2.8 Kinetics of WRN acetylation. A) Cells were incubated in growth medium with or without 1 mM MMS for 1-4 h before harvest for immunoprecipitation with anti-acetylated lysine antibody. The IP products were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (upper panel). 45 ug of each cell lysate were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (lower panel) to ensure that equal amount of total WRN was present in the IP reactions as control. For the recovery experiments, cells were washed twice with growth medium after 4 h incubation with MMS and new medium was added to the culture. Cells were harvested after 2 h or 4 h recovery. Lanes 1 is purified acetylated WRN (upper panel) or purified unmodified WRN (lower panel) that we used as marker (MKR). **B)** Quantitative graph for WRN acetylation and percentages of BrdU incorporation (N.D. signifies non-detectable).

experiments, cells were treated with MMS alone or with MMS and both inhibitors for 4 h. Interestingly, co-treatment of cells with MMS, TSA and nicotinamide results in a further increase of WRN acetylation when compared to cells treated with MMS alone (Figure 2.9A, upper panel, compare lines 3 and 4). We also treated cells with HU alone or with HU and both inhibitors. Co-treatment with HU, TSA and nicotinamide also resulted in a further amplification of WRN acetylation levels when compared to cells treated with HU alone (Figure 2.9B, upper panel, compare lanes 3 and 4). The levels of acetylated WRN also increased in cells treated with the deacetylase inhibitors alone (Figure 2.9A and 2.9B, upper panels, lanes 5). Quantitation of data from multiple independent experiments indicates that treatment with both TSA and nicotinamide results in a 5.0 fold increase in the levels of WRN acetylation (quantitations showed on Figure 2.9D and E). These results suggest that WRN is actively deacetylated *in vivo* and that acetylation of WRN is a dynamic process that, under normal conditions, is at equilibrium through the opposing actions of acetyltransferases and deacetylases. At the same time, these results also confirm that our immunoprecipitation reactions were pulling down only the acetylated form of WRN, since inhibiting deacetylation specifically increased the levels of acetylated WRN (Figure 2.9A, B and C, lower panels).

To investigate the influence of the different classes of deacetylases in WRN acetylation levels cells were treated with each of the inhibitors alone. As seen in Figure 2.9C (compare lanes 2 and 3), TSA alone increased 4.0 fold the levels of acetylated WRN (quantitation on 2.9F). However, nicotinamide alone increased 2.0 fold the levels of acetylated WRN (compare lanes 2 and 4). Thus, it seems that members of the classical HDAC family of deacetylase enzymes contribute more substantially to endogenous WRN deacetylation, at least under conditions in which DNA is not damaged.

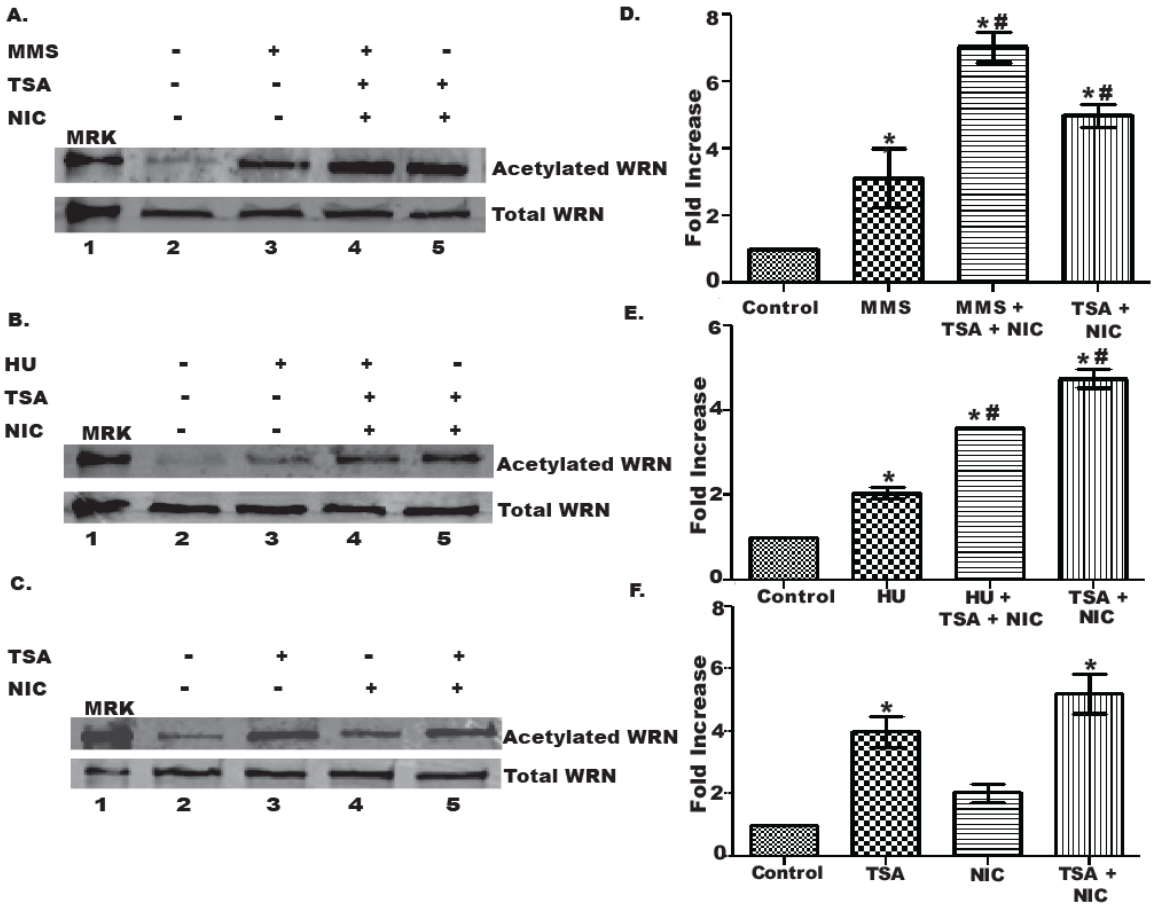

Figure 2.9 Role of HDACs and sirtuins in regulation of WRN acetylation. A) 8-D cells were incubated in growth medium with or without 1 mM MMS, 5 mM Nicotinamide (NIC), and/or 10 uM TSA for 4 h before harvest for IP with anti-acetylated lysine antibody. IP products were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (upper panel). 50 ug of each cell lysate were analyzed by Western blotting with anti-WRN antibody (lower panel). **B)** 8-D cells were incubated in growth medium with or without 2 mM HU, 5 mM NIC, and/or 10 uM TSA for 10 h before harvest for IP with anti-acetylated lysine antibody. IP products were subjected to SDS-PAGE and Western blotting with anti-WRN antibody (upper panel). 30 ug of each cell lysate were analyzed by Western blotting with anti-WRN antibody (lower panel). **C)** 8-D cells were incubated in growth medium containing 5 mM NIC and/or 10 uM TSA for 4 h. **D, E and F)** Quantitative bar graphs for WRN acetylation (mean ± SEM of 3 independent experiments. * = $P < 0.05$ when compared with control untreated cells, and # = $P < 0.05$ when compared with MMS (in D) or HU (in E) alone.

DISCUSSION

Previous studies have demonstrated that WRN is subject to post-translational modification, including phosphorylation, acetylation, and sumoylation. Here, we specifically investigate WRN acetylation in further depth. In particular, the dynamics of endogenous WRN acetylation and its relationship to DNA damage were examined. We found that WRN is acetylated under normal conditions and that acetylation of WRN significantly increases after treatment with DNA damaging agents (MMS, MMC, and cisplatin) and the replication inhibitor HU, but not after UV. These results are in agreement with the relative sensitivity of WS cells to these agents. WRN is also acetylated after treatment with the DNA damaging agent etoposide [Li et al., 2008]. Interestingly, the DNA damaging agents that induce WRN acetylation in our studies and others are known to produce lesions resulting in strong blocks to replication [Jung et al., 2007; Liu et al., 2003]. Therefore, our data strongly suggest that WRN may be acetylated in response to blockage of replication.

To establish whether WRN acetylation is actually related to the initial level of DNA damage or to a downstream effect of the damage on DNA metabolism, it was relevant to consider if increasing the frequency and persistence of DNA damage induced a further increase in the levels of acetylated WRN. Thus, we performed a series of experiments in which DNA damage removal by standard repair pathways was inhibited after treatments with appropriate DNA damaging agents. Initially, we tested the involvement of O^6-methylguanine adducts produced by MMS in inducing WRN acetylation by using O^6-benzylguanine to inactivate MGMT, the enzyme involved in repair of these lesions. However, our results showed that O^6-methylguanine lesions probably do not result in WRN acetylation, since their apparent persistence does not lead to an increase in WRN acetylation. To better define if persistence of other lesions produced by MMS results in amplification of WRN acetylation, we also targeted BER, which is responsible for repair of 7-methylguanine and 3-methyladenine lesions [Wyatt et al., 2006]. BER was inhibited with olaparib, which inhibits PARP, the enzyme involved in opening up damaged DNA to allow access to other components of the repair process. Our experiments demonstrated

an association between persistence of 7-methylguanine and 3-methyladenine lesions and WRN acetylation, since we observed a further increase on WRN acetylation when their repair was inhibited. It might be possible that the increase in WRN acetylation observed after MMS and olaparib co-treatment could be to an inhibition of deacetylases and/or an increase in acetylase activity by olaparib. However, our results suggest that this was not the case, since the cells treated with olaparib alone had comparable amounts of acetylated WRN as control untreated cells.

There are several possible explanations for the results obtained with the different inhibitors. First, it is well established that MMS produces 7-methylguanine at much higher frequency than 3-methyladenine and O^6-methylguanine, in order of abundance, respectively [Lawley et al., 1975; Cloutier et al., 2001; Wyatt et al., 2006; Zhang et al., 2006; Boysen et al., 2009]. Therefore, it could be difficult to see any changes in WRN acetylation caused by O^6-methylguanine lesions, since the frequency of these lesions is low after MMS and any change in WRN acetylation status could be undetectable. On the other hand, MMS and other agents that induce an increase in WRN acetylation levels produce lesions that result in blockage of replication [Jung et al., 2007; Liu et al., 2003]. Of the lesions formed by MMS, O^6-methylguanine disrupts hydrogen bonding with cytosine, but otherwise does not alter the double helix to any great extent. Therefore, O^6-methylguanine does not induce replication blockage, instead causing misincorporation during DNA replication and giving rise to distinctive G-C to A-T point mutations. If indeed WRN acetylation is related to a response to blockage of replication, O^6-methylguanine lesions may not induce amplification of WRN acetylation because they do not block replication. The results obtained after inhibition of repair of 7-methylguanine and 3-methyladenine lesions agrees with this notion. Several studies suggest that 7-methylguanine and 3-methyladenine present a strong block to DNA synthesis. Methylation at both the N^7 and N^3 position of purines also destabilizes the N-glycosidic bond and renders the modified bases more susceptible to being hydrolyzed into abasic sites known to block DNA synthesis [Wyatt et al., 2006; Johnson et al., 2007; O'Connor et al., 1988]. In addition, alkylations of the N^3 positions of purines, including 3-methyladenine, block DNA replication directly by occupying the minor groove of DNA,

which is normally free of methyl groups [Lindahl et al., 1993]. Thus, based on the possible relationship between WRN acetylation and blockage of replication, it is tempting to speculate that we observed a further amplification in WRN acetylation upon persistence of 7-methylguanine and 3-methyladenine because of increased frequency of encounters of replication forks with those lesions in the DNA template that, in turn, more often block DNA replication.

Although we focused primarily on MMS treatment because it elicited the largest increase in WRN acetylation, we also wanted to know if the induction and/or persistence of the lesions caused by other agents used in our initial experiments also increased WRN acetylation. Thus, we also compared the effect of bulky, covalent DNA adducts generated by cisplatin in cells deficient or proficient for NER, the repair pathway responsible for removing the majority of lesions induced by cisplatin. The results of those experiments demonstrated that cisplatin treatment of NER-deficient cells induced a further increase in the levels of WRN acetylation when compared with normal NER-proficient cells. Furthermore, the chromosome instability caused by cisplatin treatment (measured using the micronucleus assay) suggests that cisplatin lesions are likely to cause replication fork blockage and collapse. These results using strategies to inhibit DNA repair lead to several conclusions. First, WRN acetylation can be directly correlated with DNA damage induced by these agents, instead of some non-specific effect of the agent on some other aspect of cellular metabolism or physiology. Second, the increase in WRN acetylation response to repair inhibition indicates that is not the induction but the persistence of damage. Importantly, our data is consistent and extent previous studies showing WRN acetylation after DNA damage [Blander et al., 2002; Li et al., 2008; Muftuoglu et al., 2008]. Here, we took a step further and established (for the first time) a relationship between WRN acetylation and persistence of DNA damage.

In this study, we have demonstrated that WRN acetylation is a dynamic process. Specifically, we have shown that there is a time-dependent increase of WRN acetylation, reaching the maximum levels within 4 h after MMS treatement. These results are in agreement with previous studies showing temporal increase on WRN acetylation levels

after etoposide treatment [Li et al., 2008]. Interestingly, our flow cytometry analysis after MMS treatment indicates that the increase in the levels of acetylated WRN correlates to an increase in the percentage of S-phase cells. In light of these findings, it was relevant to consider if WRN acetylation has a relationship to DNA replication. Thus, the incorporation of BrdU was measured after HU and MMS treatments. Interestingly, HU and MMS treatments markedly decreased the levels of BrdU incorporation. Since WRN acetylation increased in response to HU and MMS treatments, our results indicate that WRN acetylation correlates with inhibition of DNA synthesis. Further, our findings strongly suggest that WRN acetylation observed after DNA damaging agents and replication blocking agents might arise as a response to DNA damage-induced replication stress.

Since the acetylation state of WRN is influenced by acetylases and deacetylases, we inhibited the groups of deacetylases to study their role in regulation of WRN acetylation. For those experiments, we used the drugs TSA and nicotinamide in order to inhibit various deacetylases between the different classes of deacetylases that exist in humans. The result of those experiments revealed that, even in the absence of DNA damaging treatments, deacetylase inhibitors amplified the levels of WRN acetylation by five fold. This result indicates that WRN acetylation state is determined by equilibrium between acetylation and deacetylation. Thus, disruption of this equilibrium, in this case through the inhibition of deacetylases, results in a shift towards increased WRN acetylation. These experiments also suggest that another scenario for amplification of WRN acetylation levels in response to DNA damage might be a change in the balance between acetylases and deacetylases. Our results suggest that DNA damage shifts the balance towards acetylation and perhaps increased acetylase or decreased deacetylase activity. Thus, the status of WRN acetylation might be regulated further by the presence of specific proteins involved in the acetylation process, which might favor specific acetylated or deacetylated forms of WRN.

We also studied the role of the different classes of deacetylases in regulation of WRN acetylation. To date, the classes have been divided into two families and our results

indicate that members of both families contribute to WRN deacetylation. In our studies, Class I, II and IV, that are the members of the HDAC family, appear to play a larger role, since TSA resulted in higher levels of WRN acetylation when compared to nicotinamide alone. These results indicate, for the first time, that acetylated forms of WRN are substrates for the Class I, II and IV of histone deacetylase enzymes. However, sirtuins (Class III) also play some role in regulation of WRN acetylation, since nicotinamide treatment alone at least doubled the amount of acetylated WRN when compared with untreated cells. The latter results are in agreement with recent studies showing interaction and regulation of WRN by sirtuins. Specifically, recent studies have shown that: 1) WRN interacts with SIRT1 both *in vitro* and *in vivo* and is hypoacetylated in cells overexpressing SIRT1 [Law et al., 2009; Li et al., 2008], 2) sirtuin deacetylase activity protects WRN from ubiquitination and sequential degradation by the 26S proteosome [Kahyo et al., 2008, Li et al., 2010], 3) SIRT6 collaborates with WRN at telomeric chromatin [Michishita et al., 2008], and 4) SIRT1 deacetylation regulates WRN helicase and exonuclease activity (see Figures 3.2 and 3.3 in Chapter 3). In addition, our results are consistent with studies showing WRN regulation in response to deacetylase inhibitors. Specifically, in response to TSA treatment, WRN translocates from the nucleolus to nucleosplasmic foci, a movement that correlates with WRN acetylation [Blander et al., 2002; Karmakar et al., 2005]. A recent study identified six lysine residues (K366, K887, K1117, K1127, K1389, and K1413) that are subject to acetylation on ectopically expressed, acetylated WRN [Li et al., 2010]. Thus, different deacetylases might target different acetylated lysine sites on WRN. More research needs to be done to determine which deacetylases are responsible for removing acetyl groups from individual lysines.

In summary, we report here that DNA damage and replication blocking agents induce WRN acetylation. Our results support the view that WRN is acetylated in response to blockage of replication. Cell cycle analysis, BrdU incorporation assays, and the effect of persistent DNA damage results are consistent with this possibility. Our findings regarding WRN regulation by deacetylases show for the first time that deacetylation of endogenous WRN is influenced by not only sirtuins but also HDACs. Since acetylation of WRN is a

transient process, we speculate that modification of WRN is used as a rapid way to respond to cellular stress. Collectively, our study has identified a crucial process by which WRN is regulated in response to DNA damage and blockage of replication. These results are in agreement with its putative role in DNA metabolism and maintenance of genome stability.

CHAPTER III

WRN ACETYLATION REGULATES ITS ENZYMATIC ACTIVITIES

INTRODUCTION

The amino acid sequence of WRN revealed several functional domains including its identification as a member of the RecQ family of helicases. To date, several laboratories have overproduced and purified recombinant wild type and mutant WRN proteins to characterize their catalytic activities and properties. As expected from its strong homology to RecQ helicases, WRN's central region confers ATPase activity that provides the energy for unwinding DNA with a 3'→5' directionality [Gray et al., 1997; Suzuki et al., 1997; Shen et al., 1998; Chen et al., 2002]. Furthermore, the existence of an N-terminal RNase D-type domain, not present in any other human RecQ member, confers to WRN an intrinsic 3'→5' exonuclease activity [Huang et al., 1998; Shen et al., 1998b; Mian et al., 1997]. Interestingly, the exonuclease and helicase activities of WRN have been shown to be physically and functionally separable [Huang et al., 1998]. How these functions are coordinated during DNA metabolism is unknown. However, what is well-known is that both activities (helicase and exonuclease) of WRN occur preferentially on complex DNA structures, such as those formed during replication and recombination, including forks, bubbles, and Holliday junction intermediates [Constantinou et al., 2000; Brosh et al., 2001; Mohaghegh et al., 2001; Lebel et al., 1999; Orren et al., 2002; Opresko et al., 2004]. The unwinding activity also disrupts unusual DNA structures such as G-quartets and triplexes [Brosh et al., 2001; Johnson et al., 2010]. In addition, WRN possesses DNA binding activity that appears to be dependent upon DNA structure with no apparent nucleotide sequence preference [Orren et al., 1999; Brosh et al., 2002]. The affinity of both the helicase and exonuclease for alternative DNA structures might reflect their roles in resolving specific DNA intermediates that might form during DNA metabolism.

Just downstream of the helicase domain there are additional sequences in WRN typical of some RecQ members, known as the RecQ-conserved (RQC) and the Helicase and RNase D C-terminal (HRDC) domains. These domains fold into distinct structural entities and have DNA binding affinities [Liu et al., 1999; von Kobbe et al., 2003; Hu et al., 2005; Lee et al., 2005]. In fact, experiments using truncated versions of WRN have shown that it possess four distinct DNA binding regions, including the helicase, RQC and HRDC domains [von Kobbe et al., 2003]. The exonuclease domain also possesses DNA binding affinity [Machwe et al., 2006].

