DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA-end resection in human cells

Sturzenegger, Andreas; Burdova, Kamila; Kanagaraj, Radhakrishnan; Levikova, Maryna; Pinto, Cosimo; Cejka, Petr; Janscak, Pavel

Abstract: The 5’-3’ resection of DNA ends is a prerequisite for repair of DNA double-strand breaks by homologous recombination, microhomology-mediated end joining and single-strand annealing. Recent studies in yeast have shown that following an initial DNA-end processing by the Mre11-Rad50-Xrs2 complex and Sae2, extension of resection tracts is mediated either by Exonuclease 1 or by combined activities of the RecQ-family DNA helicase Sgs1 and the helicase/endonuclease Dna2. Although human DNA2 was shown to cooperate with the BLM helicase to catalyze resection of DNA ends, it remains a matter of debate whether another human RecQ helicase, namely WRN, can substitute for BLM in DNA2-catalyzed resection. Here, we present evidence that WRN and BLM act epistatically with DNA2 to promote long-range resection of DSB ends in human cells. Our biochemical experiments show that WRN and DNA2 interact physically and coordinate their enzymatic activities to mediate 5’-3’ DNA-end resection in a reaction dependent on RPA. In addition, we present in vitro and in vivo data suggesting that BLM promotes DNA-end resection as part of the BLM-TOPOIIIα-RMI1-RMI2 complex. Our study provides new mechanistic insights into the process of DNA-end resection in mammalian cells.

DOI: https://doi.org/10.1074/jbc.M114.578823

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-98797
Journal Article
Published Version

Originally published at:
Sturzenegger, Andreas; Burdova, Kamila; Kanagaraj, Radhakrishnan; Levikova, Maryna; Pinto, Cosimo; Cejka, Petr; Janscak, Pavel (2014). DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA-end resection in human cells. Journal of Biological Chemistry, 289(39):27314-27326.
DOI: https://doi.org/10.1074/jbc.M114.578823
DNA2 Cooperates with the WRN and BLM RecQ Helicases to Mediate Long-range DNA End Resection in Human Cells*

Andreas Sturzenegger1, Kamila Burdova1, Radhakrishnan Kanagaraj1,2, Maryna Levikova1, Cosimo Pinto†, Petr Cejka‡ and Pavel Janscak†§

From the 1Institute of Molecular Cancer Research, University of Zurich, 8057 Zurich, Switzerland and the 5Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 14300 Prague, Czech Republic

Background: DNA end resection is a critical step in the homology-directed repair of DNA double strand breaks (DSBs).

Results: Human WRN helicase stimulates the DNA2-catalyzed resection of DNA ends and acts in concert with DNA2 to promote DSB repair by single strand annealing.

Conclusion: DNA2 cooperates with WRN or BLM to mediate the resection of DSBs in mammalian cells.

Significance: Defects in DNA end resection might, in part, account for the genomic instability phenotype of Werner syndrome.

The 5’-3’ resection of DNA ends is a prerequisite for the repair of DNA double strand breaks by homologous recombination, microhomology-mediated end joining, and single strand annealing. Recent studies in yeast have shown that, following initial DNA end processing by the Mre11-Rad50-Xrs2 complex and Sae2, the extension of resection tracts is mediated either by exonuclease 1 or by combined activities of the RecQ family DNA helicase Sgs1 and the helicase/endonuclease Dna2. Although human DNA2 has been shown to cooperate with the BLM helicase to catalyze the resection of DNA ends, it remains a matter of debate whether another human RecQ helicase, WRN, can substitute for BLM in DNA2-catalyzed resection. Here we present evidence that WRN and BLM act epistatically with DNA2 to promote the long-range resection of double strand break ends in human cells. Our biochemical experiments show that WRN and DNA2 interact physically and coordinate their enzymatic activities to mediate 5’-3’ DNA end resection in a reaction dependent on RPA. In addition, we present in vitro and in vivo data suggesting that BLM promotes DNA end resection as part of the BLM-TOPOIIIα-RMI1-RMI2 complex. Our study provides new mechanistic insights into the process of DNA end resection in mammalian cells.

DNA double strand breaks (DSBs)4 are a very dangerous form of DNA damage because they can cause cell death or chromosomal rearrangements, a hallmark of cancer (1). DSBs can occur accidentally during normal cellular metabolism or upon exposure of cells to exogenous agents such as ionizing radiation and radiomimetic drugs (2). There are also programmed DSBs that drive recombination events essential for physiological processes, such as meiosis and lymphocyte development (3, 4). In eukaryotic cells, DSBs are repaired by one of two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ involves ligation of the broken DNA ends and is frequently associated with a short deletion or insertion of DNA at the break site (5). In contrast, HR restores the DNA integrity accurately because it uses sister chromatids or homologous chromosomes as a template for repair (6, 7). HR is initiated by resection of the broken DNA ends to generate 3’ single-stranded (ss) DNA tails that are utilized by the RAD51 recombinase for a homology search on the donor DNA molecule (6, 7). Genetic and biochemical studies in budding yeast have shown that broken DNA ends are resected in a two-step process (8–10). DNA end resection in yeast is initiated by the Mre11-Rad50-Xrs2 complex in conjunction with Sae2 (8, 9, 11). These proteins may initiate resection of the 5’ strand of the broken DNA to remove a stretch of about 100–200 nucleotides from the DNA end (8, 9, 11). The Mre11-Rad50-Xrs2 complex also recruits the components of the long-range resection pathways Exo1 or Dna2-Sgs1 (8–10, 12, 13). Exo1 is a dsDNA-dependent 5’-3’ exonuclease that preferentially degrades DNA substrates with a 3’ ssDNA tail in a reaction stimulated by the ssDNA-binding protein RPA (13). DNA2 is a ssDNA-specific nuclease and a DNA helicase that functions in conjunction with the RecQ family DNA helicase Sgs1 and RPA to catalyze long-range DNA end resection (10, 14). In this reaction, RPA stimulates DNA unwinding by Sgs1 and promotes degradation of the 5’-terminated strand by Dna2 while protecting the growing 3’ ssDNA tail (10). DNA end resection is also the initial step in two other DSB repair pathways, single strand annealing (SSA) and microhomology-mediated end joining (8, 15).

