The Processing of Holliday Junctions by BLM and WRN Helicases Is Regulated by p53*[S]

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BLM, WRN, and p53 are involved in the homologous DNA recombination pathway. The DNA structure-specific helicases, BLM and WRN, unwind Holliday junctions (HJ), an activity that could suppress inappropriate homologous recombination during DNA replication. Here, we show that purified, recombinant p53 binds to BLM and WRN helicases and attenuates their ability to unwind synthetic HJ *in vitro*. The p53 248W mutant reduces abilities of both to bind HJ and inhibit helicase activities, whereas the p53 273H mutant loses these abilities. Moreover, full-length p53 and a C-terminal polypeptide (residues 373–383) inhibit the BLM and WRN helicase activities, but phosphorylation at Ser^{376} or Ser^{378} completely abolishes this inhibition. Following blockage of DNA replication, Ser^{15} phospho-p53, BLM, and RAD51 colocalize in nuclear foci at sites likely to contain DNA replication intermediates in cells. Our results are consistent with a novel mechanism for p53-mediated regulation of DNA recombinational repair that involves p53 post-translational modifications and functional protein-protein interactions with BLM and WRN DNA helicases.

Bloom and Werner syndromes (BS and WS)[1] are autosomal recessive disorders characterized by immune deficiency, cancer predisposition, and chromosomal instability (1). The products of the genes responsible for these disorders, BLM and WRN, are ATP-dependent DNA helicases that exhibit 3′ to 5′ polarity. Mutations in the *BLM* or *WRN* genes disrupt their helicase activity, which may be important for the phenotypic traits associated with these hereditary diseases (2).

Homologous recombination (HR) is required for genetic exchange during meiosis, repair of complex lesions in DNA, and the segregation of chromosomes at cell division. Expression of the BLM or WRN helicases in *Saccharomyces cerevisiae* containing a mutation in *sgs1*, a BLM and WRN homolog, suppresses their increased rates of illegitimate recombination and HR (3). BLM and its yeast homologue, Sgs1, functionally interact with topoisomerase III (4), whereas the WRN interaction with DNA polymerase δ is apparently required for some aspect of DNA replication and/or repair (5, 6). Recent reports indicate that both of these helicases recognize and disrupt alternative DNA structures, including G-quadruplex DNA and Holliday junctions (HJ) (7–11). HJ arise as intermediates during HR and can occur spontaneously, or during DNA replication and the repair of DNA damage (12). BLM and WRN may promote ATP-dependent translocation of HJ to eliminate DNA recombination intermediates, thereby reducing inappropriate DNA recombination *in vivo* (10, 11).

p53 suppresses genomic instability, particularly in response to DNA damage (13, 14). p53 also has been implicated in HR. Evidence of p53 modulation of HR includes the following: (*a*) overexpression of wild-type p53 (WT p53) can down-regulate the rate of HR between SV40 molecules (15); (*b*) the rate of HR is increased in p53 mutant cell lines (16–18); (*c*) p53 has 3′ to 5′ exonuclease and DNA strand transfer activities (19); and (*d*) p53 can bind and inhibit human RAD51 and bacterial RecA, central components of the HR pathway (20, 21). *In vitro*, p53 also can bind to the crossover region of HJ (22), positively or negatively supercoiled DNA (23, 24), and DNA base mismatches (25); all of these structures can be associated with HR.

p53 physically and functionally interacts with BLM and WRN *in vivo* and *in vitro* (26–28). We hypothesize that p53 may regulate HR through its modulation of the BLM and WRN helicase activities. In this study, we present the first evidence that p53 modulates the ability of BLM and WRN helicases to disrupt HJ. This property can be altered by modifications to the p53 C terminus at Ser^{376} or Ser^{378}. These modifications decrease modulation of recombination and abrogate the binding of p53 to BLM. p53-mediated inhibition of BLM or WRN helicase activity apparently occurs through direct binding to these

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[1] The abbreviations used are: BS, Bloom syndrome; WS, Werner syndrome; BLM, the product of Bloom syndrome gene; WRN, the product of Werner syndrome gene; HR, homologous recombination; HJ, Holliday junctions; APH, aphidicolin; PKC, protein kinase C; PP1, protein phosphatase 1; EMSA, electrophoretic mobility shift assay; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ATPγS, adenosine 5′-*O*-(thiotriphosphate); WT p53, wild-type p53.

helicases. In addition, we show that p53 colocalizes *in vivo* with BLM and RAD51 at putative sites of stalled DNA replication forks and HJ in cells arrested in S-phase by aphidicolin (APH). These results indicate a possible physiological mechanism for the regulation of HR by the physical and functional interaction of p53 with the BLM and WRN DNA helicases as well as their DNA substrates.

<center>EXPERIMENTAL PROCEDURES</center>

Cell Culture, Western Blot Analyses, and Immunoprecipitation—GM01310, a normal human lymphoblastoid cell line, was maintained at a density greater than 3×10^5 cells/ml in RPMI medium supplemented with 15% fetal bovine serum, penicillin, and streptomycin (Biofluids). Human WI-38 and GM08402 fibroblasts were used at early passage. Western blot analysis and immunoprecipitation were performed as described previously (27).

Proteins and Antibodies—Recombinant hexahistidine-tagged human BLM and WRN proteins were purified as described previously (29, 30). Human WT, 248W, and 273H p53 proteins were generated from recombinant baculoviruses in Sf9 insect cells, and purified using an anti-p53 (antibody PAb421, which recognizes the C terminus of p53) immunoaffinity column, as described previously (31). The concentrations of dialyzed peak fractions of p53 were determined by silver staining and quantified with a Pantropic p53 Rapid Format Assay kit (Oncogene Research Products). RuvA protein was kindly provided by Dr. Michael M. Cox (University of Wisconsin, Madison, WI). Protein kinase C (PKC) and protein phosphatase 1 (PP1) were from UBI. Anti-BLM and anti-WRN were from Santa Cruz (Santa Cruz, CA). PAb421, DO-1, Ser[15] phospho-p53, and anti-RAD51 were from Oncogene Research Products.

DNA Substrates—The synthetic X-junction (four-arm junction, blunt ends, X-12) was prepared by annealing four 50-mer oligonucleotides as described previously (10, 32). Briefly, X12-1 was 5′-^{32}P-labeled and annealed with X12-2, X12-3, and X12-4. The product was then purified by separation through a 10% TBE gel, and recovered by electroelution and dialysis. The linear blunt duplex DNA used as the nonspecific competitor was prepared by annealing X12-1 with its complement. The 28-mer M13mp18 partial duplex substrate was constructed with a 28-mer oligonucleotide complementary to position 3960–3987 in M13mp18. The substrate was labeled, annealed, and purified as described previously (33).

Helicase Assays—The BLM helicase assay reactions contained the ^{32}P-labeled X-junction in 20 mM Tris-HCl, pH 7.5, 1.25 mM MgCl$_2$, 2 mM ATP, 0.1 mg/ml BSA, and 1 mM dithiothreitol. The WRN helicase assay reactions contained 40 mM Tris borate, pH 7.4, 5 mM MgCl$_2$, 5 mM dithiothreitol, and 2 mM ATP. Reactions were initiated by the addition of BLM or WRN proteins, and they were incubated at 37 °C for 45 min. The products were separated by electrophoresis through 10% nondenaturing polyacrylamide gels at 4 °C and visualized using a PhosphorImager or film autoradiography and quantified using the ImageQuant software (Amersham Biosciences). Helicase data shown are representative of at least three independent experiments.

