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Research Article

BRCA1 Transcriptionally Regulates Damaged DNA Binding Protein (DDB2) in the DNA Repair Response Following UV-Irradiation

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KEY WORDS:

BRCA1, DDB2, XP, DNA repair, p53

ABBREVIATIONS

DDB2	damaged-DNA binding protein 2
XP	xeroderma pigmentosum

ABSTRACT

The p53 and BRCA1 tumor suppressors are involved in repair processes and may cooperate to transactivate certain genes, including p21WAF1/CIP1 and GADD45. We find that the Xeroderma Pigmentosum Complementation group E (XPE) mutated Damaged-DNA binding protein p48 (DDB2) is upregulated by BRCA1 in a p53-dependent manner following UVC, Adriamycin, or Cisplatin exposure. BRCA1 enhances p53 binding to the DDB2 promoter in vivo as well as p53-dependent transactivation of DDB2 promoter-reporter constructs through a classical p53 DNA responsive element. Antisense abrogation of BRCA1 expression abrogates upregulation of DDB2 after UVC or cisplatin exposure. Using a host cell reactivation assay, DNA repair activity is more significantly restored by introduction of BRCA1 into wt as compared to DDB2-deficient cells. Furthermore disappearance of the photoproducts cyclobutane pyrimidine dimer (CPD) and 6-4 photoproduct (6-4PP) was delayed by antisense abrogation of BRCA1 expression in UV-exposed human cells. Thus the DNA repair function of BRCA1 may be attributed in part to p53-dependent transcriptional induction of DDB2. Loss of BRCA1-dependent DDB2 repair function may contribute to cancer susceptibility and cellular sensitivity to DNA damage.

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INTRODUCTION

The breast and ovarian cancer susceptibility gene BRCA1 is involved in DNA repair, gene transcription, and transcription-coupled repair.[1] Evidence for BRCA1 involvement in DNA repair came from the observation that BRCA1 interacts with DNA repair proteins, such as Rad 50 and Rad 51, p95, and MRE11.[2,3] BACH1, a novel helicase-like protein, has been recently reported to interact with BRCA1.[4] It has also been found that BRCA1 can bind and is phosphorylated by the ATM and hCds1 checkpoint kinases.[3,5] Other evidence has implicated BRCA1 in the control of gene transcription. BRCA1 complexes with RNA polymerase II,[6] RNA helicase A,[7] histone deacetylase components, CtIP, and SWI/SNF related complex,[8-10] and BRCA1 has been shown to interact with individual DNA-binding transcription factors such as p53, c-Myc, STAT1, and ZBRK1.[11-15] Several studies have shown that the expression of p21 and GADD45, can be enhanced by BRCA1.[16-18]

The p53 tumor suppressor plays a central role in mediating the cellular response to DNA damage in mammalian cells.[19] It is believed that p53-dependent cell cycle arrest provides time to repair damage as a checkpoint response. Overexpression of BRCA1 has been shown to induce stabilization of p53 in wt p53 expressing cells.[20] Recent studies have provided evidence for the involvement of p53 in DNA repair,[21] including global genomic repair (GGR),[22] nucleotide excision repair (NER),[23] and base excision repair.[24] The connection between BRCA1 and p53 in transcriptional regulation suggests a potential link between BRCA1 function and genome integrity, possibly through control of the expression of genes that mediate checkpoint control and/or DNA repair.

The rare hereditary disease, Xeroderma Pigmentosum (XP) is characterized biochemically by defective NER, which manifests clinically as sensitivity to UV light and a high incidence of skin cancer. Based on cell fusion studies of cells from XP patients, seven NER complementation groups (A-G) and a post-replication repair-deficient variant group (XPV) have been classified.[25,26] Cell strains from a subset (Ddb-) of individuals carrying XP complementation group E (XPE) lack a damage-specific DNA binding protein p48 (DDB2) activity.[27-29] Because DDB2 was reported to recognize many types of DNA lesions[30-35] and is inducible by treatment with DNA damaging agents,[32,36,37] DDB2 was expected to play a role in damage recognition prior to NER. Several observations have challenged the role of DDB2 in NER. Among XP cells, group E have the mildest defect

as measured by UV-induced unscheduled DNA synthesis and are least sensitive to UV. Furthermore, DDB2 is not required for NER in cell-free extracts.[38,39] However, recent findings indicate that XPE cells are deficient in the GGR of cyclobutane pyrimidine dimers (CPD), and expression of DDB2 suppresses UV-induced mutagenesis,[23,40] suggesting a role of DDB2 in DNA repair.

