

The Werner syndrome protein operates in base excision repair and cooperates with DNA polymerase β

Jeanine A. Harrigan, David M. Wilson III, Rajendra Prasad¹, Patricia L. Opresko, Gad Beck, Alfred May, Samuel H. Wilson¹ and Vilhelm A. Bohr*

Laboratory of Molecular Gerontology, National Institute on Aging, NIH, Baltimore, MD, USA and

¹Laboratory of Structural Biology, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA

Received November 22, 2005; Revised and Accepted January 11, 2006

ABSTRACT

Genome instability is a characteristic of cancer and aging, and is a hallmark of the premature aging disorder Werner syndrome (WS). Evidence suggests that the Werner syndrome protein (WRN) contributes to the maintenance of genome integrity through its involvement in DNA repair. In particular, biochemical evidence indicates a role for WRN in base excision repair (BER). We have previously reported that WRN helicase activity stimulates DNA polymerase beta (pol β) strand displacement synthesis *in vitro*. In this report we demonstrate that WRN exonuclease activity can act cooperatively with pol β , a polymerase lacking 3'–5' proofreading activity. Furthermore, using small interference RNA technology, we demonstrate that WRN knockdown cells are hypersensitive to the alkylating agent methyl methanesulfonate, which creates DNA damage that is primarily repaired by the BER pathway. In addition, repair assays using whole cell extracts from WRN knockdown cells indicate a defect in long patch (LP) BER. These findings demonstrate that WRN plays a direct role in the repair of methylation-induced DNA damage, and suggest a role for both WRN helicase and exonuclease activities together with pol β during LP BER.

INTRODUCTION

One of the major theories of aging proposes that the phenotypical changes associated with age are due to the accumulation of oxidatively induced DNA base lesions (1).

Base excision repair (BER) is the cellular repair pathway responsible for the removal of these lesions and the restoration of normal DNA bases. BER is divided into two sub-pathways, in which one single nucleotide (SN) or greater than one (long patch (LP)) nucleotide is incorporated during the repair process. The major proteins involved in SN BER include DNA glycosylases, apurinic/aprimidinic endonuclease 1 (APE1), DNA polymerase β (pol β) and DNA ligase. Notably, pol β lacks 3'–5' proofreading activity, making it susceptible to incorporation errors (2), and mismatched bases have been shown to impede further nucleotide incorporation (3,4) and the ligation step (5). LP BER involves several additional proteins, including flap endonuclease 1 (FEN-1) and poly(ADP-ribose) polymerase 1 (PARP-1), among others (6–8).

Werner syndrome (WS) is a segmental progeroid syndrome in which patients appear much older than their chronological age and display many clinical features resembling those of normal aging. The protein defective in WS (WRN) is a member of the RecQ family of DNA helicases, and possesses 3'–5' exonuclease activity in addition to 3'–5' helicase activity (9). Neither the yeast or bacterial RecQ helicases possess exonuclease activity, thus making the dual catalytic activities of WRN quite unique. The N-terminal exonuclease domain of WRN shows homology to the proofreading domain of *Escherichia coli* DNA polymerase I (10) and WRN has a proposed role as an autonomous proofreading enzyme (11). Furthermore, WRN is able to degrade DNA substrates containing a 3' G:T mismatch (12,13).

We have previously shown that WRN interacts with pol β and stimulates pol β strand displacement DNA synthesis via its helicase activity (14). In this report we investigated a role for WRN exonuclease activity on 3' mismatches in cooperation with pol β *in vitro* and a cellular role for WRN in BER *in vivo*. We found that WRN exonuclease and helicase activities cooperated with pol β on BER intermediates containing

*To whom correspondence should be addressed. Tel :+1 410 558 8162; Fax : +1 410 558 8157; Email: vbohr@nih.gov
Present addresses:

Jeanine A. Harrigan, The Wellcome Trust/CRUK Gurdon Institute, University of Cambridge, Cambridge, UK

Patricia L. Opresko, Department of Environmental and Occupational Health, University of Pittsburgh, 100 Technology Drive, Pittsburgh, PA 15260, USA

© The Author 2006. Published by Oxford University Press. All rights reserved.

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oxfordjournals.org

Table 1. Oligonucleotides used in this study

Name	Sequence (5'→3')
15P	CTGCAGCTGATGCGC
15MMT	CTGCAGCTGATGCGT
15MMG	CTGCAGCTGATGCGG
15MMA	CTGCAGCTGATGCGA
17P	CTGCAGCTGATGCGCCG
17MMT	CTGCAGCTGATGCGCCT
17MMC	CTGCAGCTGATGCGCCC
17MMA	CTGCAGCTGATGCGCCA
pG18	PGTACGGATCCCCGGGTAC
34G	GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG

p, phosphate. Mismatched bases in the primer and template strands are indicated in bold.

3' mismatched substrates. In addition, using cell lines stably expressing small interfering RNAs (siRNA) against WRN, we found that WRN knockdown (WRN KD) cells display increased sensitivity to the methylating agent methyl methane-sulfonate (MMS). Furthermore, extracts from WRN KD cells displayed reduced LP BER. These results demonstrate that WRN participates in BER *in vivo* and likely promotes the efficiency of pol β -mediated BER via its helicase and exonuclease activities.

MATERIALS AND METHODS

Materials

Synthetic oligodeoxyribonucleotides were purchased from Midland Certified Reagent Co. (Table 1) or Oligos etc. Inc. (see below). MMS and methoxyamine (MX) were from Sigma. Hygromycin B was from Invitrogen and [α - 32 P]dCTP (3000 Ci/mmol) was from GE Healthcare. Recombinant human WRN (15) and pol β (16) were purified as described.

Cell lines

The siRNAs targeted against WRN mRNA (UGAAGAG-CAAGUUACUUGCUU) were cloned into the pSilencerTM 3.1-H1 hygro vector (Ambion Inc.). The pSilencerTM 3.1-H1 negative control hygro vector (Ambion) was used to express a scrambled siRNA with no significant homology to known human genes. Stable WRN KD and wild-type (WT) cells were generated by transfection of U-2 OS cells and selection by growing in 200 μ g/ml hygromycin B (W.-H. Cheng, R. Kusumoto, P.L. Opresko, X. Sui, S. Huang, M.L. Nicolette, T.T. Paull, J. Campisi, M. Seidman, V.A. Bohr, manuscript submitted). Individual clones were selected and tested for levels of WRN KD by western blot analysis and RT-PCR. The clone demonstrating the greatest level of WRN KD was chosen for further analysis. Telomerase-immortalized cell lines from a normal (GM01604) and WS (AG03141) patient were described previously (17) and cultured in DMEM containing 10% fetal bovine serum, glutamine, penicillin and streptomycin.

WRN exonuclease assays

Exonuclease reactions (10 μ l) contained WRN and various DNA substrates as indicated in exonuclease buffer [40 mM Tris (pH 8), 4 mM MgCl₂, 5 mM DTT, 0.1 μ g/ μ l BSA and 4 mM ATP]. The concentrations of the DNA substrate and

WRN are indicated in the figure legend. In addition, molar ratios were chosen based on optimal enzymatic activity under the experimental conditions employed. Samples were incubated for 15 min at 37°C and terminated by the addition of formamide stop dye (80% formamide, 0.5 \times TBE, 0.1% bromophenol blue and 0.1% xylene cyanol). Reaction products were heat denatured for 5 min at 95°C and run on a 20% denaturing polyacrylamide gel. Radioactive products were visualized using a PhosphorImager and quantitated using ImageQuant software.