Electrophoretic Mobility Gel Shift Assay (EMSA)—The DNA-binding reactions (20 μl) contained 20 mM triethanolamine-HCl, pH 7.5, 2 mM MgCl$_2$, 1 mM ATPγS, 0.1 μg/ml BSA, and 1 mM dithiothreitol, and protein concentration as indicated in the figures. Reaction mixtures were incubated at room temperature for 20 min and fixed in the presence of 0.25% glutaraldehyde for 10 min at 37 °C. The products were separated by electrophoresis through 4% nondenaturing polyacrylaminde gels at 4 °C for 3 h, and visualized using a PhosphorImager or film autoradiography.

Electron Microscopic Visualization of the p53-HJ Interaction—HJ substrates (Hol575) for electron microscopy were prepared as described previously (22). p53·DNA complexes were assembled by incubating 50 ng of DNA in a 50-μl reaction containing 10 mM HEPES, pH 7.5, and 100 mM KCl for 20 min at room temperature using a 1:6 molar ratio of HJ DNAs to p53 tetramers. The complexes were fixed with 0.6% glutaraldehyde (v/v) for 10 min at room temperature followed by gel filtration using a 1-ml Bio-Gel A-5m (Bio-Rad) column to remove free protein and fixatives. The samples were prepared for electron microscopy as described previously (34). Briefly, the samples were adsorbed to thin carbon foils supported by 400-mesh copper grids in the presence of spermidine, then washed with a water/ethanol series, air dried, and rotary shadowcast with tungsten. The grids were visualized in a Phillips CM12. Images for publication were scanned with a Nikon 4500 AF film scanner and the contrast was adjusted with Adobe Photoshop software.

ELISA and Far Western Blotting—BLM and WRN were diluted to a concentration of 2 nM in carbonate buffer (0.016 M Na$_2$CO$_3$, 0.034 M NaHCO$_3$, pH 9.6) and were then used to coat appropriate wells of a 96-well ELISA plate. WT p53 or mutant p53 proteins were incubated at 0–20 nM in a binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 5 mM ATP, 100 μg/ml BSA, and 50 mM NaCl) for 30 min at 24 °C. Then, DO-1 and secondary antibodies were added sequentially. Phosphatase substrate (Sigma) was incubated for 1 h at 37 °C. The A_{405} values, corrected for background with BSA, were expressed as the mean of three independent experiments.

Far Western blotting was performed as described by Wu *et al.* (4). BLM or WRN (200 ng) were run on a SDS-PAGE gel and transferred to Hybond-ECL filters (Amersham Biosciences). Filters were denatured and incubated with WT or mutant p53 proteins (200 ng/ml) for 1 h at 4 °C. Western analysis was then conducted to detect p53 using DO-1 as the primary antibody.

Phosphorylation-Dephosphorylation—Phosphorylation-dephosphorylation of p53 proteins was performed as described (35). p53 protein was incubated in a kinase reaction buffer with PKC (20 ng). The reactions were stopped by the addition of a peptide PKC inhibitor. The reaction mixture was then incubated in the presence or absence of phosphatase PP1 (0.02 unit). Separate aliquots were analyzed by Western blot or helicase assay.

In Vitro Protein Interaction—Glutathione *S*-transferase fusion p53 protein was produced in *Escherichia coli* and purified on glutathione-Sepharose 4B beads according to the manufacturer (Amersham Biosciences). BLM and WRN proteins were prepared using the TNT quick-coupled transcription/translation system (Promega) in the presence of [^{35}S]methionine. *In vitro* binding assay was done in immunoprecipitation buffer with rotation at room temperature for 2 h. After washing, the samples were loaded on SDS-PAGE and separated by electrophoresis.

Indirect Immunofluorescence—Cells in 4-well glass chamber slides were cultured with 5 μg/ml APH for 14 h, fixed, and stained, as described (4), using anti-BLM, anti-RAD51, and/or Ser[15] phospho-p53 antibodies. Images were analyzed by Confocal Assistant software or Laser Sharp. Quantitation of nuclear foci was determined from 100 cells for each treatment. Data were obtained from at least three independent experiments.

<center>RESULTS</center>

Modulation of the BLM and WRN Helicase Activities by p53—Because the helicase activities of BLM and WRN are necessary for the promotion of HJ branch migration (10, 11), a key step in HR, we investigated the possibility that p53 modulates the ability of BLM and WRN to disrupt a radiolabeled synthetic X-junction (X-12) substrate (blunt ends). This X-junction is a mimic of the HJ. Consistent with previous reports (9–11), both purified recombinant BLM and WRN disrupted the X-junction into one-armed (single-stranded DNA) and, to a lesser extent, into two-armed products (Fig. 1). To test the effect of p53 on BLM or WRN disrupting the X-junction, BLM or WRN were incubated with the X-junction in the presence of PAb421-immunopurified recombinant WT p53 (Fig. 1). WT p53 inhibited the activities of both BLM and WRN to similar extents. At 6 nM concentration, WT p53 inhibited both BLM and WRN helicase activities by about 80%. Two recombinant p53 mutant proteins (248W and 273H) that correspond to hotspot mutants found in human cancer were also tested. The p53 273H lacked this inhibitory activity, whereas the p53 248W mutant protein had less effect (about 50% inhibition) on BLM or WRN activity. In the absence of BLM or WRN, neither WT p53 nor mutants p53 248W and p53 273H showed intrinsic helicase activity (Fig. 1*A*).

To determine whether that inhibition of BLM and WRN helicases by p53 is structure-specific, an M13 28-bp partial duplex DNA substrate was incubated with BLM (6 nM) and increasing amounts of p53 (0, 5, 10, and 20 nM monomer). As shown in Supplemental Materials Fig. 1, p53 did not inhibit BLM unwinding of the M13 partial duplex. Consistent with a previous report that p53 does not inhibit WRN unwinding of a partial duplex substrate (33), it suggests that inhibition of BLM and WRN helicases by p53 is structure-specific.
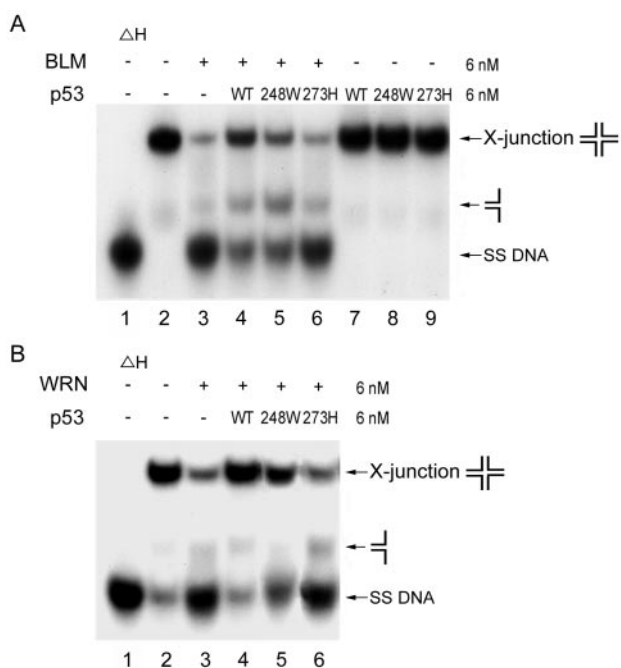
FIG. 1. **Effect of WT or mutant p53 on BLM (*panel A*) or WRN (*panel B*) helicase activity.** The helicase assay used 6 nM BLM or WRN proteins with the X-junction (1 fmol) in the absence (*lane 3*) or presence (*lanes 4–6*) of 6 nM WT, 248W, or 237H p53 proteins. p53 was incubated simultaneously with the BLM or WRN proteins and the X-junction. The appearance of the faster migrating two-armed products (small amount) and single-stranded (ss) DNA species indicates disruption of the X-junction. ΔH, heat-denatured control.