WRN deficiency causes replication abnormalities and hypersensitivity to agents that severely inhibit replication fork progression, suggesting that WRN might participate in resolution of replication blockage. It has been proposed that the first step in dealing with a blocked replication fork involves its regression. This process occurs through re-annealing of the parental strands and pairing of the daughter strands to generate a Holliday junction structure or "chicken foot intermediate" [Haber et al., 1999; Cox et al., 2002]. Fork regression would be facilitated by an enzyme that possesses both unwinding and strand annealing activity. Interestingly, similar to some recombination proteins, WRN also facilitates the pairing of complementary DNA strands, this annealing activity works in concert with its helicase activity to perform strand exchange and regress model replication forks [Machwe et al., 2005; Machwe et al., 2006]. The latter suggests that WRN, as part of its role in a genome maintenance pathway, might be involved in the correct resolution of intermediates that arise from blocked replication forks *in vivo,* caused by DNA damage. Thus, the genomic instability of WRN-deficient cells may be due to an inability to complete normal DNA replication in the absence of functional WRN.

The enzymatic activities of WRN on DNA substrates have been shown to be modulated by post-translational modifications [Kusumoto et al., 2007]. Specifically, its helicase and exonuclease activities are regulated by phosphorylation [Karmakar et al., 2002; Yannone et al., 2001; Cheng et al., 2003]. Importantly, WRN is subject to post-translational modifications following DNA damage. These modifications correlate with

WRN nuclear re-localization to replication foci, a likely site for WRN function in DNA metabolism [Blander et al., 2002; Cheng et al., 2003; Pichierri et al., 2003; Woods et al., 2004; Karmakar et al., 2005]. Collectivelly, these studies suggest that it is likely that cellular WRN trafficking and function is regulated by protein modification, and perhaps may be related to a specific DNA damage response pathway.

Our studies have demonstrated that genotoxins induce WRN acetylation (see chapter II). In order to further understand the effect of WRN acetylation on its biochemical function, unmodified WRN, acetylated WRN and deacetylated WRN protein (expressed in and purified from HEK293 cells) were compared as to their helicase, exonuclease, and fork regression activities on relevant DNA structures, with particular emphasis on WRN action on model replication forks. In this chapter, we report that acetylated WRN has significantly less exonuclease and helicase activities than unmodified WRN on simple DNA substrates (partial DNA duplexes). Interestingly, deacetylation of WRN at least partially restored the normal level of exonuclease and helicase activities. When a more rigorous examination was done on more complex DNA substrates including replication forks, surprisingly, the effect of acetylation on WRN fork regression activity was much less pronounced. Importantly, we provide evidence that the exonuclease activity of unmodified and acetylated WRN on model replication forks is comparable. Together, these experiments suggest that WRN acetylation helps to regulate WRN specificity by reducing its preference for non-physiological substrates.

RESEARCH DESIGN

SPECIFIC AIM: To investigate the effect of WRN acetylation on its biochemical functions

RATIONALE

Our previous studies demonstrate that WRN is acetylated after treatment of cells with DNA damaging agents, particularly those that block replication fork progression. It has

been shown that phosphorylation of WRN after treatment with DNA damaging agents decreases its exonuclease and helicase activities [Karmakar et al., 2002; Yannone et al., 2001; Cheng et al., 2003], indicating that WRN modification might alter its catalytic activities. However, it is unclear if acetylation alters WRN catalytic activities. To this end, we analyzed in detail how WRN acetylation influences its catalytic activities. Specifically, unmodified and acetylated WRN were compared as to their helicase, exonuclease and fork regression activities. Initially, unmodified WRN was directly compared to acetylated WRN or deacetylated WRN on simple DNA partial duplexes in regard to their helicase and exonuclease activities. Since WRN has higher specificity for complex DNA substrates, such as those form during DNA replication and recombination (including Holliday junction and forks), we also studied the fork regression and exonuclease activities on model replication forks and Holliday junctions. A mutant WRN protein containing lysine to arginine mutations at conserved acetylation sites which cannot be acetylated was analyzed, to determine if indeed WRN acetylation sites are required for WRN catalytic activities.

METHODS

Production and purification of unmodified and modified wild-type and mutant FLAG-WRN proteins. FLAG-tagged unmodified, acetylated, deacetylated, and mutant WRN proteins were overexpressed in HEK293 cells using a transient transfection assay (performed in the laboratory of our collaborator, Dr. Jianyuan Luo, University of Maryland Medical School). Unmodified FLAG-WRN and acetylated FLAG-WRN were expressed and purified as described previously (see methods section in Chapter II). To produce the FLAG-WRN 6KR mutant, site-directed mutagenesis was used to convert lysine residues (K366, K887, K1117, K1127, K1389, and K1413) to arginine.

DNA substrate construction. Oligonucleotides were purchased from Integrated DNA Technologies and their sequences are given in table 3.1. Each substrate was generated by radiolabeling the 5' end of one strand (depicted in each figure) with [ɣ-^{32}P] ATP and T4 polynucleotide kinase followed by annealing with a two-fold excess of one or more

unlabeled complementary strand(s). Annealed substrates were separated by native PAGE (12%), excised, and extracted. Labeled oligomers and annealed duplex substrates were then purified using a gel extraction kit (Qiagen).

The forks were constructed by radiolabeling one oligomer (the specific oligomer used is depicted in each figure) with [ɣ-^{32}P] ATP and T4 polynucleotide kinase, 3'-phosphatase free (Roche Molecular Biologicals, Indianapolis, IN). In an initial annealing step to form parental daughter partial duplexes, labeled strand was heated to 90°C and slow-cooled with excess complementary unlabeled daughter strand, while the other unlabeled parental strand was treated similarly in individual reactions with excess of its complementary daughter strand. The resulting lagging and leading parental-daughter partial duplexes were then mixed together at 37°C for 18 h. After separation by native 8% PAGE, the substrate was excised, extracted into TEN buffer (10mM Tris, pH 8.0, 1 mM EDTA and 10 mM NaCl), and stored at 4°C prior to use.

Helicase and Fork Regression Assays. To measure enzyme-catalyzed unwinding, the DNA substrates were incubated without or with unmodified, acetylated and deacetylated FLAG-WRN proteins (as indicated in figures) in WRN reaction buffer [40 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$ (unless otherwise indicated), 1 mM ATP (or 0.25 mM when indicated), 0.1% Nonidet P-40, 0.1 mg/ml bovine serum albumin (BSA), and 5 mM dithiothreitol] at 37°C for the specified times. Reactions were subsequently incubated with Proteinase K (1 mg/ml), SDS (0.2%) and EDTA (5 mM) for 30 min (or 1 h when indicated) at 37°C and then stopped by addition of one-sixth volume of loading dyes (30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol, and 50 mM EDTA). Samples were subjected to electrophoresis in an 8% native polyacrylamide gel in 1X Tris borate-EDTA at 100 V for 3 h at room temperature. The gel was vacuum-dried at 80°C for 1 h, and radioactive DNA products were visualized by phosphorimaging.

Exonuclease Assays. Exonuclease reactions (10 µl) containing the substrate of interest and FLAG-WRN proteins (at the indicated concentrations) in WRN reaction buffer without, or with 1 mM ATP (when indicated in figures), were preincubated on ice for 5

min, and then transferred to 37°C for the indicated times. Reactions were stopped by the addition of formamide loading buffer (95% formamide, 20 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol). DNA products were heated at 90°C and separated by denaturing (14%) PAGE. Digestion of the labeled strand by the 3' to 5' exonuclease activity of WRN proteins was visualized by phosphorimaging.

RESULTS

Acetylation alters WRN biochemical activities and specificity. Our results demonstrated that WRN is acetylated in response to DNA damaging agents and replication blocking agents. To further understand the function of WRN in DNA metabolism, we analyzed in detail how WRN acetylation influences its catalytic activities. Using the protocol depicted in Figure 3.1A, unmodified FLAG-WRN, acetylated FLAG-WRN and deacetylated FLAG-WRN were made from HEK293 cells. Western blot techniques were used to determine their protein levels and confirm the acetylated state (Figure 3.1B). First, these proteins were used to perform a helicase assay on a 21-bp partial duplex with a 49-nt 3' overhang structure constructed by annealing oligomers 21-lag and 70-lag. (Figure 3.2A, for details; see "Methods" section and Table 3.1 for nucleotide sequences). We started out using this kind of substrate, with one blunt end and the other end with a 3' single-stranded region, because the helicase activity of WRN requires a 3' single-stranded DNA region relative to the duplex DNA to be unwound. Using equivalent protein concentrations, acetylated FLAG-WRN showed significantly less helicase activity than FLAG-WRN (Figure 3.2B, lanes 5-7 vs. lanes 2-4). Interestingly, deacetylation of WRN almost completely restored the normal level of helicase activity (Figure 3.2B, lanes 8-10 vs. lanes 5-7). A quantitative graph for WRN unwinding activity is presented in Figure 3.2C. Thus, acetylation appears to suppress WRN helicase activity on simple partial duplexes.

Using the same proteins, exonuclease assays were performed on different partial duplex substrates with a recessed 3'-end structure (see structures in Figure 3.3), to determine the effect of acetylation on WRN exonuclease activity. These substrates with

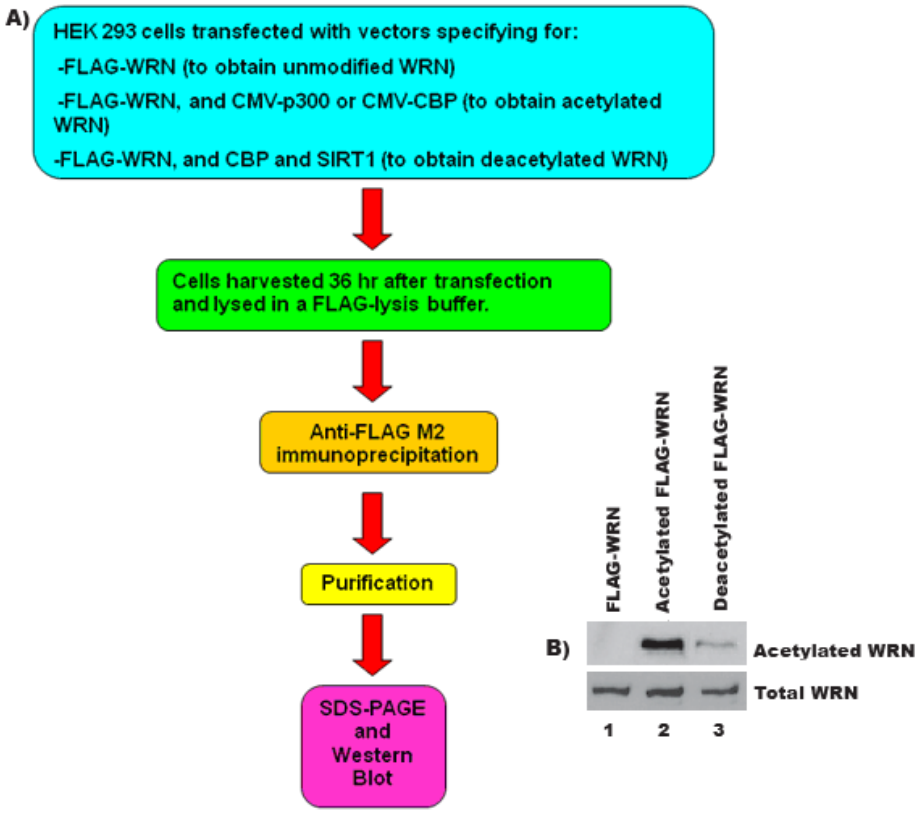

Figure 3.1 WRN acetylation assay. A) To obtain unmodified FLAG-WRN, cells were transfected with vector specifying production of FLAG-WRN. To obtain acetylated WRN, cells were co-transfected with individual vectors specifying production of FLAG-WRN and CMV-p300 or CMV-CBP (two acetyltransferases that acetylate WRN *in vivo* [Li et al., 2010]). To obtain deacetylated FLAG-WRN, cells were co-transfected with FLAG-WRN, CBP (since it is the major acetylase involved in WRN acetylayion [Li et al., 2010]) and SIRT1 (a histone deacetylase) vectors. Cells were harvested 36 h after transfection and were lysed in a FLAG-lysis buffer. After anti-FLAG M2 immunoprecipitation, the immobilized FLAG-WRN proteins were released using FLAG peptide and purified unmodified or acetylated FLAG-WRN was collected. To confirm that the system works and determine protein concentration and the level of acetylation, the purified FLAG-WRN proteins were resolved by SDS-PAGE and detected by western blot. **B)** For each transfection strategy, WRN was produced (lower panel). In the case of cotransfection with p300 or CBP, acetylated WRN was produced (upper panel, lane 2), and in the case of cotransfection with p300 and SIRT1, the levels of acetylated WRN were dramatically reduced (upper panel, lane 3).

Table 3.1

Oligonucleotides used to construct DNA substrates

21-lag GAGGATATCATGTACGATAGCP
21- lead GCTATCGTACATGATATCCTCP
K21RP3 TAGGGTTAACATCAAGTCACGP
30-lead GCTATCGTACATGATATCCTCACACTCACT
32-lag ATTCAGAGTGTGAGGATATCATGTACGATAGCP
G35left AGCTCCTAGGGTTACAAGCTTCACTAGGGTTGTCC
3(52)scr CACTCCTCTGAGTCTGGACGGCAGCTGGCCAAGTGTGAGTGTGAGTGTGAGT
5'(52) TCACTTGACAAGTGACTGTGACCTAGGCATCCTCCAGTTCCTGGAGTCAGTG
70-lag GCTATCGTACATGATATCCTCACACTCTGAATAGCCGAATTCTTAGGGTTAGGGTTAACATCAAGTCACGP
70 lead CGTGACTTGATGTTAACCCTAACCCTAAGAATTCGGCTTAAGTGAGTGTGAGGATATCATGTACGATAGC
K70P3 CGTGACTTGATGTTAACCCTAACCCTAAGAATTCGGCTTAAGTGAGTGTGAGGATATCATGTACGATAGCP
K70left fork CAGCAACATACATTGTAAGAGCATACAGACACGCACGAATTCTTAGGGTTAGGGTTAACATCAAGTCACGP
K70right fork GCTATCGTACATGATATCCTCACACTCACTTAAGCACTCAGGCACTCTAGCTCTGCTCACGACCAGACATP
C80 GCTGATCAACCCTACATGTGTAGGTAACCCTAACCCTAACCCTAAGGACAACCCTAGTGAAGCTTGTAACCCTAGGAGC TP
**base* CACTGACTCCAGGAACTGGAGGATGCCTAGGTGGCCAGCTGCCGTCCAGACTCAGAGGAGTG
HJ70M8-1 GCTATCGTACATGATATCCTCACACTCACTTAAGCCGAAGAGAATCCTGATCTCAATTGTAGTTCAGTGCP
HJ70M8-2 GCACTGAACTACAATTGAGATCAGGATTCTCTTCGGCTTCTCATTCACACTCCTATAGTACATGCTATCGP
HJ70M8-3 CGATAGCATGTACTATAGGAGTGTGAATGAGAAGCCGAATTCTTAGGGTTAGGGTTAACATCAAGTCACGP

All sequences are depicted in 5' to 3' orientation

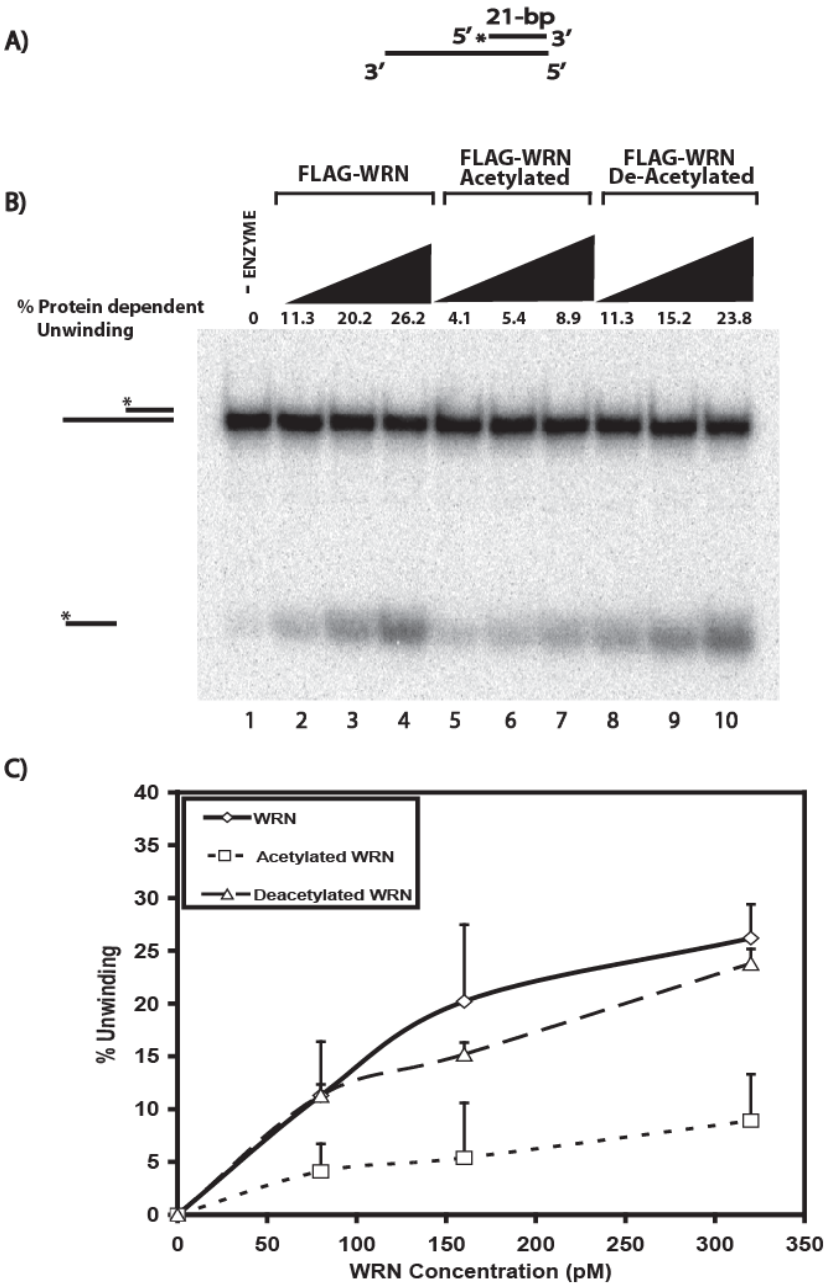

Figure 3.2 WRN helicase activity is regulated by acetylation. A) Substrate containing the 3' overhang constructed by annealing oligomers 21-lag and 70-lag. The relative position and size of the complementary region (21 bp) is indicated. **B)** Helicase assay was performed with 21-bp partial duplex using FLAG-WRN, acetylated FLAG-WRN, and deacetylated FLAG-WRN produced as explained in figure 3.1. The 3' overhang (5 fM) was incubated at 37°C for 10 min with FLAG-WRN proteins (80, 160, and 320 pM) and analyzed as described in the Methods section. **C)** Graphic representation for WRN unwinding activity (mean and S.D. for three independent experiments).

one blunt end and the other end with a recessed 3' end were selected for our initial studies because they are resistant to the helicase activity of WRN (the helicase activity requires a 3' single-stranded region). Therefore, when WRN is added to these substrates, the exonuclease activity is directed to the recessed end (Figure 3.3A). Exonuclease activity at the 3' end of the labeled strand is measured by the appearance of shorter fragments on a denaturing gel. As expected, WRN degrades the 3'-end of the labeled strand in a step wise manner in a 3' to 5' direction (Figure 3.3B, lines 2-4). Interestingly, acetylation of WRN markedly decreased its exonuclease activity (Figure 3.3B, lanes 5-7 vs. lanes 2-4). Again, deacetylation of WRN can reverse this effect (Figure 3.3B, compare lanes 8-10 vs. lanes 5-7). An eight fold molar excess of acetylated WRN is needed to obtain similar exonuclease activities between unmodified and acetylated WRN on this recessed 3' end substrate (Figure 3.3C, compare lines 2-4 to 5-7). Although an ATP requirement is connected with unwinding and the exonuclease activity of WRN is not ATP-dependent, several laboratories have shown that WRN exonuclease activity is stimulated by ATP hydrolysis on certain substrates [Kamath-Loeb et al., 1998; Machwe et al., 2002; Shen et al., 2000; Brosh et al., 2001]. Thus, we added ATP to the exonuclease reactions to investigate if ATP can stimulate the exonuclease activity of acetylated WRN. However, the exonuclease activity of acetylated WRN was not significantly stimulated even in the presence of ATP (Figure 3.3D, lane 2 vs. 3). Collectively, these results indicate that acetylation decreases WRN exonuclease activity on simple partial duplex substrates.