Pol β extension assays

Reactions were performed in exonuclease buffer containing 100 μ M dNTPs, pol β and WRN as indicated and were incubated for 15 min at 37°C. The concentrations of the DNA substrate, pol β and WRN are indicated in the figure legend and were chosen based on enzymatic activity under the reaction conditions employed. Reactions were terminated and analyzed as above for exonuclease assays. For Figure 1C, the fold increase was calculated as the % extension (total extension products/total reaction products) for lanes containing WRN and pol β divided by % extension for lanes containing pol β alone.

WRN helicase assays

Helicase reactions (10 μ l) were performed in exonuclease buffer containing 1 mM dNTPs and DNA substrates (250 fmol) as indicated. Reactions were initiated by the addition of WRN (125 fmol), incubated for 15 min at 37°C and terminated by the addition of stop dye (16 mM EDTA, 0.3% SDS, 13% glycerol, 0.05% xylene cyanol and 0.05% bromophenol blue). Reaction products were run on a 12% non-denaturing polyacrylamide gel and visualized using a PhosphorImager.

Western blotting

Cells were pelleted, resuspended in SDS-protein sample buffer and boiled for 5 min. Proteins were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad). After blocking with 2.5% non-fat milk in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) for 1 h at RT, the membrane was incubated overnight at 4°C with the following primary antibodies: anti-actin (Chemicon International, 1:2500), anti-WRN (BD Biosciences, 1:250), anti-pol β (Trevigen, 1:250) or anti-lamin A/C (Santa Cruz Biotechnology, 1:1000). After washing with PBS-T, the membrane was incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, 1:10 000) for 1 h at RT. Antigen-antibody complexes were detected by enhanced chemiluminescence using ECL Plus (Amersham Biosciences).

Cell survival assays

Cells were seeded at a density of 1×10^4 cells/well in 6-well dishes. MMS (11.8 M) was initially diluted in PBS to make a 1 M stock, and subsequent dilutions were made directly into the media. Cells were exposed to various concentrations of MMS (0–1.25 mM) for 1 h at 37°C (5% CO₂). Subsequently, cells were washed twice with PBS and fresh media was added. After incubation for 7 days, cells were counted by a

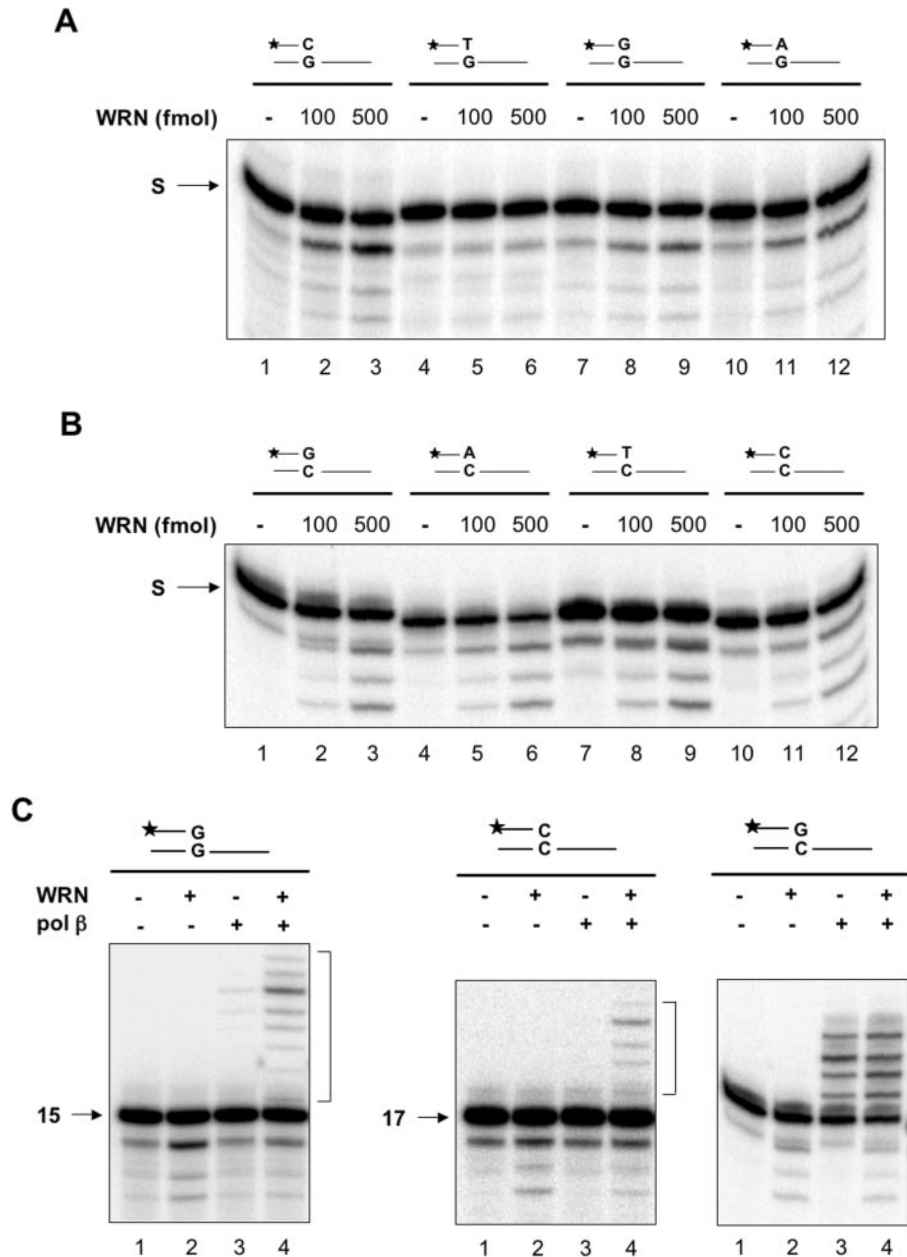


Figure 1. WRN exonuclease activity cooperates with pol β on 3' mismatches. (A) A DNA substrate (250 fmol), containing correctly base paired (15P/34G, lanes 1–3) or mispaired (15MMT/34G, lanes 4–6; 15MMG/34G, lanes 7–9; 15MMA/34G, lanes 10–12) 3' termini opposite guanine, was incubated with WRN (0, 100 or 500 fmol) for 15 min at 37°C. Products were run on denaturing polyacrylamide gels and visualized using a PhosphorImager. The 3' recessed primer is indicated by S. (B) A DNA substrate (250 fmol), containing correctly base paired (17P/34G, lanes 1–3) or mispaired (17MMA/34G, lanes 4–6; 17MMT/34G, lanes 7–9; 17MMC/34G, lanes 10–12) 3' termini opposite cytosine, was incubated with WRN (0, 100 or 500 fmol) and analyzed as above. (C) Reactions containing WRN (250 fmol, lanes 2 and 4) and pol β (10 fmol, lanes 3 and 4) were incubated with recessed substrate 15MMG/34G (250 fmol, left panel) or 17MMC/34G (250 fmol, middle panel) for 15 min at 37°C and analyzed as described above. Reactions containing WRN (300 fmol, lanes 2 and 4) and pol β (3 fmol, lanes 3 and 4) were incubated with recessed substrate 17P/34G (250 fmol, right panel) for 15 min at 37°C and analyzed as described above. The 3' recessed primer is indicated by the arrow. Extended products are indicated by the bracket.

cell lysis procedure (18) using a Z1 Coulter Particle Counter (Beckman Coulter, aperture 5 μ m). Results were expressed as the number of cells in drug-treated wells relative to cells in control wells (% control growth).