In this study, we unraveled a role for DDB2 in the BRCA1 response upon DNA damage. We found that DDB2 induction involves BRCA1 and p53 in response to UV-induced (and cisplatin-induced) DNA damage, and mapped the regulation to a p53 responsive element in the human DDB2 promoter. Our results suggest a model in which BRCA1 through p53 DNA-binding regulatory elements regulates expression of DDB2 to promote DNA repair following exposure to UVC or cisplatin. Loss of DDB2 in XPE impairs the activity of BRCA1 in DNA repair. Our results provide a novel pathway of DNA repair downstream of BRCA1, involving transcriptional of a DNA repair gene. The BRCA1-DDB2 connection suggests the possibility that BRCA1 loss of function may predispose to cancer through ineffective DNA repair, a situation similar to XPE where DDB2 function is lost through mutation.

MATERIALS AND METHODS

Cell Lines. The human cell lines Saos2 and U2OS (osteosarcoma), SW480 and DLD1 (colon carcinoma), H460 (lung cancer), PA1 (ovarian cancer), and HCC1937 (breast cancer) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). U2OS-Neo and E6 were obtained by transduction with either pCMV-Neo or E6 into U2OS followed by selection with 400 μg/ml of G418. Epstein-Barr virus-immortalized lymphoblastoid cell lines from a normal individual (ATM +/+, 2184D), and an AT homozygote (ATM -/-, 3189C) were obtained from the Human Genetic Mutant cell Repository (Camden, NJ, USA). Normal fibroblasts and the XPE strain GM01389 were obtained from the Human Genetic Mutant cell Repository (Camden, NJ).

Virus Infection. Ad-LacZ, Ad-p53, and Ad-BRCA1 were previously described.[20,41] To generate an antisense BRCA1 expressing adenovirus (pAd-AS-BRCA1(1-500)), we used the AdEasy system.[42] A HindIII/NotI fragment of human BRCA1 cDNA (1-500 bp) was removed from pCR3-BRCA1(1-500) and ligated into pAdTrack-CMV in the antisense orientation. pAdTrack-AS-BRCA1(1-500) was cotransformed into the BJ5183 bacterial strain with pAdEasy and the homologously recombined pAd-AS-BRCA1(1-500) vector was isolated. pAd-AS-BRCA1(1-500) was transfected into 293 cells and the virus was plaque purified, CsCl-banded, and titered as previously described.[41] The multiplicity of infection (MOI) was defined as the ratio of the number of plaque-forming units (PFU) used in an infection/number of cells.

Antibodies and Western Blot Analysis. Western blot analysis was carried out as previously described.[41] Blotted membranes were immunostained with anti-p53 (Ab-2, 1:500, Oncogene Science), anti-p21 (Ab-1, 1:200, Calbiochem, San Diego, CA), anti-Rb (Ab-5, 1:1000, Oncogene Science), anti-BRCA1 (Ab-1, 1:200, Oncogene Science) or anti-Actin (I-19, 1:200; Santa Cruz) antibody.

Plasmids. To make the pGL3-DDB/BS vectors, the 170 bp upstream region of the DDB2 promoter containing a putative p53-DNA binding site was amplified by PCR using primers 5´-CGAGCTCCAAGCTGGTTT-3´and 5´-GCGTCCTCCGTGTGAAG-3´ and subcloned into pGL3-basic vector (Stratagene) as a KpnI/XhoI fragment. To generate the mutant version (pGL3-DDBSmt), the p53 binding site was mutated using a site directed mutagenesis kit (Quikchange, Stratagene) and the following primers: 5´-CAAGCTGGTTTGAAAAAACCCTGGGCATGTTTGGCGGGAAGTT-3´ and 5´-AACTTCCCGCCAAACATGCCAGGGTTTTTTCAAACCAGCTTG-3´. The DDB2 expression vector was obtained by subcloning the human DDB2 cDNA into pCDNA3.1 vector. All inserts were confirmed by DNA sequencing.

Cell Survival Assay and Flow Cytometry. Five thousand cells were plated in a 24-well culture plate, and were exposed to 50 J/m2 UVC or 10 μM CDDP for 48 hrs. Attached cells were cultured for 2 weeks and stained with Coomassie Blue.[45] Cells were counted in three high power fields and the % of viable cells was obtained from the ratio of treated to untreated cells After plating 1 x 10^5 cells in a 6-well culture plate, cells were exposed to 50 J/m^2 of UV or 10 μM CDDP for 24 hrs following 16 hrs after infection of adenovirus and/or transfection of 1 μg of each plasmids as indicated in the figure legends. Flow cytometry was performed on a Coulter Epics Elite counter. DNA content analysis was performed using MacCycle software (Phoenix Flow Systems, San Diego, CA).

Reporter Assays and Host-Cell Reactivation Assay. Transfections were carried out as previously described.[41] Briefly, 5 x 10^5 cells/well in a 6-well plate were transfected by Lipofectin-DNA conjugates, harvested at 24 hrs after transfection and assayed for luciferase and β-galactosidase activity as previously described.41 For the host-cell reactivation assay, reporter DNA (pCMV-β) was exposed to doses ranging from 0 J/m^2 to 2000 J/m^2 (254 nm) in a UV Stratalinker (Stratagene) prior to transfection,[43] and transfection was performed by using Lipofectamine-2000 reagents (Gibco-BRL). Relative β-gal activity was expressed as percent activity from treated versus untreated control plasmids.