Specificity of BLM, WRN, and p53 Bound to HJ—Recombinant p53 protein produced in baculovirus exists in a tetrameric form and binds to its DNA consensus sites predominantly as a tetramer or as higher molecular weight complexes (36–40). We used EMSA to determine the specific binding of BLM, WRN, or p53 to the X-junction. Consistent with previous data (10, 11, 22), BLM induced a single-shifted band, whereas both WRN and p53 induced multiple-shifted bands (Fig. 2A). One possibility is that a single molecule of BLM binds to the X-junction, whereas WRN and p53 bind to this substrate either in different oligomeric states or at multiple sites on the DNA molecule. Whereas WT p53 bound efficiently to the X-junction, p53 248W bound to a lesser degree, and p53 273H showed no detectable binding activity (Fig. 2A). The shifted bands were competed efficiently by the unlabeled X-junction, but not by double-stranded DNA (blunt ends). The simultaneous addition of both p53 and BLM to EMSA reactions resulted in an increased intensity of the shifted bands with different mobility. The presence of both p53 and BLM in these shifted bands was confirmed by Western blot analysis with either p53 or BLM antibody, indicating the presence of a p53-BLM·X-junction complex (Fig. 2B, *bands 7* and *8*). Similar results were obtained when WRN and p53 were used on the EMSA analysis. Both proteins were detected in the shifted bands (Fig. 2C).

Electron Microscopic Visualization of the p53-HJ Interaction—To visualize HJ by electron microscopy, 4-way junctions containing 500-bp arms (Hol575) were constructed as described previously (22). The Hol575 DNA was incubated with WT p53 as well as p53 248W and p53 273H at room temperature for 20 min. The samples were fixed, processed through Bio-Gel A-5m to remove free proteins and fixatives, and prepared for EM. Examination of the WT p53 complexes with Hol575 showed that a large number of the HJ DNAs had p53 bound at the crossover point (Fig. 3A). The DNA molecules were scored (n = 300) and the results showed that 63% of the HJ had p53 bound
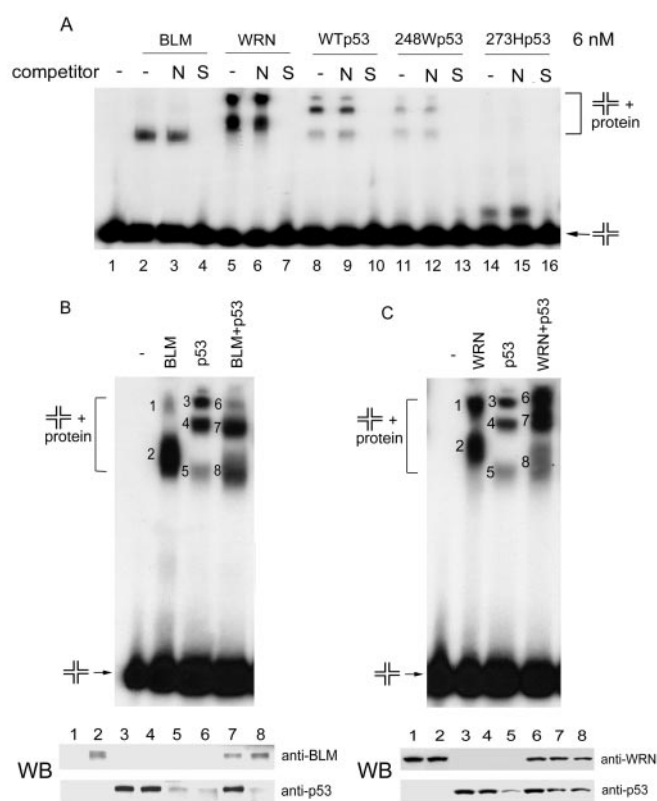

FIG. 2. *A*, the binding of BLM, WRN, WT, 248W, or 273H p53 to radiolabeled X-junction (2 fmol) was competed with double-stranded DNA or unlabeled X-junction. *N*, double-stranded DNA (20-fold excess over the radiolabeled X-junction). *S*, unlabeled X-junction (10-fold excess). *B* and *C*, 20 fmol of X-junction was incubated simultaneously with BLM or WRN (120 nM) and/or WT p53 (120 nM) and analyzed by EMSA. The shifted bands were cut and separated by SDS-PAGE following by conventional Western blotting with anti-BLM, anti-WRN, or anti-p53 (DO-1).
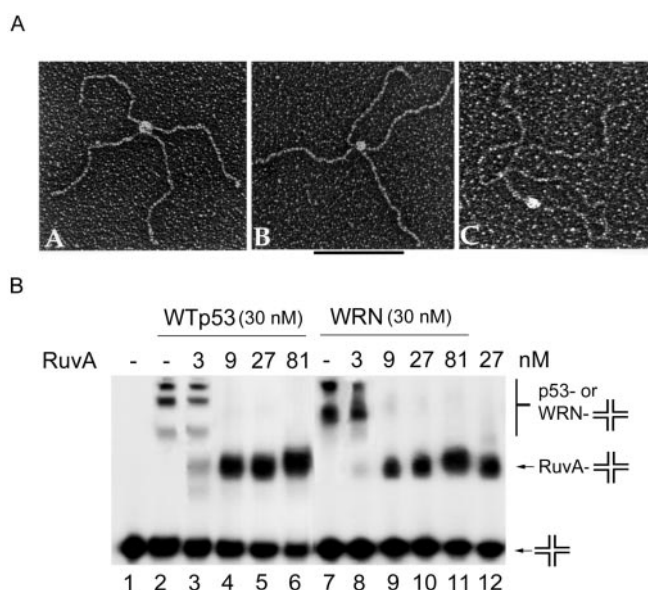

FIG. 3. *A*, visualization of p53 bound to HJ. WT p53 (*A*), p53 248W (*B*), and p53 273H proteins (*C*) were incubated with HJ containing 500-bp arms at a molar ratio of six p53 tetramers per DNA molecule. Shown in reverse contrast. *Bar* is equivalent to 500 bp. *B*, competition for binding of WRN or p53 to the X-junction by RuvA using EMSA.

