Kiran Rangaraj participated in the CCI program during the summer of 2008, where he studied protein-protein interactions involved in multiple DNA repair mechanisms in the Life Sciences Division at Lawrence Berkeley National Laboratory (LBNL). Kiran presented the work he completed there at the inaugural 2008 DOE Science and Energy Research Challenge. Kiran is currently continuing his studies at LBNL while pursuing an undergraduate degree in biochemistry at the University of California–Berkeley. He plans to enter into a graduate school program following his graduation. Kiran grew up in Milpitas, CA, and he enjoys drawing, weight training, and mountain biking.

Priscilla K. Cooper is a Senior Scientist in the Life Sciences Division of Lawrence Berkeley National Laboratory, where she heads the Department of Cancer and DNA Damage Responses. She is also a member of the University of California–San Francisco Comprehensive Cancer Center. She has a career-long interest in the biochemical and molecular basis for cellular processing of damaged DNA and the genetic control of DNA repair processes for mutation avoidance and maintenance of genomic integrity. She got a B.S. at the University of Rochester, and she earned her Ph.D. in Biological Sciences at Stanford University, investigating mechanisms of nucleotide excision repair in E. coli *and its SOS regulation. Following postdoctoral training in the Stanford University School of Medicine, she was recruited to LBNL in the early 1980s. Her current research focus is on the relationship between repair of oxidative DNA damage and normal DNA transactions in human cells, with emphasis on connections between genetic defects in repair of endogenous damage, cancer, and aging. Her laboratory studies protein biochemistry, molecular biology, cell biology, and structural biology to carry out structure-function analyses of critical multi-protein complexes functioning in DNA repair. She is the current National President of the Environmental Mutagenesis Society. She has a strong commitment to promoting basic research among undergraduates and fostering diversity in science.*

Kelly S. Trego is a postdoctoral fellow at Lawrence Berkeley National Laboratory in the laboratory of Dr. Priscilla K. Cooper. She received her Ph.D. in Molecular Genetics from The Ohio State University in 2003 in the laboratory of Dr. Deborah S. Parris. As a graduate student, she studied the interactions between proteins required for Herpes simplex virus DNA replication. Following graduation, Dr. Trego was a postdoctoral fellow in the laboratory of Dr. John J. Turchi, studying the biochemistry of mammalian DNA repair proteins and the mechanism by which proteins recognize and repair cisplatin-DNA adducts. Her work on DNA repair proteins is now focused on the biochemistry and cell biology of the novel interaction between the DNA repair protein, XPG, and the human RecQ helicase, WRN. Dr. Trego is a National Institutes of Health Ruth Kirschstein National Service Award Fellow, and current president of the Lawrence Berkeley National Laboratory Life Science Division Postdoctoral Society.

Where Multifunctional DNA Repair Proteins Meet: Mapping the Interaction Domains between XPG and WRN

Kiran Rangaraj, Priscilla K. Cooper and Kelly S. Trego

ABSTRACT

The rapid recognition and repair of DNA damage is essential for the maintenance of genomic integrity and cellular survival. Multiple complex and interconnected DNA damage responses exist within cells to preserve the human genome, and these repair pathways are carried out by a specific interplay of protein-protein interactions. Thus a failure in the coordination of these processes, perhaps brought about by a breakdown in any one multifunctional repair protein, can lead to genomic instability, developmental and immunological abnormalities, cancer and premature aging. This study demonstrates a novel interaction between two such repair proteins, Xeroderma pigmentosum group G protein (XPG) and Werner syndrome helicase (WRN), that are both highly pleiotropic and associated with inherited genetic disorders when mutated. XPG is a structure-specific endonuclease required for the repair of UV-damaged DNA by nucleotide excision repair (NER), and mutations in XPG result in the diseases Xeroderma pigmentosum (XP) and Cockayne syndrome (CS). A loss of XPG incision activity results in XP, whereas a loss of non-enzymatic function(s) of XPG causes CS. WRN is a multifunctional protein involved in double-strand break repair (DSBR), and consists of 3'–5' DNA-dependent helicase, 3'–5' exonuclease, and single-strand DNA annealing activities. Nonfunctional WRN protein leads to Werner syndrome, a premature aging disorder with increased cancer incidence. Far Western analysis was used to map the interacting domains between XPG and WRN by denaturing gel electrophoresis, which separated purified full length and recombinant XPG and WRN deletion constructs, based primarily upon the length of each polypeptide. Specific interacting domains were visualized when probed with the secondary protein of interest which was then detected by traditional Western analysis using the antibody of the secondary protein. The interaction between XPG and WRN was mapped to the C-terminal region of XPG as well as the C-terminal region of WRN. The physical interaction between XPG and WRN links NER, (made evident by the disease XP) with DSBR, which imparts additional knowledge of the overlapping nature of these two proteins and the previously distinct DNA repair pathways they are associated with. Since genomic integrity is constantly threatened by both endogenous and exogenous (internal and external) damage, understanding the roles of these proteins in coordinating DNA repair processes with replication will significantly further understanding how defects instigate physiological consequences in response to various DNA damaging sources. This ultimately contributes to our understanding of cancer and premature aging.

Introduction

The human genome is constantly subjected to both endogenous and exogenous (internal and external) damage from cellular metabolism, environmental sources of ultraviolet (UV) and ionizing radiation, and mutagenic chemicals, which generate several kinds of lesions throughout cellular DNA. Natural by-products of oxidative phosphorylation in mitochondria in addition to other cellular processes of the cell generate reactive oxygen species, which are estimated to produce 50 000–100 000 oxidative DNA lesions per mammalian cell per day, each of which could potentially cause errors in DNA replication or transcription if not repaired [1]. Such lesions may elicit point mutations or chromosomal rearrangements, prompt single-strand (ssDNA) or double-strand (dsDNA) breaks in DNA during replication, or trigger a stress response via a signal transduction pathway, all known precursors to premature aging, cancer and a number of genetic diseases. The rapid recognition and repair of DNA damage is essential for the maintenance of genomic integrity and cellular survival [2]. Multiple DNA repair pathways have evolved within cells to provide a distinct but overlapping capacity to recognize and excise lesions, in coordination with other necessary cellular processes of DNA replication, transcription and recombination such that the human genome is preserved [1]. However, the coordination of DNA repair processes may be compromised by a breakdown in any one of the numerous multifunctional proteins involved in initiating and mediating communication between repair and replication pathways, ultimately compromising the ability to restore genomic integrity. Elucidating the precise biological roles of these proteins has become an important challenge since a number of diseases and conditions have been either directly linked or associated with defects in certain repair proteins. Much of cancer and aging research focuses on understanding the involvement of these proteins within necessary repair pathways, in order to identify the exact molecular mechanisms that trigger inherited defects in DNA repair.

Ultraviolet radiation is recognized as an important health risk to the human population as noted by an ongoing epidemic of skin cancer [3]. The biochemical pathway of nucleotide excision repair (NER) is the primary mechanism in mammalian cells to remove the bulky DNA helix-distorting lesions that are caused by UV light. Eukaryotic NER can be subdivided into two subpathways — global genome repair (GGR), which removes lesions from all regions of the genome, and transcription coupled repair (TCR), which involves accelerated removal of damage from actively transcribed strands of genes [4]. The importance of GGR and TCR is attested by the existence of dramatic inherited diseases such as Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD), and Cockayne syndrome (CS) [5].

Much research has focused on XP, which is an autosomal recessive genetic disorder where patients carry mutations that partially or totally impair the efficiency of NER processes [5]. XP patients lack essential repair pathways by which the cell can remove the vast majority of UV-induced DNA damage, thus clinical features of XP include extreme sun sensitivity with marked thickening of the skin, alterations in pigmentation, and a high incidence of skin malignancies at a young age. XP patients can be subdivided into seven complementation groups XP-A through XP-G, plus one variant form XP-V, depending on which gene is affected. Each complementation group XP-A to XP-G represents separate proteins that are involved in the first steps of the NER pathway (damage recognition and incision-excision) and interact together to form recently mapped interactions with other proteins. One such gene of the complementation group G encodes for the protein XPG, which is a vital multifunctional protein currently under focus at the Cooper Lab of the Life Sciences Division at Lawrence Berkeley National Laboratory (LBNL).