It is well known that WRN prefers special DNA structures, such as those formed during replication and recombination, including forks [Opresko et al., 2003]. Importantly, WRN coordinates its annealing activity with its helicase activity to perform strand exchange and regress model replication forks [Machwe et al., 2005; Machwe et al., 2006]. The latter support the notion that WRN might be involved in the correct resolution of intermediates that arise from blocked replication forks *in vivo,* caused by DNA damage. Therefore, we wanted to explore the effect of WRN acetylation on more complex structures. To this end, a more rigorous examination was done by comparing the

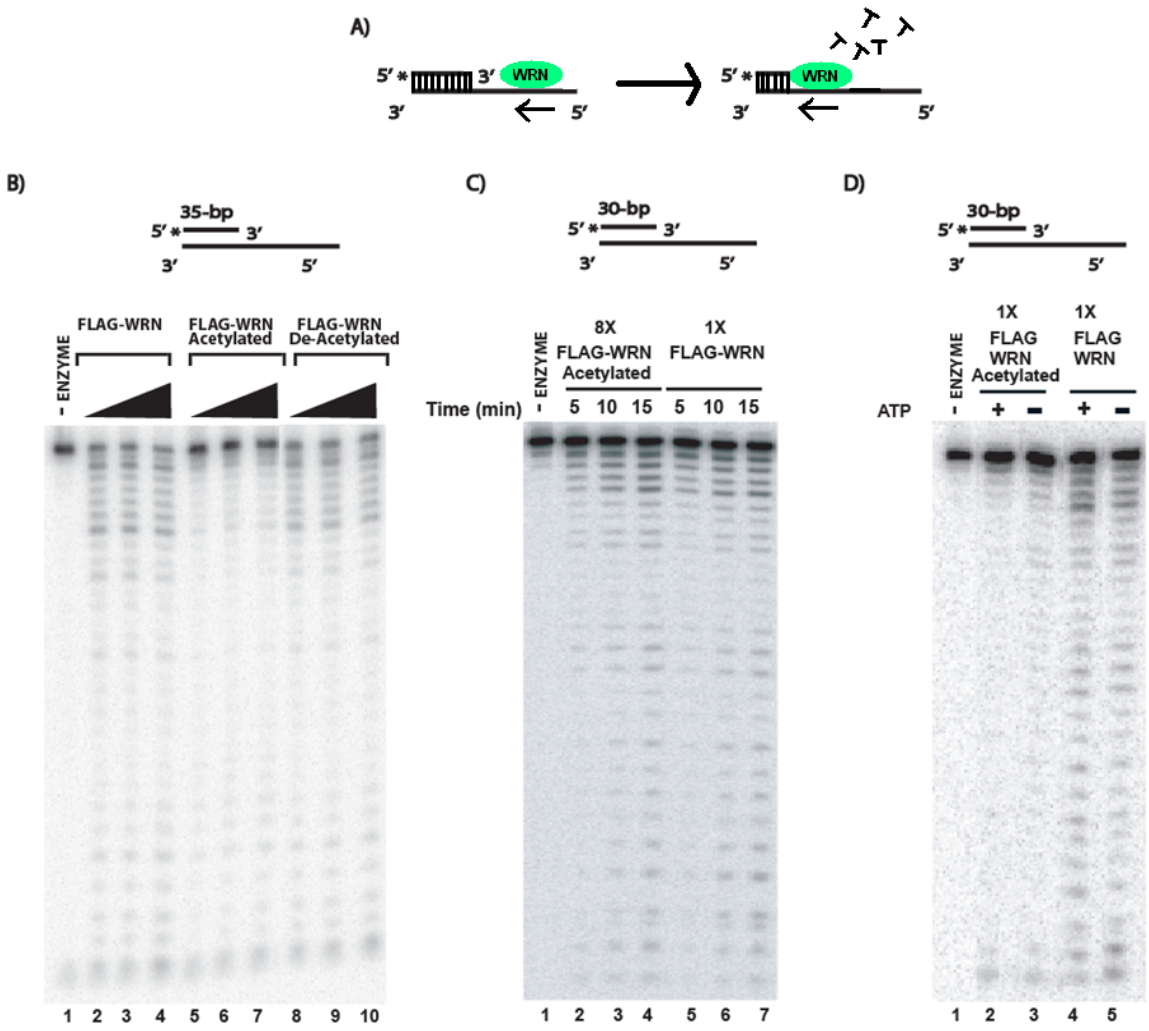

Figure 3.3 WRN exonuclease activity is regulated by acetylation. A) WRN exonuclease directionality is defined as 3' to 5' with respect to the direction that the single strand of the DNA substrate is degraded, as despicted in the figure. Exonuclease activity at the 3' end of the labeled strand is measured by the appearance of shorter fragments on a denaturing gel. **B)** Time course of the exonuclease activities of FLAG-WRN proteins on a 35-bp partial duplex with a recessed 3' end probe (0.1 nM), constructed by annealing oligomers G35left and C80, at 37°C were compared. **C)** The time course exonuclease activities of unmodified FLAG-WRN and 8-fold molar excess of acetylated FLAG-WRN protein on a 3'-end probe (0.1 nM) constructed by annealing oligomers 30-lead and K70P3. **D)** Reactions containing 3' end probe (0.1 nM) constructed by annealing oligomers 30-lead and K70P3 with or without ATP (1 mM), as indicated, were incubated at 37°C for 15 min.

unwinding strength and fork regression activities of unmodified and acetylated WRN on a series of DNA substrates constructed using the same labeled strand. Briefly, a labeled 80-nt strand was annealed with different unlabeled complementary strands to produce a 3' overhang structure, a 2-stranded fork, a 3-way junction and a model 4-stranded replication fork as well as a Holliday junction structure (see structures in Figure 3.4A and 3.5A) that were subsequently purified. The common labeled strand made it straightforward to use precisely the same molar amounts of these DNA substrates to facilitate direct comparison in enzymatic assays. For these assays, these substrates were incubated in WRN-reaction buffer containing unmodified or acetylated WRN. As shown in figure 3.4A and B, unmodified WRN unwinds the 2-stranded fork (compare lines 5 and 6) and the 3-way junction (compare lines 8 and 9) with higher efficiency than acetylated WRN, in order of preference, respectively. As expected, the 3-way junction was unwound to a variety of products that were included in the calculation. We further tested a series of 3-way junction with different sequences on the 3'-flap and, consistent with these results, acetylated WRN was less efficient than unmodified WRN (data not shown). The difference between unmodified and acetylated WRN unwinding activities was less dramatic on the 3' overhang substrate (compare lines 2 and 3). Unwinding of the Holliday junction substrate was not detected (data not shown), likely because of the length of the duplex regions of this substrate. In contrast, unmodified and acetylated WRN have comparable fork regression activity (Figure 3.4A, compare lines 11 and 12). In parallel, we measured the exonuclease activities of unmodified and acetylated WRN on the same substrates. Similar to previous results, the exonuclease activity of unmodified WRN was stronger than the exonuclease activity of acetylated WRN on the 3' overhang (Figure 3.5A, compare lines 2 and 3) and the 2-stranded fork (compare lines 5 and 6). However, the exonuclease activities of unmodified and acetylated FLAG-WRN were comparable on the 3-way junction (compare lines 8 and 9) and the model replication fork (compare lines 14 and 15). On the Holliday junction (compare lines 11 and 12), unmodified WRN appeared to be slightly mor active than acetylated WRN. A quantitative chart for WRN exonuclease activity on the different substrates is presented in Figure 3.5B. Taken together, these results suggest that acetylation of WRN alters its specificity for certain types of substrates.

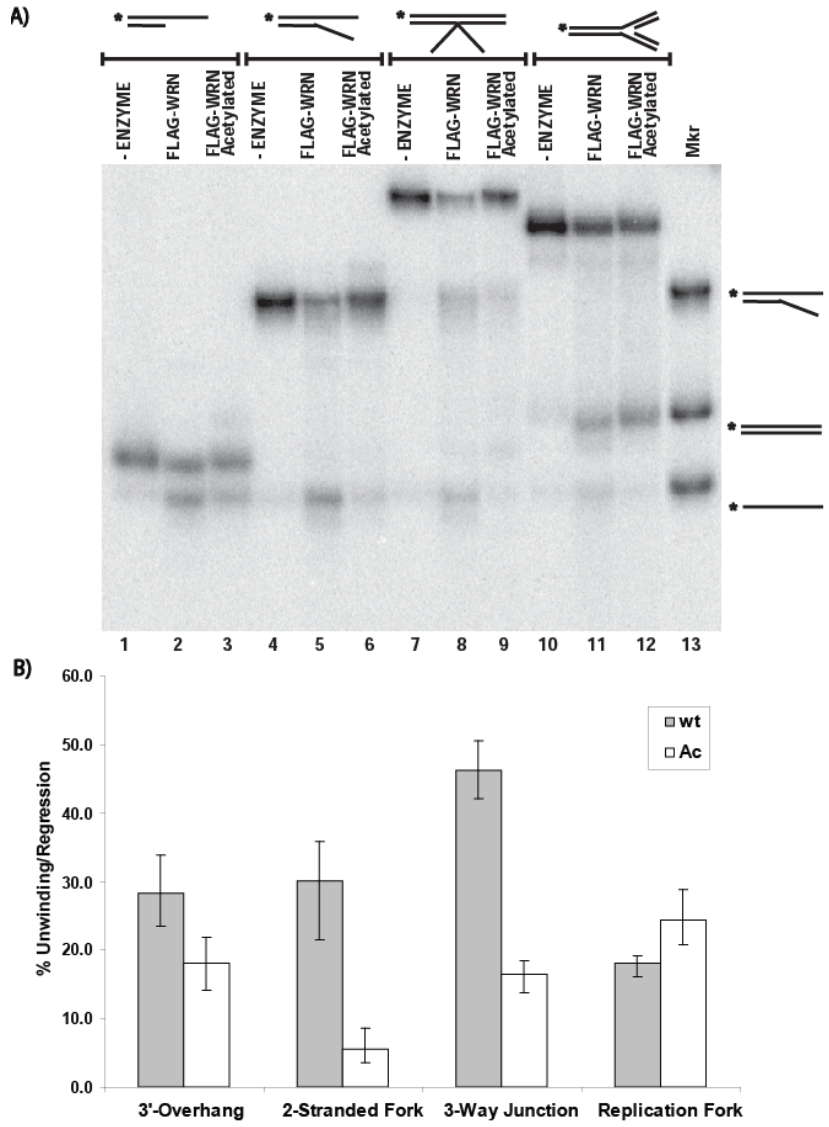

Figure 3.4 Acetylation regulates WRN specificity. A) A labeled 80-nt strand (K70P3 oligomer) was annealed with different unlabeled complementary strands to produce a 3' overhang structure (K21RP3 oligomer + K70P3 oligomer), a 2-stranded fork (K70left fork oligomer + K70P3 oligomer), and a 3-way junction (K70left fork oligomer + K70right fork oligomer + K70P3 oligomer). The model replication fork was generated by annealing parental daughter partial duplexes (labeled K70P3 + unlabeled 21-lead oligomer and unlabeled 70-lag + excess 32-lag oligomer). After individual substrates were gel-purified, these substrates (0.1 fm) were incubated at 37°C for 1 h in WRN-reaction buffer containing unmodified or acetylated WRN and analyzed as described in the Methods section. **B)** For experiment in A, percent of unwinding/regression was calculated (mean and S.D. for four independent experiments).

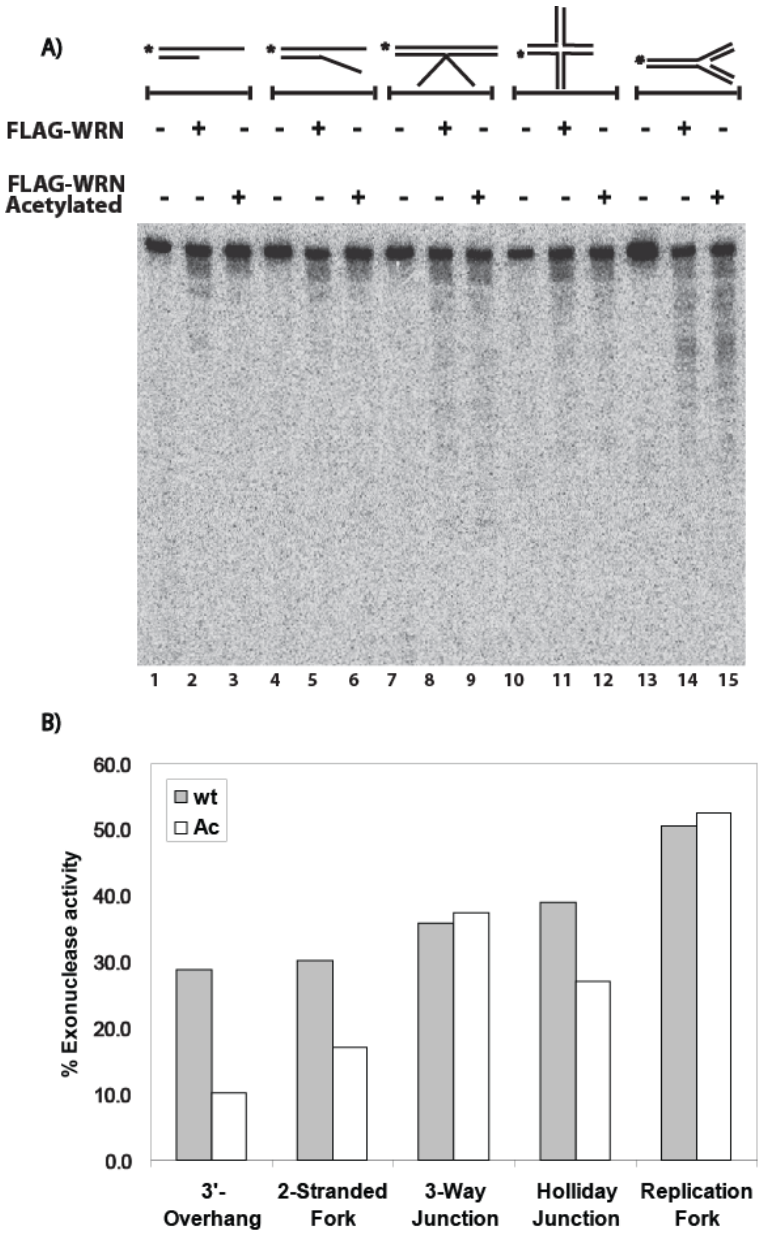

Figure 3.5 Acetylation regulates the specificity of WRN exonuclease activity. A) The substrates constructed using the same labeled 80-nt strand (as described in Figure 3.5A) were incubated at 37°C for 1 h in WRN-reaction buffer containing unmodified or acetylated WRN and analyzed as described in the Methods section. The Holliday junction was constructed by annealing of the labeled K70P3 oligomer with unlabeled partial complementary strands (HJ70M8-1 oligomer + HJ70M8-2 oligomer + HJ70M8-3 oligomer). **B)** For experiment in A, percent reduction of full length labeled strand by WRN exonuclease activity was calculated.

To confirm that unmodified and acetylated WRN have comparable fork regression activities, a different model replication fork was designed (see Figure 3.6A for details) and the fork regression activities of unmodified WRN and acetylated WRN were compared over a range of concentrations. Again, the difference in fork regression activity was much less pronounced, as manifested specifically by daughter duplex formation that is a diagnostic of fork regression (Figure 3.6B, lines 2-4 vs. 5-7). However, they have minor differences in the generation of other products that result from the helicase and exonuclease activities of WRN. After these interesting results we analyzed the exonluclease activity of WRN on this substrate. Interestingly, unmodified and acetylated WRN showed similar efficiency in exonuclease degradation of the labeled strand of this fork substrate (Figure 3.6C, lanes 2-4 vs. 5-7). Together, these results suggest that unmodified and acetylated WRN have comparable activities on four-stranded replication forks.

Recently, lysine residues subject to acetylation were identified after performing a mass spectrometry analysis of purified ectopically expressed acetylated WRN (obtained after co-transfection of FLAG-WRN with the acetylases CBP and p300 in HEK293 cells) [Li et al., 2010]. According to that study, the acetylated lysine residues in WRN are K366, K887, K1117, K1127, K1389, and K1413. Thus, as a negative control, we studied a WRN mutant containing all six lysines mutated to arginine, a conservative basic amino acid substitutions that cannot be acetylated, to examine whether any of these residues are required for WRN enzymatic function. In these experiments, the helicase and exonuclease activities of unmodified FLAG-WRN and the 6KR mutant were compared on simple partial duplexes substrates. To amplify the inherent exonuclease activity of WRN, certain reactions were carried out using Mn^{2+} instead of Mg^{2+} as a co-factor. As shown in Figure 3.7A and B, the WRN 6KR mutant maintains similar activities as the wild type (unmodified) WRN under the conditions used, suggesting that the arginine substitutions do not directly affect WRN enzymatic properties or the folding of the protein.

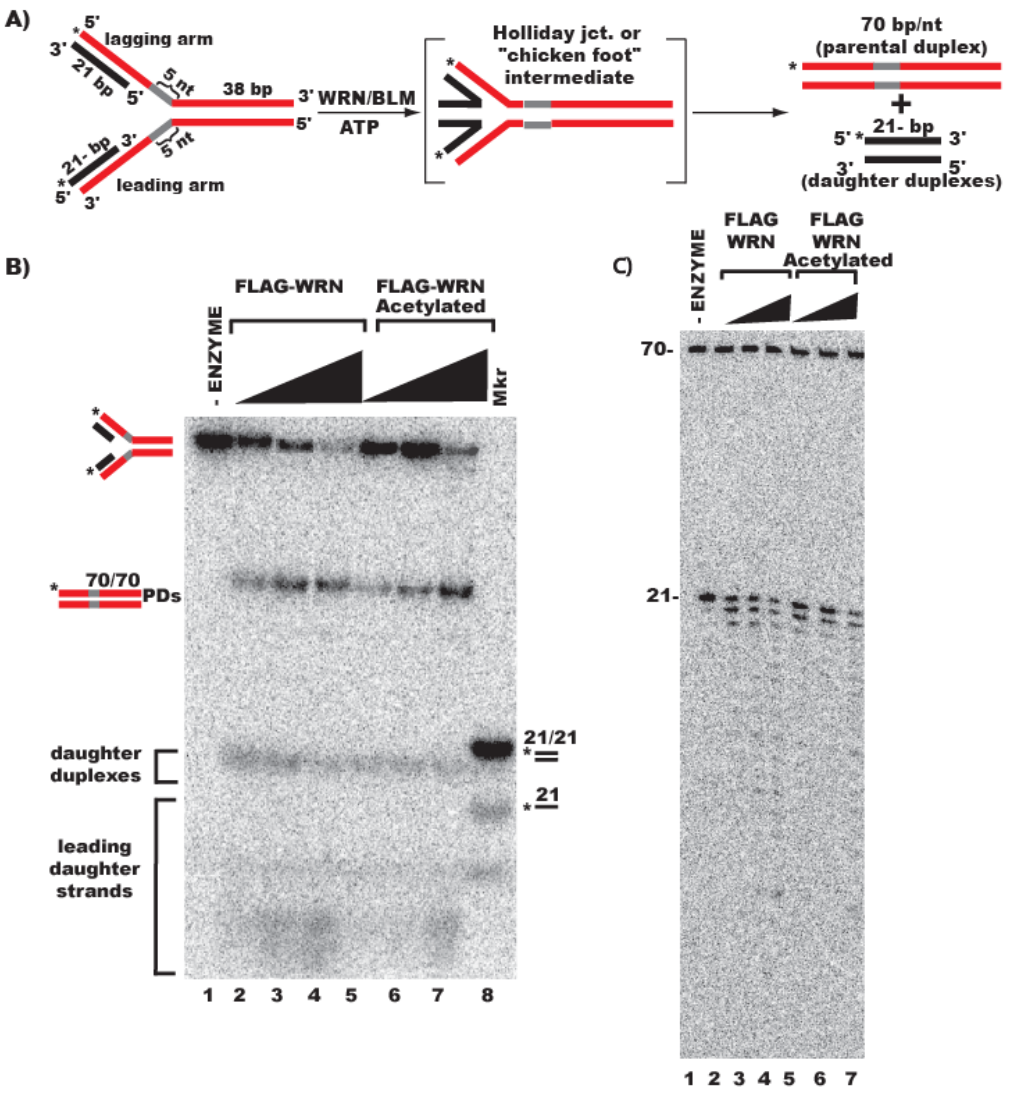

Figure 3.6 Effects of WRN acetylation on a model replication fork. A) A model replication fork was constructed containing homologous leading and lagging arms with five nucleotides of heterology at the fork junction to prevent spontaneous branch migration. Regression the fork is determined specifically by daughter duplex formation which requires unwinding and pairing of the physically unlinked daughter strands. **B)** Equal and increasing amounts of unmodified and acetylated WRN were assayed for fork regression activity on the replication fork model (4 pM) and analyzed as described in the Methods section. **C)** Exonuclease activities of unmodified and acetylated FLAG-WRN proteins on the model replication fork over 30 min at 37°C in WRN reaction buffer (including 1 mM ATP). DNA products were separated by denaturing (14%) PAGE and visualized by phosphorimaging.