somewhere on the DNA. Of these bound molecules, 70% contained p53 at the crossover point, 13% had protein bound along an arm of the HJ, and 17% had p53 at an end of one arm. The
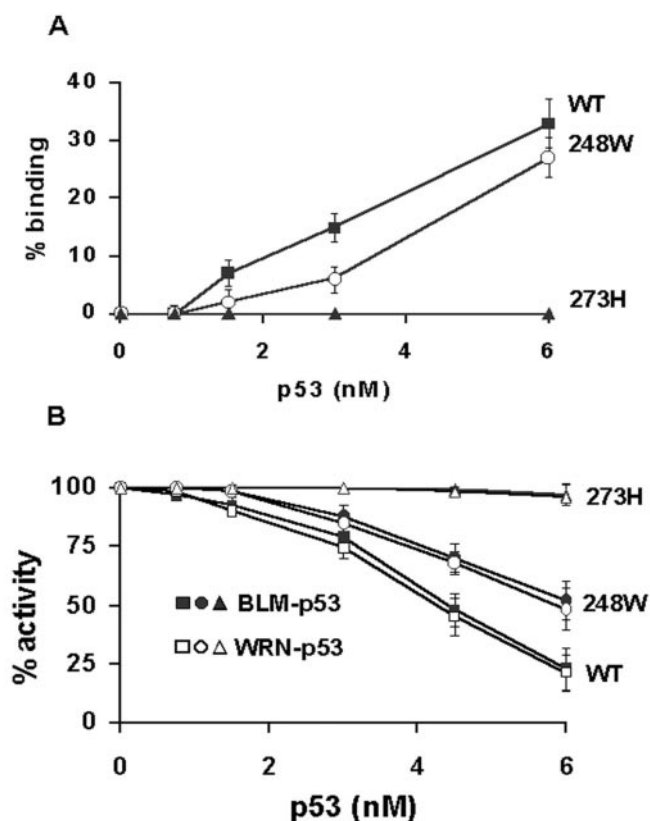
FIG. 4. **WT or mutant p53 binding to the X-junction and its effect on BLM or WRN helicase activity.** *A*, EMSA was carried out with the X-junction (2 fmol) containing the indicated concentrations of WT, 248W, or 273H p53 proteins. The percent binding was calculated from the ratio of shifted bands to free probe. Percent DNA binding is expressed as a function of p53 concentration. *B*, the X-junction (1 fmol) was incubated with 6 nM BLM or WRN proteins in the absence or presence of the indicated concentrations of WT, 248W, or 273H p53 under standard helicase assay conditions. Quantification of the products included two-armed and single-stranded DNA species. The relative percent X-junction disruption is expressed as a function of p53 concentration.

p53 248W showed slightly lower binding affinity than the WT p53. Of the molecules scored, 40% of the molecules were bound by p53. In addition, this mutant had lower affinity for the crossover point of the junction. Of the bound molecules counted, only 45% had protein at the crossover junction when compared with 70% in WT p53. The remaining molecules had p53 bound either along the arm or at the termini of the arms. Finally, the p53 273H mutant showed very low binding to the Hol575 template. Only 12% of the DNA molecules were bound by the protein and, more interestingly, none of the bound molecules had p53 at the crossover point. When compared with the WT p53, this mutant not only had reduced binding to DNA, but also had lost the specificity of the crossover point.

The *E. coli* RuvA protein specifically binds to HJ at the crossover junction (41, 42). Here, it competed efficiently with WRN or p53 for binding to the X-junction (Fig. 3*B*). In the presence of a 3-fold excess of WRN or p53 over RuvA, the RuvA·X-junction complex predominated. This indicated that the affinity of WRN and p53 for the X-junction is less than that observed with RuvA. Consistent with a previous report (11), similar results were seen with BLM (data not shown). These data indicate that p53, BLM, and WRN recognize the DNA crossover of the HJ.

Relationship between Binding to HJ and Inhibition of Helicase Activity by p53—Next, we compared the effects of various concentrations of WT, 248W, or 273H p53 for their binding to the X-junction and their inhibition of BLM or WRN helicase activity. WT p53 bound to the X-junction and inhibited the helicase activities of BLM and WRN in a dose-dependent manner (Fig. 4). Binding of the p53 248W to the X-junction and inhibition of BLM and WRN helicase activities were dose-dependent, but the magnitude of the effect was reduced significantly relative to that of WT p53. p53 273H did not significantly bind to the X-junction nor inhibit helicase activity.

In Vitro and in Vivo Interaction of p53 with BLM or WRN—To determine whether protein-protein interactions were also responsible for p53-mediated modulation of the BLM and WRN helicase activities *in vivo*, cell lysates were prepared from either untreated or irradiated (5 gray) normal lymphoblastoid cells (GM01310). The lysates were then subjected to immunoprecipitation with anti-BLM or anti-WRN antibody. The efficiency of the immunoprecipitation was assessed by analyzing supernatants of the immunoprecipitation and straight loadings of the cell lysates by Western blotting with anti-BLM or anti-WRN antibody. Only about 20% BLM or WRN proteins were immunoprecipitated by these antibodies (data not shown).

A dilution series of recombinant p53 was used as a standard for quantification of the amount of cellular p53. p53 increased to 3.3×10^4 molecules/cell 3 h after irradiation (Fig. 5*A*), whereas the amount of BLM was unchanged under these experimental conditions. Assuming that there are 4,000 molecules of BLM/cell (43), the ratio of p53 to BLM is about 8:1. Previous studies have suggested that active forms of BLM and p53 are hexameric and tetrameric, respectively (30, 36). Hence, the maximum calculated percentage of p53 that could be immunoprecipitated by anti-BLM antibodies is ~12%. We found that about 2% of the cellular p53 was immunoprecipitated by anti-BLM or anti-WRN antibody after exposure to irradiation (Fig. 5*B*). The efficiency of BLM or WRN immunoprecipitation was only 20%, thus we conclude that approximately 10% of the cellular p53 binds to either BLM or WRN under our conditions. This is close to the theoretical maximum that could be immunoprecipitated by anti-helicase antibodies, indicating that both helicases bind saturating amounts of p53. Because p53 is in large excess, these results indicate that only a fraction of p53 is involved in binding to helicases. Competitive peptides blocked co-immunoprecipitation of p53 with anti-BLM or WRN excluding the possibility that the immunoprecipitation was nonspecific (data not shown).

Direct binding of p53 to both BLM and WRN was confirmed by far Western analysis. BLM, WRN, or p53 were separated by SDS-PAGE and transferred to a nitrocellulose membrane, which was then incubated with WT or mutant p53 proteins. WT p53 and p53 248W exhibited strong binding to BLM or WRN, whereas p53 273H showed weaker, but detectable, binding. BSA was used as a negative control (Fig. 5*C*).

We next analyzed the binding affinities of WT and mutant p53 to BLM and WRN. Using an ELISA, WT p53 and p53 248W bound in a dose-dependent manner, and with similar affinities, to both BLM and WRN. In contrast, p53 273H showed a relatively weak level of binding to either BLM or WRN (Fig. 5*D*). The specificity of the interaction was demonstrated by the absence of detectable signals in wells that had been precoated with BSA only (data not shown).