XPG protein is a junction-specific endonuclease that incises the damaged DNA strand 3′ to the lesion undergoing repair [6]. The enzyme cleaves model DNA bubble structures specifically near the junction of unpaired DNA with a duplex region. Structurally, XPG shares extensive sequence homology with another structure-specific endonuclease, flap endonuclease 1 (FEN-1), exclusively through the N and I domains that together form the nuclease active site (Figure 1A). The endonuclease activity is vital to the repair of UV-damaged DNA by NER, since point mutations within XPG inactivate this function and result in XP.

XPG is structurally unique in that the endonuclease domains are separated by a "recognition" (R) domain and a "carboxyl" (C) terminus which impart XPG with additional functional abilities. Truncation mutations in XPG are more severe, resulting in a combined disease of XP with the developmental disorder Cockayne syndrome (CS), in which patients display profound postnatal neurological and developmental dysfunction, premature aging, and severe wasting that becomes evident and lethal within the first few years of life (Figure 1B). The disease severity strongly suggests that non-enzymatic functions of XPG are essential to normal

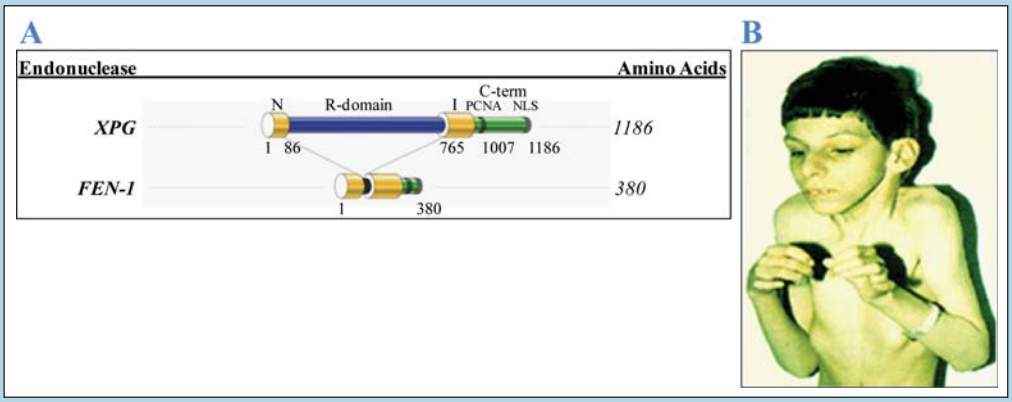

Figure 1. A) Schematic representation of the 1186-amino acid XPG protein and a comparative structure of another such endonuclease, FEN-1, where sequence homology is conserved between the catalytic sub-domains N and I of XPG and FEN-1. R- and C-terminus domains remain unique to XPG and impart XPG with additional functional capabilities. B) Cockayne syndrome sufferers have multiple systemic disorders due to a defect in the ability of cells to repair DNA that is being transcribed. Cockayne syndrome has been linked to abnormalities in XPG protein [7]. Photograph by D. Atherton. Reproduced from A. R. Lehmann, *Trends Bio-chem. Sci.*, vol. 20, pp. 402–405, 1995.

postnatal development. Scientists at the Cooper Lab have recently identified novel structural roles of the protein in both TCR and base excision repair (BER) of oxidative damage [8], [9], [10] (Cooper Lab, unpublished results). BER is a critical lesion-amending pathway involving the repair of oxidative DNA base modifications, deaminated or alkylated bases, apurinic/apyrimidic (AP) sites, and ssDNA breaks [11]. Recent studies have also suggested that XPG may have a novel replication-associated function and that it communicates with proteins known to be involved in signaling and processing of stalled or collapsed DNA replication forks and double strand break repair (DSBR), thereby extending its role in DNA damage response (Cooper Lab, unpublished results). Novel XPG binding partners were assessed using a combination approach of proteomic analysis and directed searching, and this assessment identified several proteins including Rad51, the Mre11-Rad50-Nbs1 (MRN) protein complex, as well as Werner (WRN) and Bloom (BLM) syndrome helicases, that are each functionally involved at stalled or collapsed replication forks.

One of the proteins identified was WRN, a 160 kDa (1 432-amino acid) member of the RecQ category of helicases, which are enzymes that separate the complementary strands of nucleic acid duplexes using the energy derived from adenosine triphosphate (ATP) hydrolysis [12]. RecQ helicases represent a highly conserved, five-member family that is required for the maintenance of genome integrity. This integrity is implicated by heritable cancer predisposition disorders that result from defects in any of three RecQ family members (BLM, WRN, and RecQ4) (Figure 2A) [13]. Patients lacking functional WRN protein have Werner syndrome (WS), an autosomal recessive disorder characterized at the cellular level by genomic instability. Clinical features of WS include numerous prematurely developed age-related pathologies, short stature, leg ulceration, soft-tissue calcification, and increased cancer incidence (Figure 2B). WRN has an unusual preference for unwinding DNA substrates with extensive secondary structure, such as Holliday Junctions (HJ), as well as bubbles, forks, and D-Loops, all functional intermediates in the homologous recombination repair (HRR) subpathway of DSBR. Unlike other RecQ helicases, WRN additionally possesses 3'–5' exonuclease activity [15], [16].

Recent data demonstrates that XPG strongly binds HJ substrates, and that the presence of XPG increases the binding of WRN to the DNA (K. Trego, Cooper Lab, unpublished results). The functional significance of this physical interaction was examined by testing the WRN helicase activity on a dsDNA substrate with overhanging ends. Interestingly, XPG strongly stimulated the helicase activity of WRN which, coupled with all of the other findings, suggests that the physical interaction of XPG with WRN may be important in coordinating a possible replication-associated function of XPG at stalled or collapsed replication forks with ds breaks (K. Trego, Cooper Lab, unpublished results).

Cooper Lab studies have demonstrated that XPG physically interacts with WRN protein in vivo. Therefore, this study used Far Western analysis to further examine the direct interaction of these