Cytotoxicity studies were also conducted in the presence of MX as described previously (19,20). A stock solution of 3 M MX was prepared in PBS immediately before use and dissolved to a final concentration (30 mM) in media to

which NaOH was added to adjust the pH to neutral. Dilutions of MMS as indicated were prepared in the MX-containing medium. Cells were incubated for 1 h at 37°C, washed twice with PBS and then incubated for an additional 3 h in media containing MX. Subsequently, cells were washed twice with PBS, incubated in fresh medium for 7 days at 37°C, and counted by the cell lysis procedure described above.

Cell extract preparation

Cell extracts were prepared as described previously (21). Briefly, cells were washed twice with PBS at room temperature, detached by scraping, pelleted by centrifugation and resuspended in Buffer I [10 mM Tris-HCl (pH 7.8), 200 mM KCl and protease inhibitor cocktail (Boehringer Mannheim)]. An equal volume of Buffer II [10 mM Tris-HCl (pH 7.8), 200 mM KCl, 2 mM EDTA, 40% glycerol, 0.2% Nonidet P-40, 2 mM DTT and protease inhibitor cocktail] was added. The suspension was rotated for 1 h at 4°C, and the resulting extract was clarified by centrifugation at 14 000 r.p.m. (Sorvall rotor SS-34) at 4°C for use in *in vitro* BER assays. The protein concentration of the extract was determined by Bio-Rad protein assay analysis using BSA as a standard.

In vitro SN and LP BER assay

The BER assay was performed in a final reaction volume of 20 µl. A 35 bp duplex DNA oligonucleotide substrate (250 nM) containing uracil (U) at position 15 (U strand: 5'-GCCCTGCAGGTGCAUTCTAGAGGATCCCCGGGTAC-3'; Template strand: 5'-GTACCCGGGGATCCTCTAGAGT-CGACCTGCAGGGC-3') was pre-incubated with WT or WRN KD cell extract (20 µg) for 5 min at room temp in a BER reaction mixture that contained 50 mM HEPES (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 20 mM KCl, 4 mM ATP, 5 mM phosphocreatine, 100 µg/ml phosphocreatine kinase, 0.5 mM NAD and 100 µM ddTTP. The repair reaction was initiated by the addition of 10 mM MgCl₂ and 2.2 µM [α -³²P]dCTP (specific activity, 1 × 10⁶ dpm/pmol) and incubated at 37°C. Aliquots (5 µl) were removed at the indicated periods. The reaction was terminated by the addition of an equal volume (5 µl) of DNA gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol). After incubation at 75°C for 2 min, the reaction products were separated by electrophoresis in a 15% polyacrylamide gel containing 8 M urea in 89 mM Tris-HCl (pH 8.8), 89 mM boric acid and 2 mM EDTA. The gels were scanned by PhosphorImager and the data was analyzed by ImageQuant software.

RESULTS

We have previously shown that the WRN helicase stimulates pol β strand displacement DNA synthesis (14), thereby indicating a mechanism by which WRN promotes BER. To further investigate the biochemical mechanism for WRN's role in promoting BER, we next tested for the possible contribution of the WRN exonuclease in pol β-directed BER. Given that pol β lacks proofreading activity, we hypothesized that the WRN exonuclease may cooperate with pol β to correct misincorporated bases. Therefore, using a variety of 3' mismatched substrates including purine:purine, purine:pyrimidine and pyrimidine:pyrimidine pairs, we first tested the ability of WRN to excise 3' mismatches in the context of a primer/template. The helicase is inactive on this substrate, allowing us to examine the contribution of the WRN exonuclease on pol β DNA synthesis. WRN excised cytosine (Figure 1A, lanes 2 and 3), guanine (Figure 1A, lanes 8 and 9) and adenine

Table 2. WRN excision of paired and mispaired substrates

Primer:Template	Exonuclease activity of WRN (fmol DNA/min)	
	100 fmol WRN	500 fmol WRN
C:G	1.06 ± 0.32	3.86 ± 0.81
T:G	0.13 ± 0.03	0.42 ± 0.05
G:G	0.53 ± 0.17	1.92 ± 0.47
A:G	0.65 ± 0.19	2.01 ± 0.15
G:C	0.46 ± 0.49	1.77 ± 1.04
A:C	1.11 ± 1.02	3.29 ± 1.46
T:C	0.55 ± 0.33	1.63 ± 0.83
C:C	0.50 ± 0.39	1.84 ± 0.88

Values represent the mean ± SD of three independent experiments.

(Figure 1A, lanes 11 and 12) when paired opposite guanine, but poorly excised thymine (Figure 1A, lanes 5 and 6). The specific activity of the WRN exonuclease on these 3' recessed substrates opposite G was C > A ≈ G > T (Table 2). On 3' recessed substrates containing correctly or mispaired bases opposite cytosine, the WRN exonuclease was active on all four substrates (Figure 1B). In this case, the WRN exonuclease preferred an A:C mispair, with A > G ≈ T ≈ C (Table 2). Given the ability of WRN to efficiently excise 3' mismatches, we examined for cooperativity with pol β, since a mismatched primer terminus is a poor substrate for pol β primer extension (3,4). WRN excised a G:G mispair, resulting in a predominantly 14 nt product (Figure 1C, left panel, lane 2). We observed limited extension by pol β alone (Figure 1C, left panel, lane 3), however, the presence of WRN resulted in a 15-fold increase in the percent of products extended by pol β (Figure 1C, left panel, lane 4). Similarly, on a recessed substrate containing a 3' C:C mispair, WRN was able to excise the mismatch (Figure 1C, middle panel, lane 2) allowing for incorporation by pol β (Figure 1C, middle panel, lane 4). WRN increased the percent of extended products 10-fold (Figure 1C, middle panel, lane 4) compared to pol β alone (Figure 1C, middle panel, lane 3). As expected, on a recessed substrate with correctly paired 3' termini, pol β extension was similar in the absence or presence of WRN (Figure 1C, right panel, lanes 3 and 4). These results suggest that the WRN exonuclease has the capability to excise mismatches following misincorporation of nucleotides by pol β.