Modifications to the p53 C Terminus Attenuate Its Inhibition of BLM and WRN Helicase Activities—The p53 C terminus is required for binding to both BLM or WRN (26–28) and may be post-translationally modified (44, 45). Therefore, we determined whether modification of the p53 C terminus would alter its ability to modulate the helicase activities of BLM and WRN in a model system. PKC phosphorylates p53 *in vitro* at Ser[378]
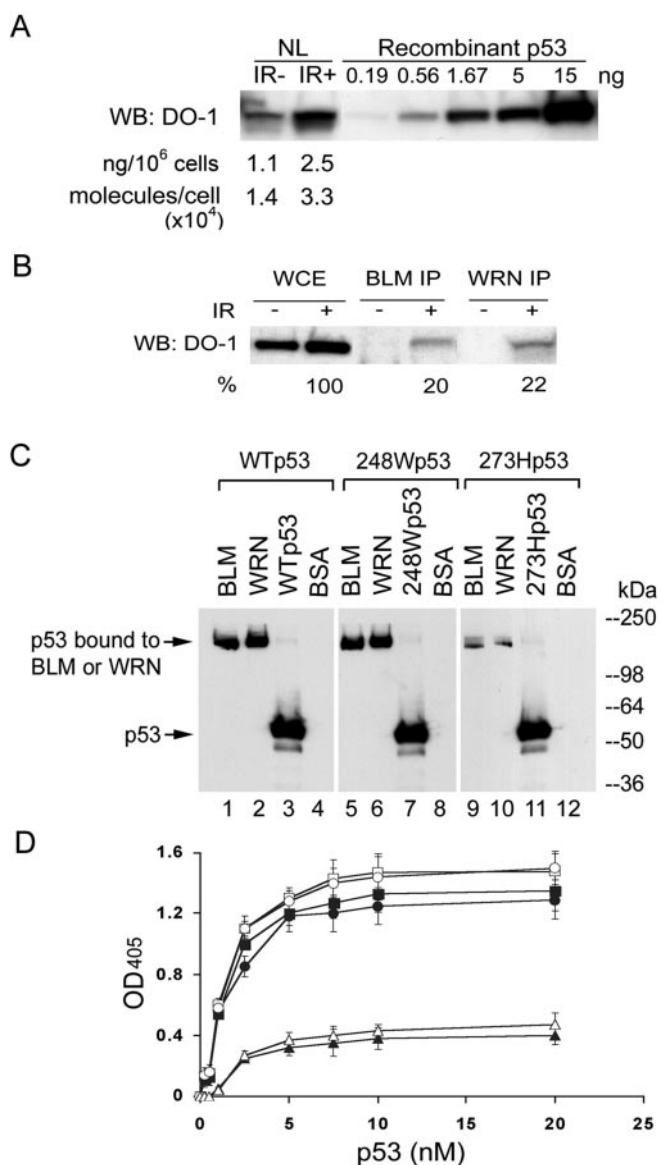
FIG. 5. *In vivo* and *in vitro* **interaction between p53 and BLM or WRN.** *A*, normal lymphoblastoid cell lines (GM01310, NL) were treated with or without 5 gray γ-radiation and incubated for 2 h. Cell lysates were analyzed by Western blotting (*WB*) with anti-p53 antibody (DO-1) to quantify the cellular p53 amount, using recombinant p53 as standards. *B*, cell lysates (10-fold amounts of WCE) were subjected to immunoprecipitation with anti-BLM or anti-WRN antibody, followed by Western blotting with anti-p53 antibody (DO-1) to quantitate immunoprecipitated p53. *WCE*, whole cell extracts. *C*, detection of p53 interaction with BLM and WRN by far Western blotting. BLM or WRN were fixed to a nitrocellulose filter and incubated with purified WT p53, p53 248W, or p53 273H. The filter was then probed using the anti-p53 DO-1 antibody. WT and mutant p53 were loaded directly as positive controls (*lanes 3, 7,* and *11*) and BSA was used as a negative control (*lanes 4, 8,* and *12*). *D*, p53 binding to BLM or WRN was quantified by ELISA. BLM- or WRN-precoated wells were incubated with WT or mutant p53 proteins. Bound p53 protein was detected using the DO-1 antibody. The A_{405} values were corrected for background binding in the BSA-coated wells. Symbols used are: WT p53-BLM, ■; WT p53-WRN, □; p53 248W-BLM, ●; p53 248W-WRN, ○; p53 273H-BLM, ▲; p53 273H-WRN, △.

within the PAb421 antibody epitope, thereby reducing PAb421 reactivity (35, 46). PP1 dephosphorylates the PKC-reactive site in p53 (35). Consistent with these reports, phosphorylation of purified recombinant p53 protein by PKC significantly reduced the reactivity of p53 to PAb421, but not to DO-1, an antibody that targets the N terminus of p53 (Supplemental Materials). Incubation of the PKC-treated p53 protein with phosphatase PP1 effectively restored PAb421 reactivity.
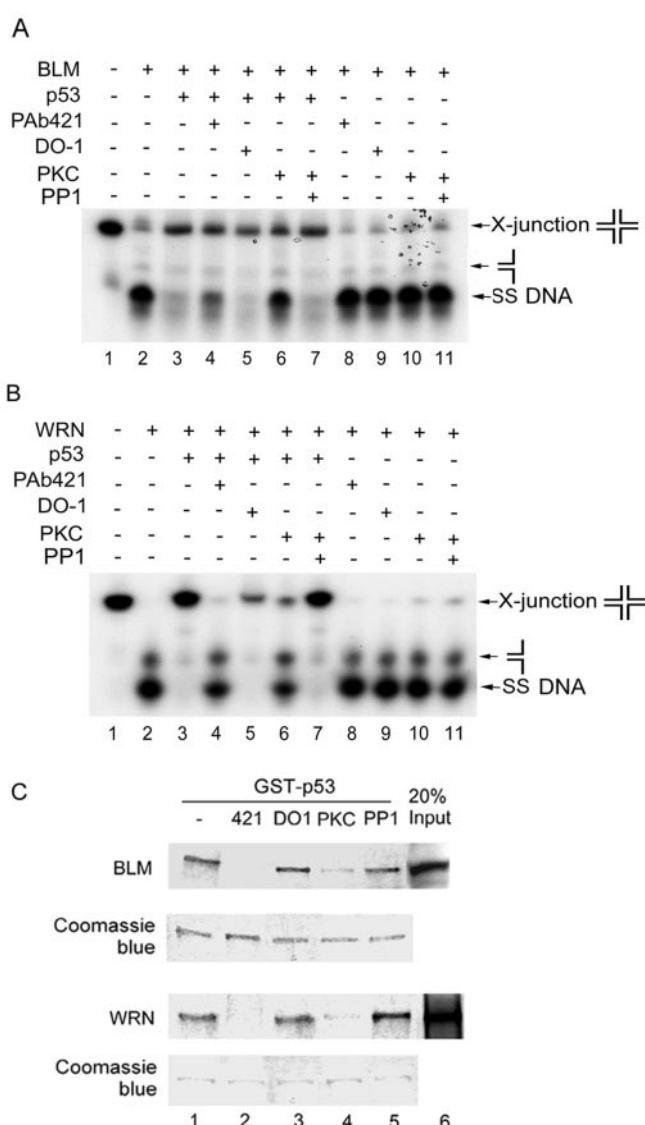

FIG. 6. *A* and *B*, effect of modified p53 on BLM or WRN helicase activity. BLM or WRN proteins (9 nM) were incubated with the X-junction (1 fmol) in the presence of WT p53 (9 nM), pAb421 (50 ng), DO-1 (50 ng, *lane 5*), PKC-phosphorylated p53 (9 nM p53, 20 ng of PKC), and/or PP1-dephosphorylated p53 (9 nM p53, 20 ng of PKC, 0.02 unit) under standard helicase reaction conditions. *C*, interaction of modified p53 with BLM or WRN. 2 μg of glutathione *S*-transferase-p53 fusion proteins were modified by PAb421, DO-1, PKC, or PKC + PP1, as described as above, then incubated with 5 μl of *in vitro* translated BLM or WRN proteins labeled with [^{35}S]methionine to determine the binding affinity of p53 with BLM and WRN. A 20% input of the BLM and WRN proteins is included in *lane 6* (from the same blot). Glutathione *S*-transferase-p53 protein input was verified by Coomassie Blue staining.