The molecular machinery of DNA end resection appears to be largely conserved between yeast and man (15–19). However, it remains a matter of debate which DNA helicase mediates DNA2-catalyzed resection in mammalian cells. Mammals pos-
The Role of WRN and BLM in DNA End Resection

**EXPERIMENTAL PROCEDURES**

**Antibodies and siRNA**—Primary antibodies used for immunoblotting were as follows: mouse monoclonal anti-WRN (BD Biosciences, catalog no. 611169), rabbit polyclonal anti-DNA2 (Abcam, catalog no. ab96488), rabbit polyclonal anti-BLM (Abcam, catalog no. ab476), rabbit polyclonal anti-TFIIH (Santa Cruz Biotechnology, catalog no. sc293), mouse monoclonal anti-FLAG (Sigma, catalog no. F1804), and rabbit polyclonal anti-RMI1 (Proteintech, catalog no. 14630-1-AP). Anti-FLAG M2 magnetic beads (Sigma) were used for immunoprecipitation. Primary antibodies used for immunofluorescence staining were as follows: mouse monoclonal anti-RPA2 (Abcam, catalog no. ab2175) and rabbit monoclonal anti-γ-H2AX (Cell Signaling Technology, catalog no. 97185). Rabbit polyclonal anti-WRN antibody used for immunoprecipitation has been described previously (24).

All siRNA oligoduplexes used in this study were purchased from Microsynth. The sequences of the sense strands of these duplexes were as follows: siLuc, 5′-CGUACGCGGAAUCUGCGAdTdT-3′; siWRN, 5′-UAGGGAACUACUCUUGGCA-AdTdT-3′; siBLM, 5′-CCGAUCAUGUGAAGCUAAGAdTdT-3′; siDNA2, 5′-UACCACUCUAACUGUAAGGACAdTdT-3′; siEXO1, 5′-CAGCAACUCAUUACUGCAUAdTdT-3′; siRMI1, 5′-AGGCAACUCAUGUAAGTdTdT-3′ (25); siCtIP, 5′-UCCCAAAACUUAUCCAUAdTdT-3′ (26); and siRMI1, 5′-AGCCUUCACGAAUGUUGAAdTdT-3′ (27).

**Plasmid Constructions**—The human DNA2 (hDNA2) ORF was amplified by PCR without the initiation and stop codons to generate a fragment including ggatcc-hDNA2-ctcgag. After digestion with BamHI and XhoI, the hDNA2 fragment was cloned into pFLAG-CMV2 (Sigma) digested with BglII/Sall (pFLAG-CMV2-hDNA2). The human WRN (hWRN) ORF was inserted into pcDNA3.1/Hygro− (Invitrogen) via the Nhel and Dral sites (pcDNA3.1-hWRN). The siRNA-resistant form of this construct was generated by changing four nucleotides in the siWRN-targeting region (T270C, A273G, G276C, and A279G) using the QuikChange site-directed mutagenesis kit (Stratagene).

**Protein Purifications**—Wild-type and mutant forms of WRN, BLM, EXO1, and RPA were produced and purified as described previously (28–31). The TOPOIIα–RMI1–RMI2 (TRR) complex was a gift from Drs. Kata Sarlos and Ian Hickson (University of Copenhagen, Denmark). DNA2 was produced as a fusion with a His8 tag (N terminus) and a FLAG tag (C terminus) in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen). The transfer vector for bacmid preparation was a gift from Dr. Judith L. Campbell (32). The transfer vectors for nuclease-deficient (D227A) and helicase-deficient (K654R) mutants of DNA2 were generated using the QuikChange site-directed mutagenesis kit (Stratagene). SF9 cells expressing DNA2 fusion proteins were harvested 52 h after infection (typically a 800-ml culture) and washed with PBS. All subsequent steps were carried out at 4°C. Pelleted cells were resuspended in lysis buffer (25 mM Tris-HCl (pH 7.5), 2 mM β-mercaptoethanol, 10 mM EDTA-free protease inhibitor (Roche), 1 mM phenylmethylsulfonyl fluoride, 30 μg/ml leupeptin, and 15 mM imidazole) and incubated for 20 min under continuous stirring. Subsequently, glycerol and 5 mM NaCl were added slowly to final concentrations of 15% (v/v) and 300 mM, respectively, while mixing the sample. The cell suspension was then incubated for an additional 30 min under continuous stirring. The cell lysate was centrifuged at 55,000 × g for 30 min to obtain soluble extract, which was then incubated with 5 ml of nickel-nitrotriacetic acid-agarose beads (Qiagen) for 1 h batchwise. The resin was washed extensively with lysis buffer containing 10% (v/v) glycerol and 1 mM NaCl. The protein was eluted with lysis buffer supplemented with 10% (v/v) glycerol, 100 mM NaCl, and 250 mM imidazole. Fractions containing detectable amounts of protein, as measured by Bradford assay, were pooled, diluted 1:1 with TBS buffer (50 mM Tris-HCl (pH 7.5) and 150 mM NaCl) and incubated batchwise with 1 ml of anti-FLAG M2 affinity resin (Sigma) for 30 min. The resin was then transferred to a gravity flow column and washed with TBS-PI buffer (TBS buffer containing 1 mM β-mercaptoethanol and 5 μg/ml leupeptin). Elution of the protein was achieved by adding TBS-PI buffer supplemented with 200 μg/ml 3× FLAG peptide (Sigma). Fractions containing DNA2 were pooled, diluted with 0.5 volumes of water and 1 volume of AQ buffer (25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol, and 5 mM β-mercaptoethanol) and loaded onto a 1-ml HiTrap Q column (GE Healthcare) pre-equilibrated with AQ buffer. The column was washed with AQ buffer and DNA2 was eluted by AQ buffer supplemented with 600 mM NaCl. Fractions containing DNA2 were identified by SDS-PAGE, pooled, and stored at −80°C. The activity of purified recombinant DNA2 proteins was tested using a Y structure oligonucleotide duplex with single-stranded arms (10). In agreement with previous reports, wild-type DNA2 was found to be capable of degrading both ssDNA arms of this structure (data not shown) (10, 17). In the presence of RPA, the cleavage of the 3′ ssDNA arm by DNA2 was inhibited, and DNA2 degraded preferentially the 5′ ssDNA arm (data not shown) (10, 17). The DNA2-D227A mutant did not contain any nuclease activity, which indicated that the nuclease activity of our wild-type DNA2 preparation was inherent to DNA2 (data not shown).
Nuclease and Helicase Assays—To test the activity of purified DNA2, we used a 31-bp forked duplex with 19-nt ssDNA arms, as described previously (10). The helicase activity of WRN and BLM was tested using a 29-bp forked duplex generated by annealing of the following oligonucleotides: f-9 (5′-ACTAT-CATTG AGTGTTAA CTGACGCTCT GAACTAGT GAGTGA-3′) and f-10 (5′-GAGGT-CAGTGAATTG GAGCTCGCAG TCAATG-3′). Both DNA substrates were radiolabeled at the end of the 5′ ssDNA arm.