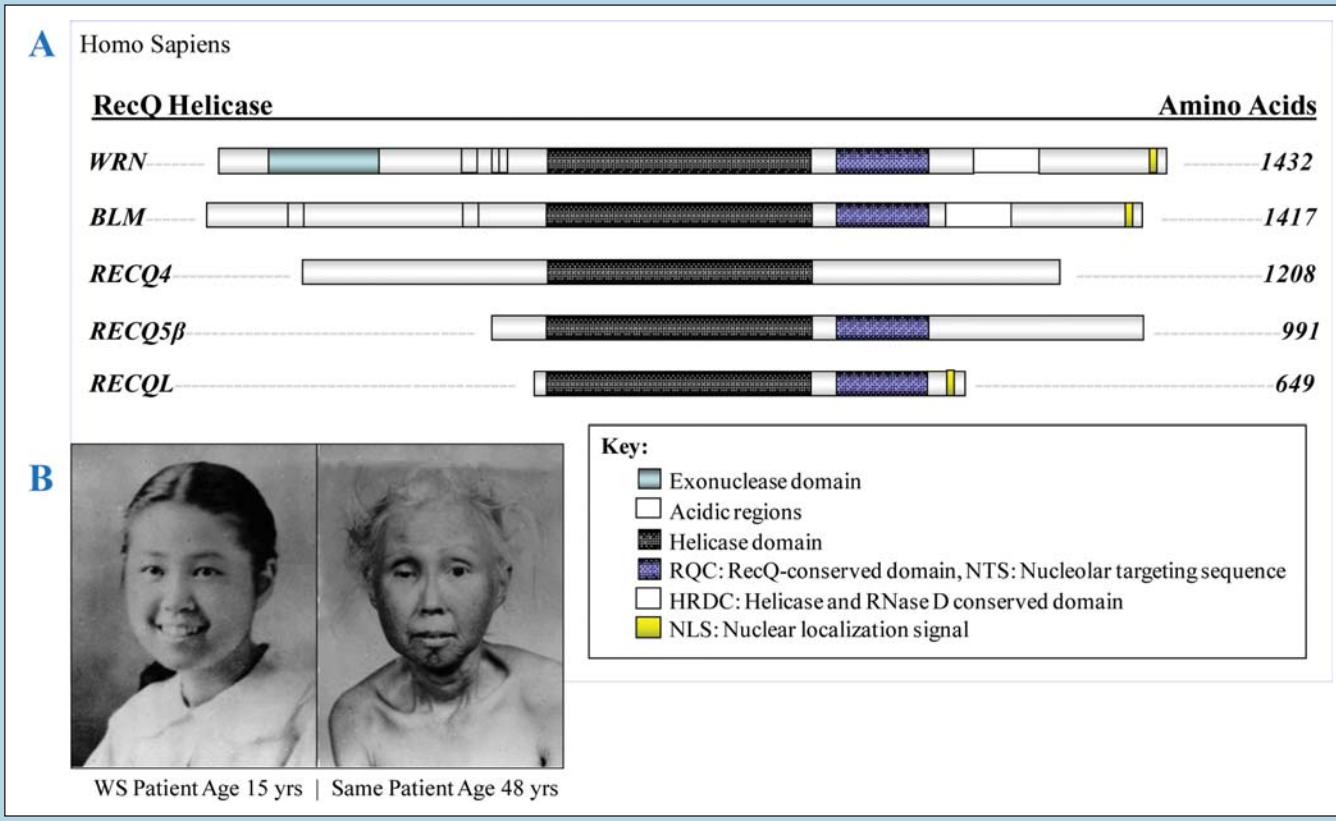

Figure 2. A) Schematic representation of the highly conserved, five-member RecQ family of DNA helicases for *H. Sapiens*, to which WRN protein belongs. RecQ helicases are critical in the maintenance of genome integrity as implicated by heritable cancer predisposition disorders that result by defects in BLM, RECQ4, and WRN. Amino acid designations of each are shown on the right and each respective domain within the proteins correspond to the colors explained in the key below. WRN protein uniquely imparts an exonuclease domain in the amino-(N)-terminal region. B) Nonfunctional WRN protein leads to Werner's syndrome (WS), a premature aging disorder with increased cancer incidence [14].

two proteins in vitro, since in vivo interactions may be mediated via an accessory protein [17], [18]. The Far Western technique allowed mapping of the precise domains between XPG and WRN proteins that are responsible for this novel interaction. The analysis involved separating full length and recombinant XPG and WRN deletion constructs based primarily upon the length of each polypeptide through denaturing gel electrophoresis. Thus, specific interacting domains could be visualized when probed with the secondary protein of interest. Results defined, for the first time, interaction domains between XPG and WRN that have imparted additional knowledge on the multifunctional role these proteins play in the maintenance of genomic stability.

Materials and Methods

Buffers

6X Sodium Dodecyl Sulfate (SDS) Sample Buffer was prepared using 70% 0.5 M Tris, pH 6.8, and 30% glycerol, with 0.35 M SDS, 0.6 M (D,L)-1,4-dithiothreitol (DTT) from Soltec Ventures, Inc., and 0.18 mM Bromophenol Blue dye (BPB). Reagents were combined in a 50 mL conical flask, then vortexed prior to being stored at –20 °C in 1 mL aliquots. All reagents excluding DTT were purchased from Sigma-Aldrich Corporation.

5X Tris-Glycine SDS Running Buffer for SDS Polyacrylamide Gel Electrophoresis (PAGE) was prepared using deionized water with 125 mM Tris-base, 0.96 M glycine from Bio-Rad Laboratories, Inc., and 17 mM of SDS. One liter of the 5X stock was made, from which dilutions to 1X were performed prior to gel running.

Standard Tris-Glycine Transfer Buffer for SDS-PAGE was prepared using deionized water containing 20% methanol, with 25 mM Tris-base and 190 mM glycine.

Proteins

Full length XPG protein and XPG protein domains were purified in the Cooper Lab as previously described or were obtained from Miaw-Sheue Tsai of the Expression and Molecular Biology (EMB) Core (Life Sciences Division, LBNL, Berkeley, CA 94720) [19].

All full-length WRN fractions and WRN domain constructs were kindly provided by Steven M. Yannone (Life Sciences Division, Department of Molecular Biology, LBNL, Berkeley, CA 94720), J. Jefferson P. Perry (Department of Molecular Biology and Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037), and L. David Finger (City of Hope, Beckman Research Institute, Duarte, CA 91010). Protein purification was conducted as previously described [20]. Protein constructs were appended with an N-terminal His_6 tag, cloned into a vector and recombinantly overexpressed in bacterial or insect-cells. Cells were resuspended in Tris-HCl buffer (pH 7.5) and affinity-purified from sonicated cell lysate by conventional nickel-nitrilotriacetic acid agarose resin column chromatography. Subsequent column-chromatography methods were used to purify the protein constructs to near homogeneity.

Experimental Method

To obtain the Far Western Assay, 1.5–6.0 µg of each polypeptide (full-length, truncation and control proteins) was denatured by boiling for five minutes in a 1:6 volumetric dilution of 6X SDS-sample buffer upon a 95 °C heat block, then resolved by SDS-PAGE using a precast 1.0 mm x 10 or 12 well, Novex® 4–12% or 4–20% Tris-Glycine Gel (Invitrogen Corp.). Molecular weight quantification of the proteins was achieved using Kaleidoscope Prestained Standards (Bio-Rad Laboratories). Proteins were transferred overnight to Bio-Rad Trans-Blot nitrocellulose filters using the corresponding Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell at 4 °C, after which the filters were briefly immersed in 1X Phosphate Buffered Saline (PBS) (Omni Pur) then Ponceau S Solution (Sigma-Aldrich) to provide temporary protein visualization and gauge the efficiency of transfer. Brief subsequent immersions in PBS allowed destaining of the background for increased visual clarity. Filters were immersed twice in denaturation buffer (6 M guanidine-HCl in PBS) for 10 minutes and then incubated six times for 10 minutes each in serial dilutions (1:1) of denaturation buffer in PBS to allow progressive protein renaturation. Filters were blocked in PBS containing 5% nonfat powdered milk for three hours at room temperature to prevent non-specific background binding of the antibodies to the membrane, prior to being incubated in full-length XPG or WRN proteins (10.0 pmole/mL) in PBS supplemented with 0.5% powdered milk, 0.1 or 0.5% Tween-20 (Sigma-Aldrich), 1.0 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) overnight at 4 °C with gentle agitation. Following overnight incubation, the filters were extensively washed four times for 10 minutes each in PBS containing 0.1–1.0% Tween-20, after which conventional Western blotting was performed to detect the presence of WRN or XPG using mouse monoclonal 4H12 Ab-66606 αWRN (Abcam) at a 1/666 dilution, or mouse 8H7 Ab-1 αXPG (NeoMarkers) at a 1/5 000 dilution, respectively, as primary antibody. After a one-hour incubation with the primary antibody, the filters were washed three times for five to ten minutes each with PBS containing 0.1–0.5% Tween-20. Enhanced Chemiluminescent (ECL) anti-mouse IgG/horseradish peroxidase conjugate (GE Healthcare) was applied as secondary antibody at a 1/(10 000) dilution for 45 minutes and detected by ECL using either Thermo Scientific, SuperSignal West Pico, or GE Healthcare-Amersham, Advance Western Blotting Detection kits. Filters were then exposed to film (GE Healthcare-Amersham Hyperfilm ECL) at 1–30 second exposure times to obtain the best image. Certain amendments to the stated protocol are described in the figure legends where appropriate.