The PKC-phosphorylated p53 protein exhibited reduced inhibition of BLM or WRN helicase activity relative to unmodified p53, whereas dephosphorylation of PKC-treated p53 protein by PP1 restored its activity (Fig. 6, *A* and *B*). With PKC-phosphorylated p53 protein at a concentration of 9 nM, ~28% inhibition of BLM and 21% inhibition of WRN helicase activities were observed. Approximately 93% inhibition of both BLM and WRN helicase activities was achieved using dephosphorylated PKC-treated p53, similar to the 94% inhibition seen when using unmodified WT p53. In addition, PAb421, but not DO-1, an antibody to the N terminus of p53, also blocked the inhibitory effect of p53 on BLM and WRN helicase activities. Compared with 45% inhibition of BLM, the PAb421·p53 complex gave only 12% inhibition of WRN.
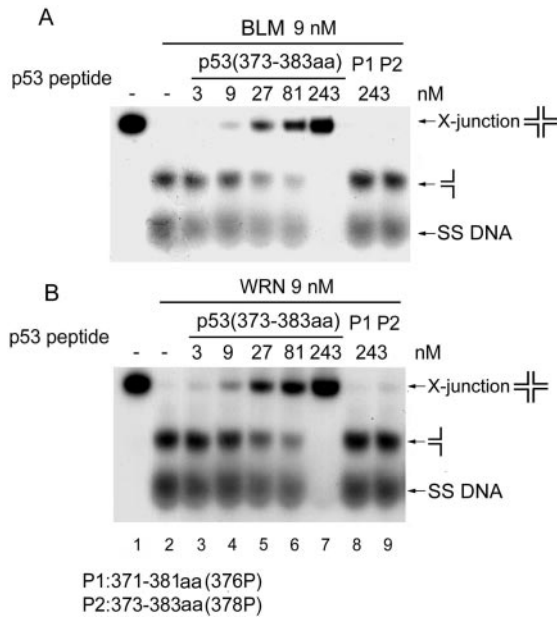
FIG. 7. *A* and *B*, effects of C-terminal peptides of p53 on BLM or WRN helicase activity. BLM or WRN proteins were incubated with the X-junction (1 fmol) in the presence of C-terminal peptides of p53 under standard helicase reaction conditions.
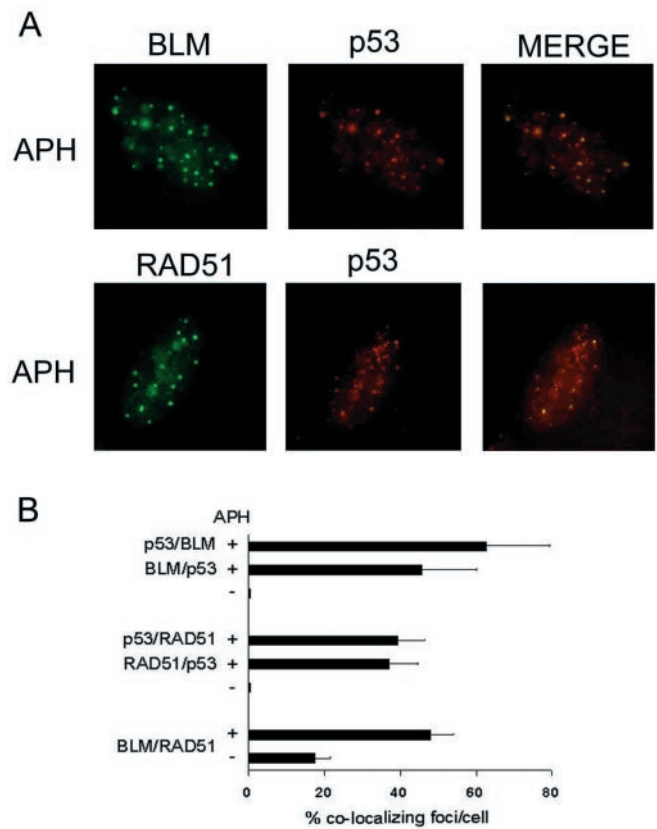

FIG. 8. **Increasing colocalization of p53 with BLM or RAD51 in cells treated with APH.** *A*, WI-38 cells were stained with anti-BLM, anti-RAD51, and/or anti-Ser[15] phospho-p53 antibodies, and nuclei were stained with 4,6-diamidino-2-phenylindole. Untreated cells were used as controls. *B*, quantitation of nuclear foci (mean ± S.D.) was determined from 100 cells analyzed by Confocal Assistant software or Laser Sharp. Data were obtained from at least three independent experiments. Student's *t* test was used for analyzing the statistical significance of colocalization between p53-BLM and BLM-p53 ($p < 0.5$), p53-RAD51 and RAD51-p53 ($p > 0.5$), and BLM-RAD51 treated and untreated ($p < 0.01$) groups.

To examine whether modification of the p53 C terminus altered binding to HJ, the binding affinities of PKC-phosphorylated p53 and dephosphorylated PKC-treated p53 to the X-junction were determined by EMSA. The PKC-phosphorylated p53 and dephosphorylated PKC-treated p53 had similar affinities toward the X-junction as unmodified WT p53 (Supplemental Materials). To explore the mechanism of p53 inhibition of BLM and WRN helicase activities, we examined binding affinities between C-terminal-modified p53 and BLM or WRN proteins. In agreement with the results of the helicase assays, modification of the recombinant glutathione *S*-transferase-p53 fusion protein, through either PAb421 antibody or PKC phosphorylation, attenuated its ability to bind to BLM or WRN proteins (Fig. 6*C*).

To further determine the requirement of the p53 C terminus for the modulation of BLM and WRN helicase activity, a synthetic p53 peptide corresponding to residues 373–383 was used in the helicase assay. This p53 peptide exhibited a concentration-dependent inhibition of BLM and WRN helicase unwinding of the X-junction (Fig. 7, *A* and *B*). In contrast, the p53 peptide phosphorylated at Ser[376] (P1) or Ser[378] (P2) was noninhibitory, even at a high concentration (Fig. 7, *A* and *B*). These three short peptides did not compete with p53 for binding to the X-junction, whereas the longer p53 peptide (319–393 amino acids) competed efficiently (Supplemental Materials). Taken together, we conclude that a p53 C-terminal region containing residues 373–383 is required for the interaction with and modulation of the branch migration activities of the BLM and WRN helicases on HJ.

p53, BLM, and RAD51 Colocalize to Nuclear Foci—RAD51 protein catalyzes a key step in HR, and it accumulates in nuclear foci that are thought to correspond to sites of stalled replication forks and recombinational repair of DNA double-strand breaks (47). BLM and RAD51 form a complex and colocalize to nuclear foci in cells arrested in S-phase using either APH or hydroxyurea (47, 48), suggesting that these proteins cooperate in the repair of breaks arising at stalled replication forks. The effects of p53 on BLM disruption of HJ presented here suggest that p53 may be involved in this process *in vivo*. To test this hypothesis, we examined the localization of p53,

BLM, and RAD51 in cells treated with APH. Cell cycle distribution was determined by flow cytometry (data not shown). Consistent with previous reports (47, 48), BLM and RAD51 nuclear foci increased and colocalized in S-phase cells after treatment with APH (Fig. 8). About 45% of the APH-treated cells displayed Ser[15] phospho-p53 nuclear foci, but none of the untreated cells did. Quantitative analysis of the confocal microscopic pixels indicated that 63 and 39% of Ser[15] phospho-p53 foci colocalized with BLM and RAD51, respectively (Fig. 8*B*). Similarly, Ser[15] phospho-p53, BLM, and RAD51 were found to colocalize after S-phase arrest in U2OS cells, derived from a human osteogenic sarcoma that contains WT p53 (data not shown). These colocalization data provide *in vivo* evidence that p53 may play a role in a recombinational repair pathway that also includes BLM and RAD51.