Nuclease and helicase assays were performed in buffer containing 25 mM Tris acetate (pH 7.5), 2 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/ml BSA, 10.7 mM phosphocreatine, 0.02 mg/ml creatine phosphokinase, and 1 mM ATP. Reactions contained 2 nM DNA substrate and the indicated concentrations of DNA2 or WRN/BLM. Where indicated, RPA solution (150 mM EDTA, 2% (w/v) SDS, 30% (v/v) glycerol, and 25 mM Tris-acetate (pH 7.5), 2 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/ml BSA, 10.7 mM phosphocreatine, 0.02 mg/ml creatine phosphokinase, and 1 mM ATP. Reactions contained 2 nM DNA substrate (molecules), 8 nM DNA2, 350 nM RPA (100% DNA strand coverage, assuming all DNA was single-stranded), and various concentrations of WRN or BLM as indicated. EXO1 was present at a concentration of 20 nM. The reactions were assembled on ice and initiated by the addition of ATP. Reaction mixtures (15 μl) were incubated at 37 °C for 60 min in the case of protein titration experiments. In time course experiments, 15-μl reaction aliquots were withdrawn at defined time points as indicated. Reactions were terminated as described for the helicase assays. The samples were subjected to electrophoresis in a 1% agarose gel run in 1× TAE buffer. Gels were post-stained with SYBR Gold (Invitrogen) and analyzed using Multimaging Light Cabinet (Alpha Innotech). To monitor resection by hybridization of radiolabeled oligonucleotide probes, terminated reactions (21 μl) were divided equally into two tubes. 5′ end-labeled oligonucleotide probes were then added to a final concentration of 5 nM. This mixture was heated in an oven to 75 °C for 5 min and then slowly cooled down to room temperature over 2.5 h. Reaction products were separated by electrophoresis in a 1% agarose gel. Gels were dried on Whatman MM3 paper and analyzed by phosphorimaging using a Typhoon 9400 scanner (GE Healthcare). Images were quantified using ImageQuant TL software. The relative concentration of the resection products generated in WRN-DNA2 or BLM-DNA2 reactions was calculated as a percentage of the product generated in a reaction containing 20 nM EXO1 at the 2-min time point, which led to 100% resection within the region probed with radiolabeled oligonucleotides. Usually, the EXO1 reaction was loaded on each gel in triplicates. The following oligonucleotides were used for the preparation of the hybridization probes: oligo#224, 5′-GGCGGTCGTCTTTTACAA-GCTGTT-3′ (it anneals to the 3′-terminated strand; annealing position, 112–133 nt upstream of the StuI cleavage site; the complementary sequence is underlined); oligo#227, 5′-GGGA-TAGTTAAAGCCGCTTGGA-3′ (it anneals to the 3′-terminated strand and inserted; annealing position, 353–374 nt upstream of the StuI cleavage site); and oligo#237, 5′-GTTCCGGGGCTGGCT-TAACATTG-3′ (it anneals to the 5′-terminated strand; annealing position, 122–133 nt upstream of the StuI cleavage site). Oligonucleotides were 5′ end-labeled using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs). The two non-complementary dG residues at the 5′ end of the oligonucleotides were added to ensure equal labeling efficiency.

Cell Culture and Transfection—U2OS and HEK293 cells were grown in DMEM (Sigma) supplemented with 10% fetal calf serum (Invitrogen) and streptomycin/penicillin (100 units/ml). Plasmid DNA was transfected using standard linear polyethyleneamine method. Lipofectamine RNAiMAX (Invitrogen) was used for siRNA transfection. To generate HEK293 clones stably expressing FLAG-DNA2, cells were cotransfected with pFLAG-CMV2-hDNA2 and pBABE-puro (Addgene) and subjected to puromycin (1 μg/ml) selection. Puromycin-resistant clones were tested for expression of FLAG-DNA2 by Western blotting.