Results

To identify the XPG protein domain that mediates the interaction with WRN, in vitro, a series of XPG deletion constructs were previously recombinantly expressed and purified using conventional column chromatography to near homogeneity (Figure 3A). Deletion constructs together isolate each domain region within XPG (N, R, I and C-terminal domains), in order to potentially identify a single key domain or group of domains required for the binding of WRN (Figure 3B). XPG constructs were immobilized to a nitrocellulose filter and stained for total protein visibility (Figure 3C). The filter was then incubated in buffer containing the second protein of interest, WRN, and then washed to remove any unbound protein. The presence of WRN was detected using conventional Western analysis with WRN antibodies (Figure 3C). When WRN was used to probe nitrocellulose bound full length XPG and XPG deletion constructs, an immunoreactive band was detected where full length XPG , the positive control Replication Protein A (RPA), and

constructs XFX and Exon-15 had migrated, as well as the positive control, Replication Protein A (RPA). A previously established interaction between WRN and RPA allows for the immunoreactive band of RPA to confirm the ability of WRN protein to bind to the filter and be detected by antibodies during the Western analysis. The interaction viewed between WRN and the XPG domain regions appeared to be specific, as WRN did not bind to the negative control protein, bovine serum albumin (BSA), which was run in parallel on the same blot. WRN protein also did not bind to the XPG deletion constructs XPGΔC, XFXΔC, and R-domain.

Importantly, the Far Western analysis identified the binding of WRN to full-length XPG, XFX, and Exon-15 domains, which together isolate the C-terminus of XPG as the key interaction domain with WRN (Figures 3A, 3B, 3C). As the binding of WRN to the full-length protein was expected, more critical are the deletion constructs which maintain the interaction. The first interacting

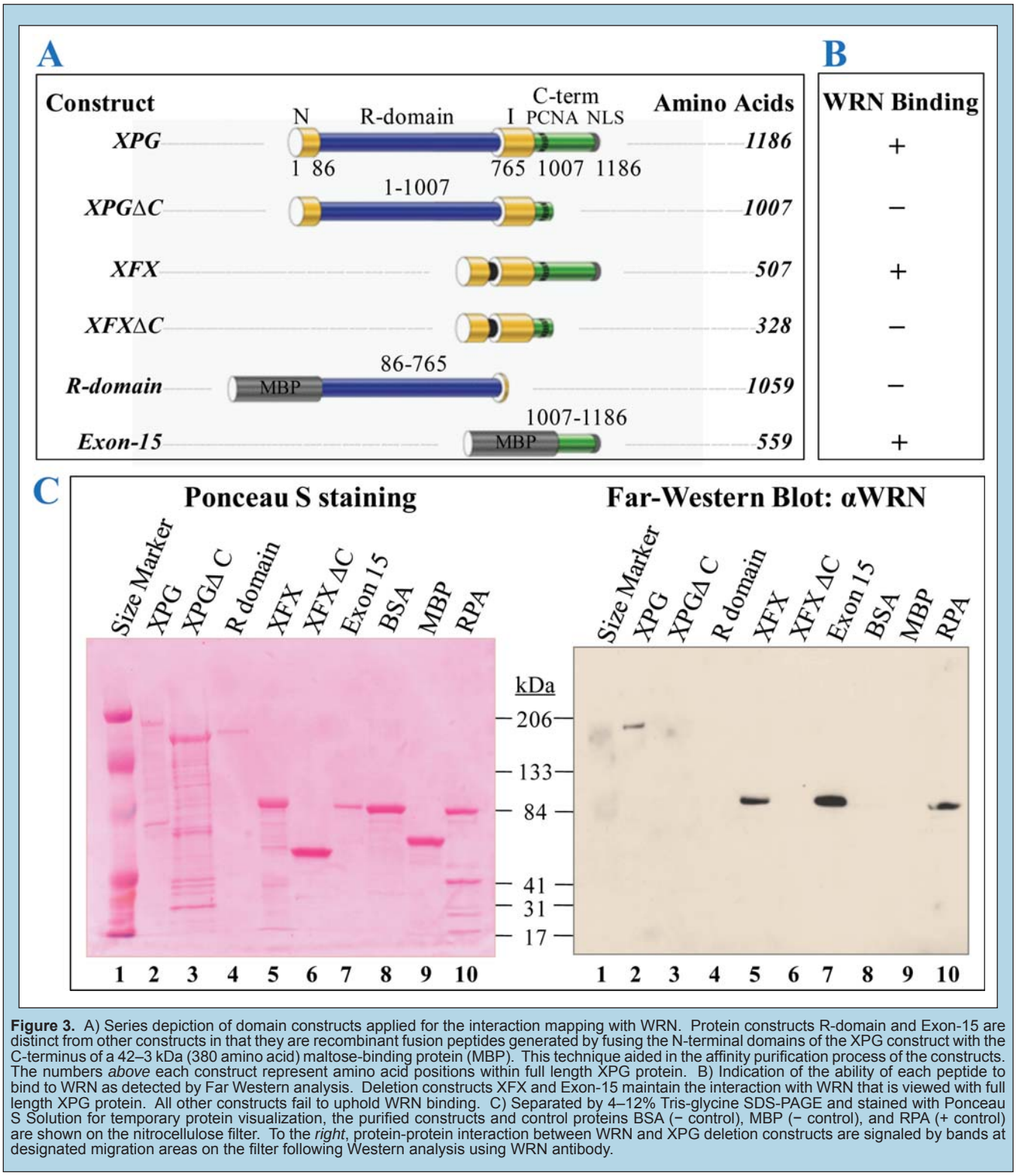

Figure 3. A) Series depiction of domain constructs applied for the interaction mapping with WRN. Protein constructs R-domain and Exon-15 are distinct from other constructs in that they are recombinant fusion peptides generated by fusing the N-terminal domains of the XPG construct with the C-terminus of a 42–3 kDa (380 amino acid) maltose-binding protein (MBP). This technique aided in the affinity purification process of the constructs. The numbers *above* each construct represent amino acid positions within full length XPG protein. B) Indication of the ability of each peptide to bind to WRN as detected by Far Western analysis. Deletion constructs XFX and Exon-15 maintain the interaction with WRN that is viewed with full length XPG protein. All other constructs fail to uphold WRN binding. C) Separated by 4–12% Tris-glycine SDS-PAGE and stained with Ponceau S Solution for temporary protein visualization, the purified constructs and control proteins BSA (− control), MBP (− control), and RPA (+ control) are shown on the nitrocellulose filter. To the *right*, protein-protein interaction between WRN and XPG deletion constructs are signaled by bands at designated migration areas on the filter following Western analysis using WRN antibody.

construct, XFX, preserves all domains of full-length XPG with the exclusion of the R-domain, which when coupled with the negative binding to the R-domain construct, indicates that the R-domain region is not required for WRN binding. Furthermore, Exon-15, a 559-aa deletion construct that comprises only the C-terminus (residues 1 007–1 186), bound to WRN and was found to be necessary and sufficient for interaction with WRN protein.

In order to further understand the nature of the XPG and WRN protein-protein interaction, in vitro, the regions of WRN protein that mediate the interaction with XPG were also sought. A reciprocal Far Western analysis was designed to meet this objective, and full-length XPG was used to probe nitrocellulose-bound WRN and recombinant WRN protein constructs (Figure 4). The WRN protein consists of four domains: 1) a central conserved helicase domain found in all RecQ helicases, 2) a unique N-terminal exonuclease domain, 3) a C-terminal region consisting of the RecQ-conserved domain/nucleolar targeting sequence (RQC/NTS) and 4) helicase and RNaseD conserved (HRDC) domains.