DISCUSSION

p53-mediated Inhibition of BLM and WRN Helicase Activities—We report evidence that p53 inhibits the helicase activities of both BLM and WRN. WT p53 inhibits BLM and WRN helicase disruption of the X-junction more efficiently than p53 248W, whereas p53 273H lacks this activity. Mutations in p53 including codons 248 and 273 are observed frequently in human cancers (49, 50). The influence of these missense mutations on DNA binding and protein conformation is still unclear. Most of the mutants at codons 248 and 273 do not display any obvious change in their protein con-

formation, as determined by reactivity to antibodies PAb240 or PAb1620, or by binding to hsp70 protein (51). Analysis of the crystal structure of p53 reveals that both 248 and 273 residues contact the DNA directly (52). WT p53 exerts an inhibitory effect on the exonuclease activity of WRN, whereas the p53 273H mutant does not (33). Recent studies indicate that WT p53 inhibits recombinational processes when encountering mismatches in heteroduplexes, but p53 273H is significantly defective in this function (18). Our results indicate that WT p53 can regulate members of the RecQ helicase family involved in recombinational repair, but that the p53 273H mutant lacks this function. These data are consistent with the hypothesis that WT p53 plays a functional role in the helicase-HR pathway.

Effects of Modifications to the p53 C Terminus on Helicase Activity—The p53 C terminus contains several important phosphorylation sites that affect p53-mediated function (44, 45). For example, p53 can be regulated positively or negatively by reversible PKC modifications *in vitro*, affecting the latent or active state of the protein (35, 45), although it is uncertain whether or not PKC phosphorylates p53 *in vivo* (53–55). Certain types of cellular stress, *e.g.* ionizing irradiation, lead to rapid dephosphorylation of p53 at Ser^{376} (46, 56). Recent reports have shown that p53 binds to BLM or WRN *in vivo* and *in vitro* and that p53 lacking the C terminus does not bind to these helicases (26–28). Based on the data presented here, the p53 C terminus is involved in the inhibition of the BLM and WRN helicase activities. p53-mediated inhibition of BLM or WRN helicase activity is reduced by modification of p53 through either the binding of PAb421, a p53-specific antibody that binds to a C-terminal epitope, or phosphorylation at Ser^{376} and Ser^{378}, which inhibits its binding to BLM or WRN. Furthermore, a p53 C-terminal polypeptide (residues 373–383) is sufficient to inhibit BLM or WRN helicase activity, whereas a peptide phosphorylated at Ser^{376} or Ser^{378} lacks this activity. Taken together, our data provide direct evidence that posttranslational modification of the p53 C terminus regulates its interaction with these DNA helicases. The fact that this small C-terminal peptide inhibits helicase activity and that the inhibition can be reversed by phosphorylation indicates that the p53 C terminus contains an active site. Post-translational modification of p53 in response to DNA strand breaks may be a molecular switch that regulates the functional interaction between p53 and DNA helicases.

Insight into the Mechanism of p53-mediated BLM and WRN Helicase Activities—BLM specifically binds to HJ, but fails to form a stable complex with linear, blunt-ended duplex DNA that contains a sequence identical to that of one of the "arms" of HJ (11). This indicates that BLM binds strongly to the crossover region of HJ. WRN also binds to HJ (10). The activity of WRN on recombination intermediates is due, at least in part, to the recognition of the junction within the duplex DNA substrate.

Here, we report that the binding affinity of WT p53 to a mimic of the HJ is higher than that of the p53 248W mutant, and that p53 273H lacks this binding ability. Because abilities of WT p53, p53 248W, and p53 273H proteins to bind to the X-junction correlate with their capacity to inhibit BLM and WRN helicase activities, it is possible that p53 binding to the DNA substrate may be required for p53-mediated inhibition of the helicase activity. However, both helicases also interact physically with WT p53, as shown by far Western blotting and ELISA, indicating that p53 may also modulate the helicase activity by binding directly to the BLM or WRN proteins. Consistent with this latter hypothesis, modification of the p53 C terminus leads to an attenuation of p53-mediated inhibition

on BLM and WRN helicase activities, but does not impair its binding to the X-junction. These findings are strong evidence that inhibition of BLM and WRN helicase activities by p53 is mediated by direct interaction with BLM and WRN, and not with the HJ substrate.

p53 as a Cofactor in BLM-RAD51 HR Pathway—RAD51 is a central component of the HR pathway that is involved in DNA double-strand break repair (47). One major role for HR that has emerged in recent years is to facilitate the reinitiation of replication following replication fork collapse. Removal of HJ is necessary following such repair. Interaction between BLM and RAD51 may, therefore, serve to recruit BLM to the sites of recombinational repair (47, 57). BLM disrupts HJ by branch migration, and the loss of BLM would give rise to excessive recombination, corresponding to the genome-wide hyper-recombination and genomic instability in BLM-deficient cells. p53 binds to RAD51 and is involved in recombinational repair (20), and p53-deficient cells show hyper-recombination (16–18, 21). One model is that p53 or other proteins, *e.g.* RAD51 (47), recruit BLM and WRN to HJ and participate in the assembly of the multiprotein HR complex (28). The physical and functional interactions between these DNA helicases and p53 may be regulated either by its post-translational modification, consistent with the data shown here, or by other proteins in the HR complex. Further studies are needed to refine this model. Consistent with previous reports that WRN copurifies with a DNA replication complex (58) and binds to p53 (26, 27), p53 may regulate the anti-recombinase functions of the human RecQ helicase family members that are critical for the maintenance of genomic stability.