Electrophoretic Mobility Shift and Supershift Assays. The anti-p53 antibody pAb421 (Ab-1, Oncogene Science) was used to activate sequence-specific DNA-binding by p53 in electrophoretic mobility shift assays as described.[44] The oligonucleotide probe contained the following sequences from the human DDB2 promoter: 5´-TTTGAACAAGCCCCTGGGCAT-GTTTGGC-3´.

Northern Blotting. RNA isolation and Northern blotting was performed as described.[41] A EcoRI fragment from pCDNA3-V5-DDB2 was used as a probe for DDB2 mRNA expression. A Not I fragment from pCEP4-WAF1 was used as probe for $p21^{WAF1}$ mRNA expression.[41] A 2.1 kb Hind III fragment from pCEP4-KILLER was used as probe for human KILLER/DR5.[45] Quantitation of hybridization intensity was performed using Imagequant software (Molecular Dynamics).

In Situ Detection of DNA Photoproducts. This procedure was described previously.[46-47] Briefly, 2 x 10^4 cells seeded/well in 24 well culture plates were infected with Ad-LacZ or Ad-AS-BRCA1 at 40 MOI for 24 hrs, then irradiated with 20 J/m^2 UVC. Cells were fixed immediately, at 6 hrs, 24 hrs, and 48 hrs after irradiation with cold (4°C) methanol-acetic acid (3:1) for one hour. Cells were then treated with 0.5% Triton-X100 for 5 min and 0.07 M NaOH in 70% ethanol for 3 min. After washing 4X with PBS for 5 min each wash, cells were incubated with 20% goat serum for 30 min at room temperature. The monoclonal antibodies, 64 M-2 (1/200) and TDM-2 (1/1500) were used for detection of 6-4PP and CPD, respectively.[49] Cells were treated with primary antibodies for 30 min at 37°C. Cells were incubated with goat anti-mouse immunoglobulin G (H + L) conjugated with biotin (PIERCE) at 20 μg/ml final concentration for 30 min. After washing with PBS, cells were incubated with streptavidin-FITC (PIERCE) for 30 min. Cells were treated with 100 μg/ml Rnase A and 0.2 μg/ml propidium iodide for 15 min at room temperature. To determine global genomic repair, 6-4PP or CPD positive cells were visualized under fluorescence microscopy (ZEISS, Germany).

Chromatin Immunoprecipitation Assay. Cells in 100 mm dishes were fixed by the addition of 1% formaldehyde/PBS for 10 min and then treated with 0.125 M Glycine for 5 min. After washing with PBS, the cells were resuspended in 1 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 50 mM NaF, 0.2 mM Na orthovanadate, 5 μM trichostatin A, 0.5 mM PMSF, 15 μM pepstatin A and E-64, and 0.1 μg/ml leupeptin and aprotinin). The cells were sonicated on ice to an average length of 200–1000 bp, and cleared by centrifugation at 14,000 rpm for 10 min. The chromatin solution was precleared for 1 hour at 4°C using 50 μl of ChIP protein A/G agarose beads (Equal amounts of protein A and protein G agarose in 10 mM Tris (pH 8.0), 1 mM EDTA, 200 μg/ml sonicated salmon sperm DNA, 500 μg/ml BSA, 0.05% sodium azide) and 1 μg of mouse IgG. A total of 10 μl of each antibody against p53 (Ab-1 and Ab-2, Calbiochem) and 50 μl of ChIP protein A/G was added to