In addition to full-length WRN, preliminary Far Western assays included protein constructs, Exo-1, a peptide containing the exonuclease domain within residues 1–333, and H-32, which covers the final 290 residues of the 362 aa C-terminal region (Figures 4, 5A). Immunoreactive responses occurred as expected at sites where full-length WRN protein and the positive control proteins Cockayne Syndrome B (CSB), RPA, and full-length XPG had migrated. These signals confirm the efficiency of the incubation with XPG protein and Western probing with XPG antibody. In addition to these interactions, XPG binding to H-32 was found, but also for the negative control BSA, which was run parallel on the same blot along with another negative control protein, glutathione-S-transferase (GST) that showed no binding band. The interaction band with BSA confounds other results because it suggests a non-specific interaction due to error in the Far Western methodology. BSA had been applied as a negative control in previous Far Western assays of XPG constructs and had performed consistently (Figure 3C), which called into question the validity of the H-32 interaction band, which indicated the WRN C-terminus as being the domain responsible for the interaction with XPG. GST, however, performed as a negative control which prompted further investigation into the cause of the interaction band with BSA.

To identify the basis for BSA reactivity, a second Far Western analysis was designed to examine whether the immunoreactive response to BSA was source-dependent. This was achieved by using two new sources of BSA along with the same WRN constructs and control proteins used in the previous Far Western analysis. Second sources of BSA were Standard BSA (1:10), in addition to an SDS-PAGE high-range protein standard (Bio-Rad, Cat. #161-0303), which contains a BSA molecular weight determinant for blotting applications with several other such protein markers (Figure 5B). Positive interactions among both new sources of BSA in this Far Western assay suggested non source-dependent reactivity and supported determining if the basis of the repeated immunoreactive response was due to a protein-protein interaction between XPG and BSA. This investigation was conducted by designing a control Far Western experiment (Figure 6A). The results demonstrated equivalent positive bands for four distinct sources of BSA, thereby eliminating the possibility of source-dependent reactivity. The results also showed that the positive signals were not due to interactions between BSA and XPG, since immunoreactive bands of equal intensity were found for BSA on the half of the control blot that had not been exposed to XPG. By eliminating the possibility of protein-protein interaction as being the cause for BSA reactivity, cross-reactivity between BSA and the antibodies used for the Western detection became the single unexamined cause. A second BSA control experiment was designed to isolate which antibody was causing the cross-reactivity with BSA (Figure 6B). The control experiment determined the primary 8H7 αXPG antibody as the cause for cross-reactivity, since sections two and four of the membrane only received secondary anti-mouse IgG/horseradish peroxidase conjugate and showed no such interaction. This result establishes that the BSA interaction band found in preliminary Far Western assays with WRN constructs were non-XPG dependent, validating the result that the WRN C-terminal construct, H-32, was interacting with XPG.

After the preliminary results were validated, a final Far Western analysis was designed to include six additional WRN C-terminal

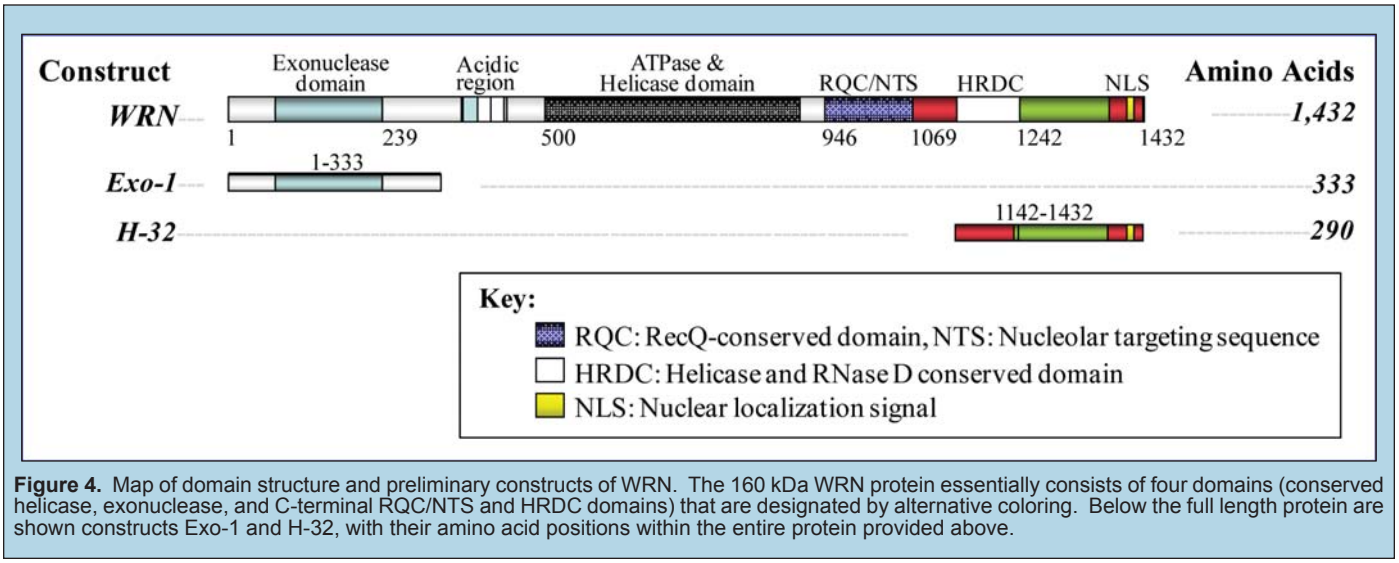

Figure 4. Map of domain structure and preliminary constructs of WRN. The 160 kDa WRN protein essentially consists of four domains (conserved helicase, exonuclease, and C-terminal RQC/NTS and HRDC domains) that are designated by alternative coloring. Below the full length protein are shown constructs Exo-1 and H-32, with their amino acid positions within the entire protein provided above.