Based on our findings that the WRN exonuclease removed several 3' mismatches in the context of a recessed DNA substrate, we next wanted to determine whether the WRN helicase could unwind gap substrates containing a 3' mismatch. As shown in Figure 2A, WRN was able to displace the downstream oligonucleotide from substrates with either correctly paired nucleotides (lane 2) or G:G (lane 4) and A:G (lane 6) mismatches. The gapped substrates unwound by WRN migrated to the same position as a recessed substrate, which was used as a marker (Figure 2A, lane 7). Following our observations that WRN unwound from a gap containing a 3' mismatch, we next evaluated both exonuclease and helicase activities of WRN on the same substrates, with and without pol β. We found that similar to the recessed substrates, WRN was able to degrade from gapped substrates containing correctly paired 3' termini (Figure 2B, lanes 2 and 4) or A:G mispairs (Figure 2B, lanes 6 and 8). In addition, pol β incorporated predominantly 1 nt (Figure 2B, lanes 3 and 7). However, the presence of WRN resulted in a stimulation of

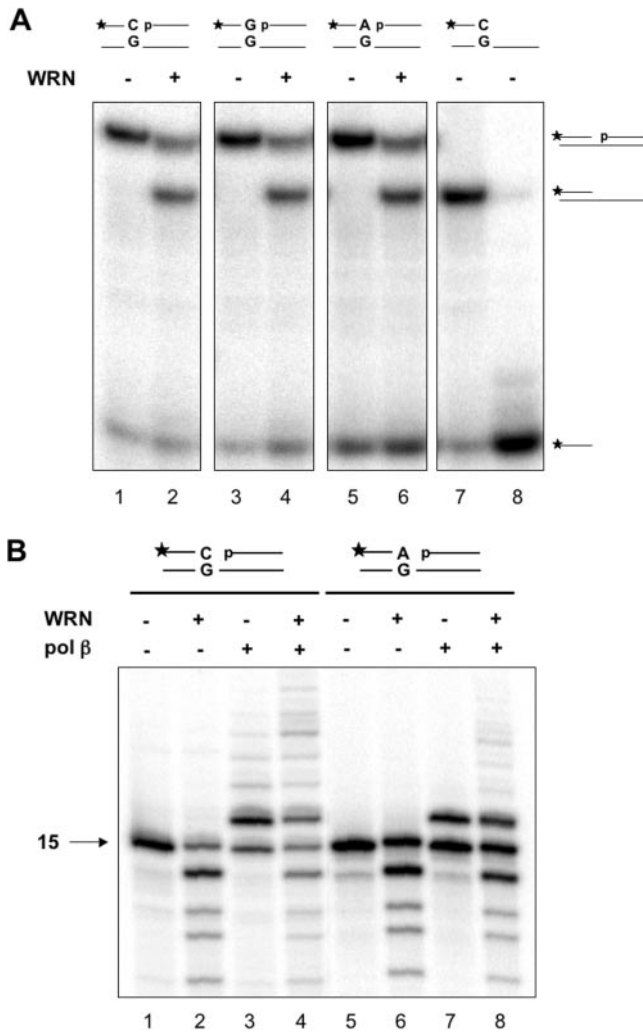


Figure 2. WRN exonuclease and helicase act on gapped substrates with 3' internal mismatches together with pol β . (A) A DNA substrate (250 fmol), containing correctly base paired (15P/pG18/34G, lanes 1 and 2) or mispaired (15MMG/pG18/34G, lanes 3 and 4; 15MMA/pG18/34G, lanes 5 and 6) nucleotides opposite guanine in the context of a 1 nt gap, was incubated with WRN (125 fmol) for 15 min at 37°C. Reaction products were run on a native polyacrylamide gel and visualized using a PhosphorImager. Lane 7, 15P/34G (250 fmol). Lane 8, heat denatured 15P/34G (250 fmol). (B) Reactions containing WRN (250 fmol, lanes 2, 4, 6 and 8) or pol β (10 fmol, lanes 3, 4, 7 and 8) were incubated for 15 min at 37°C with gapped DNA substrates (250 fmol) containing correctly paired (15P/pG18/34G, lanes 1–4) or mispaired (15MMA/pG18/34G, lanes 5–8) nucleotides. Products were separated on a denaturing polyacrylamide gel and visualized using a PhosphorImager. The position of the 15 nt labeled oligonucleotide is indicated. Extended products are indicated by the bracket.

pol β strand displacement synthesis and longer products (Figure 2B, lanes 4 and 8). These results demonstrate that both WRN helicase and exonuclease activities can potentially function together on BER strand break intermediates containing correct or mispaired 3' termini.

Based on our *in vitro* data that WRN helicase and exonuclease activities cooperate with pol β on BER intermediates, we next focused on whether WRN participates with pol β *in vivo* during BER. We therefore generated stable cell lines containing either an siRNA targeting WRN mRNA (WRN KD) or an siRNA negative control (WT) with a

sequence that has no significant similarity to human genes (see Materials and Methods). We determined by real-time RT-PCR that WRN mRNA levels were decreased 3-fold in WRN KD cells compared to WT cells (data not shown). In addition, the WRN KD cells contained undetectable levels of WRN protein compared to WT cells, while levels of actin were similar between the two cell lines (Figure 3A). The reduction in WRN levels in the stable cell lines was maintained even after 20 passages (data not shown). Furthermore, while WRN protein levels were significantly reduced in the KD cells, pol β levels were not affected (Figure 3B). To confirm that the WRN KD cells behaved similar to WS cells, we measured cellular survival by colony formation following treatment with mitomycin C (MMC) or camptothecin (CPT). WRN KD cells were more sensitive to both MMC and CPT (data not shown). Similar results were also observed following treatment of WT and WRN KD cells with MMC or CPT and measurement of cellular proliferation using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, data not shown). These results are consistent with previously published reports demonstrating that cells lacking the WRN protein display increased sensitivity to CPT and DNA cross-linking agents (22–25). In summary, these results demonstrate that the WRN KD cells have significantly reduced levels of WRN protein, and that knock-down of WRN confers sensitivity to DNA damaging agents similar to that of WS cells.

As pol β -mediated BER is important for the repair of alkylation-induced DNA base damage, we investigated the influence of WRN on cellular survival in the presence of the methylating agent MMS. MMS is a S_N2 methylating agent and can generate 7-methylguanine, 3-methyladenine, 1-methyladenine and 3-methylcytosine lesions (26). Following incubation of cells with MMS, and measurement of cellular survival, we found that WRN KD cells were more sensitive to MMS compared to WT cells (Figure 4A). The lethal dose at 37% (LD37) for WRN KD cells was 0.68 mM MMS and 1.15 mM MMS for WT cells. Therefore, the down-regulation of WRN resulted in a 1.7-fold increased sensitivity to MMS. We also investigated the sensitivity of WS fibroblasts to MMS. Similar to the results observed with WRN KD cells, we found that WS cells were hypersensitive to MMS compared to normal cells (Figure 4B). The LD37 for WS and WT cells was 0.15 and 0.75 mM MMS, respectively. Thus, WS cells displayed a 5-fold higher sensitivity to MMS compared to WT cells. As MMS-mediated DNA damage is repaired, at least in part, by SN BER, these results suggest that WRN plays a role in SN BER. To investigate a role for WRN in LP BER of MMS-induced DNA lesions, we performed survival assays in the presence of MX. MX can react with the aldehydic C1' atom of an abasic site rendering it refractory to β -elimination by pol β dRP lyase activity (19), thus blocking SN BER. Incubation of WRN KD cells with MMS in the presence of MX resulted in decreased cellular survival compared to WT cells (Figure 4C). WRN KD cells were 1.6-fold more sensitive to MMS in the presence of MX compared to WT cells, as determined by the LD37 (0.35 and 0.58 mM MMS for WRN KD and WT cells, respectively). Thus together, these results suggest that WRN is also important for the repair of MMS-induced DNA damage via both the SN and LP pathway of BER.