Immunoprecipitation—HEK293 cells were transfected with the pcDNA3.1-hWRN and/or pFLAG-CMV2-hDNA2 vectors. Cells were harvested to lysis buffer (50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 15 mM sodium pyrophosphate, and 1% Triton X-100) and incubated on ice for 30 min. The lysates were centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were incubated overnight at 4 °C with 5 μl of anti-FLAG M2 agarose beads (Sigma) and washed three times with 1% Triton-TAE. The washed beads were resuspended in TAE buffer and added to the TBE gel. The samples were subjected to electrophoresis on a 12% denaturing gel and stained with SYBR Gold (Invitrogen) and imaged using Multimaging Light Cabinet (Alpha Innotech).
0.5% (v/v) Nonidet P-40) supplemented before use with protease (Complete EDTA-free, Roche) and phosphatase (PhosSTOP, Roche) inhibitors, 2 mM MgCl₂ and benzonase (50 units/ml). Cell extracts (1 mg of protein) were subjected to immunoprecipitation using anti-FLAG M2 magnetic beads (10 μl) or Protein A/G Plus UltraLink Resin (10 μl, Thermo Scientific) coated with rabbit polyclonal anti-WRN antibody (10 μg), which was carried out overnight at 4°C. Immunoprecipitates were washed four times with lysis buffer. Bound proteins were eluted by Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting.

To test the interaction between purified WRN and DNA2 proteins, 500 ng of each protein was mixed in 200 μl of NET-N100 buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 0.5% (v/v) Nonidet P-40) and incubated at 4°C for 4 h. As a control, DNA2 was incubated in the absence of WRN. The protein mixtures were subsequently subjected to immunoprecipitation using anti-WRN antibody (4 μg), which was carried out at 4°C for 2 h. Immunoprecipitates were washed four times with NET-N100 buffer. Bound proteins were eluted by Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting.

**GST Pulldown Assay**—GST-tagged fragments of WRN were produced in the *Escherichia coli* BL21-CodonPlus(DE3)-RII strain (Stratagene) and bound to GSH Sepharose 4B (GE Healthcare) as described previously (24). As a control, beads were coated with GST protein only. The beads were incubated with 500 ng of purified His₆-DNA2-FLAG protein in 400 μl of NET-N100 buffer at 4°C for 2 h. After extensive washing with NET-N100 buffer, proteins bound to the beads were analyzed by Western blotting. Blots were first stained in Ponceau S solution (0.1% (w/v) Ponceau S and 5% (v/v) acetic acid) to visualize WRN fragments and subsequently probed with anti-FLAG antibody.

**Reverse Transcription and Quantitative Real-time PCR**—Total RNA was isolated from cells using the RNaseasy mini kit (Qiagen). 200 ng of RNA was used for cDNA synthesis using a high-capacity cDNA reverse transcription kit (Applied Biosystems). The target gene expression level was determined by quantitative real-time PCR that was performed on a ABI Prism 7300 (Applied Biosystems) using SYBR Select Master Mix (Applied Biosystems). The following primer pairs were used to determine EXO1 mRNA levels: 5'-ACCTCTAAGG AACAAGGTTC-3' (forward) and 5'-AGGAGGAAGGC TTTCTAGAATC-3' (reverse). The housekeeping gene RPLPO, used as a control, was amplified with the following primers: 5'-CCAGTCTGGA GAAACTGCTG-3' (forward) and 5'-CGAGCGGCTG CACCTTATGGG-3' (reverse). The Pfaffl equation was used for normalization and calculation of relative EXO1 expression levels in comparison with the control gene (33).

**SA-GFP Reporter Assay**—SA-GFP reporter assays were performed as described previously (34, 35). HEK293/SA-GFP cells were seeded in poly-L-lysine-coated 6-well plates at a density of 0.5 million cells/well. U2OS/SA-GFP cells were seeded in 6-well plates at a density of 0.25 million cells/well. The next day, cells were transfected with appropriate siRNA (40 nm) using Lipofectamine RNAiMAX (Invitrogen). After 24 h, siRNA-transfected cells were transferred into a 12-well plate, with 200,000 cells/well for HEK293/SA-GFP and 100,000 cells/well for U2OS/SA-GFP. 44 h after siRNA transfection, cells were transfected with 0.6 μg of the I-SceI expression vector pCBASce (36) using linear polyethyleneimine and, 6 h later, with appropriate siRNA (20 nm) using the standard calcium phosphate method. 52 h after I-SceI transfection, cells were harvested and subjected to flow cytometry analysis using LSRII (BD Biosciences) and FlowJo software to determine the percentage of GFP-positive cells. The mean values obtained with control siRNA (siLuc) samples were 0.9% for HEK293/SA-GFP cells and 2.0% for U2OS/SA-GFP cells. To test the effect of ectopic expression of WRN on SSA repair efficiency of WRN-depleted HEK293/SA-GFP cells, the mutant form of the pcDNA3.1-hWRN construct harboring silent mutations in the SWRN-targeting region (0.6 μg) was cotransfected with pCBASce (0.6 μg). The plasmid pcDNA3.1 was used as a control vector in these experiments. Cells were subjected to flow cytometry analysis at 52 h after plasmid transfection.

**Immunofluorescence Assays**—U2OS cells transfected with the indicated siRNAs were cultured on glass coverslips. 48 h after siRNA transfection, cells were treated with 1 μM camptotheacin (CPT) for 1 h. After pre-extraction for 5 min on ice in 25 mM HEPES (pH 7.4) buffer containing 0.5% (v/v) Triton X-100, 50 mM NaCl, 1 mM EDTA, 3 mM MgCl₂, and 0.3 M sucrose, cells were fixed with 4% (v/v) formaldehyde for 15 min at room temperature. Subsequently, cells were permeabilized by soaking in 0.2% (v/v) Triton X-100 for 5 min at room temperature. After blocking in PBS containing 10 mg/ml BSA for 30 min at room temperature, fixed cells were incubated for 2 h at room temperature with the indicated primary antibodies. The slides were washed with PBS and incubated for 1 h at room temperature with secondary antibodies diluted in blocking solution (Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen)). After washing with PBS, coverslips were mounted using Vectashield containing DAPI (Vector Laboratories). Automated image acquisition was performed using an Olympus IX70 microscope equipped with the ScanR imaging platform. A ×40/1.3 numerical aperture objective was used. 10 z stacks at a spacing of 0.3 μm were taken, and 100 images were acquired for each sample. Analysis was performed using ScanR analysis software. Nuclei were identified on the basis of the DAPI signal, and RPA foci were identified on the basis of edge-based subobject counts. At least 1000 cells were analyzed for each condition.