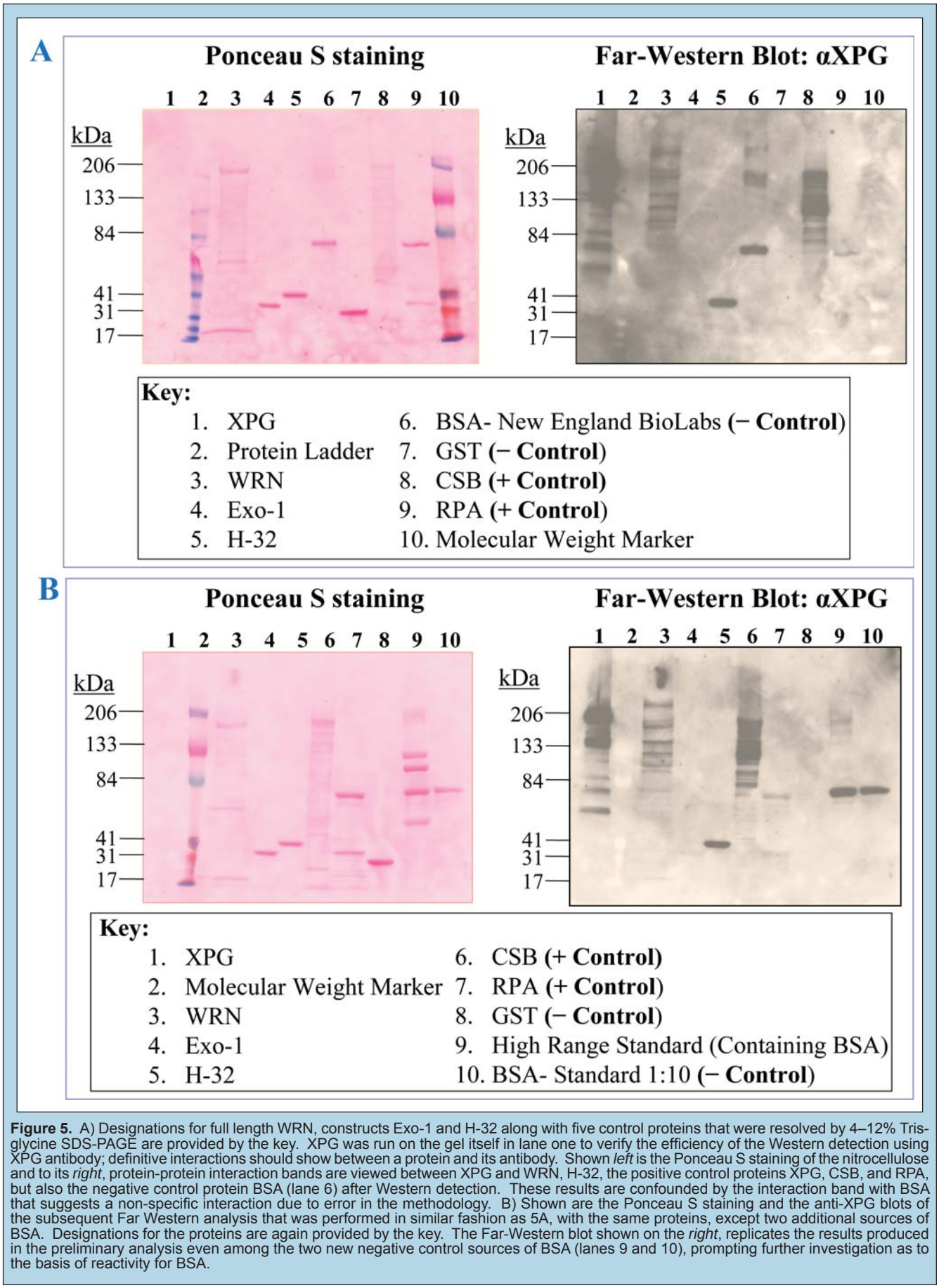

Figure 5. A) Designations for full length WRN, constructs Exo-1 and H-32 along with five control proteins that were resolved by 4–12% Tris-glycine SDS-PAGE are provided by the key. XPG was run on the gel itself in lane one to verify the efficiency of the Western detection using XPG antibody; definitive interactions should show between a protein and its antibody. Shown *left* is the Ponceau S staining of the nitrocellulose and to its *right*, protein-protein interaction bands are viewed between XPG and WRN, H-32, the positive control proteins XPG, CSB, and RPA, but also the negative control protein BSA (lane 6) after Western detection. These results are confounded by the interaction band with BSA that suggests a non-specific interaction due to error in the methodology. B) Shown are the Ponceau S staining and the anti-XPG blots of the subsequent Far Western analysis that was performed in similar fashion as 5A, with the same proteins, except two additional sources of BSA. Designations for the proteins are again provided by the key. The Far-Western blot shown on the *right*, replicates the results produced in the preliminary analysis even among the two new negative control sources of BSA (lanes 9 and 10), prompting further investigation as to the basis of reactivity for BSA.

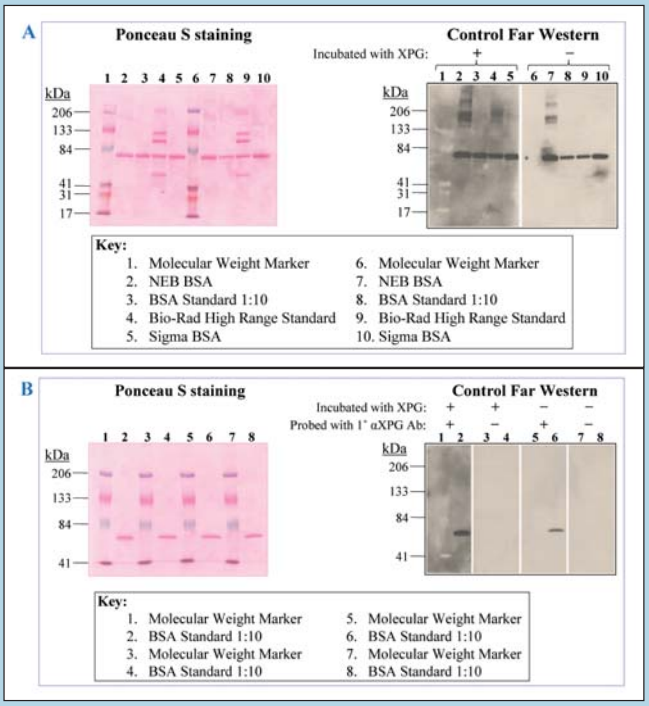

Figure 6. A control Far Western experiment was designed to examine the possibility of non-XPG based interactions as the cause for the immunoreactive response received in the Far Western blots shown in Figures 5A and B. A. Four distinct sources of BSA were loaded twice in a sequence specified by the key onto a 7% gel with 4% stack, resolved by SDS-PAGE, transferred to a nitrocellulose filter and blocked. The filter was then cut in half (between lanes 5 & 6). One half of the membrane underwent overnight incubation with XPG protein, and the other half was incubated without XPG protein. Western analysis was conducted on both halves using XPG antibody. B. A second control experiment was designed in order to isolate which antibody was causing the cross-reactivity with BSA. One source of BSA was loaded in an every-other lane sequence onto a 7% gel with 4% stack and subjected to SDS-PAGE. Following the 3 hour block, the nitrocellulose filter was cut in half, and as before, only one half of the filter received treatment with XPG. This half appears on the left of the Control Far Western diagram. Furthermore, each half of the filter was cut in half again, thereby forming 4 quarters, 2 of which that had received treatment with XPG. Only one quarter from each half of the filter was allowed to incubate with primary XPG antibody, in order to isolate the possible cross-reactivity of the primary verses secondary (anti-mouse IgG/horse radish peroxidase) antibodies. These quarters appear as the 1st and 3rd quarters of the nitrocellulose filter shown in the diagram. Positive interactions are viewed only on the quarters of the nitrocellulose filter that received primary XPG antibody treatment regardless of exposure to XPG protein, which determined the primary antibody as the source of the cross-reactivity.

domain deletion constructs for finer interaction mapping of this region (Figure 7A). Thus far, most of the WRN-interacting proteins have been shown to bind to more than one region within WRN protein, thus the eight total recombinant truncated WRN proteins used in the Far Western assay enabled precise mapping of the XPG binding domains of WRN (Figure 7A) [21], [22]. The inability to produce a soluble and purified helicase domain construct precludes the ability to test this region. The WRN C-terminal deletion constructs enable isolation of the RQC (aa 949-1069; construct 69), the HRDC (1142-1242; construct HRDC), and the nuclear localization signal (NLS) (aa 1243-1432; construct P-28) domains, as well as all the regions between these complexes (constructs 42, 70, 82, and H-32). Subtractive analysis can be used to identify two independent interaction domains for XPG in the WRN protein, based on the results of the Far Western assay (Figure 7B).