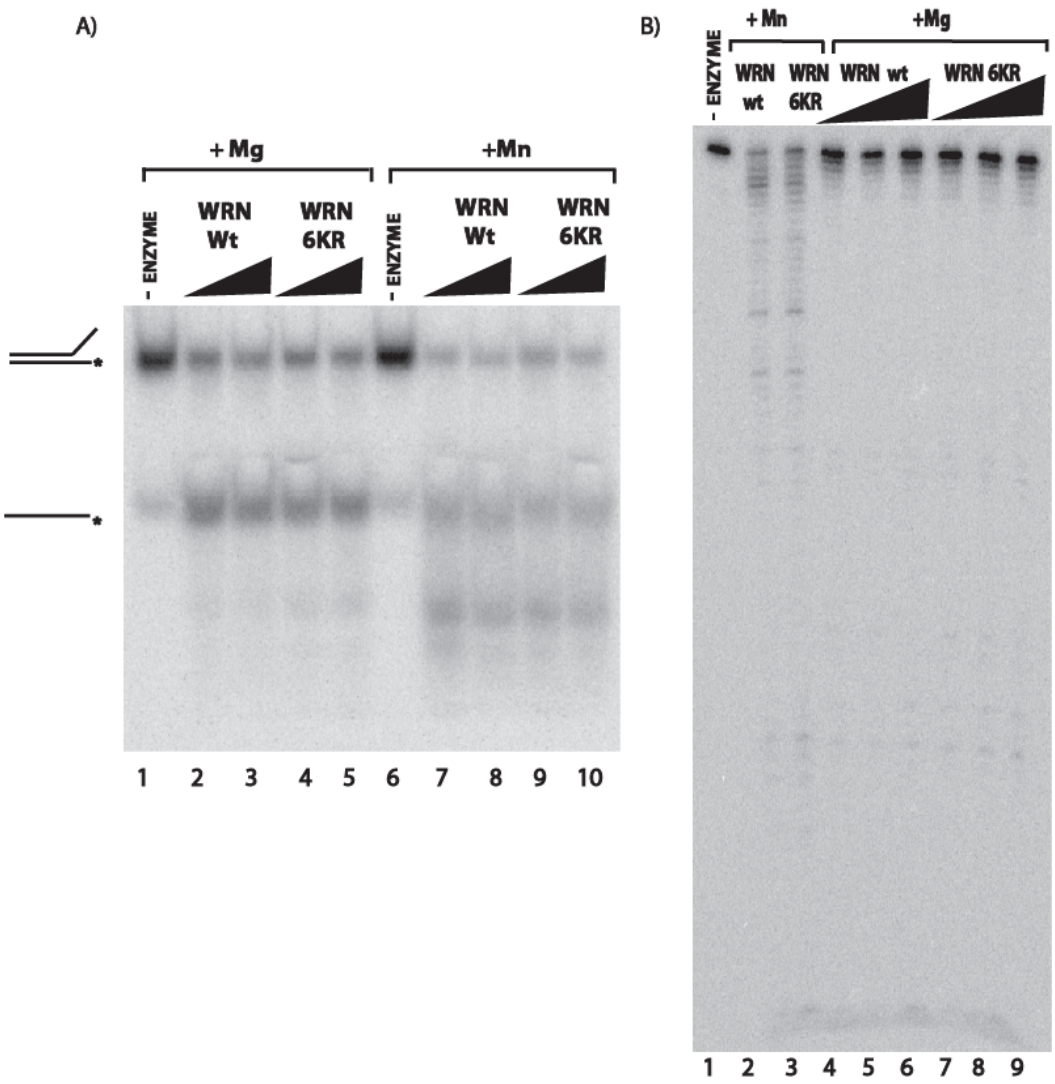

Figure 3.7 WRN 6KR-mutant retains similar activities as the WRN-wt. A) Helicase assay was performed with a partial duplex, containing a 31 and 21 nt 5' and 3' single-stranded arms (constructed by annealing oligomers 3(52)scr and *base), using FLAG-WRN (WRN-wt) and the FLAG-6KR-mutant (WRN 6KR) produced as explained in the Methods section. The partial duplex was incubated at 37°C for 30 min with FLAG-WRN proteins in WRN reaction buffer (including 1 mM $MgCl_2$ or 1 mM $MnCl_2$, as depicted, and 0.25 mM ATP) and analyzed by native PAGE. **B)** Exonuclease activities of unmodified and 6KR-mutant FLAG-WRN proteins on a partial duplex (constructed by annealing oligomers 5' (52) and *base) over 20 min at 37°C in WRN reaction buffer (including 1 mM $MnCl_2$ instead of $MgCl_2$ when indicated). DNA products were separated by denaturing (8%) PAGE and visualized by phosphorimaging.

DISCUSSION

The genomic instability and hypersensitivity to DNA damaging agents of WS cells are thought to be caused by DNA metabolic defects that result from absence of WRN function. This notion has been supported by the identification of 3'→ 5' helicase and 3'→ 5' exonuclease activities of WRN. Notably, WRN has been reported to possess strand annealing and strand exchange activities. Most recently, this notion has been strengthened by the discovery of a new activity in WRN, when Machwe et al. reported that WRN regresses model replication forks [Machwe et al., 2006]. Collectively, these biochemical properties of WRN suggest that it has an important role in DNA metabolism. Interestingly, the enzymatic activities of WRN on DNA substrates have been shown to be modulated by phosphorylation [Karmakar et al., 2002; Yannone et al., 2001; Cheng et al., 2003]. However, how WRN activities are regulated by acetylation remain to be elucidated. In this study, we report the impact of acetylation on WRN catalytic activities using helicase, exonuclease, and fork regression assays.

Initially, WRN helicase and exonuclease activities of unmodified and acetylated WRN were compared using simple DNA partial duplexes structures. Our experiments clearly demonstrate that acetylated WRN has markedly less helicase and exonuclease activities than unmodified WRN indicating that WRN acetylation reduces both helicase and exonuclease activities, at least on those simple substrates. Deacetylation of WRN could reverse this effect. However, it is believed that WRN physiological substrates are those formed during replication and recombination, including forks, since WRN has highest preference for those kinds of structures and possesses replication fork regression activity. Thus, a more depth analysis on various DNA structures, including replication forks, was done. For a 2-stranded fork and a 3-way junction substrate, the unwinding activity of unmodified WRN was substantially higher when compared with acetylated WRN. The difference in unwinding activities was less dramatic in the 3' overhang substrate. However, the results of experiments performed using a replication fork showed approximately equivalent fork regression activities between both unmodified and acetylated WRN. Notably, the exonuclease activity on replication forks between

unmodified and acetylated WRN was also very similar. Collectively, our data strongly suggest that WRN acetylation helps to regulate WRN specificity for certain types of substrates.

Our studies here suggest that the effect of acetylation on WRN enzymatic function is DNA structure-dependent, with little or no effect on either regression or exonuclease activity on four-stranded replication forks. Since our previous studies demonstrated that WRN is acetylated in response to DNA damage and blockage of replication (see Chapter II), we speculate that WRN acetylation plays an important role in regulation of WRN function to resolve replication blockage. Specifically, acetylation may reduce WRN affinity for inappropriate DNA structures, while maintaining specificity for replication fork structures that are the putative physiological targets for WRN action. In support of a fundamental role of WRN during DNA replication, replication forks have been shown to be preferential targets for WRN function, since WRN acts more efficiently on forked DNA than double-stranded duplex DNA [Brosh et al., 2002; Compton et al., 2008] and regresses model replication forks *in vitro* [Machwe at al., 2006 and 2007], an important process to gain access to the replication-blocking lesion, allowing processive replication to resume once the blocking lesion is removed. The hypersensitity to replication blocking agents, prolonged S-phase and reduced lifespan observed in WRN-deficient cell lines are consistent with our notion as well.

Li and colleagues identified six lysine residues subject to acetylation on WRN [Li et al., 2010]. Moreover, they demonstrated that WRN acetylation was at the lowest level detected when all of the six lysines are mutated to arginines, by comparison with single, double, triple, and quadruple mutants, suggesting that all six lysines are involved in WRN acetylation. As part of this study, we analyzed if those six lysine residues are required for WRN enzymatic function. The results of our experiments revealed that these specific lysines are not required for WRN unwinding and exonuclease activities, indicating that these residues do not appear to be involved in catalysis and/or protein folding. The fact that arginine substitutions do not alter WRN's catalytic activities on simple DNA partial duplexes structures contrasts with the effect on these activities when WRN is acetylated

on those lysine residues. Arginine residues cannot be acetylated but retain the positive charge, as for lysine residues. The positive charges of these amino acids are often involved in helping mediate binding to the negatively charged backbone of DNA. Acetylation of lysine eliminates this charge and therefore may alter DNA binding strength or specificity. Thus, our experiments suggest that acetylation of lysine residues on WRN might alter DNA binding affinity and enzymatic activities in such a way to lower the affinity for non-target DNA structures in favor of more physiological structures. It will be important to address in future experiments whether the relevance of individual lysine residues for DNA structure selectivity is altered by acetylation. Addressing these issues should also help to understand how WRN interacts with different DNA structures.

In summary, we have demonstrated that WRN acetylation helps to regulate WRN specificity for certain types of substrates, suggesting that WRN acetylation may increase specificity by reducing preference for non-physiological substrates. Importantly, our findings have identified that acetylation is likely involved in regulation of the DNA metabolic function of WRN.

CHAPTER IV

WRN INTERACTION WITH RPA IN RESPONSE TO AGENTS THAT BLOCK REPLICATION

INTRODUCTION

Existing evidence suggests an association of WRN with the process of DNA replication. Specifically, WS cells have dramatically reduced replicative capacity leading to very early cellular senescence; they grow slowly and have been reported to have a longer S phase and replication initiation and elongation abnormalities [Martin et al., 1970; Takeuchi et al., 1982b; Salk et al., 1985; Poot et al., 1992; Yan et al., 1998]. In addition, WS cells are hypersensitive to DNA damaging agents that tend to block progression of replication forks such as interstrand crosslinking agents including mitomycin C and cisplatin, topoisomerase inhibitors such as camptothecin, and DNA replication inhibitors including HU [Gebhart et al., 1988; Ogburn et al., 1997; Poot et al., 1999, 2001; Pichierri et al., 2001]. However, sensitivity of WS cells to DNA damaging agents does not appear to reflect a direct role in an established DNA repair pathway. Instead, sensitivity to both DNA damaging agents and HU suggests that WRN plays a role in responding to replication blockage by lesions or other obstructions.

A role for WRN in DNA replication likely involves key physical and functional interactions with other proteins involved directly or indirectly in completing duplication of the genome. In support of this notion, it has been reported that WRN associates or interacts directly with factors involved in DNA replication such as PCNA (which functions as a clamp to improve DNA polymerase processivity), FEN-1 (which processes the 5' ends of Okazaki fragments in lagging strand DNA synthesis) [Lebel et al., 1999; Brosh et al., 2001b and 2002b; Rodriguez-Lopez et al., 2003; Sharma et al., 2005], and Topo I (which relaxes superhelical tension generated during DNA replication) [Lebel et al., 1999; Lane et al., 2003]. In addition, WRN interacts with DNA polymerase δ

[Kamath-Loeb et al., 2000 and 2001; Szekely et al., 2000], a major replicative DNA polymerase, and with RPA [Shen et al., 1998a and 2003, Brosh et al., 1999, Doherty et al., 2005, Sommers et al., 2005], a protein heterotrimer that protects single-stranded DNA and binds to gaps at blocked replication forks. Taken together, these observations suggest that loss of these interactions in WRN-deficient cells might disrupt key replication-related pathways. Specifically, it has been suggested that WRN is involved either in preventing the collapse of stalled replication forks, or in the resolution of intermediates present at blocked forks. Consistent with this idea, recent studies have shown that: 1) WRN expression is upregulated during S and G2 phases in highly proliferative transformed cell lines [Kawabe et al., 2000b], 2) upon replication arrest, WRN is redistributed to distinct nuclear foci (associated with ongoing and/or blocked DNA synthesis) and is modified [Constantinou et al., 2000; Sakamoto et al., 2001], 3) WS cells have defective elongation showing marked asymmetry of replication forks from individual bidirectional origins [Rodriguez-Lopez et al., 2002], and 4) WRN can coordinate its unwinding and pairing activities to regress a model replication fork substrate [Machwe et al., 2006]. Thus, the nature of genomic instability, increased cancer and premature aging observed in WS may be the result of improper resolution of blocked replication and illegitimate recombination caused by loss of WRN.

RPA is a single-stranded DNA binding protein complex composed of three structural subunits, RPA70, RPA32, and RPA14, that is involved in DNA replication, repair, and recombination [Iftode et al., 1999; Wold et al., 1997]. It has been shown that RPA binds to single-stranded gaps generated by stalled replication forks, helping to protect these regions and leading to the initiation of downstream pathways [Raderschall et al., 1999; Wold et al., 1997]. The initiation of those pathways requires the recruitment and activation of proteins such as ATR/ATRIP, RAD17 complexes, and RAD51 [Dart et al., 2004; Zou et al., 2003a and 2003b; Binz et al., 2004; Jackson et al., 2002; Golub et al., 1998], which are critical players for the DNA damage response. Importantly, DNA damage induces hyperphosphorylation (≥5 residues) of the N-terminal region of the 32 kDa subunit of RPA [Binz et al., 2004; Din et al., 1990; Dutta et al., 1992; Mitsis et al.,

1995,; Oakley et al., 2001; Shao et al., 1999], presumably to regulate its function in DNA metabolism.

Several studies have shown that purified recombinant RPA and WRN interact with each other and that RPA can stimulate WRN unwinding strength *in vitro* [Brosh et al., 1999; Shen et al., 1998; Doherty et al., 2005], and it has been suggested that an interaction between WRN and RPA might occur *in vivo* within cells. Importantly, RPA colocalizes with WRN in nuclear foci after treatments that induce blockage of replication such as HU [Constantinou et al., 2000; Sakamoto et al., 2001; Ammazzalorso et al., 2010]. Together, this evidence suggests that RPA and WRN might functionally interact at stalled replication forks. Thus, we were interested to examine the intracellular interaction between WRN and RPA, particularly in response to blockage of replication. Whether WRN-RPA association occurs via a direct stable interaction was also analyzed. The results of these experiments indicate that WRN and RPA form a direct association under normal physiological conditions *in vivo* and treatments that block replication fork progression result in an increased association between them. Thus, our findings further support the idea that WRN and RPA are involved in DNA replication by working in a complex at blocked or stalled replication forks.

RESEARCH DESIGN

SPECIFIC AIM: To study if genotoxins influence WRN interaction with RPA

Aim a. To investigate if DNA damaging treatments (known as blockers of replication) regulate the intracellular interaction between WRN and RPA

Aim b. To analyze whether WRN and RPA association occurs via a direct interaction

RATIONALE

The evidence of replication problems in WS cells and the sensitivity of these cells to inhibitors of replication suggest a direct physical and functional association of WRN with the process of DNA replication. To play such a role WRN must be located at forks or recruited to them when needed. Consistent with this idea, WRN is subject to translocation from nucleolus to discrete sites, called nuclear foci, in response to DNA damage and replication blockers. These discrete subnuclear regions are considered as sites of DNA damage and blocked replication and therefore their formation is an important stage in DNA metabolism [Nelms et al., 1998]. Interestingly, WRN co-localizes with RPA in these nuclear foci in response to treatment with the replication inhibitor HU [Constantinou et al., 2000]. Taken together, these facts support the notion of an association between WRN and RPA at blocked replication forks. Thus, based on previous evidence showing *in vitro* interaction between WRN and RPA, we hypothesized in this study that these factors interact directly within cells, particularly in response to replication blockers. Up to this point, an *in vivo* interaction between WRN and RPA had not been demonstrated. Therefore, we wanted to address if indeed an intracellular interaction between WRN and RPA exists *in vivo*, perhaps specifically in response to replication fork blockage. If this hypothesis is true, blockage of replication by DNA damage should induce or increase WRN-RPA association. First, we used co-immunoprecipitation experiments to investigate if WRN and RPA associate within cells (Aim a). For these experiments, we treated cells with MMS and HU, agents known to induce blockage of replication, and studied their effect in the association between WRN and RPA. Then, we analyzed if WRN-RPA association occurs thru a direct protein-protein interaction (Aim b).

Aim a. To investigate if DNA damaging treatments (known as blockers of replication) regulate the intracellular interaction between WRN and RPA. The first specific question we wanted to address in regard to WRN and RPA interaction was: Do WRN and RPA interact within cells? First, co-immunoprecipitation experiments were used to examine possible association between WRN and RPA. The second question we

wanted to address was: Do DNA damaging treatments (that block replication fork progression) alter the nature of WRN interaction with RPA? For these experiments, we chose the DNA alkylating agent methylmethanesulfonate (MMS), which methylates the DNA bases, producing 7-methylguanine, 3-methyladenine and O6-methylguanine [Wyatt et al., 2006]. Importantly, WRN-deficient cells are hypersensitive to MMS and it induces specific lesions (7-methylguanine and 3-methyladenine) that physically block replication fork elongation in cells [Imamura et al., 2002; Harrigan et al., 2006, Groth et al., 2010]. Additionally, we used the replication inhibitor HU, which depletes deoxyribonucleotide pools by inhibition of the enzyme ribonucleotide reductase, theoretically blocking progression of all replication forks [Skog et al., 1992, Pichierri et al., 2001]. As for MMS, WS cells are hypersensitive to HU, most likely by apoptosis of cells with stalled replication forks [Pichierri et al., 2001]. Thus, we used MMS and HU treatments to determine if DNA damaging treatments that block replication influence the interaction between WRN and RPA.

Aim b. To analyze whether WRN and RPA association occurs via a direct interaction. Although a co-immunoprecipitation experiment from cells might indicate an association between two proteins, it does not prove a direct interaction between them. Within a protein complex, proteins might interact directly or indirectly via one or more bridging molecules, such as other proteins or nucleic acids (DNA or RNA). Thus, we wanted to analyze whether WRN and RPA association occurs via a direct interaction. Therefore, we tested whether purified WRN and RPA could directly bind to each other by Far Western analysis.