**RESULTS**

**DNA2 Can Mediate DNA End Resection in Conjunction with WRN Helicase**—To test whether the human WRN helicase can mediate resection of broken DNA ends in concert with DNA2, we purified these proteins to homogeneity and analyzed their activities in vitro (Fig. 1A). WRN and BLM unwind DNA in the 3'-5' direction and require a 3' ssDNA tail for loading onto the DNA substrate (37, 38). Therefore, we generated a derivative of the pUC19 plasmid in which a Stul site was flanked on each side by four recognition sites for the nicking endonuclease Nt.BbvCl. Cleavage of this pUC19 derivative with Stul and Nt.BbvCl resulted in a 2.7-kb-long linear DNA molecule ending with 3'
overhangs of 26 nt in length, whereas its cleavage by Stul alone gave rise to a linear DNA molecule with blunt ends. Processing of the DNA substrates was monitored by agarose gel electrophoresis followed by SYBR gold staining (Fig. 1B). In addition, 32P-labeled synthetic oligonucleotides complementary to the 3′-terminated strand were used as hybridization probes to detect ssDNA generated by resection at specific positions (Fig. 1B) (10). We found that WRN, together with DNA2 and RPA, could catalyze efficient 5′ end resection on the 3′-tailed substrate but not the blunt-ended substrate (Fig. 1C). As expected, no reaction products were detected with an oligonucleotide probe complementary to the 5′-terminated strand, indicating that the observed DNA resection activity is limited to the 5′ strand (Fig. 1D). Of note, the end product of the resection reaction on the 3′-tailed DNA substrate appeared as a discrete band on SYBR gold-stained gel that was clearly shifted with respect to the unprocessed dsDNA substrate, indicating extensive resection (Fig. 1C, top panel, compare lanes 2 and 7). In contrast, no gradual shift in the electrophoretic mobility of the resection product was apparent after annealing of the radioactive probes. This is most likely due to the fact that DNA2 nuclease generates short oligonucleotides that can reanneal to the resected DNA along with the radioactive probe, leading to a DNA molecule with an electrophoretic mobility similar to that of the DNA substrate. Together, these results clearly demonstrate that DNA2, in conjunction with WRN and RPA, can catalyze extensive 5′ end resection, providing that the DNA substrate contains a 3′ ssDNA overhang.

To further characterize the DNA end resection reaction mediated by WRN-DNA2, reactions with the 3′-tailed substrate were carried out at various WRN concentrations, whereas DNA2 was kept at a concentration of 8 nM. We observed that the amount of resection product increased gradually with WRN concentration, reaching a plateau at about 8 nM (Fig. 1E, lanes 2–7, and Fig. 1F). Quantitative analysis of gel images revealed that about 35% of the DNA substrate was resected to the position of 133 nt from the 3′ end and that about 15% of the DNA substrate was resected to the position of 374 nt...
To this end, HEK293 cells were transfected with plasmids expressing WRN and FLAG-tagged DNA2, respectively, and complex formation between these proteins was tested by immunoprecipitation using beads conjugated with anti-FLAG M2 antibody. We found that WRN coimmunoprecipitated with FLAG-DNA2, indicating that these proteins form a complex in vivo (Fig. 3A, lane 3). This interaction was specific because anti-FLAG beads did not immunoprecipitate WRN from an extract lacking FLAG-DNA2 (Fig. 3A, lane 1). To further investigate complex formation between WRN and DNA2, we generated a stable HEK293 cell line expressing FLAG-DNA2. By immunoprecipitation using anti-FLAG M2 beads or anti-WRN antibody, we found that FLAG-DNA2 formed a complex with endogenous WRN in these cells (Fig. 3, B and C). Western blot analysis indicated that the level of FLAG-DNA2 in these cells was only slightly higher than that of endogenous DNA2, suggesting that WRN and DNA2 form a complex under physiological conditions (Fig. 3B, top panel). Interaction between FLAG-DNA2 and endogenous BLM was also detected as expected (Fig. 3B) (17). The cellular concentration of these protein complexes was not altered when cells were subjected to treatment with CPT, which causes breakage of DNA replication forks (Fig. 3B, lanes 2–5) (42). This suggests that the interaction of DNA2 with WRN and BLM in the cell is not dependent on DNA damage.

To test whether WRN and DNA2 interact directly, purified proteins were mixed and incubated at 4 °C for 4 h. Complex formation between WRN and DNA2 was tested by immunoprecipitation using anti-WRN antibody. We found that DNA2 coimmunoprecipitated with WRN. DNA2 was not present in the immunoprecipitated material whether WRN was omitted, confirming a direct protein-protein interaction (Fig. 3D). To map the interaction site of DNA2 on WRN, we tested binding of purified His<sub>6</sub>-DNA2-FLAG protein to various WRN fragments covering the entire WRN polypeptide (Fig. 3E). The WRN fragments were produced in E. coli as fusions with a GST tag and isolated on GSH-Sepharose beads. Using a GST pulldown assay, we found that DNA2 bound specifically to a WRN fragment including the core helicase domain (helicase/Zn<sup>2+</sup>-binding domains) and the winged helix domain (Fig. 3F, compare lanes 1 and 4) a binding site of a number of other proteins shown to interact with WRN (43, 44). DNA2 was also bound to a fragment containing only the helicase core or to the C-terminal portion of WRN starting at the beginning of the winged helix domain (Fig. 3F, compare lanes 1, 3, 4, and 5). In contrast, DNA2 did not bind the N-terminal portion of WRN containing the exonuclease domain (Fig. 3F, lane 2). Collectively, these results suggest that there are at least two DNA2-interaction sites on WRN: one located in the central helicase domain and the other in the C-terminal region of WRN.