The interactions among the three WRN C-terminal truncation constructs 69, 42, and HRDC together isolate one of the domains of interaction. Constructs 69 and HRDC do not possess immunoreactive bands, thereby eliminating these domains as being interactive with XPG. However, a strong interaction appears for 42, a deletion construct that essentially bridges the 73 residues that separate constructs 69 and HRDC, inclusive of these regions. Thus, an independently interacting region within the WRN C-terminus

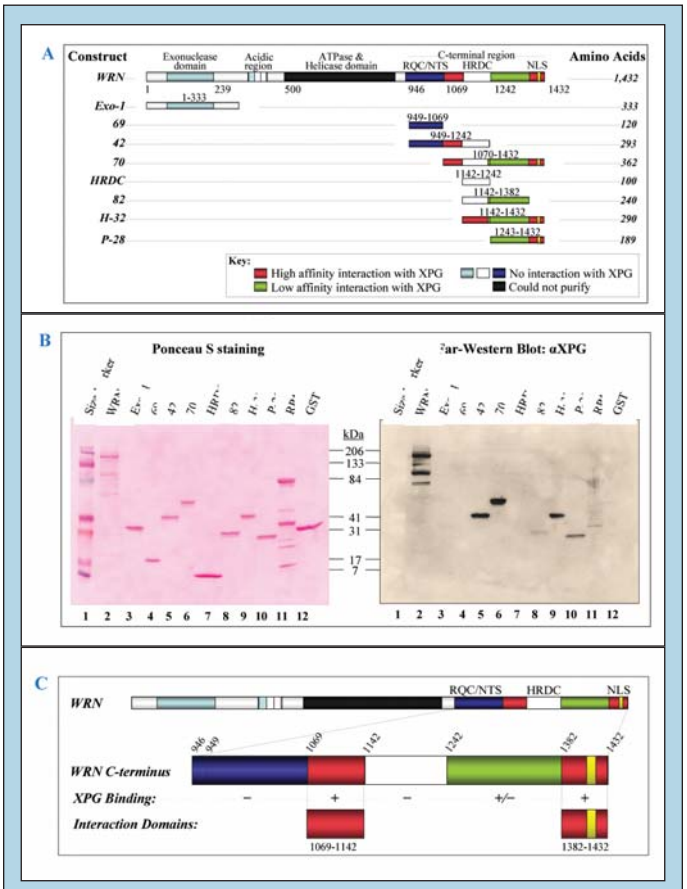

Figure 7. Preliminary results (Figures 5A and B) prompted further investigation of the interacting sub-domains of the WRN C-terminus with XPG using 6 additional C-terminal deletion constructs to the already tested H-32. A. Eight WRN constructs were used to map the domains responsible for interaction with XPG. The sub-domains enable isolation of the C-terminal RQC, HRDC, NLS domains, in addition to the regions between these complexes. Inability to produce a soluble and purified helicase domain construct precludes the ability to identify all interaction sites for XPG on WRN. All full-length WRN fractions and WRN domain constructs were kindly provided by the lab of Steven M. Yannone (Life Sciences Division, LBNL). B. Full length WRN, Exo-1 and the total 7 C-terminal sub-constructs, as well as positive control protein RPA, and negative control protein GST, were resolved by 4–20% Tris-glycine SDS-PAGE in the order designated by the construct map. The proteins were blotted to nitrocellulose, blocked and incubated with purified XPG protein prior to Western analysis using XPG antibody, resulting in the blot which appears on the right-hand side of the diagram. Positive interactions are viewed for full length WRN, constructs 42, 70, H-32, P-28, and weakly pronounced for 82. C. Schematic representation of the C-terminal domain of WRN and the regions of the C-terminus that enable the interaction with XPG. The ability of XPG to bind to each C-terminal domain is designated by (+) interaction, (−) no interaction, and (+/−) weak interaction. Both conclusive interaction sites are diagrammatically displayed in red at the *bottom*. The first interaction site appears as amino acids 1 069–1 142, which resides between the RQC and HRDC domains on WRN. The second, a 50 residue interaction site comprising amino acids1 382–1 432 exists at the very C-terminal end of WRN. This interaction site may begin prior to the last 50 residues (amino acid 1 382) based on the weak interaction viewed in the green region.

was found to reside between the RQC and HRDC domains, which comprises amino acids 1069–1142 (Figure 7C).

Additional XPG interactions with deletion constructs 82, H-32 and P-28 support the existence of a second binding site for XPG within the C-terminus. Protein construct 82 harbors a complete HRDC complex plus the next 140 of the remaining 190 5' C-terminal residues (aa 1142–1382), and displays a very weak interaction band. However, the H-32 fraction maps essentially the same region within the C-terminus as 82, in addition to the final 50 C-terminal residues (aa 1142–1432), and displays a much stronger interaction with XPG. This result suggests that the final 50 residues or amino acids 1382–1432 comprise the majority of a second XPG binding domain within the WRN C-terminus (Figure 7C). A strongly interacting P-28 construct, which maps the C-terminal region 5' to the HRDC complex, confirms this result. The weak interaction with 82 indicates that the second XPG binding domain may begin prior to the final 50 residues of WRN. Additionally, the relatively weaker interaction of XPG to P-28 as compared to H-32 could be due to ineffective folding of the shorter form which excludes the HRDC domain, thereby compromising XPG binding ability. This suggests that an intact HRDC domain may increase the WRN C-terminal ability to assume the native conformation viewed in the full length protein. The largest deletion construct of the WRN C-terminus, 70 (aa 1070–1432), which includes both interaction sites confirms these results by demonstrating the strongest immunoreactive band on the blot. Hence, WRN possesses two independent XPG interacting regions within the C-terminus.

Conclusion

These studies identify for the first time the C-terminal domain as the key region of XPG that is necessary and sufficient for a physical interaction with WRN, as well as two independent interaction sites of WRN that complement this interface. The fact that WRN preferentially unwinds secondarily structured DNA substrates, which are functional intermediates of the HRR subpathway of DSBR, and that WRN is responsible for the recruitment of other proteins that mediate DNA repair in order to optimize DSBR, provides the possibility of a novel replication-associated role for XPG within this repair pathway (Figure 8). This proposed role is supported by interactions of XPG with proteins Rad 51, BLM and the MRN protein complex that are also involved in the processing of stalled or collapsed DNA replication forks within HRR. The stable interaction between XPG and WRN links the repair of UV-induced DNA damage (NER), which has been made evident by the disease XP, with DSBR, which imparts additional knowledge of the overlapping nature of these two proteins and the previously distinct repair pathways they are associated with.

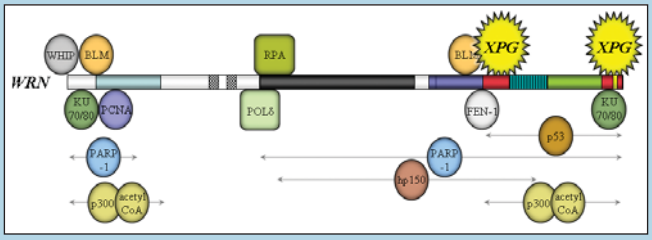

Figure 8. Select documented protein binding partners for WRN and novel interacting domains with XPG. WRN domains are again designated by the same colors used in Figure 4. Protein binding partners indicated by rectangular symbols interact with WRN, though the precise location of the interaction domain within the helicase has not been identified. Binding proteins indicated by round symbols are positioned at approximate, identified binding sites (additional binding sites may exist). Horizontal arrows indicate the limits of binding domains where the site of interaction is broad ranging or mapped less accurately. XPG is shown at its novel binding domains within the WRN C-terminus based on the results of this study.

The direct interaction, in vitro, of the XPG C-terminus with WRN is particularly interesting, given that novel structural roles of XPG in BER are mediated by this same region. Therefore, future research will focus on finely defining the interaction motifs of the XPG C-terminus with WRN protein, as well as with all other proteins identified as C-terminal binding. This investigation will involve sub-cloning and expressing three sequential MBP fused C-terminal sub-domains through polymerase chain reaction (PCR). Far Western analysis will be used to determine whether the numerous proteins that interact with XPG utilize the same region of its C-terminus or whether the interactions are mutually exclusive.

Further understanding the protein structure of the C-terminal region of XPG is additionally important due to the regions numerous interactions with BER proteins, and its likely role in CS. However, the domain has been difficult to study due to its extended and flexible conformation (Cooper Lab, unpublished results). Future studies will attempt to determine the protein structure of the C-terminal domain of XPG by forming a complex between Exon-15 (XPG C-terminus) and the smallest interacting domain of WRN that includes both regions of interaction (construct 70), in order to form a rigid structure capable of study. Initial analysis will involve using multi-angle light scattering (MALS) to determine if the XPG C-terminal extended structure has change conformation following incubation with construct 70, based upon the relative radius of the complex in solution. The precise protein structure in solution will then be determined using small angle X-ray scattering (SAXS).