METHODS

WRN-RPA Co-immunoprecipitation Experiments. A co-immunoprecipitation assay is used to target a known protein that is believed to be a member of a larger complex of proteins (using an antibody specific for the known protein) to pull the entire protein complex out of solution and thereby identify other members of the complex. For our IP experiments, we used an SV40-transformed fibroblast cell line, 1-O, that was obtained

from J. Christopher States, University of Louisville [States et al., 1993]. Methylmethanesulfonate (MMS), phenylmethylsulfonylfluoride (PMSF), HU, and protease inhibitor cocktail were purchased from Sigma-Aldrich, and cell culture media and reagents were purchased from Invitrogen. Cells were grown in MEM-α Glutamax medium supplemented with 10% FBS, 1% HEPES, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For DNA damaging treatments, cells were incubated in medium containing 1 mM MMS for 4 h or 2 mM HU for 10 h before harvesting. For co-immunoprecipitation experiments, cells were lysed by sonication in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA) supplemented with protease inhibitor cocktail, 1 mM PMSF and 10 units/ml of DNase I (New England Biolabs). After centrifugation at 21,000 × g for 12 min at 4°C, supernatants were isolated and their protein concentrations measured. Samples (800 μg of protein each) were pre-cleared with Protein G Plus/Protein A agarose beads (Calbiochem) and 1 μg of normal mouse IgG (Santa Cruz) for 30 min, then incubated with mouse monoclonal anti-RPA32 antibody (Calbiochem) for 15 h at 4°C. The samples were subsequently mixed with 30 μl of Protein G Plus/Protein A bead suspension at 4°C for 3 h. After collection by centrifugation and removal of supernatant, the beads were then washed three times with RIPA buffer supplemented with protease cocktail inhibitors, 1 mM PMSF and 200 μg/ml ethidium bromide. After the final wash, equal portions of RIPA and 2X SDS sample buffer were added to the beads and immunoprecipitated proteins were released by heating at 95°C for 5 min. Equal volumes of each sample were resolved by SDS-PAGE (6% or 12% for WRN or RPA, respectively). Proteins were transferred to PVDF membranes (Bio-Rad) by electroblotting. The membranes were blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris, pH 7.4, 140 mM NaCl and 0.1% Tween-20) and analyzed by Western analysis with rabbit anti-WRN (Santa Cruz) or mouse anti-RPA32 (Calbiochem) antibodies for 18 h at 4°C followed by chemiluminescent detection using ECL Plus (GE Healthcare).

Far Western assays. Far Western blot analysis is a method used to study protein-protein interactions using an immobilized protein on a membrane to capture potential binding

partners that are then detected on the membrane using specific antibodies. Purified RPA (60 and 120 ng or 0.5 and 1 pmol, respectively) or recombinant WRN (30, 60 and 90 ng or 180, 360, and 540 fmol, respectively) and corresponding concentrations of bovine serum albumin (BSA) were spotted directly onto nitrocellulose membranes. After allowing the applied samples to dry for 15 min at 4°C, membranes were blocked for 1 h at 4°C with 5% nonfat dry milk in TBST. The membranes were then incubated in 5 ml of TBST-5% milk solution (including 25 mM NaCl or 100 mM NaCl as indicated) containing purified WRN (400 ng = 2.4 pmol) or RPA (360 ng = 3 pmol) for 3 h at 4°C. After washing three times for 10 min each with TBST, membranes were subjected to immunodetection by 1) incubation for 1 h with anti-WRN or anti-RPA32 antibody, 2) incubation for 1 h with appropriate HRP-linked secondary antibodies, 3) chemiluminescent development using ECL Plus (GE Healthcare) and 4) visualization by autoradiography. Films were scanned to assess the level of protein binding, with comparison to RPA standards spotted separately on the same membranes.

RESULTS

Blockers of replication enhance intracellular association of WRN with RPA.

WRN has an important role in the maintenance of genomic integrity [Sidorova et al., 2008], and accumulating evidence suggests its involvement in a DNA metabolic pathway that allows the cell to responds to replication blockage to maintain chromosomal stability. RPA participates in DNA replication and one of its roles is to bind and protect ssDNA formed during unwinding of the parental duplex and as a result of blockage of replicative DNA synthesis [Wold et al., 1997; Raderschall et al., 1999]. Interestingly, previous studies have shown WRN and RPA co-localization in nuclear foci in response to treatment with HU, indicating that these proteins function at the same site and suggesting that they might interact within a complex at blocked replication forks [Constantinou et al., 2000]. If so, it should be possible to detect interactions between WRN and RPA within cells in response to DNA damaging treatments known to block replication. To explore this possibility, we used co-immunoprecipitation methods that we developed earlier (see chapter II) to identify proteins that are putative members within a complex. If

WRN and RPA are part of the same complex, by targeting one of them with an antibody it is possible to pull the intact protein complex out of the cell (and thereby identify the other one as a member of the same complex). For these experiments, we use human fibroblasts treated either for 10 h with or without HU, an agent that blocks DNA replication by exhausting deoxynucleotide pools within cells, or for 4 h with or without the alkylating agent MMS, that produces methylated bases that block DNA replication. Both agents alter the subnuclear localization profile of WRN [Wyatt et al., 2006, Karmakar et al., 2005]. Since WRN and RPA possess DNA binding activities [Orren et al., 1999; Brosh et al., 2002; Bochkareva et al., 2002], it was possible that association might occur indirectly through DNA. Therefore, we used two strategies to minimize the possibility that protein interactions were mediated through DNA bridging: 1) DNase I was employed during lysis and immunoprecipitation to thoroughly digest DNA from protein samples, and 2) ethidium bromide was included during washing of the immunoprecipitate to intercalate DNA strands and thereby destabilize potential protein-DNA interactions. A schematic representation of the protocol used is shown in Figure 4.1. Briefly, we used an antibody against the RPA32 subunit for immunoprecipitation and antibodies against WRN and RPA32 for immunodetection. Analysis of immunoprecipitates by immunoblotting with RPA antibody demonstrated that the level of RPA was equal in the immunoprecipitated fraction in HU- and MMS-treated and untreated cells (Figure 4.2A, lower panel), indicating that we immunoprecipitated equivalent amounts of RPA in each sample and that RPA expression or abundance not changed by HU or MMS treatment. Even in untreated cells, we were able to detect endogenous WRN co-immunoprecipitated along with RPA, demonstrating an association between WRN and RPA even in the absence of exogenous damage (Figure 4.2, upper panel, lanes 2 and 5). Interestingly, the amount of WRN precipitated with RPA is substantially higher in MMS-treated cells (Figure 4.2A, upper panel, lanes 2 vs. 3). Similar results were obtained with HU-treated cells (Figure 4.2A, lanes 5 vs. 6). Quantitation of data from multiple independent experiments indicates that MMS results in a 4.4 fold increase and HU results in a 2.0 fold increase in the levels of WRN interacting with RPA (Figure 4.2B). Thus, WRN and RPA are co-immunoprecipitated

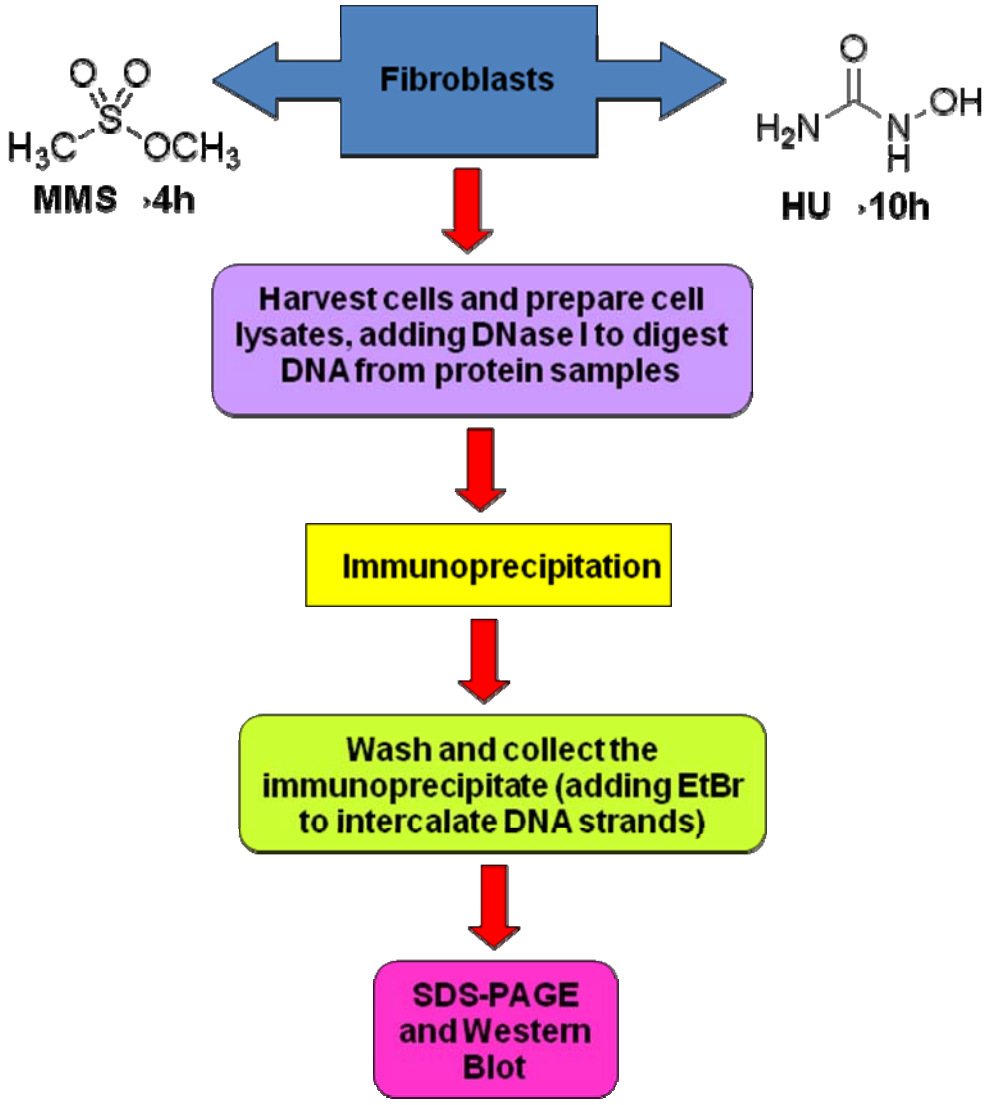

Figure 4.1 Schematic representation of the procedure used

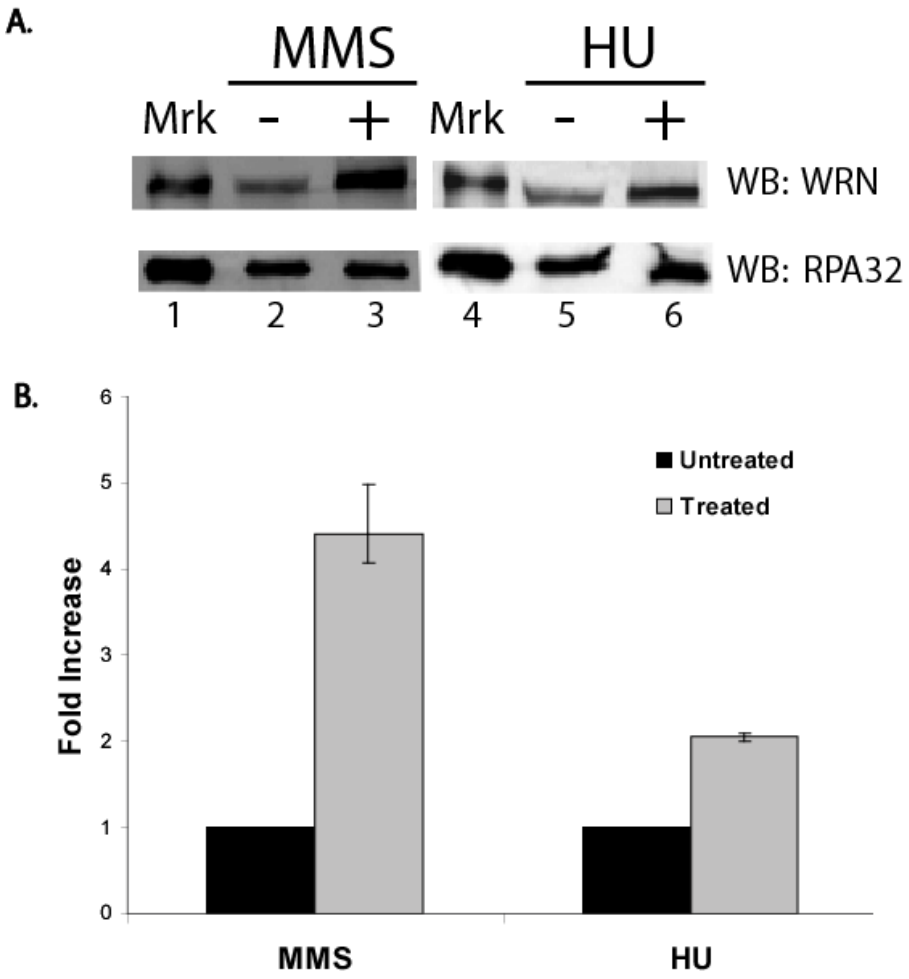

Figure 4.2 DNA damage enhances the co-immunoprecipitation of WRN with RPA. A) Cells incubated with or without MMS (1 mM) for 4 h or HU (2 mM) for 10 h were prepared for immunoprecipitation using anti-RPA32 subunit monoclonal antibody as described in Methods. Aliquots (40 µl) of the resuspended immunoprecipitated fractions from untreated (*lanes 2 and 5*), MMS-treated (*lane 3*), and HU-treated (*lane 6*) lysates were analyzed by SDS-PAGE (6%) and Western blotting using anti-WRN antibody and chemiluminescent detection (*upper panel*). In parallel, aliquots (2.5 µl) of these same immunoprecipitated fractions were analyzed by SDS-PAGE (12%) and Western blotting using anti-RPA32 antibody (*lower panel*). Purified WRN and RPA were loaded as protein markers (*Mkr*, *lanes 1 and 4*). **B)** Quantitative bar graph for WRN-RPA interaction under conditions described above (mean and S.D. for three independent experiments).

from cell lysates, suggesting that they are associated within the same complex *in vivo*. More importantly, this interaction is significantly increased following treatments known to block DNA replication.

WRN and RPA association occurs via a direct interaction. The experiments above show an association between WRN and RPA *in vivo.* However, proteins might interact directly or indirectly, via one or more linked proteins. Thus, we wanted to analyze whether WRN and RPA association occurs via a direct interaction. Thus, we tested whether purified WRN and RPA could bind to each other by a Far Western dot blotting method that is used to analyze specific protein-protein interactions. Briefly, several concentrations of one protein were immobilized on nitrocellulose membranes. The membranes were blocked with 5% milk solution to saturate any non-specific binding sites and then incubated in buffer containing the other protein of interest. As a control for non-specific binding, the same amounts of BSA were separately spotted onto the same membrane. Then, membranes were subjected to immunodetection by incubation with anti-WRN or anti-RPA32 antibodies to assess binding of the proteins. Figure 4.3A shows that when RPA was spotted onto the membrane, WRN binds to RPA in a manner dependent on the RPA concentration. Similar results were obtained when WRN was spotted onto the membrane. Specifically, RPA bound exclusively to WRN in amounts dependent on WRN concentration (Figure 4.3B). No non-specific binding of WRN or RPA to BSA was observed. Additionally, we tested the stability of WRN-RPA interaction. To this end, we analyzed the effect of increasing concentrations of NaCl on the WRN-RPA interaction. Interestingly, similar amounts of RPA bound to WRN at NaCl concentrations of 25 mM and (more physiologically relevant) 100 mM (Figure 4.3B). Therefore, we can conclude that WRN and RPA association occurs via a direct, salt tolerant interaction.

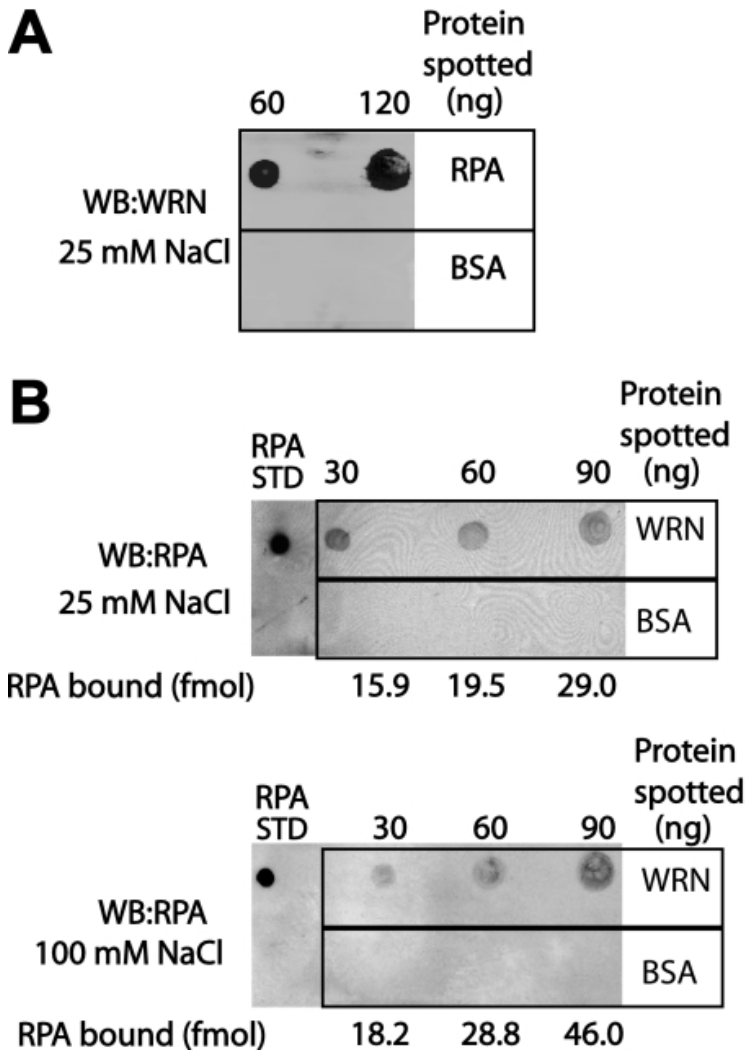

Figure 4.3 RPA and WRN directly interact with each other. For Far Western analysis, purified RPA (**A**), WRN-E84A (**B**), and BSA (both, as a control for non-specific binding) were immobilized on nitrocellulose membranes at the concentrations indicated above each panel. After blocking, membranes were then incubated in buffer containing 25 or 100 mM NaCl as indicated at left and either (**A**) WRN-E84A (400 ng = 2.4 pmol) or (**B**) RPA (360 ng = 3 pmol). As described in Methods, immunodetection and autoradiography were used to assess binding of the protein in solution to the immobilized protein on the membrane. The amounts of RPA bound to immobilize WRN (indicated below corresponding spots in *B*) were determined by comparison to an RPA standard (50 fmol) spotted on each membrane.

DISCUSSION

To safeguard genome stability, cells rely on an accurate response to replication stress. The observed enhanced genomic instability and diminished replicative lifespan of WS cells highlight the importance of WRN in DNA metabolism and maintenance of genomic stability. Several lines of evidence support the view that WRN might play a critical role in the response to replication stress, specifically in the response to stalled replication forks. In agreement with this notion, WRN co-localizes with replication factors, including RPA, in response to treatment with DNA damaging agents and the replication inhibitor HU [Constantinou et al., 2000]. Therefore, it should be possible to detect interactions between WRN and RPA *in vivo*, particularly in response to replication blocking agents.

In this study we clearly demonstrate, by co-immunoprecipitation experiments, an association between WRN and RPA *in vivo*. Importantly, we also demonstrated that WRN-RPA interaction significantly increased after MMS and HU, agents known to block replication. This is the first time that co-immunoprecipitation of endogenous WRN and RPA has been demonstrated, as well as its enhancement by genotoxins. These results indicate that, in response to replication blockage, WRN and RPA associate in the same protein complex *in vivo*. This binding appeared not to be mediated by DNA, because the interaction persists in the presence of ethidium bromide and DNase I and is substantially increased after MMS or HU treatment. Our results are consistent with studies demonstrating that WRN and RPA orthologs in *C.elegans* cooperate at blocked replication forks after HU, and that WRN is required for the efficient formation of RPA foci in response to DNA replication inhibition [Yan et al., 1998, Lee et al., 2010].