**WRN-DNA2 Reseets DNA Ends More Efficiently Than BLM-DNA2**—Next, we set out to compare WRN and BLM with respect to their abilities to resect DNA ends in concert with DNA2 and RPA in vitro. Using a Y structure oligonucleotide duplex (29 bp) with single-stranded arms (30 nt each), we found that our preparations of WRN and BLM exhibited similar levels of specific helicase activity (Fig. 4A). For resection reactions, we used the 3'-tailed DNA substrate that was readily processed by...
The extent of DNA resection at various reaction time points was monitored by annealing of radiolabeled oligonucleotide probes. These experiments clearly showed that WRN-DNA2 resected the DNA substrate at a much higher rate compared with BLM-DNA2 (Fig. 4, B and C). Notably, WRN-DNA2-catalyzed resection to the position of 374 nt away from the 3' end was faster than BLM-DNA2-catalyzed resection to the position of 133 nt (Fig. 4, B and C). We also compared the activities of WRN-DNA2 and BLM-DNA2 on blunt-ended DNA substrate in the presence of RPA. We found that this DNA substrate was largely refractory not only to processing by WRN-DNA2 but also to processing by BLM-DNA2 (Fig. 4D, compare lanes 4 and 5 to lanes 10 and 11). Taken together, we show that WRN-DNA2 resects DNA ends more efficiently than BLM-DNA2 in vitro.

Dissection of Pathways Involved in DNA End Resection in Human Cells—To assess whether WRN is involved in DNA end resection in vivo, we investigated the effect of its depletion on the efficiency of SSA-mediated repair of endonuclease-induced DSBs in cells that were either proficient or deficient for EXO1 and DNA2, respectively. For this epistasis analysis, we initially used the human embryonic kidney cell line HEK293 stably transduced with the SA-GFP reporter cassette consisting of two truncated GFP gene alleles (5' GFP and Sce3' GFP) that form a direct sequence repeat (280 bp) separated by a region of about 2.4 kb (Fig. 5A) (34, 45). SSA-mediated recombination between these homologous sequences triggered by a DSB generated in the distal GFP allele by the I-SceI endonuclease results in the formation of a functional GFP gene (Fig. 5A). This requires extensive DNA end resection to expose the complementary ssDNA regions for annealing. The proteins of interest were depleted from HEK293/SA-GFP cells by RNA interference. Cells were subsequently transfected with an I-SceI expression vector to create a DSB in the reporter cassette, and the percentage of GFP-positive cells arising upon SSA-mediated repair was determined by flow cytometry 2 days after plasmid transfection. We found that cells depleted of either EXO1, WRN, or DNA2 exhibited a marked reduction in the frequency of SSA repair events (55, 65, and 75%, respectively) compared with mock-depleted cells (Fig. 5, B and C).
BLM was found to be associated with a significant increase in SSA repair efficiency (140%) compared with control cells (Fig. 5, B and C). Of note, the SSA repair defect of WRN-depleted cells could be rescued by ectopic expression of the siRNA-resistant form of WRN, excluding an off-target effect of the WRN siRNA used in this study (Fig. 5D). Combined depletion of EXO1 and WRN or EXO1 and DNA2 further decreased the repair efficiency compared with the respective single depletions, whereas codepletion of DNA2 and WRN did not (Fig. 5, B and C). In addition, combined depletion of EXO1 and BLM had nearly the same effect on the SSA repair efficiency as EXO1 depletion (Fig. 5, B and C). Therefore, these findings suggest that HEK293 cells have at least two pathways for long-range resection of DSB ends: one mediated by EXO1 and the other dependent upon DNA2 and WRN.

To substantiate these findings, we performed a similar set of experiments using U2OS/SA-GFP cells (35). This analysis indicated that combined depletion of EXO1 and DNA2 almost completely abolished (reduced by 91%) SSA-mediated DSB repair in U2OS/SA-GFP cells, as did depletion of MRE11 (by 89%) or CtIP (by 82%), suggesting that long-range DNA end resection in U2OS cells is largely dependent on EXO1 and DNA2 (Fig. 5, E–G). However, in contrast to the results obtained with HEK293/SA-GFP cells, we observed a significant reduction in SSA repair efficiency not only after depletion of WRN (by 57%) but also after depletion of BLM (by 59%) (Fig. 5, E and F). Codepletion of BLM and WRN further decreased the repair efficiency to a level comparable with that in DNA2-depleted (by 73%) cells (Fig. 5, E and F). Moreover, combined depletion of DNA2 with either BLM or WRN had nearly the
same inhibitory effect on SSA repair as DNA2 depletion (Fig. 5, E and F). On the contrary, codepletion of EXO1 with either WRN or BLM caused a much higher reduction in repair efficiency than depletion of DNA2 alone, and triple depletion of EXO1, BLM, and WRN brought repair efficiency down to the level measured in cells depleted of EXO1 and DNA2 (Fig. 5, E and F). Collectively, these data suggest that, in U2OS cells, both WRN and BLM assist DNA2 to mediate long-range resection of broken DNA ends.