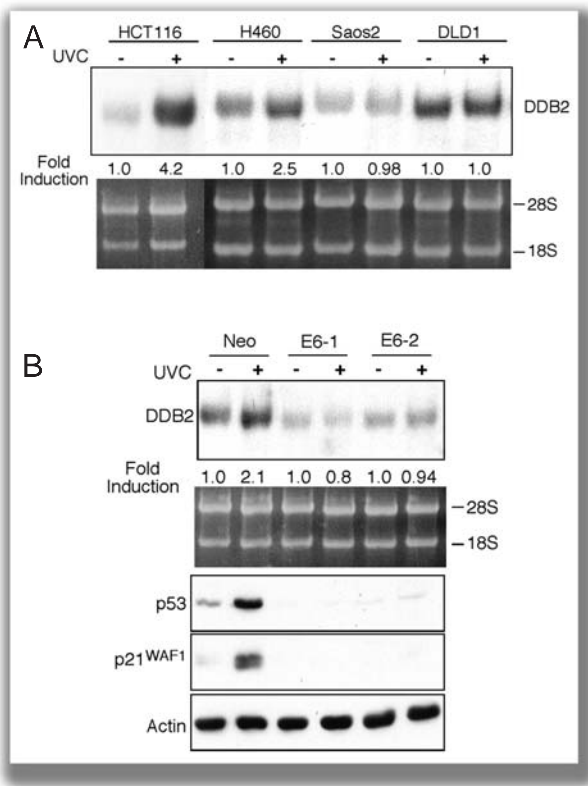

Figure 1. wt p53-dependent induction of DDB2. A. HCT116 (wt-p53), H460 (wt-p53), DLD1 (mt-p53), and Saos2 (p53-null) cell lines were exposed to 20 J/m^2 of UVC, and DDB2 mRNA expression was analyzed 16 hrs by Northern blotting (upper panel). The fold increase in mRNA expression, as compared to no treatment (-), is indicated below the DDB2 hybridizations. An ethidium bromide stain of the RNA is shown to document loading of the RNA (lower panel). B. HPV16-E6 stably introduced into wt p53-expressing U2OS cells eliminates p53 and prevents DDB2 induction after UVC exposure. U2OS clones transfected with either pCMV-Neo (Neo) or -E6 (E6-1, -2) were exposed to 20 J/m^2 UVC, and analyzed for DDB2 mRNA expression by Northern blot (upper panel). The protein expression of p53 and p21^{WAF1} in the respective clones was determined by Western blot (lower panel). Actin protein expression is shown as a control for protein loading of cell lysates.

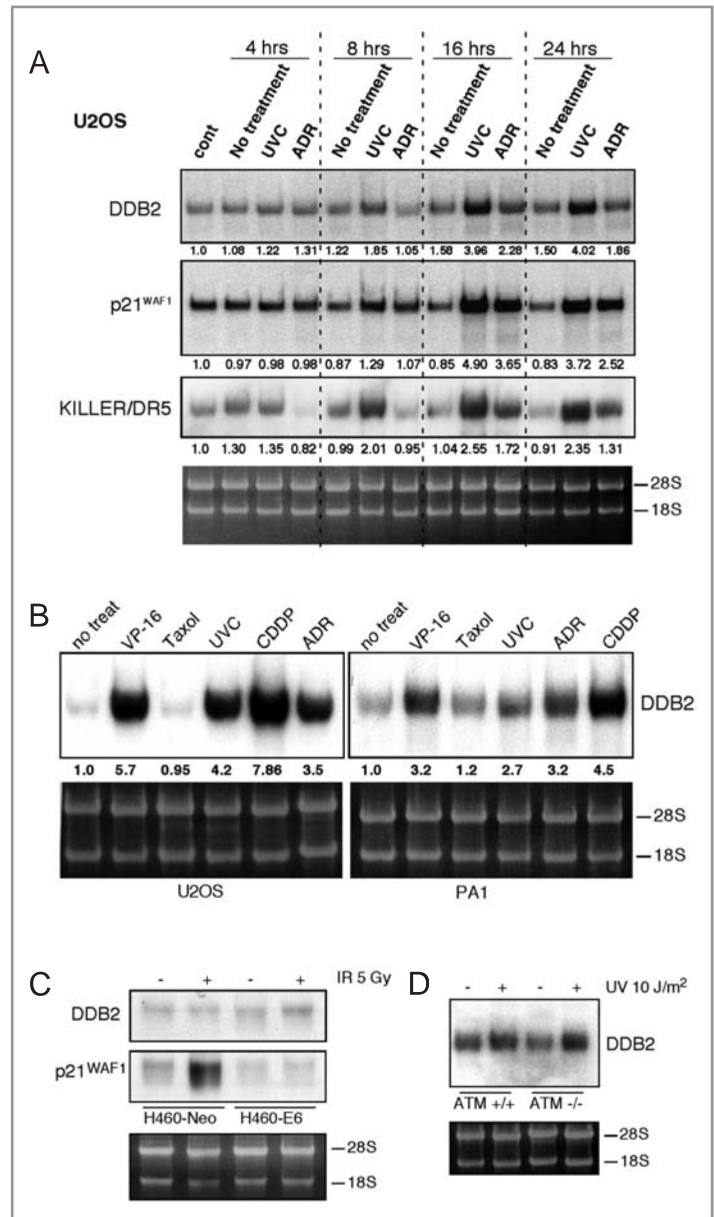

Figure 2. p53-dependent upregulation of DDB2 mRNA upon exposure to DNA damaging agents. A. Northern analysis of total RNA for DDB2, p21^{WAF1}, and KILLER/DR5 harvested at increasing time points (as indicated) following 20 J/m^2 of UVC or 0.2 µg/ml of adriamycin treatment. The fold increase in mRNA expression compared to no treatment is shown. B. Various DNA damaging agents induce DDB2 expression. U2OS or PA1 cells were exposed to DNA damaging agents for 16 hrs (0.2 µM VP-16, 1 µM Taxol, 10 µM CDDP, or 0.2 µg/ml Adriamycin) and DDB2 mRNA expression was examined. C. Ionizing radiation (IR) failed to induce DDB2 in wt p53-expressing H460 cells. H460-Neo and E6 transfectants were exposed to 5 Gy of IR and DDB2 or p21WAF1 mRNA expression was examined. D. Induction of DDB2 after UVC exposure is ATM-independent. Expression of DDB2 is induced after UVC exposure in normal (2184D, ATM +/+) and AT homozygous cells (3189C, ATM-/-). An ethidium bromide stain is shown (panels A-D) to document equivalent RNA loading.