Although our results support the notion that WRN and RPA play a part in the same protein complex, they do not prove a direct physical association between them. Hence, we tested whether recombinant WRN and purified RPA could bind to each other by Far Western dot blotting analysis. The results demonstrated that WRN bound RPA directly. Notably, the interaction is stable even at physiologically relevant salt concentrations. Taken together, our data strongly suggest that co-immunoprecipitation of WRN and RPA

is mediated by a direct interaction between them. Importantly, our results are consistent with previous reports showing a direct interaction between purified WRN and RPA. According to these studies, the interaction between WRN and RPA occurs thru the N-terminal region of WRN (aa239-499) and the RPA70 subunit (aa100-300) [Brosh et al., 1999; Doherty et al., 2005; Shen et al., 2003].

It is believed that during DNA replication different DNA lesions can pose a serious threat to genome integrity by interfering with fork stability. Those lesions will influence how the blocked replication fork structure will be processed to restart DNA replication. Proposed models for resolution of replication blockage suggest that one of the first steps involves fork regression to generate a Holliday junction or "chicken foot" intermediate [Haber et al., 1999; Cox et al., 2002]. The unwinding and annealing activities of WRN suggest that WRN would be suitable to perform fork regression. In response to HU treatments, WRN is subject to translocation from nucleolus to nuclear foci (considered as sites of blocked replication) where it co-localizes with RPA [Constantinou et al., 2000]. This evidence suggests that WRN and RPA might interact at blocked replication forks, facilitating WRN function in proper resolution of replication blockage. Consistent with this notion, several studies have shown that: 1) RPA enhances WRN unwinding strength [Shen et al., 1998a; Brosh et al., 1999], 2) WRN has a preferential action on complex DNA structures, including replication forks [Huang et al., 2000; Brosh et al., 2001; Opresko et al., 2004; Orren et al., 2002], 3) WRN specifically regresses replication forks [Machwe et al., 2006], and 4) WRN displaces RPA from a replication fork substrate independently of its catalytic activity and subsequently remodels/regresses this replication fork [Machwe et al., 2011]. Based on these findings, we propose a possible scenario as to how WRN and RPA might function cooperatively to resolution of stalled replication forks. Upon replication blockage, RPA binds to resulting single-stranded DNA gaps and helps to recruit WRN to blocked forks via a direct interaction between these two proteins. WRN regresses the fork and displaces RPA in the process. Subsequently, the regressed fork is subsequently processed and replication is restarted. Since we previously demonstrated that WRN is modified, specifically acetylated, in response to DNA damage and/or replication blockage, and WRN acetylation [Blander et

al., 2002] and phosphorylation [Pichierri et al., 2003] correlates with its relocalization from nucleolus to nuclear foci where it colocalizes with RPA, it is possible that a modified form of WRN might mediate or enhance the WRN-RPA interaction.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The primary objective of our studies was to further clarify the role of WRN in response to agents that damage DNA and/or block replication, with a particular emphasis on the relationship of acetylation of WRN with its function in DNA metabolism. In 2002, Blander et al. made the observation that WRN can be acetylated *in vivo* by the acetyltransferase p300 [Blander et al., 2002], and that this modification correlates with its translocation from the nucleolus into nucleoplasmic foci. These findings suggested that WRN acetylation could play an important role in regulation of WRN function, a concept that has been reinforced by the experiments presented here. It is also noteworthy that WRN is also subject to phosphorylation and sumoylation.

In Chapter II, we addressed the dynamics of endogenous WRN acetylation and its relationship to DNA damage. To this end, we measured the levels of WRN acetylation after DNA damaging agents and replication blocking agents. The results of those experiments revealed that WRN is detectably acetylated under normal conditions. However, certain DNA damaging agents (MMS, MMC, cisplatin, but not UV) and inhibitors of DNA replication such as HU significantly increase WRN acetylation. Our results are consistent with a study showing WRN acetylation after treatments with DNA damaging agents, such as MMC [Karmakar et al., 2005]. Importantly, our results using inhibitors of standard repair pathways to suppress DNA damage removal extend those observations in two ways. First, our results confirm that WRN acetylation itself is, at least in part, related directly to DNA damage and not some non-specific effect of the treatment on some other aspect of cellular metabolism or physiology. Second, since we demonstrated that inhibition of repair of certain lesions induced further amplification of WRN acetylation, our results revealed that it is not the induction of damage but its persistence that optimally enhances WRN acetylation. Thus, our results suggest that WRN acetylation is a downstream effect of certain types of DNA lesions on DNA metabolism. Since all of the agents that enhance WRN acetylation in our studies are

known to either directly cause or produce lesions that result in blockage of replication, the results of our experiments strongly suggest that WRN might be acetylated in response to stalled or blocked replication. In agreement with this notion, we showed that increases in the levels of acetylated WRN correlate with inhibition of DNA synthesis and an increase in the percentage of S-phase cells.

Since the acetylation state of proteins in humans is regulated by acetylases and deacetylases, we used deacetylase inhibitors to investigate the role of deacetylases in regulation of WRN acetylation. The results of our experiments revealed that WRN is actively deacetylated *in vivo* and that, even in untreated cells, WRN acetylation state is determined by an equilibrium between acetylation and deacetylation. These results are in agreement with studies showing WRN regulation in response to deacetylase inhibitors, since it has been shown that WRN translocates from the nucleolus to nucleoplasmic foci in response to TSA treatment [Blander et al., 2002]. Since our previous studies demonstrated enhancement of WRN acetylation after DNA damaging and replication blocking treatments, a possible explanation for our results is that blockage of replication by DNA damage or other circumstances shifts the equilibrium towards acetylation. Previous studies have shown that WRN function is influenced by the sirtuin family of deacetylase enzymes, including SIRT1 [Li et al., 2008 and 2010; Law et al., 2009; Kahyo et al., 2008; Michishita et al., 2008]. Notably, we demonstrated for the first time that members of the classical HDAC family of deacetylase enzymes appear to play a role in WRN deacetylation. In fact, our results suggest that HDACs may play the predominant role, since inhibition of HDACs (using TSA) resulted in higher levels of WRN acetylation when compared to inhibition of sirtuins (using nicotinamide).

To investigate the effect of WRN acetylation on its biochemical function, unmodified, acetylated, and deacetylated WRN were compared as to their helicase, exonuclease and fork regression activities on different DNA structures. Our data demonstrated that acetylated WRN has dramatically less helicase and exonuclease activities than unmodified WRN on simple DNA substrates. Conversely, deacetylation of WRN restored the helicase and exonuclease activities to near normal levels. These

experiments initially suggested that WRN acetylation might regulate WRN enzymatic function in a negative manner. However, since WRN has preference for complex DNA structures, such as DNA replication and recombination intermediates, we explored WRN action on those kinds of substrates. When a more rigorous examination was performed on complex DNA substrates (such as replication forks), the effect of acetylation on WRN fork regression activity and exonuclease activity on those substrates was substantially less pronounced. Collectively, our results suggest that WRN acetylation regulates WRN specificity by reducing its preference for non-physiological DNA substrates. This supports the idea that WRN acetylation is likely to be critical for its contribution to genomic integrity surveillance. Since our data indicate that WRN acetylation might increase WRN specificity by reducing preference for non-physiological substrates, further DNA binding assays are required to explore if the binding of acetylated WRN to the different DNA structures correlates with the results obtained in our helicase, exonuclease, and fork regression assays. Based on our results, we speculate that WRN acetylation (as well as other post-translational modifications) may serve as a rapid way to respond to cellular stress and restart replication. By this reasoning, acetylation may be involved in reducing WRN affinity for nucleolar DNA while maintaining affinity for replicative DNA structures associated with replication foci. Consistent with this notion, previous studies have shown that WRN acetylation correlates with its translocation to nuclear foci in response to DNA damage and replication blockers [Blander et al., 2002; Karmakar et al., 2005]. WRN also assists the pairing of complementary DNA strands. Thus, unmodified and acetylated WRN should be compared in future experiments to investigate the effect of acetylation on WRN annealing activity.

In our experiments, we measured overall acetylation of WRN. Interestingly, our results suggest that the status of WRN acetylation might be regulated by different conditions, such as DNA damage and disruption of the equilibrium between acetylases and deacetylases. However, it is unclear if independent acetylation events occur on different lysine sites upon different conditions and/or DNA damaging treatments. A recent study identified putative acetylated lysine residues (K366, K887, K1117, K1127, K1389, and K1413) in WRN using ectopically expressed WRN and the acetylases p300

and CBP [Li et al., 2010]. However, it would be informative and more physiologically relevant to identify WRN acetylation sites on endogenous WRN, using mass spectrometry analysis. Such an analysis would explore if, indeed, all or a subset of these residues are the actual sites subject to acetylation in the native environment of cells--i.e, with endogenous levels of WRN and the relevant acetylases. The same technique can be used after treatment of cells with the different DNA damaging agents, or the deacetylase inhibitors, to explore differential patterns of acetylation between the different treatments. The immunoprecipitation protocols developed for these studies should be useful for isolating modified and unmodified WRN for these types of experiments. If the different treatments target different residues, those studies should provide valuable insight in regard to functional differences between the various acetylation events and if there is any hierarchy in the acetylation of different lysine residues. In addition, site-directed mutagenesis can be used to mutate the putatively acetylated lysines to investigate which modifications affect WRN biochemical activities. Specifically, those mutants can be used in DNA binding assays as well as helicase and exonuclease assays, to determine if specific mutations (or combinations of mutations) affect the interaction of lysine residues with the negatively charged backbone of DNA. To directly determine the effect of WRN acetylation on its cellular function, WRN cDNAs containing lysine to arginine (a conservative basic substitution that cannot be acetylated) point mutations at putative acetylation sites can be constructed and transfected into WRN-deficient cells to compare the ability of wild-type and acetylation-deficient WRN to complement the hypersensitivity of WS cells to HU and DNA damaging agents as well as other phenotypes of WRN-deficient cells.

The data described herein suggest that WRN is regulated in response to DNA damage and replication blocking agents. To confirm this relationship and investigate it further, we analyzed if DNA damage regulates the nature of WRN's potential interactions in response to treatments that block replication. Specifically, we investigated the potential interaction between WRN and the single-stranded DNA binding protein complex, RPA, and whether it is altered in response to treatment with HU or MMS. Our experiments revealed that WRN and RPA associate with each other even under normal physiological

conditions *in vivo*. Interestingly, treatments that block replication fork progression induce an increased association between these two factors. As we also demonstrated that purified WRN and RPA bind to one another, this association *in vivo* is also likely to be direct. The results of our experiments confirm previous findings showing interaction between purified WRN and RPA [Brosh et al., 1999; Doherty et al., 2005; Shen et al., 2003]. Importantly, our results substantially extended those findings by showing endogenous WRN-RPA interaction in the native environment of cells, as well as its enhancement by treatments that block replication. It is known that RPA is hyperphosphorylated in response to treatment with DNA damaging agents and replication blocking agents [Oakley et al., 2001; Shao et al., 1999; Anantha et al., 2009]. Thus, future co-immunoprecipitation experiments could be performed to analyze if WRN specifically or preferentially interacts with the hyperphosphorylated form of RPA. On the other hand, the addition of acetyl groups to lysine residues on WRN after DNA damage may create a new surface for protein association(s). Thus, it will be interesting to explore as well whether and how acetylated forms of WRN interact with RPA and if this is influenced by treatment with agents that block replication.

In summary, through this study, we provided some interesting and revealing results that support the likely importance of WRN regulation in response to DNA damage and blockage of replication. Collectively, our results suggest that WRN acetylation is a downstream effect of DNA damage on DNA metabolism which influences WRN function, including altering its specificity by reducing preference for non-physiological substrates. Thus, the results of the studies presented in this work have identified unique mechanisms by which WRN is regulated in response to DNA damage. Importantly, our results are consistent with evidence pointing to a role for WRN in response to blocked replication, including its recruitment to sites of ongoing and/or blocked replication upon DNA damage and its ability to regress model replication forks. Based on our findings and the existing evidence, we propose a possible scenario for how loss of WRN function (possibly caused by problems with regulation of WRN acetylation) in DNA metabolism might result in the cancer and premature aging phenotypes typically associated with WS (Figure 5.1). An inability to properly resolve blocked replication forks, due to loss or

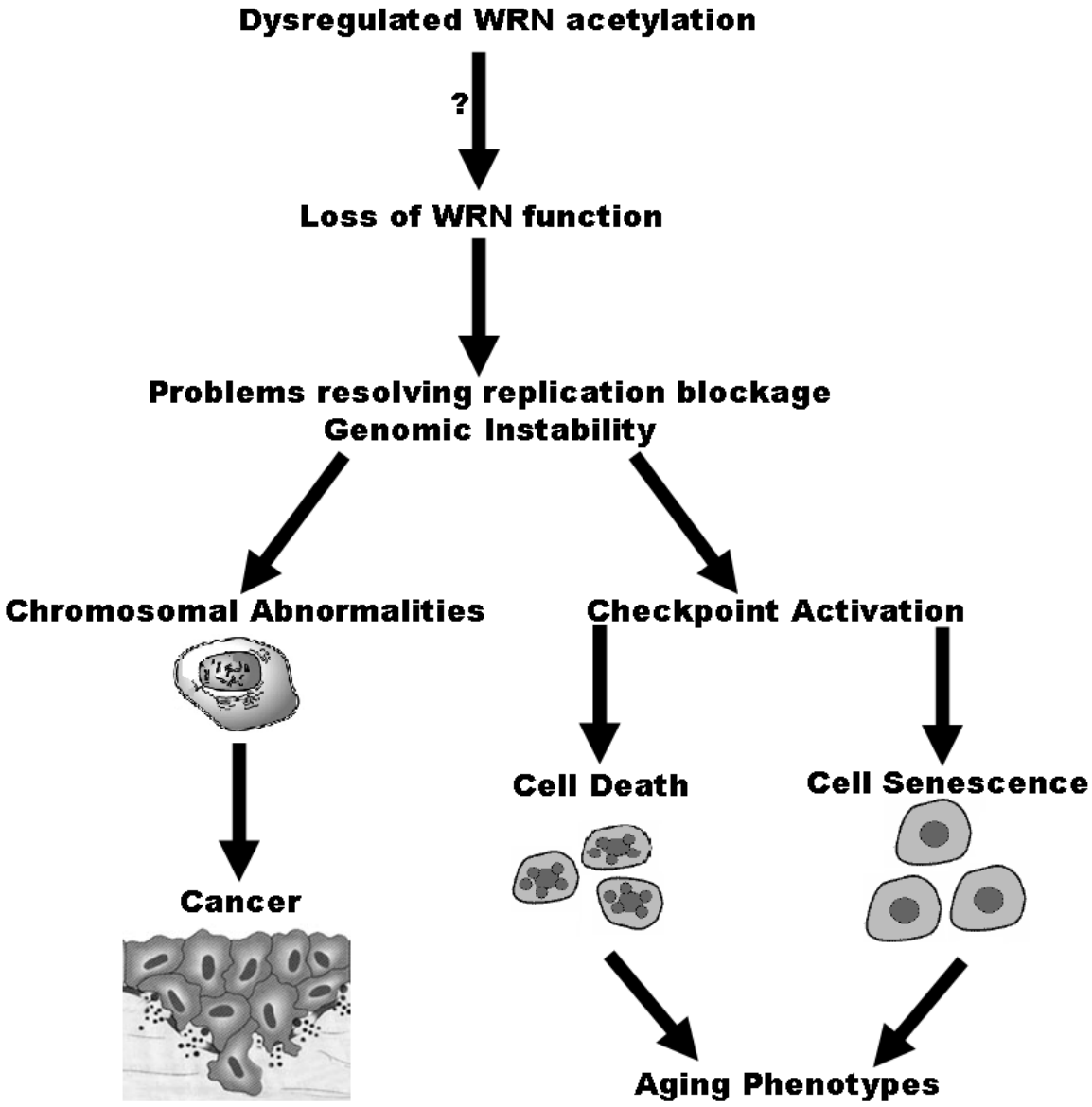

Figure 5.1 Schematic representation of the downstream effects caused by loss of WRN function. Loss of WRN function (possibly caused by problems with regulation of WRN acetylation) in DNA metabolism might result in the inability of cells to properly resolve replication blockage and thereby increase genomic instability. These DNA metabolic problems might cause chromosomal abnormalities and activate checkpoints that, in turn, might trigger cell death and cellular senescence; processes that result in the accelerated development of age-related phenotypes and elevated cancer frequency associated with WS.

dysfunction of WRN, may further delay or cause collapse of DNA replication, triggering overall genomic instability and activation of checkpoint pathways that, in turn, might trigger cellular senescence and cell death; two mechanisms that are thought to drive certain aging processes. Genomic instability may also contribute to chromosomal aberrations, potentially driving carcinogenesis. This model would be consistent with the increased cancer incidence and aging phenotypes in WS. However, further research is needed to determine if, indeed, acetylation of WRN is essential to minimize large-scale chromosomal aberrations and prevent the development of age-related phenotypes. To investigate the role of acetylated WRN in the pathogenesis of aging, WRN cDNAs containing lysine mutations at putative acetylation sites can be constructed and transfected into WRN-deficient cells to compare the ability of wild-type and acetylation-deficient WRN to complement the premature cellular senescence of WS cells as well as other age-related phenotypes of WRN-deficient cells. The same strategy can be used to measure genomic instability, using techniques such as the micronucleus assay, to determine whether and to what extent loss of WRN acetylation might contribute to the development of cancer. Alternatively, loss of function or dysregulation of acetylases and deacetylases that disrupt the equilibrium in the process of WRN acetylation might also be used as a possible strategy. Thus, creating a cell culture model in which the balance between acetylation and deacetylation is altered by knocking down and/or overexpressing specific deacetylases and acetylases involved in the WRN acetylation process, such as p300, may help to determine the importance of acetylated WRN in processes such as cellular senescence and carcinogenesis. To examine whether WRN acetylation was required for suppression of age-related and cancer phenotypes at the physiological level, specialized transgenic mouse models would have to be developed in which mutated WRN genes, incapable of acetylation as described above but otherwise catalytically unaffected, are re-introduced into mice lacking functional WRN and telomerase; notably, telomerase deficiency is necessary to reveal WRN-related cancer and aging phenotypes in mice (Chang et al. 2004). Although many questions regarding WRN acetylation and its function remain to be answered, our findings provided mechanistic insights into the role of WRN in DNA metabolism. Importantly, our work revealed that WRN acetylation is a fascinating area of research to keep our attention well into the future.

APPENDIX

LIST OF ABBREVIATIONS

ATM: Ataxia telangiectasia mutated protein kinase

ATP: Adenosine triphosphate

ATR: ATM and Rad3-related protein kinase

BER: Base Excision Repair

BLM: Bloom Syndrome Protein

BN: Binucleated

bp: Base pairs

BrdU: Bromodeoxyuridine

CBP: CREB-binding protein

cDNA: complementary DNA

DNA: Deoxyribonucleic acid

DNA-PK: DNA-dependent protein kinase

EDTA: Ethylenediaminetetraacetic acid

FEN-1: Flap Endonuclease I

fmol: femtomol

HAT: Histone acetyltransferase

HDACs: Histone Deacetylases

HEK293: Human Embryonic Kidney 293 cells

HRDC: Helicase and RNase D C-terminal

HU: Hydroxyurea

IP: Immunoprecipitation

MGMT: Methylguanine methyltransferase

MMC: Mitomycin C

MMS: Methylmethanesulfonate

MN: Micronucleus

MRK: Marker

NAD^+: Nicotinamide adenine dinucleotide

N.D.: Non-detectable

NER: Nucleotide Excision Repair

NER+: Nucleotide Excision Repair Proficient

NER-: Nucleotide Excision Repair Deficient

NIC: Nicotinamide

O^6-BG: O^6-benzylguanine

PARP: Poly (ADP-ribose) polymerase

PCNA: Proliferating Cell Nuclear Antigen

pmol: picomol

PMSF: Phenylmethylsulfonylfluoride

PVDF: Polyvinylidene Fluoride

RPA: Replication Protein A

RECQ: RecQ helicase

RQC: RecQ-conserved

S.D.: Standard Deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIRT1: Sirtuin 1

ssDNA: Single-stranded DNA

SUMO: Small Ubiquitin-like Modifier

TOPO I: Topoisomerase I

TSA: Trichostatin A

UV: Ultraviolet

WB: Western Blot

WRN: Werner Protein

WS: Werner Syndrome

REFERENCES

Ammazzalorso F, Pirzio LM, Bignami M, Franchitto A, Pichierri P. ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. EMBO J. 2010 Sep 15;29(18):3156-69.