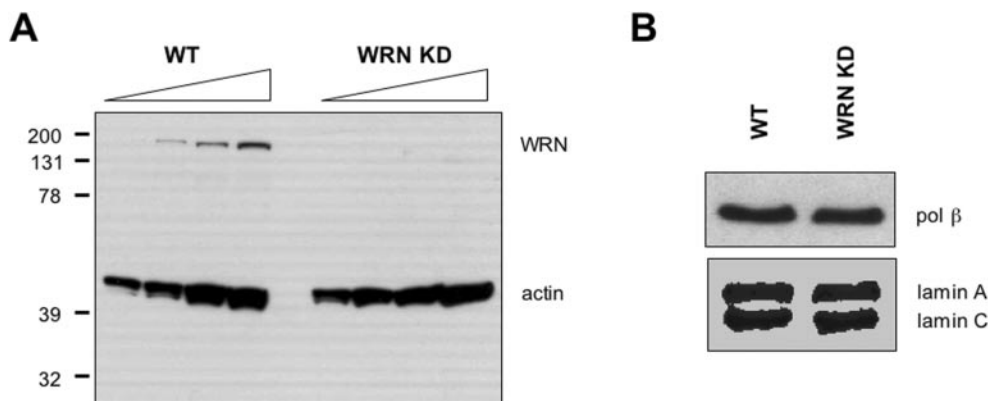


Figure 3. Characterization of WRN KD cells. (A) Levels of WRN. Pellets from WT and WRN KD cells were lysed in sample buffer and increasing volumes (1, 2, 5 or 10 μ l) as indicated were loaded on 10% SDS-PAGE gels. Western blots were performed using anti-WRN and anti-actin primary antibodies as indicated. (B) Levels of pol β . Equal concentrations of WT and WRN KD extracts were loaded on SDS-PAGE gels. Western blots were performed using anti-pol β and anti-lamin A/C primary antibodies as indicated.

To further examine the contribution of WRN in BER, we developed a modified BER assay in which the first nucleotide incorporated was [32 P]dCMP and the second incorporated by LP BER was a chain terminating ddNMP instead of a normal dNMP. This assay specifically examines pol β -mediated LP BER activity as ddNTPs can be utilized by pol β , but not by the replicative polymerases. In addition, since the substrate is a 35 bp oligonucleotide duplex, contributions by PCNA-dependent polymerases are excluded. Thus, the labeled *in vitro* BER products are the fully repaired and ligated 35mer SN BER product, the 16mer product representing a LP BER intermediate (2 nt incorporated), and the 15mer product (1 nt incorporated) representing either SN or LP BER intermediates (Figure 5A). Since the fate of the 15mer product could eventually be either SN or LP BER, quantification of this product was not included (see below). In the experiment shown in Figure 5B, the BER reaction was conducted with extracts from WT and WRN KD cells and the reaction products were analyzed after a 10 min incubation. The results indicated that the amount of the SN BER product in the WRN KD extract was slightly reduced (1.2-fold) compared with WT extract, and the LP BER (2 nt incorporated) product was reduced 2-fold (Figure 5B, compare lanes 1 and 2). A time-course of *in vitro* BER with WT and WRN KD extracts revealed that WRN KD extracts had reduced levels (\sim 2-fold) of the LP BER intermediate (16mer) at each time point examined (Figure 5C and D). The quantitation of LP BER products is shown in Figure 5D and demonstrates a deficiency in LP BER in the WRN KD extracts compared with the WT extracts. While the experiment shown in Figure 5D represents one of four similar assays, the average fold-decrease in the LP BER product (16mer), as a function of WRN down-regulation, is shown in Figure 5E. Thus, our results demonstrate a role for WRN in cell extract-mediated LP BER.

DISCUSSION

In this study, we characterized WRN exonuclease activity on 3' mismatches in cooperation with pol β and examined a cellular role for WRN in BER. We found that the WRN

exonuclease removed 3' mismatches in the context of recessed and gapped substrates. In addition, WRN and pol β acted cooperatively on BER intermediates containing 3' mismatches. We also demonstrated that a down-regulation of WRN expression resulted in elevated cellular sensitivity to MMS and greatly reduced LP BER *in vitro* using whole cell extract assays. These findings provide further support that WRN participates in pol β -directed repair of DNA damage.

We have previously demonstrated a physical and functional interaction between WRN and pol β (14). Furthermore, we have recently shown that WRN unwinds several single-strand break BER intermediates, and that this unwinding activity is regulated by the presence of APE1 and pol β (27). As WRN also possesses 3'-5' exonuclease activity, and pol β lacks proofreading activity, we examined in this report for cooperativity between WRN and pol β on 3' mismatch-containing substrates. The WRN exonuclease degraded most of the 3' mispairs examined, and in doing so, was able to promote incorporation by pol β . Furthermore, both WRN exonuclease and helicase activities operated together, and in cooperation with pol β , to facilitate processing of strand break intermediates. As shown in Figure 2B, the primer strand was degraded by WRN, albeit to a lesser extent, even in the presence of ongoing DNA synthesis by pol β . This may indeed be reflective of an initial 3' end processing by exonucleases, such as WRN, on certain DNA lesions *in vivo*, or alternatively, degradation of the 3' termini may be limited by the cellular localization of WRN and/or the concentration of WRN relative to pol β at the site of the DNA damage. In a similar fashion, the 5' termini may determine whether pol β inserts predominantly 1 nt, or strand displacement synthesis is required to facilitate repair. For example, if the 5' dRP is oxidized and refractory to pol β dRP lyase activity, WRN helicase activity could stimulate pol β strand displacement synthesis *in vivo* and promote LP BER. In conclusion, while the WRN exonuclease did not preferentially excise 3' mismatches, these findings coupled with our previous results suggest a model whereby the two catalytic activities of WRN may be important and may cooperate during pol β -directed BER.

As shown here for WRN, APE1 (28) and TREX1 (29,30) have also been found to excise nucleotides misincorporated