the chromatin solution and incubated overnight at 4°C. Beads were washed twice with RIPA and IP wash buffer (100 mM Tris (pH8.5), 500 mM LiCl, 1% NP-40, 1% deoxycholate), and two more times with RIPA for 3 min each wash. Beads were resuspended in 300 µl of cross-link reversal buffer (125 mM Tris (pH6.8), 10% 2-mercaptoethanol, 4% SDS) and boiled for 30 minutes. DNA was extracted by phenol-chloroform and purified by ethanol precipitation. Resuspended DNA was amplified by PCR using specific primers for the human p21 promoter site-1 (forward, 5′-TCC-CTCCATCCCTATGCT-3′; reverse, 5′-GGCAAGGTTTACCTGGG-3′) and the human DDB2 promoter (forward, 5′-CGAGCT-CAAGCTG-GTTT-3′; reverse, 5′-GCGTCCTCCGTGTGAAG-3′).

RESULTS

Induction of DDB2 Requires wt p53 Expression. First we determined whether the induction of DDB2 was p53-dependent using several wt or mutant-p53 expressing cell lines exposed to UVC. As shown in Figure 1A, only the wt p53-expressing cell lines, HCT116 and H460, induced DDB2 mRNA following UV exposure. No DDB2 mRNA upregulation was observed in the mutant-p53 expressing or p53-null cell lines, DLD1 and Saos2, respectively. To confirm these observations, we tested the induction of DDB2 in U2OS-Neo and -E6 transfectants with or without UVC exposure. As expected, DDB2 as well as p21^{WAF1} induction was abrogated in the U2OS-E6 cells, in which p53 is degraded by HPV-E6, as compared to U2OS-Neo cells (Fig. 1B). Thus, it is clear that functional p53 is required for upregulation of DDB2 in response to UVC damage.

UV Exposure Enhances DDB2 Expression in an ATM-Independent Manner. It has been shown that DDB2 is involved in GGR upon certain forms of DNA damage such as UV irradiation.[40] To examine the effect of