REFERENCES

1. German, J., Ellis, N. A., and Proytcheva, M. (1996) *Clin. Genet.* **49,** 223–231
2. Chakraverty, R. K., and Hickson, I. D. (1999) *Bioessays* **21,** 286–294
3. Yamagata, K., Kato, J.-I., Shimamoto, A., Goto, M., Furuichi, Y., and Ikeda, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8733–8738
4. Wu, L., Davies, S. L., North, P. S., Goulaouic, H., Riou, J. F., Turley, H., Gatter, K. C., and Hickson, I. D. (2000) *J. Biol. Chem.* **275,** 9636–9644
5. Kamath-Loeb, A. S., Johansson, E., Burgers, P. M., and Loeb, L. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4603–4608
6. Szekely, A. M., Chen, Y. H., Zhang, C., Oshima, J., and Weissman, S. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 11365–11370
7. Fry, M., and Loeb, L. A. (1999) *J. Biol. Chem.* **274,** 12797–12802
8. Sun, H., Karow, J. K., Hickson, I. D., and Maizels, N. (1998) *J. Biol. Chem.* **273,** 27587–27592
9. Mohaghegh, P., Karow, J. K., Brosh, J. R., Jr., Bohr, V. A., and Hickson, I. D. (2001) *Nucleic Acids Res.* **29,** 2843–2849
10. Constantinou, A., Tarsounas, M., Karow, J. K., Brosh, R. M., Bohr, V. A., Hickson, I. D., and West, S. C. (2000) *EMBO Reports* **1,** 80–84
11. Karow, J. K., Constantinou, A., Li, J. L., West, S. C., and Hickson, I. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6504–6508
12. Sharples, G. J., Ingleston, S. M., and Lloyd, R. G. (1999) *J. Bacteriol.* **181,** 5543–5550
13. Vogelstein, B., Lane, D., and Levine, A. J. (2000) *Nature* **408,** 307–310
14. Oren, M. (1999) *J. Biol. Chem.* **274,** 36031–36034
15. Wiesmuller, L., Cammenga, J., and Deppert, W. W. (1996) *J. Virol.* **70,** 737–744
16. Bertrand, P., Rouillard, D., Boulet, A., Levalois, C., Soussi, T., and Lopez, B. S. (1997) *Oncogene* **14,** 1117–1122
17. Mekeel, K. L., Tang, W., Kachnic, L. A., Luo, C. M., DeFrank, J. S., and Powell, S. N. (1997) *Oncogene* **14,** 1847–1857
18. Dudenhoffer, C., Kurth, M., Janus, F., Deppert, W., and Wiesmuller, L. (1999) *Oncogene* **18,** 5773–5784
19. Mummenbrauer, T., Janus, F., Muller, B., Wiesmuller, L., Deppert, W., and Grosse, F. (1996) *Cell* **85,** 1089–1099
20. Buchhop, S., Gibson, M. K., Wang, X. W., Wagner, P., Sturzbecher, H. W., and Harris, C. C. (1997) *Nucleic Acids Res.* **25,** 3868–3874
21. Sturzbecher, H.-W., Donzelmann, B., Henning, W., Knippschild, U., and Buchhop, S. (1996) *EMBO J.* **15,** 1992–2002
22. Lee, S., Cavallo, L., and Griffith, J. (1997) *J. Biol. Chem.* **272,** 7532–7539
23. Palecek, E., Vlk, D., Stankova, V., Brazda, V., Vojtesek, B., Hupp, T. R., Schaper, A., and Jovin, T. M. (1997) *Oncogene* **15,** 2201–2209
24. Mazur, S. J., Sakaguchi, K., Appella, E., Wang, X. W., Harris, C. C., and Bohr, V. A. (1999) *J. Mol. Biol.* **292,** 241–249
25. Degtyareva, N., Subramanian, D., and Griffith, J. D. (2001) *J. Biol. Chem.* **276,** 8778–8784

26. Blander, G., Kipnis, J., Leal, J. F., Yu, C. E., Schellenberg, G. D., and Oren, M. (1999) *J. Biol. Chem.* **274,** 29463–29469
27. Spillare, E. A., Robles, A. I., Wang, X. W., Shen, J. C., Schellenberg, G. D., and Harris, C. C. (1999) *Genes Dev.* **13,** 1355–1360
28. Wang, X. W., Tseng, A., Ellis, N. A., Spillare, E. A., Linke, S. P., Robles, A. I., Seker, H., Yang, Q., Hu, P., Beresten, S., Bemmels, N. A., Garfield, S., and Harris, C. C. (2001) *J. Biol. Chem.* **276,** 32948–32955
29. Brosh, R. M., Jr., Orren, D. K., Nehlin, J. O., Ravn, P. H., Kenny, M. K., Machwe, A., and Bohr, V. A. (1999) *J. Biol. Chem.* **274,** 18341–18350
30. Karow, J. K., Newman, R. H., Freemont, P. S., and Hickson, I. D. (1999) *Curr. Biol.* **9,** 597–600
31. Bargonetti, J., Friedman, P. N., Kern, S. E., Vogelstein, B., and Prives, C. (1991) *Cell* **65,** 1083–1091
32. McGlynn, P., and Lloyd, R. G. (1999) *Nucleic Acids Res.* **27,** 3049–3056
33. Brosh, R. M., Jr., Karmakar, P., Sommers, J. A., Yang, Q., Wang, X. W., Spillare, E. A., Harris, C. C., and Bohr, V. A. (2001) *J. Biol. Chem.* **276,** 35093–35102
34. Griffith, J. D., and Christiansen, G. (1978) *Annu. Rev. Biophys. Bioeng.* **7,** 19–35
35. Takenaka, I., Morin, F., Seizinger, B. R., and Kley, N. (1995) *J. Biol. Chem.* **270,** 5405–5411
36. Halazonetis, T. D., and Kandil, A. N. (1993) *EMBO J.* **12,** 5057–5064
37. Kern, S. E., Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991) *Science* **252,** 1708–1711
38. Hainaut, P., and Milner, J. (1993) *Cancer Res.* **53,** 4469–4473
39. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1993) *Nucleic Acids Res.* **21,** 3167–3174
40. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* **71,** 875–886
41. Adams, D. E., and West, S. C. (1995) *Mutat. Res.* **337,** 149–159
42. Rafferty, J. B., Sedelnikova, S. E., Hargreaves, D., Artymiuk, P. J., Baker, P. J., Sharples, G. J., Mahdi, A. A., Lloyd, R. G., and Rice, D. W. (1996) *Science* **274,** 415–421
43. Sanz, M. M., Proytcheva, M., Ellis, N. A., Holloman, W. K., and German, J. (2000) *Cytogenet. Cell Genet.* **91,** 217–223
44. Appella, E., and Anderson, C. W. (2001) *Eur. J. Biochem.* **268,** 2764–2772
45. Jayaraman, L., and Prives, C. (1999) *Cell Mol. Life Sci.* **55,** 76–87
46. Waterman, M. J., Stavridi, E. S., Waterman, J. L., and Halazonetis, T. D. (1998) *Nat. Genet.* **19,** 175–178
47. Wu, L., Davies, S. L., Levitt, N. C., and Hickson, I. D. (2001) *J. Biol. Chem.* **276,** 19375–19381
48. Bischof, O., Kim, S. H., Irving, J., Beresten, S., Ellis, N. A., and Campisi, J. (2001) *J. Cell Biol.* **153,** 367–380
49. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) *Science* **253,** 49–53
50. Hainaut, P., and Hollstein, M. (2000) *Adv. Cancer Res.* **77,** 81–137
51. Ory, K., Legros, Y., Auguin, C., and Soussi, T. (1994) *EMBO J.* **13,** 3496–3504
52. Cho, Y., Gorina, S., Jeffrey, P., and Pavletich, N. P. (1994) *Science* **265,** 346–355
53. Chernov, M. V., Bean, L. J., Lerner, N., and Stark, G. R. (2001) *J. Biol. Chem.* **276,** 31819–31824
54. Milne, D. M., McKendrick, L., Jardine, L. J., Deacon, E., Lord, J. M., and Meek, D. W. (1996) *Oncogene* **13,** 205–211
55. Chernov, M. V., Ramana, C. V., Adler, V. V., and Stark, G. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2284–2289
56. Webley, K., Bond, J. A., Jones, C. J., Blaydes, J. P., Craig, A., Hupp, T., and Wynford-Thomas, D. (2000) *Mol. Cell. Biol.* **20,** 2803–2808
57. Wu, L., and Hickson, I. D. (2001) *Science* **292,** 229–230
58. Lebel, M., Spillare, E. A., Harris, C. C., and Leder, P. (1999) *J. Biol. Chem.* **274,** 37795–37799

The Processing of Holliday Junctions by BLM and WRN Helicases Is Regulated by p53

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