The Far Western technique used in this study enabled the direct in vitro mapping of interaction sites between DNA repair proteins XPG and WRN that extend our understanding of these proteins' involvement in vital DNA damage repair pathways. The novel physical interaction of XPG with WRN suggests that WRN protein might be important in coordinating the possible replication-associated function of XPG at stalled or collapsed replication forks with ds breaks. Since genomic integrity is constantly threatened by both endogenous and exogenous damage, understanding the roles of these proteins in coordinating DNA repair processes with replication will significantly further understanding how their defection instigates physiological consequences in response to various DNA damaging sources. By piecing together the processes of these multifunctional proteins that restore and maintain genomic integrity, we are brought closer to understanding and preventing cancer and premature aging.

Acknowledgements

This research was conducted at the Cooper Lab of the Life Sciences Division at LBNL, and was supported by the Center for Science and Engineering Education and the U.S. Department of Energy (DOE), Office of Science. I would like to take this opportunity to express my sincere gratitude to my mentor Kelly Trego, for her patience and enlightening instruction, and also for

giving me a great deal of direction, inspiration and wisdom during the few weeks of the internship, as well as my supervisor Priscilla Cooper, for not only selecting me for this internship, but also for giving me the opportunity to continue conducting research as I attend UC Berkeley. With her continued support I can continue to learn and fulfill my desire to pursue a career in the sciences. I would also like to thank Miaw-Sheue Tsai, Steve Yannone, Jeff Perry, and Dave Finger for making a large part of this study possible, as well as Cliff Ng, Altaf Sarker, Jill Fuss, and Susan Tsutakawa for their knowledge and assistance regarding different aspects of this research. Additionally, I would like to thank the DOE Office of Science, and the Community College Internship Program for giving me the opportunity to participate in a truly fulfilling research experience.

References

[1] R.M. Brosh, Jr. and V.A. Bohr, "Survey and Summary, Human premature aging, DNA repair and RecQ helicases," *Nucleic Acids Research*, vol. 35, no. 22, pp. 7527–7544, 2007.

[2] S.M. Yannone, S. Roy, D.W. Chan, M.B. Murphy, S. Huang, J. Campisi and D.J. Chen, "Werner Syndrome Protein is Regulated and Phosphorylated by DNA-dependent Protein Kinase," *Journal of Biological Chemistry*, vol. 276, no. 41, pp. 38242–38248, 2001.

[3] J.O. Fuss and P.K. Cooper, "DNA Repair: Dynamic Defenders against Cancer and Aging," *Public Library of Science Biology*, vol. 4, no. 6, pp. 899–903, 2006.

[4] E.C. Friedberg, G.C. Walker, W. Siede, R.D. Wood, R.A. Schultz and T. Ellenberger, *DNA Repair and Mutagenesis*, 2nd ed., Washington, D.C: American Society for Microbiology, 2005.

[5] M.P. Gallego and A. Sarasin, "Transcription-coupled repair of 8-oxoguanine in human cells and its deficiency in some DNA repair diseases," *Biochimie*, vol. 85, no. 11, pp. 1073–1082, 2003.

[6] A. O'Donovan, A.A. Davies, J.G. Moggs, S.C. West and R.D. Wood., "XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair," *Nature*, vol. 371, pp. 432–435, Sept.1994.

[7] A.R. Lehmann, "Nucleotide excision repair and the link with transcription," *Trends in Biochemical Sciences*, vol. 20, no. 10, pp. 402–405, Oct. 1995.

[8] T. Bessho, "Nucleotide excision repair 3' endonuclease XPG stimulates the activity of base excision repair enzyme thymine glycol DNA glycosylase," *Nucleic Acids Research*, vol. 27, no. 4, pp. 979–983, 1999.

[9] A. Klungland, I. Rosewell, S. Hollenbach, E. Larsen, G. Daly, B. Epe, E. Seeberg, T. Lindahl and D.E. Barnes, "Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13300–13305, Nov. 1999.

[10] M. Weinfeld, J.Z. Xing, J. Lee, S.A. Leadon, P.K. Cooper and X.C. Le., "Factors influencing the removal of thymine glycol from DNA in gamma-irradiated human cells," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 68, pp. 139–149, 2001.

[11] T.K. Hazra, A. Das, S. Das, S. Choudhury, Y.W. Kow and R. Roy, "Oxidative DNA damage repair in mammalian cells: A new perspective," *DNA Repair, (Amst.)*, vol. 6, no. 4, pp. 470–480, April 2007.

[12] P. Soultanas and D.B. Wigley, "Unwinding the 'Gordian knot' of helicase action," *Trends in Biochemical Sciences*, vol. 26, no. 1, pp. 47–54, Jan. 2001.

[13] I.D. Hickson, "RecQ Helicases: Caretakers of the Genome," *Nature Reviews Cancer*, vol. 3, pp. 169–178, 2003.

[14] C.J. Epstein, G.M. Martin, A.L. Schulz and A.G. Motulsky, "Werner's syndrome a review of its symptomatology, natural history pathologic features, genetics and relationship to the natural aging process," *Medicine (Baltimore)*, vol. 45, no. 3, pp. 177–221, 1966.

[15] M.D. Gray, J.C. Shen, A.S. Kamath-Loeb, A. Blank, B.L. Sopher, G.M. Martin, J. Oshima and L.A. Loeb, "The Werner syndrome protein is a DNA helicase," *Nature Genetics*, vol. 17, no. 1, pp. 100–103, Sept. 1997.

[16] S. Huang, B. Li, M.D. Gray, J. Oshima, I.S. Mian and J. Campisi, "The premature ageing syndrome protein, WRN, is a 3'→5' exonuclease, *Nature Genetics*, vol. 20, no. 2, pp. 114–116, Oct. 1998.

[17] L. Wu, D.L. Davies, P.S. North, H. Goulaouic, J.F. Riou, H. Turley, K.C. Gatter and I.D. Hickson, "The Bloom's syndrome gene product interacts with topoisomerase III," Journal of Biological Chemistry, vol. 275, no. 13, pp. 9636–9644, March 2000.

[18] G. Pedrazzi, C.Z, Bachrati, N. Selak, I. Studer, M. Petkovic, I.D. Hickson, J. Jiricny and I. Stagljar, "The Bloom's syndrome helicase interacts directly with the human DNA mismatch repair protein hMSH6," *Journal of Biological Chemistry*, vol. 384, no. 8, pp. 1155–1164, Aug. 2003.

[19] A.H. Sarker, S.E. Tsutakawa, S. Kostek, C. Ng, D.S. Shin, M. Peris, E. Campeau, J.A. Tainer, E. Nogales and P.K. Cooper, "Recognition of RNA polymerase II and transcription bubbles by XPG, CSB, and TFIIH: insights for transcription-coupled repair and Cockayne Syndrome," *Molecular Cell*, vol. 28, no. 2, pp. 187–198, Oct. 2005.

[20] J.J.P. Perry, S.M. Yannone, L.G. Holden, C. Hitomi, A. Asaithamby, S. Han, P.K. Cooper, D.J. Chen and J.A. Tainer, "WRN exonuclease structure and molecular mechanism imply an editing role in DNA end processing," *Nature Structural and Molecular Biology*, vol. 13, no. 5, pp. 414–422, May 2006.

[21] D. Salk, K. Au, H. Hoehn and G.G. Martin, "Cytogenetic aspects of Werner syndrome," *Advances in Experimental Medicine and Biology*, vol. 190, pp. 541–546, 1985.

[22] C. von Kobbe, J.A. Harrigan, V. Schreiber, P. Stiegler, J. Piotrowski, L. Dawut and V.A. Bohr, "Poly(ADP-ribose) polymerase 1 regulates both the exonuclease and helicase activities of the Werner syndrome protein," *Nucleic Acids Research*, vol. 32, no. 13,. pp. 4003–4014, Aug. 2004.