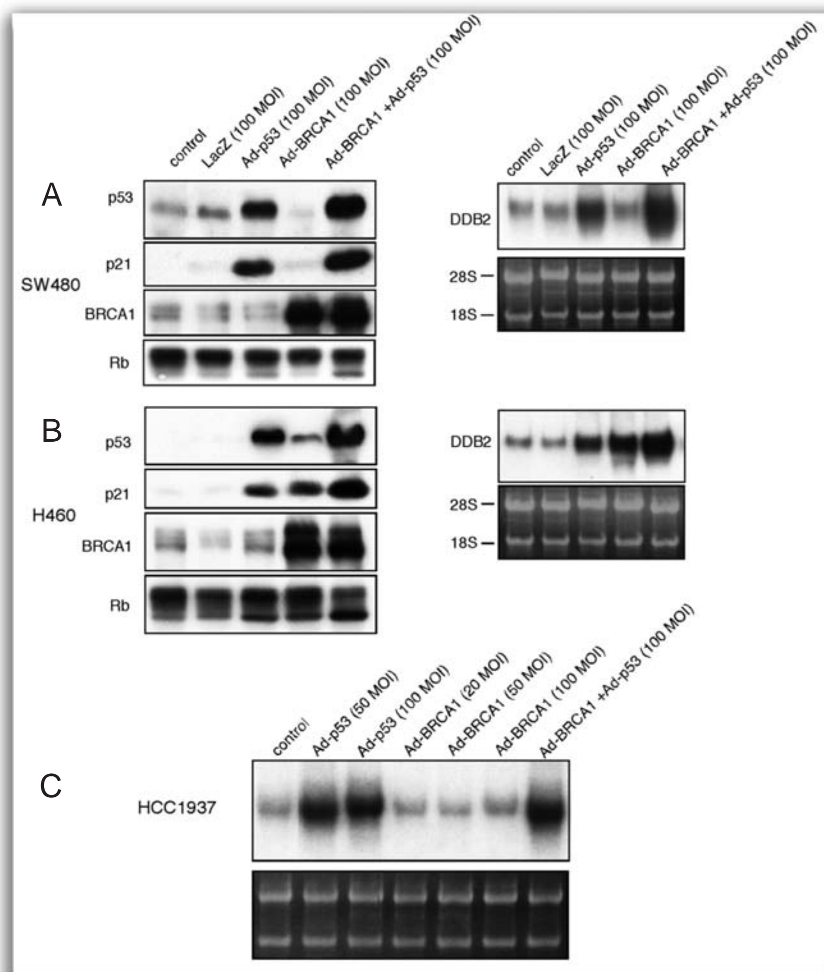

Figure 3. Overexpression of BRCA1 enhances DDB2 gene expression in a p53-dependent manner. Mutant- or wt-p53 expressing cells, SW480 (A) or H460 (B) were infected with Ad-LacZ, Ad-p53, Ad-BRCA1 or the combination of Ad-p53 and Ad-BRCA1 at 100 MOI as ndicated. Cells were harvested at 24 hrs after infection for Western or Northern blotting. C. HCC1937 cells were infected with Ad-LacZ, Ad-p53, Ad-BRCA1 or the combination of Ad-p53 and Ad-BRCA1 at 20-100 MOI as indicated. Cells were harvested at 24 hrs after infection for Northern blotting.

UVC and other DNA damaging agents on the induction of DDB2 mRNA, we performed Northern blot analysis using U2OS cells. As shown in Figure 2A, DDB2 was upregulated up to 3.96-fold at 16 hrs after UVC exposure. This DDB2 induction was somewhat stronger than that observed following Adriamycin treatment. In contrast, the p53 target gene p21^{WAF1} was induced similarly following either UVC or Adriamycin exposure (4.9-fold or 3.65-fold, respectively; Fig. 2A middle panel). The expression pattern of another p53 target gene which is involved in apoptosis, KILLER/DR5, after UVC or Adriamycin exposure also appeared similar to DDB2 induction (Fig. 2A, lower panel). VP-16, Adriamycin and Cisplatinum (CDDP), upregulated DDB2 mRNA in both U2OS and PA1 cell lines, suggesting that DDB2 is a target in the cellular response to a variety of DNA damaging agents, in addition to UVC (Fig. 2B). In the case of exposure to ionizing radiation, DDB2 induction was not observed appreciably as revealed by Northern blot using H460-Neo and E6 cell lines (Fig. 2C).

p53 stabilization following UV exposure occurs in a manner independent of the ATM kinase. We therefore confirmed that the p53-dependent induction of DDB2 following UVC exposure does not require the presence of wt ATM. As shown in Figure 2D, both ATM+/+ and ATM-/- cells showed similar levels of upregulation of DDB2 mRNA upon UVC exposure. These results indicate that the induction of DDB2 is not mediated through the ATM kinase.

Induction of DDB2 Involves BRCA1. Because BRCA1 has been shown to stimulate the p53-dependent transcriptional response, we wondered whether induction of DDB2 by BRCA1 might contribute to the DNA repair process. We first tested whether BRCA1 could induce DDB2 in wt or mutant-p53 expressing cell lines by BRCA1 overexpression using Ad-BRCA1. In the case of the mutant-p53 expressing cell line SW480, BRCA1 failed to appreciably induce DDB2, or p21^{WAF1} to significant levels (Fig. 3A). In contrast, in the case of the wt p53 expressing cell line H460, DDB2 and p21^{WAF1} were strongly induced by BRCA1 overexpression (Fig. 3B). In order to investigate whether induction of DDB2 is dependent on the existence of wt BRCA1 in the cells, we infected HCC1937 cell line expressing both mutant-p53 and muntant-BRCA1 with Ad-p53 and/or Ad-BRCA1. Our results reveal that when wt-p53 is expressed at high levels DDB2 expression can be upregulated even when BRCA1 is mutant. It is possible that wt BRCA1 may serve as a coactivator for p53-dependent induction of DDB2 at more physiological p53 levels following DNA damage or that the BRCA1 mutant in HCC1937 is capable of affecting DDB2 expression in the presence of exogenous wt p53.

We therefore generated an antisense BRCA1-expressing adenovirus (Ad-AS-BRCA1) to evaluate whether BRCA1 is required for DDB2 induction upon DNA damage. We first infected H460 or U2OS cells with Ad-AS-BRCA1 in the presence or absence of Ad-BRCA1 to test whether the Ad-AS-BRCA1 reagent could inhibit endogenous or exogenously overexpressed BRCA1. As shown in Figure 4A, Ad-AS-BRCA1 infection led to suppressed expression of endogenous as well as exogenously overexpressed BRCA1 (Lane 2 vs. 4, Lane 5 vs. 7). Expression of BRCA1 protein became undetectable following infection using 50 MOI of Ad-AS-BRCA1 in both H460 and U2OS cell lines (Lane 2 vs. 4, Lane 1 vs. 7). In the case of U2OS cells, as little as 20 MOI of Ad-AS-BRCA1 was sufficient to substantially decrease endogenous BRCA1 expression (Lane 6). There was some difference in the efficacy of Ad-AS-BRCA1 reduction of BRCA1 expression between the two cell lines, possibly related to their susceptibility to adenovirus infection. We also examined the effect of Ad-AS-BRCA1 on the level of endogenous BRCA1 expression after DNA damage. As shown in Figure 4B, the endogenous level of BRCA1 was clearly suppressed by increasing amounts of Ad-AS-BRCA1 following either UV or CDDP exposure. In order to evaluate whether Ad-AS-BRCA1 could abrogate an endogenous activity of BRCA1, we exposed the cells to UVC or CDDP and measured SubG1 phase to examine the susceptibility to DNA damage after following infection of Ad-LacZ, Ad-BRCA1, or Ad-AS-BRCA1. Elimination of BRCA1 protein clearly sensitized the cells to both UVC and CDDP in U2OS cells (Fig. 4C). We also observed increased susceptibility to UV exposure in Ad-AS-BRCA1 infected-H460 cells (MacLachalan et al. unpublished data). Using the Ad-AS-BRCA1 reagent we next determined the effect of UVC or CDDP on the induction of DDB2 in the presence or absence of BRCA1 protein. Interestingly, p21^{WAF1} induction by UV exposure appeared not as significantly affected by abrogation of BRCA1, but induction of DDB2 by UV exposure appeared totally inhibited (Fig. 4D).