To bolster our conclusion that DNA2, WRN, and BLM have an epistatic relationship in DSB end resection, we extended our analysis to measurement of RPA focus formation in U2OS cells treated with CPT. As expected, 1 h after addition of CPT, RPA formed numerous foci in γ-H2AX-positive cells, which were dependent on the presence of CtIP (Fig. 6). Depletion of DNA2 resulted in a marked reduction in the number of RPA foci per cell compared with mock-depleted cells (Fig. 6). Cells depleted of BL or WRN displayed a mild decrease in RPA focus frequency compared with mock-depleted cells (Fig. 6). In contrast, combined depletion of BL and WRN caused approximately the same reduction in RPA focus frequency as depletion of DNA2 alone. Moreover, cells depleted of DNA2 and BL or DNA2 and WRN displayed an RPA foci frequency comparable with that of DNA2-depleted cells (Fig. 6). These data further support the conclusion that DNA2, WRN, and BLM operate in the same DNA end resection pathway.

Role of the BLM-TOPOIIIα-RMI1-RMI2 Complex in DNA End Resection—In human cells, BLM exists in a complex with TOPOIIIα, RMI1, and RMI2, which is known to catalyze double Holliday junction dissolution during HR (46–49). Studies in yeast have shown that Top3α and Rml1 are also required for DNA-end resection in vivo and stimulate DNA end resection by...
Our discovery of the involvement of WRN in DNA end resection is consistent with the findings that WRN interacts physically with the MRN complex and accumulates at sites of DSBs in human cells (50, 51). Moreover, it has been demonstrated that WRN depletion leads to a marked reduction in the frequency of RPA and BrdU/ssDNA foci formed in response to ionizing radiation, indicative of a resection defect (52). A similar phenotype has been observed in DNA2-depleted cells (18). Although the previous studies did not address the relationship between WRN and DNA2, they demonstrated that these enzymes act synergistically with EXO1 to promote DNA end resection in human cells (18, 52). A role for WRN as a critical DNA end resection factor is also consistent with the cellular phenotype of Werner syndrome, a severe premature aging disorder caused by inherited mutations in the WRN gene (53). Cells derived from Werner syndrome patients are characterized by non-homologous chromosome exchanges, termed variegated translocation mosaicism, and large chromosomal deletions that may result from aberrant DSB repair by NHEJ as a consequence of a defect in DNA end resection (54–56). Indeed, it is becoming clear that NHEJ accounts for most chromosomal translocations in humans (57). Moreover, a role for DNA end resection as the critical determinant of DSB repair pathway choice is well established (58). Accumulating evidence suggests that defects in homology-directed repair pathways, which are dependent on DNA end resection, result in overuse of NHEJ for repair, leading to accumulation of chromosomal rearrangements (57). However, it should be noted that WRN is also known to promote DSB repair by the classical Ku-dependent NHEJ (C-NHEJ) pathway to suppress microhomology-mediated end joining (59, 60). This alternative end joining pathway is capable of producing chromosomal translocations, particularly when Ku-dependent NHEJ is deficient (57). Moreover, WRN has been shown to be involved in the resolution HR intermediates (61, 62). Therefore, it seems that the genomic instability in Werner syndrome is a consequence of multiple defects in DNA repair pathways.

Our finding that the TRR complex stimulates DNA end resection by BLM-DNA2 in vitro is consistent with previous reports showing that the association of BLM with TOPOIIIα and RMI1 enhances its DNA unwinding activity, which drives the BLM-DNA2-catalyzed resection reaction (17, 63). Similarly, RMI1 and RM12 have been shown to enhance the efficiency of the BLM-TOPOIIIα-mediated double Holliday junction dissolution reaction (48, 49, 64). It has also been shown that RMI1 forms a complex with RPA and that this interaction is essential for the stimulatory effect of RPA on double Holliday junction dissolution by the BTRR complex (65). Therefore, it is possible that a physical interaction between RMI1 and RPA
The Role of WRN and BLM in DNA End Resection

loaded on the 3′-terminated DNA strand during DNA2-catalyzed resection might enhance the DNA unwinding processivity of the BTRR complex and, hence, increase the efficiency of the resection reaction. However, it should be noted that the stimulatory effect of the TRR complex on DNA end resection by BLM-DNA2 in vitro was rather modest under our experimental conditions. On the contrary, RM1 depletion in U2OS/SA-GFP cells reduced the efficiency of SSA-mediated DSB repair to levels displayed by BLM- or DNA2-deficient cells, suggesting that BLM requires RM1 to promote DNA end resection in vivo. Of note, it has been shown that silencing of RM1 or RM2 expression by RNA interference destabilizes both BLM and TOPOIIIα (47, 49). Therefore, it is evident that, in addition to being important for the functional attributes of the BTRR complex, RM1 and RM2 are indispensable for the structural integrity of its components in vivo.

Although BLM depletion compromised SSA-mediated DSB repair in U2OS/SA-GFP cells, it had an opposite effect on SSA in HEK293/SA-GFP cells. Similarly, the efficiency of SSA-mediated DSB repair in HEK293/SA-GFP cells was elevated significantly upon depletion of RM1 (data not shown). These findings suggest that, in HEK293 cells, the BTRR complex might act as an SSA suppressor, most likely through unwinding of the annealed intermediate formed following DNA end resection. Strikingly, we found that BLM concentration in HEK293 cells was much higher than in U2OS cells (data not shown). Therefore, it appears that the BTRR complex exerts an inhibitory effect on SSA when its concentration in the cell exceeds certain threshold.

Acknowledgments—We thank Kata Sarlos and Ian D. Hickson for the purified TRR complex, Judith L. Campbell for the transfer vector for preparation of the bacmid expressing His6-hDNA2-FLAG, Jeremy M. Stark for the HEK293/SA-GFP and U2OS/SA-GFP cell lines, Stefano Ferrari and Stephanie Bregenhorn for help with protein purification, and Christiane Koenig for technical assistance.