Appella E, Anderson CW . Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem. 2001 May;268(10):2764-72.

Bachrati CZ, Hickson ID. RecQ helicases: suppressors of tumorigenesis and premature aging.Biochem J. 2003 Sep 15;374(Pt 3):577-606.

Bachrati CZ, Hickson ID. RecQ helicases: guardian angels of the DNA replication fork. Chromosoma. 2008 Jun;117(3):219-33.

Bieliauskas AV, Pflum MK. Isoform-selective histone deacetylase inhibitors. Chem Soc Rev. 2008 Jul;37(7):1402-13.

Binz SK, Sheehan AM, Wold MS. Replication protein A phosphorylation and the cellular response to DNA damage. DNA Repair (Amst). 2004 Aug-Sep;3(8-9):1015-24.

Blander G, Zalle N, Daniely Y, Taplick J, Gray MD, Oren M.DNA damage-induced translocation of the Werner helicase is regulated by acetylation. J Biol Chem. 2002 Dec 27;277(52):50934-40.

Bochkareva E, Korolev S, Lees-Miller SP, Bochkarev A. Structure of the RPA trimerization core and its role in the multistep DNA-binding mechanism of RPA. EMBO J. 2002 Apr 2;21(7):1855-63.

Boysen G, Pachkowski BF, Nakamura J, Swenberg JA. The formation and biological significance of N7-guanine adducts. Mutat Res. 2009 Aug 2;678(2):76-94.

Brosh RM Jr, Orren DK, Nehlin JO, Ravn PH, Kenny MK, Machwe A, Bohr VA. Functional and physical interaction between WRN helicase and human replication protein A. J Biol Chem. 1999 Jun 25;274(26):18341-50.

Brosh RM Jr, Majumdar A, Desai S, Hickson ID, Bohr VA, Seidman MM. Unwinding of a DNA triple helix by the Werner and Bloom syndrome helicases. J Biol Chem. 2001a Feb 2;276(5):3024-30.

Brosh RM Jr, von Kobbe C, Sommers JA, Karmakar P, Opresko PL, Piotrowski J, Dianova I, Dianov GL, Bohr VA. Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity. EMBO J. 2001b Oct 15;20(20):5791-801.

Brosh RM Jr, Waheed J, Sommers JA. Biochemical characterization of the DNA substrate specificity of Werner syndrome helicase. J Biol Chem. 2002a Jun 28;277(26):23236-45.

Brosh RM Jr, Driscoll HC, Dianov GL, Sommers JA. Biochemical characterization of the WRN-FEN-1 functional interaction. Biochemistry. 2002b Oct 8;41(40):12204-16.

Cen Y. Sirtuins inhibitors: the approach to affinity and selectivity. Biochim Biophys Acta. 2010 Aug;1804(8):1635-44.

Cepeda V, Fuertes MA, Castilla J, Alonso C, Quevedo C, Soto M, Pérez JM. Poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors in cancer chemotherapy. Recent Pat Anticancer Drug Discov. 2006 Jan;1(1):39-53.

Chang S, Multani AS, Cabrera NG, Naylor ML, Laud P, Lombard D, Pathak S, Guarente L, DePinho RA. Essential role of limiting telomeres in the pathogenesis of Werner syndrome. Nat Genet. 2004 Aug;36(8):877-82.

Chao C, Wu Z, Mazur SJ, Borges H, Rossi M, Lin T, Wang JY, Anderson CW, Appella E, Xu Y. Acetylation of mouse p53 at lysine 317 negatively regulates p53 apoptotic activities after DNA damage. Mol Cell Biol. 2006 Sep;26(18):6859-69.

Chen L, Oshima J.Werner Syndrome. J Biomed Biotechnol. 2002;2(2):46-54.

Cheng WH, von Kobbe C, Opresko PL, Fields KM, Ren J, Kufe D, Bohr VA.Werner syndrome protein phosphorylation by abl tyrosine kinase regulates its activity and distribution. Mol Cell Biol. 2003 Sep;23(18):6385-95.

Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science. 2009 Aug 14;325(5942):834-40.

Close P, Creppe C, Gillard M, Ladang A, Chapelle JP, Nguyen L, Chariot A. The emerging role of lysine acetylation of non-nuclear proteins. Cell Mol Life Sci. 2010 Apr;67(8):1255-64.

Cloutier JF, Castonguay A, O'Connor TR, Drouin R. Alkylating agent and chromatin structure determine sequence context-dependent formation of alkylpurines. J Mol Biol. 2001 Feb 16;306(2):169-88.

Compton SA, Tolun G, Kamath-Loeb AS, Loeb LA, Griffith JD. The Werner syndrome protein binds replication fork and holliday junction DNAs as an oligomer. J Biol Chem. 2008 Sep 5;283(36):24478-83.

Constantinou A, Tarsounas M, Karow JK, Brosh RM, Bohr VA, Hickson ID, West SC. Werner's syndrome protein (WRN) migrates Holliday junctions and co-localizes with RPA upon replication arrest. EMBO Rep. 2000 Jul;1(1):80-4.

Cox MM. The nonmutagenic repair of broken replication forks via recombination. Mutat Res. 2002 Dec 29;510(1-2):107-20.

Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. J Cell Physiol. 2000 Jul;184(1):1-16.

Dart DA, Adams KE, Akerman I, Lakin ND. Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. J Biol Chem. 2004 Apr 16;279(16):16433-40.

Din S, Brill SJ, Fairman MP, Stillman B. Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. Genes Dev. 1990 Jun;4(6):968-77.

Doherty KM, Sommers JA, Gray MD, Lee JW, von Kobbe C, Thoma NH, Kureekattil RP, Kenny MK, Brosh RM Jr. Physical and functional mapping of the replication protein a interaction domain of the werner and bloom syndrome helicases. J Biol Chem. 2005 Aug 19;280(33):29494-505.

Dolan ME, Moschel RC, Pegg AE. Depletion of mammalian O6-alkylguanine-DNA alkyltransferase activity by O6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. Proc Natl Acad Sci U S A. 1990 Jul;87(14):5368-72.

Dolan ME, Pegg AE. O6-benzylguanine and its role in chemotherapy. Clin Cancer Res. 1997 Jun;3(6):837-47.

Dusre L, Covey JM, Collins C, Sinha BK. DNA damage, cytotoxicity and free radical formation by mitomycin C in human cells. Chem Biol Interact. 1989;71(1):63-78.

Dutta A, Stillman B. cdc2 family kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. EMBO J. 1992 Jun;11(6):2189-99.

Federico M, Bagella L. Histone deacetylase inhibitors in the treatment of hematological malignancies and solid tumors. J Biomed Biotechnol. 2011;2011:475641.

Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2(5):1084-104.

Fenech M. The micronucleus assay determination of chromosomal level DNA damage. Methods Mol Biol. 2008;410:185-216.

Gebhart E, Bauer R, Raub U, Schinzel M, Ruprecht KW, Jonas JB. Spontaneous and induced chromosomal instability in Werner syndrome. Hum Genet. 1988 Oct;80(2):135-9.

Gerson SL. Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol. 2002 May 1;20(9):2388-99.

Golub EI, Gupta RC, Haaf T, Wold MS, Radding CM. Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. Nucleic Acids Res. 1998 Dec 1;26(23):5388-93.

Goto M, Rubenstein M, Weber J, Woods K, Drayna D. Genetic linkage of Werner's syndrome to five markers on chromosome 8. Nature. 1992 Feb 20;355(6362):735-8.

Goto M. Hierarchical deterioration of body systems in Werner's syndrome: implications for normal ageing. Mech Ageing Dev. 1997 Dec;98(3):239-54.

Gray MD, Shen JC, Kamath-Loeb AS, Blank A, Sopher BL, Martin GM, Oshima J, Loeb LA. The Werner syndrome protein is a DNA helicase. Nat Genet. 1997 Sep;17(1):100-3.

Groth P, Ausländer S, Majumder MM, Schultz N, Johansson F, Petermann E, Helleday T. Methylated DNA causes a physical block to replication forks independently of damage

signalling, O(6)-methylguanine or DNA single-strand breaks and results in DNA damage. J Mol Biol. 2010 Sep 10;402(1):70-82.

Haber JE. DNA recombination: the replication connection. Trends Biochem Sci. 1999 Jul;24(7):271-5.

Hanaoka F, Yamada M, Takeuchi F, Goto M, Miyamoto T, Hori T. Autoradiographic studies of DNA replication in Werner's syndrome cells. Adv Exp Med Biol. 1985;190:439-57.

Harrigan JA, Wilson DM 3rd, Prasad R, Opresko PL, Beck G, May A, Wilson SH, Bohr VA. The Werner syndrome protein operates in base excision repair and cooperates with DNA polymerase beta. Nucleic Acids Res. 2006 Jan 30;34(2):745-54.

Honma M, Tadokoro S, Sakamoto H, Tanabe H, Sugimoto M, Furuichi Y, Satoh T, Sofuni T, Goto M, Hayashi M. Chromosomal instability in B-lymphoblasotoid cell lines from Werner and Bloom syndrome patients. Mutat Res. 2002 Sep 26;520(1-2):15-24.

Horton JK, Stefanick DF, Naron JM, Kedar PS, Wilson SH. Poly(ADP-ribose) polymerase activity prevents signaling pathways for cell cycle arrest after DNA methylating agent exposure. J Biol Chem. 2005 Apr 22;280(16):15773-85.

Hu JS, Feng H, Zeng W, Lin GX, Xi XG. Solution structure of a multifunctional DNA- and protein-binding motif of human Werner syndrome protein. Proc Natl Acad Sci U S A. 2005 Dec 20;102(51):18379-84.

Huang S, Li B, Gray MD, Oshima J, Mian IS, Campisi J. The premature ageing syndrome protein, WRN, is a 3'-->5' exonuclease. Nat Genet. 1998 Oct;20(2):114-6.

Huang S, Beresten S, Li B, Oshima J, Ellis NA, Campisi J. Characterization of the human and mouse WRN 3'-->5' exonuclease. Nucleic Acids Res. 2000 Jun 15;28(12):2396-405.

Huang S, Lee L, Hanson NB, Lenaerts C, Hoehn H, Poot M, Rubin CD, Chen DF, Yang CC, Juch H, Dorn T, Spiegel R, Oral EA, Abid M, Battisti C, Lucci-Cordisco E, Neri G, Steed EH, Kidd A, Isley W, Showalter D, Vittone JL, Konstantinow A, Ring J, Meyer P, Wenger SL, von Herbay A, Wollina U, Schuelke M, Huizenga CR, Leistritz DF, Martin GM, Mian IS, Oshima J. The spectrum of WRN mutations in Werner syndrome patients. Hum Mutat. 2006 Jun;27(6):558-67.

Huen MS, Chen J. The DNA damage response pathways: at the crossroad of protein modifications.Cell Res. 2008 Jan;18(1):8-16.

Iftode C, Daniely Y, Borowiec JA. Replication protein A (RPA): the eukaryotic SSB. Crit Rev Biochem Mol Biol. 1999;34(3):141-80.

Imamura O, Fujita K, Itoh C, Takeda S, Furuichi Y, Matsumoto T. Werner and Bloom helicases are involved in DNA repair in a complementary fashion. Oncogene. 2002 Jan 31;21(6):954-63.

Jackson SP. Sensing and repairing DNA double-strand breaks. Carcinogenesis. 2002 May;23(5):687-96

Jamieson ER, Jacobson MP, Barnes CM, Chow CS, Lippard SJ. Structural and kinetic studies of a cisplatin-modified DNA icosamer binding to HMG1 domain B. J Biol Chem. 1999 Apr 30;274(18):12346-54.

Johnson RE, Yu SL, Prakash S, Prakash L. A role for yeast and human translesion synthesis DNA polymerases in promoting replication through 3-methyl adenine. Mol Cell Biol. 2007 Oct;27(20):7198-205.

Johnson JE, Cao K, Ryvkin P, Wang LS, Johnson FB. Altered gene expression in the Werner and Bloom syndromes is associated with sequences having G-quadruplex forming potential. Nucleic Acids Res. 2010 Mar;38(4):1114-22.

Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov. 2002 Apr;1(4):287-99.

Jung Y, Lippard SJ. Direct cellular responses to platinum-induced DNA damage. Chem Rev. 2007 May;107(5):1387-407.

Kahyo T, Mostoslavsky R, Goto M, Setou M. Sirtuin-mediated deacetylation pathway stabilizesWerner syndrome protein. FEBS Lett. 2008 Jul 23;582(17):2479-83.

Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. DNA Repair (Amst). 2007 Aug 1;6(8):1079-99.

Kamath-Loeb AS, Shen JC, Loeb LA, Fry M. Werner syndrome protein. II. Characterization of the integral 3' --> 5' DNA exonuclease. J Biol Chem. 1998 Dec 18;273(51):34145-50.

Kamath-Loeb AS, Johansson E, Burgers PM, Loeb LA. Functional interaction between the Werner Syndrome protein and DNA polymerase delta. Proc Natl Acad Sci U S A. 2000 Apr 25;97(9):4603-8.

Kamath-Loeb AS, Loeb LA, Johansson E, Burgers PM, Fry M. Interactions between the Werner syndrome helicase and DNA polymerase delta specifically facilitate copying of tetraplex and hairpin structures of the d(CGG)n trinucleotide repeat sequence. J Biol Chem. 2001 May 11;276(19):16439-46.

Karmakar P, Piotrowski J, Brosh RM Jr, Sommers JA, Miller SP, Cheng WH, Snowden CM, Ramsden DA, Bohr VA.Werner protein is a target of DNA-dependent protein kinase in vivo and in vitro, and its catalytic activities are regulated by phosphorylation. J Biol Chem. 2002 May 24;277(21):18291-302.

Karmakar P, Bohr VA. Cellular dynamics and modulation of WRN protein is DNA damage specific. Mech Ageing Dev. 2005 Nov;126(11):1146-58.

Kawabe Y, Seki M, Seki T, Wang WS, Imamura O, Furuichi Y, Saitoh H, Enomoto T. Covalent modification of the Werner's syndrome gene product with the ubiquitin-related protein, SUMO-1. J Biol Chem. 2000a Jul 14;275(28):20963-6.

Kawabe T, Tsuyama N, Kitao S, Nishikawa K, Shimamoto A, Shiratori M, Matsumoto T, Anno K, Sato T, Mitsui Y, Seki M, Enomoto T, Goto M, Ellis NA, Ide T, Furuichi Y, Sugimoto M. Differential regulation of human RecQ family helicases in cell transformation and cell cycle. Oncogene. 2000b Sep 28;19(41):4764-72.

Kusumoto R, Muftuoglu M, Bohr VA.The role of WRN in DNA repair is affected by post-translational modifications. Mech Ageing Dev. 2007 Jan;128(1):50-7.

Laine JP, Opresko PL, Indig FE, Harrigan JA, von Kobbe C, Bohr VA. Werner protein stimulates topoisomerase I DNA relaxation activity. Cancer Res. 2003 Nov 1;63(21):7136-46.

Law IK, Liu L, Xu A, Lam KS, Vanhoutte PM, Che CM, Leung PT, Wang Y. Identification and characterization of proteins interacting with SIRT1 and SIRT3: implications in the anti-aging and metabolic effects of sirtuins. Proteomics. 2009 May;9(9):2444-56.

Lawley PD, Orr DJ, Jarman M. Isolation and identification of products from alkylation of nucleic acids: ethyl- and isopropyl-purines. Biochem J. 1975 Jan;145(1):73-84.

Lebel M, Spillare EA, Harris CC, Leder P. The Werner syndrome gene product co-purifies with the DNA replication complex and interacts with PCNA and topoisomerase I. J Biol Chem. 1999 Dec 31;274(53):37795-9.

Lee JW, Kusumoto R, Doherty KM, Lin GX, Zeng W, Cheng WH, von Kobbe C, Brosh RM Jr, Hu JS, Bohr VA. Modulation of Werner syndrome protein function by a single mutation in the conserved RecQ domain. J Biol Chem. 2005 Nov 25;280(47):39627-36

Lee SJ, Gartner A, Hyun M, Ahn B, Koo HS. The Caenorhabditis elegans Werner syndrome protein functions upstream of ATR and ATM in response to DNA replication inhibition and double-strand DNA breaks. PLoS Genet. 2010 Jan;6(1):e1000801.

Li K, Casta A, Wang R, Lozada E, Fan W, Kane S, Ge Q, Gu W, Orren D, Luo J. Regulation of WRN protein cellular localization and enzymatic activities by SIRT1-mediated deacetylation. J Biol Chem. 2008 Mar 21;283(12):7590-8.

Li K, Wang R, Lozada E, Fan W, Orren DK, Luo J. Acetylation of WRN protein regulates its stability by inhibiting ubiquitination. PLoS One. 2010 Apr 23;5(4):e10341.

Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993 Apr 22;362(6422):709-15

Liu JS, Kuo SR, Melendy T. Comparison of checkpoint responses triggered by DNA polymerase inhibition versus DNA damaging agents. Mutat Res. 2003 Nov 27;532(1-2):215-26.

Liu L, Scolnick DM, Trievel RC, Zhang HB, Marmorstein R, Halazonetis TD, Berger SL. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. Mol Cell Biol. 1999 Feb;19(2):1202-9.

Liu Z, Macias MJ, Bottomley MJ, Stier G, Linge JP, Nilges M, Bork P, Sattler M. The three-dimensional structure of the HRDC domain and implications for the Werner and Bloom syndrome proteins. Structure. 1999 Dec 15;7(12):1557-66.

Lord CJ, Ashworth A. Targeted therapy for cancer using PARP inhibitors. Curr Opin Pharmacol. 2008 Aug;8(4):363-9.

Machwe A, Xiao L, Theodore S, Orren DK. DNase I footprinting and enhanced exonuclease function of the bipartite Werner syndrome protein (WRN) bound to partially melted duplex DNA. J Biol Chem. 2002 Feb 8;277(6):4492-504.

Machwe A, Xiao L, Groden J, Matson SW, Orren DK. RecQ family members combine strand pairing and unwinding activities to catalyze strand exchange. J Biol Chem. 2005 Jun 17;280(24):23397-407.

Machwe A, Xiao L, Orren DK. Length-dependent degradation of single-stranded 3' ends by the Werner syndrome protein (WRN): implications for spatial orientation and coordinated 3' to 5' movement of its ATPase/helicase and exonuclease domains. BMC Mol Biol. 2006a Feb 17;7:6.

Machwe A, Xiao L, Groden J, Orren DK. The Werner and Bloom syndrome proteins catalyze regression of a model replication fork. Biochemistry. 2006b Nov 28;45(47):13939-46.

Machwe A, Xiao L, Lloyd RG, Bolt E, Orren DK. Replication fork regression in vitro by the Werner syndrome protein (WRN): holliday junction formation, the effect of leading arm structure and a potential role for WRN exonuclease activity. Nucleic Acids Res. 2007;35(17):5729-47.

Machwe A, Lozada E, Wold MS, Li GM, Orren DK. Molecular cooperation between the Werner syndrome protein and replication protein A in relation to replication fork blockage. J Biol Chem. 2011 Feb 4;286(5):3497-508.

Markovitsi D, Gustavsson T, Banyasz A. Absorption of UV radiation by DNA: spatial and temporal features. Mutat Res. 2010 Apr-Jun;704(1-3):21-8.

Marks PA. The clinical development of histone deacetylase inhibitors as targeted anticancer drugs. Expert Opin Investig Drugs. 2010 Sep;19(9):1049-66.

Martin GM, Sprague CA, Epstein CJ. Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype. Lab Invest. 1970 Jul;23(1):86-92.

Martin GM.Genetic syndromes in man with potential relevance to the pathobiology of aging. Birth Defects Orig Artic Ser. 1978;14(1):5-39.

Martin GM, Oshima J. Lessons from human progeroid syndromes. Nature. 2000 Nov 9;408(6809):263-6.