BRCA1 Enhances DDB2 iIduction Through a p53-Dependent Mechanism. In order to identify p53 DNA-binding consensus elements within the DDB2 genomic regulatory region, we searched the public DNA database. We found a perfect match to the 20 bp p53-DNA consensus sequence at 140 bp upstream of the initiation codon of human DDB2 (Fig.

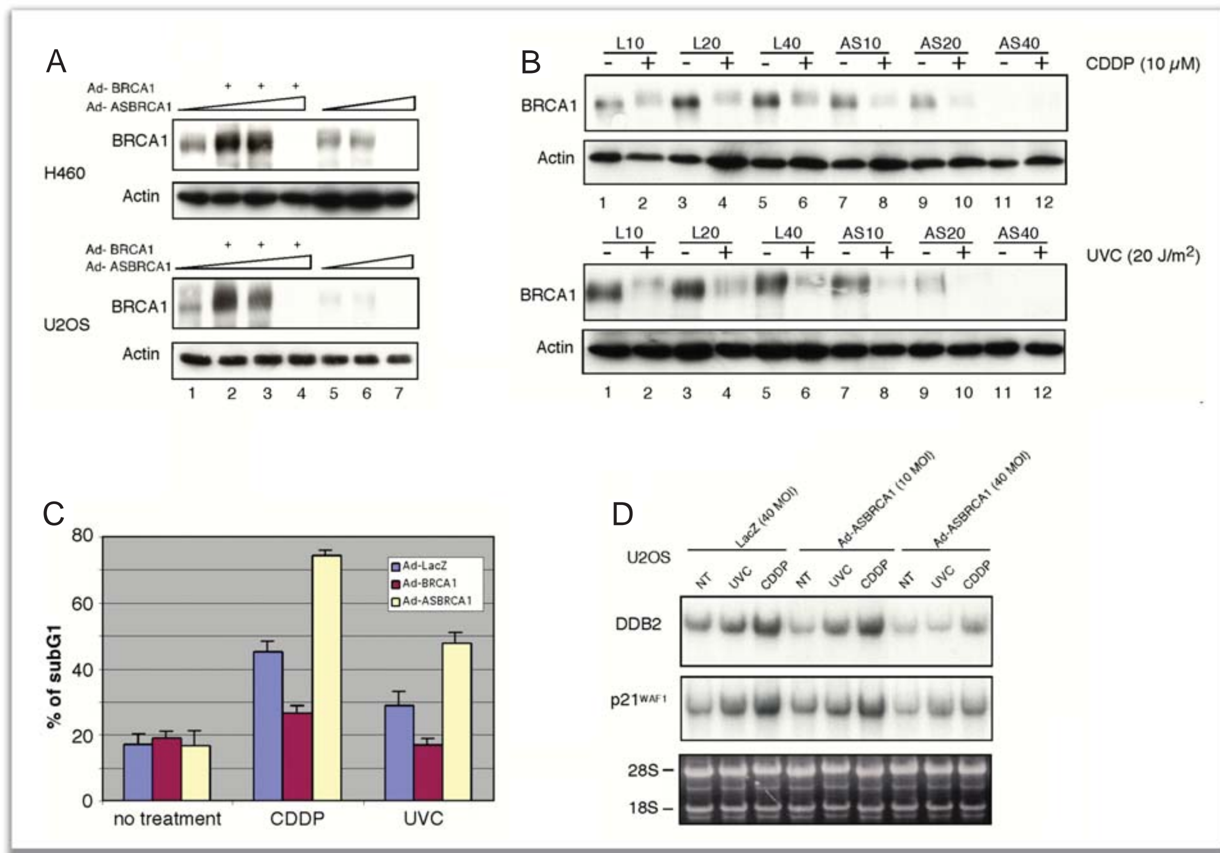

Figure 4. Abrogation of endogenous BRCA1 expression inhibits DDB2 induction following UV or cisplatin exposure. A. H460 or U2OS cells were infected with increasing amounts of Ad-AS-BRCA1 (10 MOI, lanes 2 and 5; 20 MOI, lanes 3 and 6; 50 MOI, lanes 4 and 7) in the presence or absence of 50 MOI of Ad-BRCA1 (indicated as "+") and the expression of BRCA1 was examined. Actin is shown to document protein loading. B, D. U2OS cells were exposed to 10 μM CDDP or 20 J/m^2 UVC for 16 hrs following infection of Ad-LacZ or Ad-AS-BRCA1 at 10-40 MOI as indicated and harvested for Western blotting (B) or Northern blotting (D). Actin protein expression and an ethidium bromide stain are shown to document protein and RNA loading, respectively. C. U2OS cells were exposed to 20 J/m^2 of UVC or 10 μM of CDDP for 24 hrs following infection of Ad-LacZ, -BRCA1, or -AS-BRCA1. Cells were harvested, and the subG1 fraction was analyzed.

5A). We found that this putative p53-binding site can bind to wt p53 (Fig. 5B). We generated a promoter-reporter plasmid containing the wt p53-DNA binding site and a plasmid (pGL3-DDBSmt) containing point mutations within the consensus (Fig. 5C). We examined the transcriptional activity of the wt and mutant DDB2 promoter-reporter constructs in the presence or absence of wt p53 using SW480 cells. As shown in Figure 5D, the wt DDB2 promoter-reporter plasmid pGL3-DDB/BS showed a 36-fold induction in luciferase activity in the presence of wt p53 whereas the mutant pGL3-DDBSmt construct showed no transcriptional activation by wt p53. To elucidate the effect of BRCA1 on the transactivation of DDB2, we cotransfected the wt p53 with or without a human BRCA1 expression vector into SW480 cells. Although BRCA1 could not promote luciferase activity in the absence of wt p53, we observed significantly enhanced luciferase activity after introduction of BRCA1 in the presence of wt p53 (Fig. 5E). To confirm these results, we transfected BRCA1 into wt p53 expressing U2OS cells. As expected, basal promoter activity was observed, presumably in part due to endogenous p53, and this activity was enhanced by