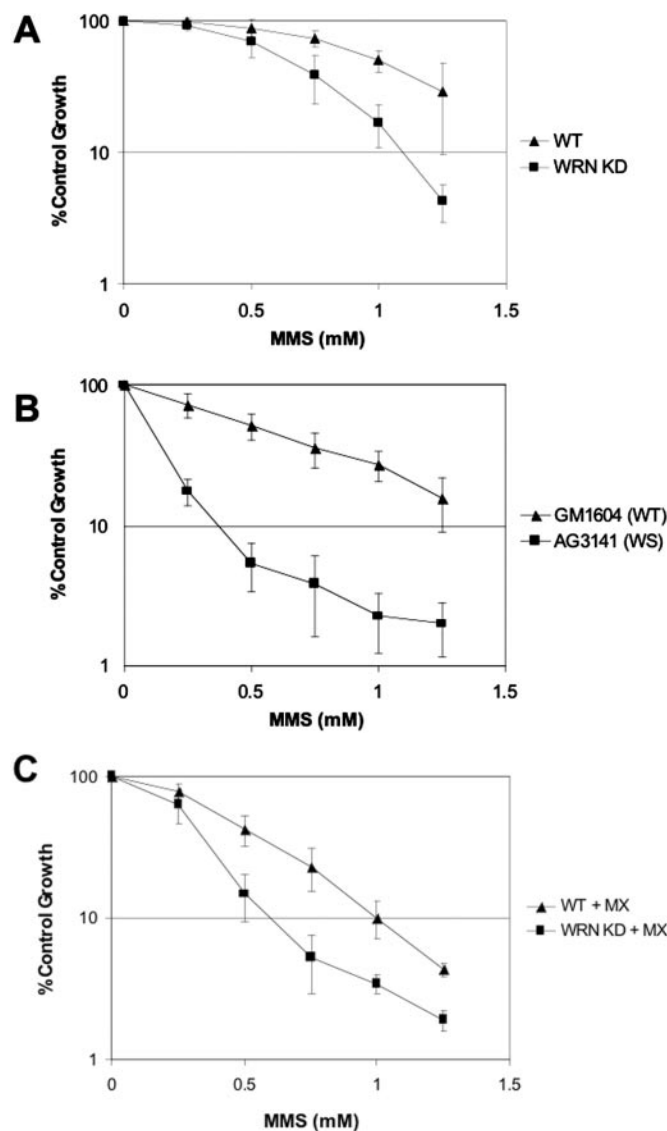


Figure 4. WRN KD cells show elevated sensitivity to methylation damage. (A) WT and WRN KD cells were incubated in the absence or presence of MMS (0–1.25 mM) for 1 h at 37°C. Survival curves were generated as described in Materials and Methods. Values represent the mean \pm SD of four experiments. (B) WT (GM1604) and WS (AG03141) cells were incubated in the absence or presence of MMS (0–1.25 mM) for 1 h at 37°C. Survival curves were generated as described in Materials and Methods. Values represent the mean \pm SD of three or four experiments. (C) WT and WRN KD cells were incubated with MMS as indicated in the presence of 30 mM MX for 1 h at 37°C. Cells were then incubated for an additional 3 h at 37°C with MX (30 mM) alone. Survival curves were generated as described in Materials and Methods. Values represent the mean \pm SEM of three experiments.

by pol β . Thus, these proteins may provide redundant or compensatory editing activities for this error-prone polymerase. Which exonuclease is called upon *in vivo* may be dictated by the intracellular environment, protein–protein interactions, DNA sequence, or the nucleotide mismatch. For example, we note that the mismatch least excised by WRN, T:G, is a preferred mismatch of APE1 (28,31). Cooperativity between WRN and pol β may also be important in certain sequence contexts, such as trinucleotide repeat sequences and guanine-rich regions, which form secondary structures such as

hairpins and G4 quadruplexes. Expansion of triplet repeats by pol β has been shown to be due to its limited strand displacement activity (32), and WRN is efficient at catalyzing the unwinding of many alternate DNA structures including Holliday junctions, triple helices and G4 quadruplexes (33,34). In the event that pol β misincorporates a nucleotide, the WRN exonuclease could excise the mismatch. Thus, WRN may serve to facilitate strand displacement and enhance the fidelity of DNA repair via its combined helicase and exonuclease activities, particularly during LP repair synthesis.

As the understanding of LP BER is continually emerging, as is the number of proteins involved in this repair pathway, which specific protein or protein complex is called upon *in vivo* will likely be determined by the DNA damaging agent and the specific lesion generated. PARP-1 has been identified as a component of a BER protein complex (7) and has been shown to stimulate LP BER in the presence of FEN-1 (8). In addition, it has recently been shown that FEN-1 stimulates LP BER *in vitro* by creating short 1 nt gap products which are favorable substrates for pol β gap-filling activity (35). WRN physically and functionally interacts with many of the proteins involved in LP BER, including pol β , PARP-1 and FEN-1 (36), and in this report we demonstrate for the first time that extracts down-regulated for WRN protein expression are deficient in LP BER. This also represents the first reported association between a human disorder and a specific deficiency in LP BER.

We also demonstrate that both WS and WRN KD cells were hypersensitive to MMS. Consistent with our findings in human cells, Imamura *et al.* (37) observed that WRN^{-/-} chicken DT40 cells displayed elevated sensitivity to MMS. Furthermore, it was recently shown using antisense oligonucleotides that WRN KD cells were hypersensitive to the methylating agents methyl-lexitropsin and temozolomide compared to isogenic controls (38). Together these results suggest that WRN participates in the repair of methylation-induced DNA damage and thus confers cellular survival. This is most likely through a role of WRN in pol β -mediated BER.

In support of this conclusion, we found that extracts from WRN KD cells have reduced SN and LP BER activities *in vitro*, which are consistent with the *in vivo* cellular sensitivity assays. The observation that WRN KD cells were more sensitive to MMS in the presence of MX suggests that WRN participates in LP BER. Consistently, we observed a significant decrease in LP BER using WRN KD extracts *in vitro*. The sensitivity of WRN KD cells to MMS alone, compared to isogenic controls, indicates a role for WRN in SN BER. In addition, extracts from WRN KD cells had slightly lower SN BER of a uracil-containing substrate. The observation that the survival studies with MMS alone were more dramatic than the *in vitro* BER results may reflect an additional role for WRN in the processing of stalled replication forks and/or DNA double-strand break (DSB) repair. MMS-induced DNA damage can result in the stalling of replication forks (39,40) and the formation of DSBs, as evidenced by the phosphorylation of histone H2AX (41,42). In addition, when cells lacking pol β are treated with MMS, unrepaired single-strand breaks are converted to DSBs during S phase, and become substrates for homologous recombination (43). Thus, if WRN participates in the BER of MMS-induced DNA damage,