Mian IS. Comparative sequence analysis of ribonucleases HII, III, II PH and D. Nucleic Acids Res. 1997 Aug 15;25(16):3187-95.

Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TL, Barrett JC, Chang HY, Bohr VA, Ried T, Gozani O, Chua KF. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature. 2008 Mar 27;452(7186):492-6.

Mitsis PG. Phosphorylation and localization of replication protein A during oogenesis and early embryogenesis of Drosophila melanogaster. Dev Biol. 1995 Aug;170(2):445-56.

Mohaghegh P, Karow JK, Brosh Jr RM Jr, Bohr VA, Hickson ID. The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. Nucleic Acids Res. 2001 Jul 1;29(13):2843-9.

Moradei O, Maroun CR, Paquin I, Vaisburg A. Histone deacetylase inhibitors: latest developments, trends and prospects. Curr Med Chem Anticancer Agents. 2005 Sep;5(5):529-60.

Muftuoglu M, Kusumoto R, Speina E, Beck G, Cheng WH, Bohr VA. Acetylation regulates WRN catalytic activities and affects base excision DNA repair. PLoS One. 2008 Apr 9;3(4):e1918.

Murakami J, Lee YJ, Kokeguchi S, Tsujigiwa H, Asaumi J, Nagatsuka H, Fukui K, Kuroda M, Tanaka N, Matsubara N. Depletion of O6-methylguanine-DNA methyltransferase by O6-benzylguanine enhances 5-FU cytotoxicity in colon and oral cancer cell lines. Oncol Rep. 2007 Jun;17(6):1461-7.

Nakura J, Miki T, Ye L, Mitsuda N, Zhao Y, Kihara K, Yu CE, Oshima J, Fukuchi KI, Wijsman EM, Schellenberg GD, Martin GM, Murano S, Hashimoto K, Fujiwara Y, Ogihara T. Narrowing the position of the Werner syndrome locus by homozygosity analysis-extension of homozygosity analysis. Genomics, 1996 Aug 15;36(1):130-41

Naryzhny SN, Lee H. The post-translational modifications of proliferating cell nuclear antigen: acetylation, not phosphorylation, plays an important role in the regulation of its function. J Biol Chem. 2004 May 7;279(19):20194-9.

Nelms BE, Maser RS, MacKay JF, Lagally MG, Petrini JH. In situ visualization of DNA double-strand break repair in human fibroblasts. Science. 1998 Apr 24;280(5363):590-2.

Oakley GG, Loberg LI, Yao J, Risinger MA, Yunker RL, Zernik-Kobak M, Khanna KK, Lavin MF, Carty MP, Dixon K. UV-induced hyperphosphorylation of replication protein

a depends on DNA replication and expression of ATM protein. Mol Biol Cell. 2001 May;12(5):1199-213.

O'Connor TR, Boiteux S, Laval J. Ring-opened 7-methylguanine residues in DNA are a block to in vitro DNA synthesis. Nucleic Acids Res. 1988 Jul 11;16(13):5879-94.

Ogburn CE, Oshima J, Poot M, Chen R, Hunt KE, Gollahon KA, Rabinovitch PS, Martin GM. An apoptosis-inducing genotoxin differentiates heterozygotic carriers for Werner helicase mutations from wild-type and homozygous mutants. Hum Genet. 1997 Dec;101(2):121-5.

Opresko PL, Cheng WH, von Kobbe C, Harrigan JA, Bohr VA. Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. Carcinogenesis. 2003 May;24(5):791-802.

Opresko PL, Otterlei M, Graakjaer J, Bruheim P, Dawut L, Kolvraa S, May A, Seidman MM, Bohr VA. The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. Mol Cell. 2004 Jun 18;14(6):763-74.

Orren DK, Brosh RM Jr, Nehlin JO, Machwe A, Gray MD, Bohr VA. Enzymatic and DNA binding properties of purified WRN protein: high affinity binding to single-stranded DNA but not to DNA damage induced by 4NQO. Nucleic Acids Res. 1999 Sep 1;27(17):3557-66.

Orren DK, Theodore S, Machwe A. The Werner syndrome helicase/exonuclease (WRN) disrupts and degrades D-loops in vitro. Biochemistry. 2002 Nov 19;41(46):13483-8.

Orren DK. Werner syndrome: molecular insights into the relationships between defective DNA metabolism, genomic instability, cancer and aging. Front Biosci. 2006 Sep 1;11:2657-71.

Petrucco S. Sensing DNA damage by PARP-like fingers. Nucleic Acids Res. 2003 Dec 1;31(23):6689-99.

Pichierri P, Franchitto A, Mosesso P, Palitti F. Werner's syndrome protein is required for correct recovery after replication arrest and DNA damage induced in S-phase of cell cycle. Mol Biol Cell. 2001 Aug;12(8):2412-21.

Pichierri P, Rosselli F, Franchitto A.Werner's syndrome protein is phosphorylated in an ATR/ATM-dependent manner following replication arrest and DNA damage induced during the S phase of the cell cycle. Oncogene. 2003 Mar 13;22(10):1491-500.

Plummer ER. Inhibition of poly(ADP-ribose) polymerase in cancer. Curr Opin Pharmacol. 2006 Aug;6(4):364-8.

Polo SE, Jackson SP. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev. 2011 Mar 1;25(5):409-33.

Poot M, Hoehn H, Rünger TM, Martin GM. Impaired S-phase transit of Werner syndrome cells expressed in lymphoblastoid cell lines. Exp Cell Res. 1992 Oct;202(2):267-73.

Poot M, Gollahon KA, Rabinovitch PS. Werner syndrome lymphoblastoid cells are sensitive to camptothecin-induced apoptosis in S-phase. Hum Genet. 1999 Jan;104(1):10-4.

Poot M, Yom JS, Whang SH, Kato JT, Gollahon KA, Rabinovitch PS. Werner syndrome cells are sensitive to DNA cross-linking drugs. FASEB J. 2001 May;15(7):1224-6.

Raderschall E, Golub EI, Haaf T. Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):1921-6.

Rodríguez-López AM, Jackson DA, Iborra F, Cox LS. Asymmetry of DNA replication fork progression in Werner's syndrome. Aging Cell. 2002 Oct;1(1):30-9. 49

Rodríguez-López AM, Jackson DA, Nehlin JO, Iborra F, Warren AV, Cox LS. Characterisation of the interaction between WRN, the helicase/exonuclease defective in progeroid Werner's syndrome, and an essential replication factor, PCNA. Mech Ageing Dev. 2003 Feb;124(2):167-74.

Sakamoto S, Nishikawa K, Heo SJ, Goto M, Furuichi Y, Shimamoto A. Werner helicase relocates into nuclear foci in response to DNA damaging agents and co-localizes with RPA and Rad51. Genes Cells. 2001 May;6(5):421-30.

Salk D, Bryant E, Hoehn H, Johnston P, Martin GM. Growth characteristics of Werner syndrome cells in vitro. Adv Exp Med Biol. 1985;190:305-11.

Sancar A. Excision repair in mammalian cells. J Biol Chem. 1995 Jul 7;270(27):15915-8.

Schellenberg GD, Martin GM, Wijsman EM, Nakura J, Miki T, Ogihara T. Homozygosity mapping and Werner's syndrome. Lancet. 1992 Apr 18;339(8799):1002.

Shao RG, Cao CX, Zhang H, Kohn KW, Wold MS, Pommier Y. Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. EMBO J. 1999 Mar 1;18(5):1397-406.

Sharma S, Otterlei M, Sommers JA, Driscoll HC, Dianov GL, Kao HI, Bambara RA, Brosh RM Jr. WRN helicase and FEN-1 form a complex upon replication arrest and

together process branchmigrating DNA structures associated with the replication fork. Mol Biol Cell. 2004 Feb;15(2):734-50.

Sharma S, Sommers JA, Gary RK, Friedrich-Heineken E, Hübscher U, Brosh RM Jr.The interaction site of Flap Endonuclease-1 with WRN helicase suggests a coordination of WRN and PCNA. Nucleic Acids Res. 2005 Dec 2;33(21):6769-81.

Shen JC, Gray MD, Oshima J, Loeb LA. Characterization of Werner syndrome protein DNA helicase activity: directionality, substrate dependence and stimulation by replication protein A. Nucleic Acids Res. 1998a Jun 15;26(12):2879-85

Shen JC, Gray MD, Oshima J, Kamath-Loeb AS, Fry M, Loeb LA. Werner syndrome protein. I. DNA helicase and dna exonuclease reside on the same polypeptide. J Biol Chem. 1998b Dec 18;273(51):34139-44.

Shen JC, Loeb LA. Werner syndrome exonuclease catalyzes structure-dependent degradation of DNA. Nucleic Acids Res. 2000 Sep 1;28(17):3260-8.

Shen JC, Lao Y, Kamath-Loeb A, Wold MS, Loeb LA. The N-terminal domain of the large subunit of human replication protein A binds to Werner syndrome protein and stimulates helicase activity. Mech Ageing Dev. 2003 Aug-Sep;124(8-9):921-30

Sidorova JM. Roles of the Werner syndrome RecQ helicase in DNA replication. DNA Repair (Amst). 2008 Nov 1;7(11):1776-86.

Sinha RP, Häder DP. UV-induced DNA damage and repair: a review. Photochem Photobiol Sci. 2002 Apr;1(4):225-36.

Skog S, Heiden T, Eriksson S, Wallström B, Tribukait B. Hydroxyurea-induced cell death in human T lymphoma cells as related to imbalance in DNA/protein cycle and deoxyribonucleotide pools and DNA strand breaks. Anticancer Drugs. 1992 Aug;3(4):379-86.

Sommers JA, Sharma S, Doherty KM, Karmakar P, Yang Q, Kenny MK, Harris CC, Brosh RM Jr. p53 modulates RPA-dependent and RPA-independent WRN helicase activity. Cancer Res. 2005 Feb 15;65(4):1223-33.

States JC, Quan T, Hines RN, Novak RF, Runge-Morris M. Expression of human cytochrome P450 1A1 in DNA repair deficient and proficient human fibroblasts stably transformed with an inducible expression vector. Carcinogenesis. 1993 Aug;14(8):1643-9.

Suzuki N, Shimamoto A, Imamura O, Kuromitsu J, Kitao S, Goto M, Furuichi Y. DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. Nucleic Acids Res. 1997 Aug 1;25(15):2973-8.

Szekely AM, Chen YH, Zhang C, Oshima J, Weissman SM. Werner protein recruits DNA polymerase delta to the nucleolus. Proc Natl Acad Sci U S A. 2000 Oct 10;97(21):11365-70.

Takeuchi F, Hanaoka F, Goto M, Akaoka I, Hori T, Yamada M, Miyamoto T.Altered frequency of initiation sites of DNA replication in Werner's syndrome cells. Hum Genet. 1982a;60(4):365-8.

Takeuchi F, Hanaoka F, Goto M, Yamada M, Miyamoto T. Prolongation of S phase and whole cell cycle in Werner's syndrome fibroblasts. Exp Gerontol. 1982b;17(6):473-80.

Tang Y, Zhao W, Chen Y, Zhao Y, Gu W. Acetylation is indispensable for p53 activation. Cell. 2008 May 16;133(4):612-26.

Thomas W, Rubenstein M, Goto M, Drayna D. A genetic analysis of the Werner syndrome region on human chromosome 8p. Genomics. 1993 Jun;16(3):685-90.

von Kobbe C, Thomä NH, Czyzewski BK, Pavletich NP, Bohr VA. Werner syndrome protein contains three structure-specific DNA binding domains. J Biol Chem. 2003 Dec 26;278(52):52997-3006.

Walkinshaw DR, Yang XJ. Histone deacetylase inhibitors as novel anticancer therapeutics. Curr Oncol. 2008 Oct;15(5):237-43.

Wang X, Peterson CA, Zheng H, Nairn RS, Legerski RJ, Li L. Involvement of nucleotide excision repair in a recombination-independent and error-prone pathway of DNA interstrand cross-link repair. Mol Cell Biol. 2001 Feb;21(3):713-20.

Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: What are the cancer relevant targets? Cancer Lett. 2009 May 8;277(1):8-21.

Wold MS. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Biochem. 1997;66:61-92.

Woods YL, Xirodimas DP, Prescott AR, Sparks A, Lane DP, Saville MK. p14 Arf promotes small ubiquitin-like modifier conjugation of Werners helicase. J Biol Chem. 2004 Nov 26;279(48):50157-66.

Wyatt MD, Allan JM, Lau AY, Ellenberger TE, Samson LD.3-methyladenine DNA glycosylases: structure, function, and biological importance. Bioessays. 1999 Aug;21(8):668-76.

Wyatt MD, Pittman DL. Methylating agents and DNA repair responses: Methylated bases and sources of strand breaks. Chem Res Toxicol. 2006 Dec;19(12):1580-94.

Yan H, Chen CY, Kobayashi R, Newport J. Replication focus-forming activity 1 and the Werner syndrome gene product. Nat Genet. 1998 Aug;19(4):375-8.

Yang XJ. Lysine acetylation and the bromodomain: a new partnership for signaling. Bioessays. 2004 Oct;26(10):1076-87.

Yannone SM, Roy S, Chan DW, Murphy MB, Huang S, Campisi J, Chen DJ.Werner syndrome protein is regulated and phosphorylated by DNA-dependent protein kinase. J Biol Chem. 2001 Oct 12;276(41):38242-8.

Ye L, Nakura J, Mitsuda N, Fujioka Y, Kamino K, Ohta T, Jinno Y, Niikawa N, Miki T, Ogihara T. Genetic association between chromosome 8 microsatellite (MS8-134) and Werner syndrome (WRN): chromosome microdissection and homozygosity mapping. Genomics. 1995 Aug 10;28(3):566-9.

Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, Martin GM, Mulligan J, Schellenberg GD. Positional cloning of the Werner's syndrome gene. Science. 1996 Apr 12;272(5259):258-62.

Yu CE, Oshima J, Wijsman EM, Nakura J, Miki T, Piussan C, Matthews S, Fu YH, Mulligan J, Martin GM, Schellenberg GD. Mutations in the consensus helicase domains of the Werner syndrome gene. Werner's Syndrome Collaborative Group. Am J Hum Genet. 1997 Feb;60(2):330-41.

Zhang F, Bartels MJ, Pottenger LH, Gollapudi BB, Schisler MR. Simultaneous quantitation of 7-methyl- and O6-methylguanine adducts in DNA by liquid chromatography-positive electrospray tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Apr 3;833(2):141-8.

Zhao Y, Lu S, Wu L, Chai G, Wang H, Chen Y, Sun J, Yu Y, Zhou W, Zheng Q, Wu M, Otterson GA, Zhu WG. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21 (Waf1/Cip1). Mol Cell Biol. 2006 Apr;26(7):2782-90.

Zheng H, Wang X, Warren AJ, Legerski RJ, Nairn RS, Hamilton JW, Li L. Nucleotide excision repair- and polymerase eta-mediated error-prone removal of mitomycin C interstrand cross-links. Mol Cell Biol. 2003 Jan;23(2):754-61.

Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003a Jun 6;300(5625):1542-8.

Zou L, Liu D, Elledge SJ. Replication protein A-mediated recruitment and activation of Rad17 complexes. Proc Natl Acad Sci U S A. 2003b Nov 25;100(24):13827-32.

VITA

ENERLYN MELIZA LOZADA SANTIAGO

Place of birth: San Juan, Puerto Rico
Date of birth: July 17, 1979

EDUCATION

2004 - Present **Ph.D. Student**, University of Kentucky, Lexington, Kentucky, US

1997 - 2002 **B.S. General Sciences**, University of Puerto Rico, Río Piedras, Puerto Rico

PROFESSIONAL POSITIONS

2005 - Present **Graduate research assistant.** University of Kentucky, Kentucky, US

2002 – 2004 **Biochemical Technician I.** Abbott Diagnostic Laboratories, Barceloneta, Puerto Rico

2001 **Research assistant.** University of Puerto Rico, Río Piedras, Puerto Rico

2000 **Research assistant.** University of Kentucky, Kentucky, US

2000 **Research assistant.** University of Puerto Rico, Río Piedras, Puerto Rico

AWARDS AND HONORS

2010	Excellence in Science Award
2009	Excellence in Science Award
2008	Excellence in Science Award
2006	Lyman T. Johnson Academic Year Fellowship Award
2005	Lyman T. Johnson Academic Year Fellowship Award
2004	Lyman T. Johnson Academic Year Fellowship Award
1997	Academic Excellent Award
1994	Academic Excellent Award

PROFESSIONAL MEMBERSHIPS

2009 - Present	Delta Epsilon Iota Academic Honor Society
2008 - Present	American Association for the Advancement of Science
2008 - Present	National Scholars Honor Society
2000 - 2002	American Chemical Society

CONFERENCES

2010	Annual Midwest DNA Repair Symposium, Louisville, Kentucky, US
2007	Annual Clinical and Translational Science Spring Conference, Lexington, Kentucky, US
2007	Annual Midwest DNA Repair Symposium, Columbus, Ohio, US

POSTERS PRESENTATIONS

Machwe A, Edwards D, **Lozada E**, Orren DK. Roles of RecQ helicases in supressing genomic instability, cancer and aging. Integrated Biomedical Sciences Orientation, Lexington, Kentucky, US. **2010**

Lozada E, Luo J, Orren DK. Genotoxin-induced WRN modification and effects on its DNA metabolic function. Midwest DNA Repair Symposium, Louisville, Kentucky, US. **2009**

Keirsey J, **Lozada E** , Kavecansky J, Bhattacharyya S, Russell B, Grierson P, Sandy A, Orren DK, Groden J. Novel phosphorylation sites in BLM regulate it's helicase activity and localization after HU treatment. Molecular and Clinical mechanisms in Bloom's Syndrome and Related Disorders Meeting, Chicago, IL, US. **2008**

Lozada E, Luo J, Orren DK. Acetylation of Werner Syndrome protein and the effects on its enzymatic function. Annual Clinical and Translational Science Spring Conference, Lexington, Kentucky, US. **2007**

SEMINARS

Genotoxin-induced acetylation of the Werner Syndrome Protein and effect on its DNA metabolic function. University of Kentucky, Lexington, Kentucky, US. **2011**

Regulation of Werner Syndrome Protein by acetylation. University of Kentucky, Lexington, Kentucky, US. **2009**

PUBLICATIONS

Lozada E, Luo J, Orren DK. Genotoxin-induced acetylation of the Werner Syndrome Protein (WRN) and effect on its DNA metabolic function. Manuscript in preparation, **2011**.

Machwe A, **Lozada E**, Wold MS, Li GM, Orren DK. Molecular cooperation between the Werner syndrome protein and replication protein A in relation to replication fork blockage. J Biol Chem. **2011** Feb 4;286(5):3497-508.

Li K, Wang R, **Lozada E**, Fan W, Orren DK, Luo J. Acetylation of WRN protein regulates its stability by inhibiting ubiquitination. PLoS One. **2010** Apr 23;5(4):e10341.

Li K, Casta A, Wang R, **Lozada E**, Fan W, Kane S, Ge Q, Gu W, Orren D, Luo J. Regulation of WRN protein cellular localization and enzymatic activities by SIRT1-mediated deacetylation. J Biol Chem. **2008** Mar 21;283(12):7590-8.

Machwe A, **Lozada E**, Xiao L, Orren DK. Competition between the DNA unwinding and strand pairing activities of the Werner and Bloom syndrome proteins. BMC Mol Biol. **2006** Jan 13;7:1.

ACADEMIC TRAINING/PROFESSIONAL EDUCATION

1999 **School of Leaders**, Diocese of Arecibo, Arecibo, PR

1999 **Organ and tissue donation and transplantation Training,** Foundation of the Kidney and Catastrophic Illnesses Gloria Menéndez, Manatí, PR

2002 **OSHA Safety Training,** Abbott Diagnostic Laboratories, Barceloneta, PR

2003 **Massage Therapist**. Millenium Beauty Academiy, Hatillo, PR

2004 **Advanced Radiation Training**, Radiation Safety Department, University of Kentucky, Lexington, Kentucky, US

2010 **Graduate Certification in Business Administration**, University of Kentucky, Lexington, Kentucky, US