REFERENCES

1. Jackson, S. P., and Bartek, J. (2009) The DNA-damage response in human biology and disease. Nature 461, 1071–1078
2. Khanna, K. K., and Jackson, S. P. (2001) DNA double-strand breaks: signaling, repair and the cancer connection. Nat. Genet. 27, 247–254
3. Longhese, M. P., Bonetti, D., Guerini, I., Manfrini, N., and Clerici, M. (2009) DNA double-strand breaks in meiosis: checking their formation,
The Role of WRN and BLM in DNA End Resection

sequences. Mol. Cell Biol. 24, 9305–9316
46. Wu, L., and Hickson, I. D. (2003) The Bloom’s syndrome helicase suppresses crossing over during homologous recombination. Nature 426, 870–874
47. Yin, J., Sobeck, A., Xu, C., Meetei, A. R., Hoatlin, M., Li, L., and Wang, W. (2005) BLAP75, an essential component of Bloom’s syndrome protein complexes that maintain genome integrity. EMBO J. 24, 1465–1476
48. Wu, L., Bachrati, C. Z., Ou, J., Xu, C., Yin, J., Chang, M., Wang, W., Li, L., Brown, G. W., and Hickson, I. D. (2006) BLAP75/RMI1 promotes the BLM-dependent dissolution of homologous recombination intermediates. Proc. Natl. Acad. Sci. U.S.A. 103, 4068–4073
49. Xu, D., Guo, R., Bachrati, C. Z., Yang, J., Enomoto, T., Brown, G. W., Hoatlin, M. E., Hickson, I. D., and Wang, W. (2008) RMI, a new OB-fold complex essential for Bloom syndrome protein to maintain genome stability. Genes Dev. 22, 2843–2855
50. 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
51. Lan, L., Nakajima, S., Komatsu, K., Nussenzweig, A., Shimamoto, A., Oshima, J., and Yasui, A. (2005) Accumulation of Werner protein at DNA double-strand breaks in human cells. J. Cell Sci. 118, 4153–4162
52. Tomimatsu, N., Mukherjee, B., Deland, K., Kurimasa, A., Bolderson, E., Khanna, K. K., and Burma, S. (2012) Exo1 plays a major role in DNA end resection in humans and influences double-strand break repair and damage signaling decisions. DNA Repair 11, 441–448
53. Yu, C. E., Oshima, J., Fu, Y. H., Wijisman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G. M., Mulligan, J., and Schellenberg, G. D. (1996) Positional cloning of the Werner’s syndrome gene. Science 272, 258–262
54. Salk, D., Au, K., Hoehn, H., and Martin, G. M. (1981) Cytogenetics of Werner’s syndrome cultured skin fibroblasts: variegated translocation mosaicism. Cytogenet. Cell Genet. 30, 92–107
55. Fukuchi, K., Martin, G. M., and Monnat, R. J., Jr. (1989) Mutator phenotype of Werner syndrome is characterized by extensive deletions. Proc. Natl. Acad. Sci. U.S.A. 86, 5893–5897
56. Melcher, R., von Golitschek, R., Steinlein, C., Schindler, D., Neitzel, H., Kainer, K., Schmid, M., and Hoehn, H. (2000) Spectral karyotyping of Werner syndrome fibroblast cultures. Cytogenet. Cell Genet. 91, 180–185
57. Bunting, S. F., and Nussenzweig, A. (2013) End-joining, translocations and cancer. Nat. Rev. Cancer 13, 443–454
58. Symington, L. S., and Gautier, J. (2011) Double-strand break end resection and repair pathway choice. Annu. Rev. Genet. 45, 247–271
59. Chen, L., Huang, S., Lee, L., Davalos, A., Schiestl, R. H., Campisi, J., and Oshima, J. (2003) WRN, the protein deficient in Werner syndrome, plays a critical structural role in optimizing DNA repair. Aging Cell 2, 191–199
60. Perry, J. J., Yannone, S. M., Holden, L. G., Hitomi, C., Asaithamby, A., Han, S., Cooper, P. K., Chen, D. J., and Tainer, J. A. (2006) WRN exonuclease structure and molecular mechanism imply an editing role in DNA end processing. Nat. Struct. Mol. Biol. 13, 414–422
61. 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
62. Swanson, C., Saintigny, Y., Emond, M. J., and Monnat, R. J., Jr. (2004) The Werner syndrome protein has separable recombination and survival functions. DNA Repair 3, 475–482
63. Bussen, W., Raynard, S., Bussygina, V., Singh, A. K., and Sung, P. (2007) Holliday junction processing activity of the BLM-Topo IIIα-BLAP75 complex. J. Biol. Chem. 282, 31484–31492
64. Raynard, S., Bussen, W., and Sung, P. (2006) A double Holliday junction dissolvasome comprising BLM, topoisomerase IIIα, and BLAP75. J. Biol. Chem. 281, 13861–13864
65. Xue, X., Raynard, S., Bussygina, V., Singh, A. K., and Sung, P. (2013) Role of replication protein A in double Holliday junction dissolution mediated by the BLM-Topo IIIα-RMI1-RMI2 protein complex. J. Biol. Chem. 288, 14221–14227
DNA2 Cooperates with the WRN and BLM RecQ Helicases to Mediate Long-range DNA End Resection in Human Cells
Andreas Sturzenegger, Kamila Burdova, Radhakrishnan Kanagaraj, Maryna Levikova, Cosimo Pinto, Petr Cejka and Pavel Janscak

J. Biol. Chem. 2014, 289:27314-27326.
doi: 10.1074/jbc.M114.578823 originally published online August 13, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.578823

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 36 of which can be accessed free at http://www.jbc.org/content/289/39/27314.full.html#ref-list-1