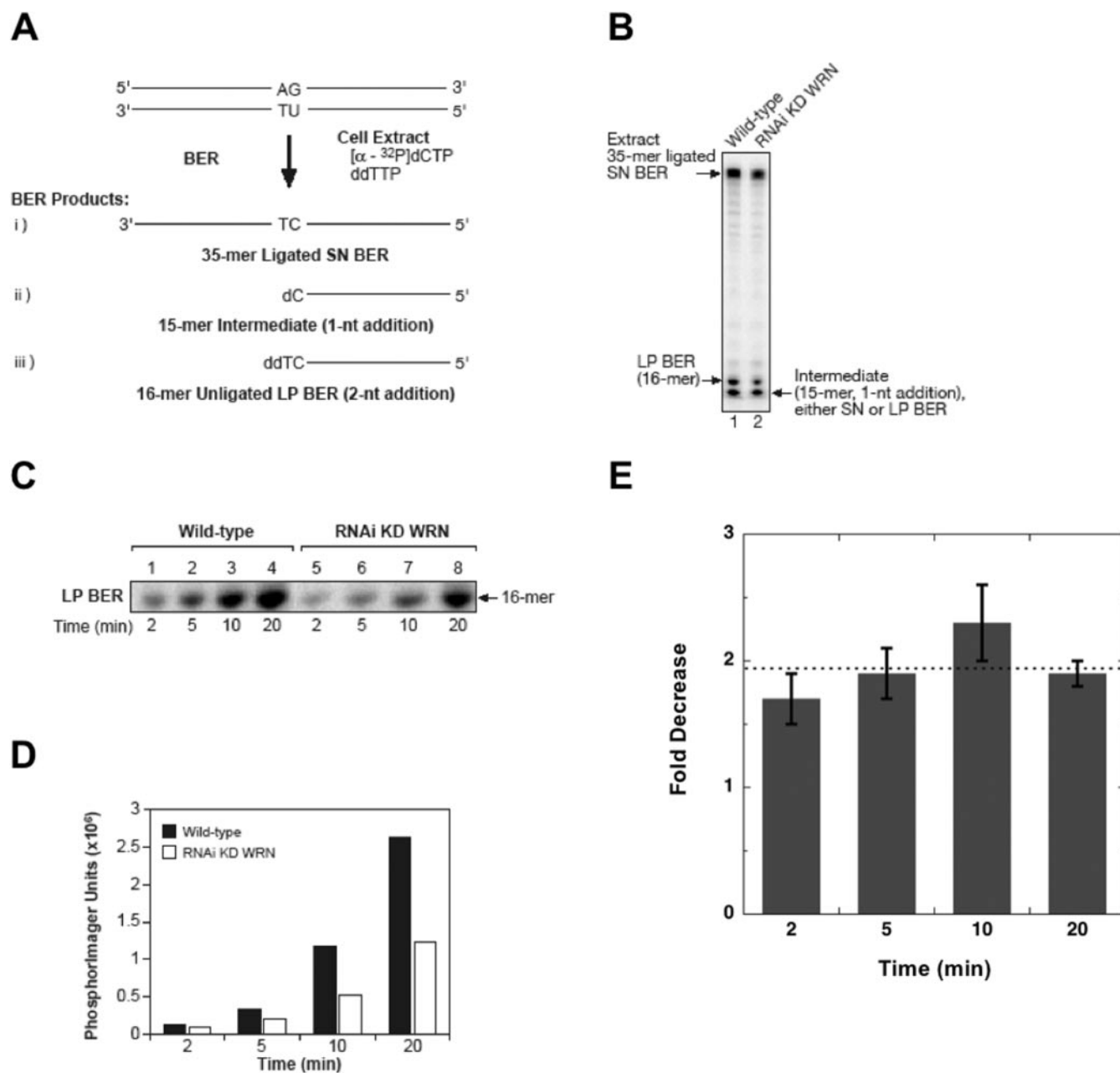


Figure 5. Comparison of uracil-DNA mediated BER in extracts from WT and WRN KD cells. (A) Substrate DNA and predicted BER reaction products and intermediates. The sizes of intermediates (LP BER, 2 nt addition; SN or LP BER, 1 nt addition) and complete BER (ligated SN BER) product are shown. (B and C) Phosphorimages of denaturing polyacrylamide gels showing *in vitro* BER products. The reaction conditions and products analyses are described in Materials and Methods. A 35 bp duplex substrate containing a uracil residue at position 15 was incubated with [32 P]dCTP and ddTTP and WT or WRN KD cell extracts, as indicated. The positions of the BER products are indicated. (D) Quantitation of LP BER products generated in (C). The gel was scanned using a phosphorimager and the BER products were quantified. Products (arbitrary phosphorimager units) were plotted as a function of incubation time. (E) The average fold-decrease in WRN KD compared to WT cell extracts from four experiments is plotted. The dotted line represents the average decrease in LP BER as a function of WRN down-regulation.

as suggested by this study, the absence of WRN may lead to increased replication fork arrest and/or DSBs during replication. As WRN has also been shown to participate in DSB repair (25,44), the decreased cellular survival of WRN KD cells observed here may also be due in part to WRN's role in DSB repair.

Recently Szekeley *et al.* (45) reported that the KD of WRN in primary human fibroblasts results in increased DNA damage following oxidative stress, particularly in non-dividing cells.

As BER is important for the repair of both oxidation and alkylation-mediated DNA damage, our results presented here are consistent with their findings, and with a specific role for WRN in BER.

ACKNOWLEDGEMENTS

The authors thank Marit Otterlei and Julie Horton for helpful discussions. Nadja Souza-Pinto, Heng-Kuan Wong and Tina

Thorslund are thanked for critical reading of the manuscript. We thank Jerry Shay (University of Texas Southwestern Medical Center, Dallas, Texas) for kindly providing GM01604 and AG03141 cell lines. Support for this research and Funding to pay the Open Access publication charges for this article was provided by the Intramural Program of the National Institute on Aging, NIH.

Conflict of interest statement. None declared.

REFERENCES

- Ames, B.N. and Shigenaga, M.K. (1992) Oxidants are a major contributor to aging. *Ann. N. Y. Acad. Sci.*, **663**, 85–96.
- Osheroff, W.P., Jung, H.K., Beard, W.A., Wilson, S.H. and Kunkel, T.A. (1999) The fidelity of DNA polymerase beta during distributive and processive DNA synthesis. *J. Biol. Chem.*, **274**, 3642–3650.
- Kosa, J.L. and Sweasy, J.B. (1999) The E249K mutator mutant of DNA polymerase beta extends mispaired termini. *J. Biol. Chem.*, **274**, 35866–35872.
- Yang, L., Beard, W., Wilson, S., Roux, B., Broyde, S. and Schlick, T. (2002) Local deformations revealed by dynamics simulations of DNA polymerase Beta with DNA mismatches at the primer terminus. *J. Mol. Biol.*, **321**, 459–478.
- Bhagwat, A.S., Sanderson, R.J. and Lindahl, T. (1999) Delayed DNA joining at 3' mismatches by human DNA ligases. *Nucleic Acids Res.*, **27**, 4028–4033.
- Fan, J. and Wilson, D.M., III (2005) Protein–protein interactions and posttranslational modifications in mammalian base excision repair. *Free Radic. Biol. Med.*, **38**, 1121–1138.
- Lavrik, O.I., Prasad, R., Sobol, R.W., Horton, J.K., Ackerman, E.J. and Wilson, S.H. (2001) Photoaffinity labeling of mouse fibroblast enzymes by a base excision repair intermediate. Evidence for the role of poly(ADP-ribose) polymerase-1 in DNA repair. *J. Biol. Chem.*, **276**, 25541–25548.
- Prasad, R., Lavrik, O.I., Kim, S.J., Kedar, P., Yang, X.P., Vande Berg, B.J. and Wilson, S.H. (2001) DNA polymerase beta-mediated long patch base excision repair. Poly(ADP-ribose) polymerase-1 stimulates strand displacement DNA synthesis. *J. Biol. Chem.*, **276**, 32411–32414.
- Harrigan, J.A. and Bohr, V.A. (2003) Human diseases deficient in RecQ helicases. *Biochimie*, **85**, 1185–1193.
- Mushegian, A.R., Bassett, D.E., Jr, Boguski, M.S., Bork, P. and Koonin, E.V. (1997) Positionally cloned human disease genes: patterns of evolutionary conservation and functional motifs. *Proc. Natl Acad. Sci. USA*, **94**, 5831–5836.
- Shevelev, I.V. and Hubscher, U. (2002) The 3'–5' exonucleases. *Nature Rev. Mol. Cell Biol.*, **3**, 364–376.
- Kamath-Loeb, A.S., Shen, J.C., Loeb, L.A. and Fry, M. (1998) Werner Syndrome Protein. II. characterization of the integral 3'→5' DNA exonuclease. *J. Biol. Chem.*, **273**, 34145–34150.
- Huang, S., Beresten, S., Li, B., Oshima, J., Ellis, N.A. and Campisi, J. (2000) Characterization of the human and mouse WRN 3'→5' exonuclease. *Nucleic Acids Res.*, **28**, 2396–2405.
- Harrigan, J.A., Opresko, P.L., von Kobbe, C., Kedar, P.S., Prasad, R., Wilson, S.H. and Bohr, V.A. (2003) The Werner syndrome protein stimulates DNA polymerase beta strand displacement synthesis via its helicase activity. *J. Biol. Chem.*, **278**, 22686–22695.
- Orren, D.K., Brosh, R.M., Jr, Nehlin, J.O., Machwe, A., Gray, M.D. and Bohr, V.A. (1999) 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.*, **27**, 3557–3566.
- Nguyen, L.H., Barsky, D., Erzberger, J.P. and Wilson, D.M., III (2000) Mapping the protein–DNA interface and the metal-binding site of the major human apurinic/apyrimidinic endonuclease. *J. Mol. Biol.*, **298**, 447–459.
- Ouellette, M.M., McDaniel, L.D., Wright, W.E., Shay, J.W. and Schultz, R.A. (2000) The establishment of telomerase-immortalized cell lines representing human chromosome instability syndromes. *Hum. Mol. Genet.*, **9**, 403–411.
- Butler, W.B. (1984) Preparing nuclei from cells in monolayer cultures suitable for counting and for following synchronized cells through the cell cycle. *Anal. Biochem.*, **141**, 70–73.
- Horton, J.K., Prasad, R., Hou, E. and Wilson, S.H. (2000) Protection against methylation-induced cytotoxicity by DNA polymerase beta-dependent long patch base excision repair. *J. Biol. Chem.*, **275**, 2211–2218.
- Horton, J.K., Baker, A., Berg, B.J., Sobol, R.W. and Wilson, S.H. (2002) Involvement of DNA polymerase beta in protection against the cytotoxicity of oxidative DNA damage. *DNA Repair (Amst)*, **1**, 317–333.
- Biade, S., Sobol, R.W., Wilson, S.H. and Matsumoto, Y. (1998) Impairment of proliferating cell nuclear antigen-dependent apurinic/apyrimidinic site repair on linear DNA. *J. Biol. Chem.*, **273**, 898–902.
- Poot, M., Gollahon, K.A. and Rabinovitch, P.S. (1999) Werner syndrome lymphoblastoid cells are sensitive to camptothecin-induced apoptosis in S phase. *Hum. Genet.*, **104**, 10–14.
- Poot, M., Yom, J.S., Whang, S.H., Kato, J.T., Gollahon, K.A. and Rabinovitch, P.S. (2001) Werner syndrome cells are sensitive to DNA cross-linking drugs. *FASEB J.*, **15**, 1224–1226.
- Saintigny, Y., Makienko, K., Swanson, C., Emond, M.J. and Monnat, R.J., Jr (2002) Homologous recombination resolution defect in werner syndrome. *Mol. Cell Biol.*, **22**, 6971–6978.
- Cheng, W.H., von Kobbe, C., Opresko, P.L., Arthur, L.M., Komatsu, K., Seidman, M.M., Carney, J.P. and Bohr, V.A. (2004) Linkage between Werner syndrome protein and the Mre11 complex via Nbs1. *J. Biol. Chem.*, **279**, 21169–21176.
- Sedgwick, B. (2004) Repairing DNA-methylation damage. *Nature Rev. Mol. Cell Biol.*, **5**, 148–157.
- Ahn, B., Harrigan, J.A., Indig, F.E., Wilson, D.M., III and Bohr, V.A. (2004) Regulation of WRN helicase activity in human base excision repair. *J. Biol. Chem.*, **279**, 53465–53474.
- Chou, K.M. and Cheng, Y.C. (2002) An exonucleolytic activity of human apurinic/apyrimidinic endonuclease on 3' mispaired DNA. *Nature*, **415**, 655–659.
- Hoss, M., Robins, P., Naven, T.J., Pappin, D.J., Sgouros, J. and Lindahl, T. (1999) A human DNA editing enzyme homologous to the *Escherichia coli* DNAQ/MutD protein. *EMBO J.*, **18**, 3868–3875.
- Mazur, D.J. and Perrino, F.W. (1999) Identification and expression of the TREX1 and TREX2 cDNA sequences encoding mammalian 3'→5' exonucleases. *J. Biol. Chem.*, **274**, 19655–19660.
- Hadi, M.Z., Ginalski, K., Nguyen, L.H. and Wilson, D.M., III (2002) Determinants in nuclease specificity of Ape1 and Ape2, human homologues of *Escherichia coli* exonuclease III. *J. Mol. Biol.*, **316**, 853–866.
- Hartenstine, M.J., Goodman, M.F. and Petruska, J. (2002) Weak strand displacement activity enables human DNA polymerase beta to expand CAG/CTG triplet repeats at strand breaks. *J. Biol. Chem.*, **277**, 41379–41389.
- Brosh, R.M., Jr, Majumdar, A., Desai, S., Hickson, I.D., Bohr, V.A. and Seidman, M.M. (2001) Unwinding of a DNA triple helix by the werner and bloom syndrome helicases. *J. Biol. Chem.*, **276**, 3024–3030.
- Mohaghegh, P., Karow, J.K., Brosh, J.R., Jr, Bohr, V.A. and Hickson, I.D. (2001) The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. *Nucleic Acids Res.*, **29**, 2843–2849.
- Liu, Y., Beard, W.A., Shock, D.D., Prasad, R., Hou, E.W. and Wilson, S.H. (2005) DNA polymerase beta and flap endonuclease I enzymatic specificities sustain DNA synthesis for long patch base excision repair. *J. Biol. Chem.*, **280**, 3665–3674.
- Opresko, P.L., Cheng, W.H., von Kobbe, C., Harrigan, J.A. and Bohr, V.A. (2003) Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. *Carcinogenesis*, **24**, 791–802.
- Imamura, O., Fujita, K., Itoh, C., Takeda, S., Furuichi, Y. and Matsumoto, T. (2002) Werner and Bloom helicases are involved in DNA repair in a complementary fashion. *Oncogene*, **21**, 954–963.
- Blank, A., Bobola, M.S., Gold, B., Varadarajan, S., Kolstoe, D., Meade, E.H., Rabinovitch, P.S., Loeb, L.A. and Silber, J.R. (2004) The Werner syndrome protein confers resistance to the DNA lesions N3-methyladenine and O6-methylguanine: implications for WRN function. *DNA Repair (Amst)*, **3**, 629–638.
- Boiteux, S. and Laval, J. (1982) Mutagenesis by alkylating agents: coding properties for DNA polymerase of poly (dC) template containing 3-methylcytosine. *Biochimie*, **64**, 637–641.

40. Larson, K., Sahn, J., Shenkar, R. and Strauss, B. (1985) Methylation-induced blocks to *in vitro* DNA replication. *Mutat. Res.*, **150**, 77–84.
41. Nikiforov, A., Svetlova, M., Solovjeva, L., Sasina, L., Siino, J., Nazarov, I., Bradbury, M. and Tomilin, N. (2004) DNA damage-induced accumulation of Rad18 protein at stalled replication forks in mammalian cells involves upstream protein phosphorylation. *Biochem. Biophys. Res. Commun.*, **323**, 831–837.
42. Liu, J.S., Kuo, S.R. and Melendy, T. (2003) Comparison of checkpoint responses triggered by DNA polymerase inhibition versus DNA damaging agents. *Mutat. Res.*, **532**, 215–226.
43. Pascucci, B., Russo, M.T., Crescenzi, M., Bignami, M. and Dogliotti, E. (2005) The accumulation of MMS-induced single strand breaks in G1 phase is recombinogenic in DNA polymerase beta defective mammalian cells. *Nucleic Acids Res.*, **33**, 280–288.
44. Prince, P.R., Emond, M.J. and Monnat, R.J., Jr (2001) Loss of Werner syndrome protein function promotes aberrant mitotic recombination. *Genes Dev.*, **15**, 933–938.
45. Szekeley, A.M., Bleichert, F., Numann, A., Van, K.S., Manasanch, E., Ben, N.A., Canaan, A. and Weissman, S.M. (2005) Werner protein protects nonproliferating cells from oxidative DNA damage. *Mol. Cell. Biol.*, **25**, 10492–10506.