increasing the dose BRCA1 (Fig. 5F). We also observed an enhanced transactivation of the DDB2-promoter-reporter after UV exposure (Fig. 5G). In order to ascertain whether BRCA1 could be detected along with p53 at the endogenous DDB2 promoter following DNA damage, we performed a chromatin immunoprecipitation (ChIP) assay (Fig. 5H). Although we could not co-immunoprecipitate either the DDB2 or the p21 promoter with anti-BRCA1 antibody (data not shown), elimination of BRCA1 protein expression by antisense BRCA1 (Ad-AS-BRCA1 infection) clearly suppressed p53 binding to both the DDB2 and the $p21^{WAF1}$ promoter (Fig. 5H, lane 10 and 11 vs. lane 4, 5, 8). These results suggest that BRCA1 is involved in enhancing p53 binding to p53-DNA consensus sequences upon DNA damage in vivo (Fig. 5H). This is likely due to the stabilization of p53 protein by BRCA1. The results demonstrate the existence of a p53 DNA-binding sequence within the human DDB2 promoter region which may mediate the p53-dependent transactivation of the DDB2 gene. Moreover, BRCA1 appears to enhance DDB2 transactivation in a p53-dependent manner through the p53 DNA-binding element within the DDB2 promoter.

DDB2 is Required for Cell Survival and In Vivo DNA Repair Activity After UVC Exposure. To examine the role of DDB2 in GGR and in cell survival upon UVC exposure, we tested cell survival using fibroblasts derived from the XP complementation group E (XPE). Cell survival was determined at 14 days after 50 J/m^2 UVC or 0.2 μM of CDDP exposure of wt or mutant DDB2-expressing cells. As shown in Figure 6A, the XPE fibroblasts carrying mutant-DDB2 (GM01389 cells) showed significantly fewer cells than the wt DDB2-expressing cells after UV exposure (Fig. 6A). Most of GM01389 cells were killed by CDDP exposure, but approximately 20% of DDB2 wt cells were viable (Fig. 6B). GM01389 cells contain a L350P change in one allele and an Asn-349 deletion in the second allele of DDB2.[48] These results indicate that DDB2 may be required for cell survival in response to UVC and CDDP.

To examine DDB2 function using an in vivo system, we used the host-cell reactivation assay.[43] We transfected a UVC-irradiated β-galactosidase expressing vector into the mutant DDB2 expressing XPE GM01389 cells or wt fibroblasts, and measured β-gal activity at 24 hrs later. The wt DDB2-expressing fibroblasts restored β-galactosidase activity more effectively than the mutant DDB2-expressing XPE GM01389 fibroblasts (Fig. 6C), indicating

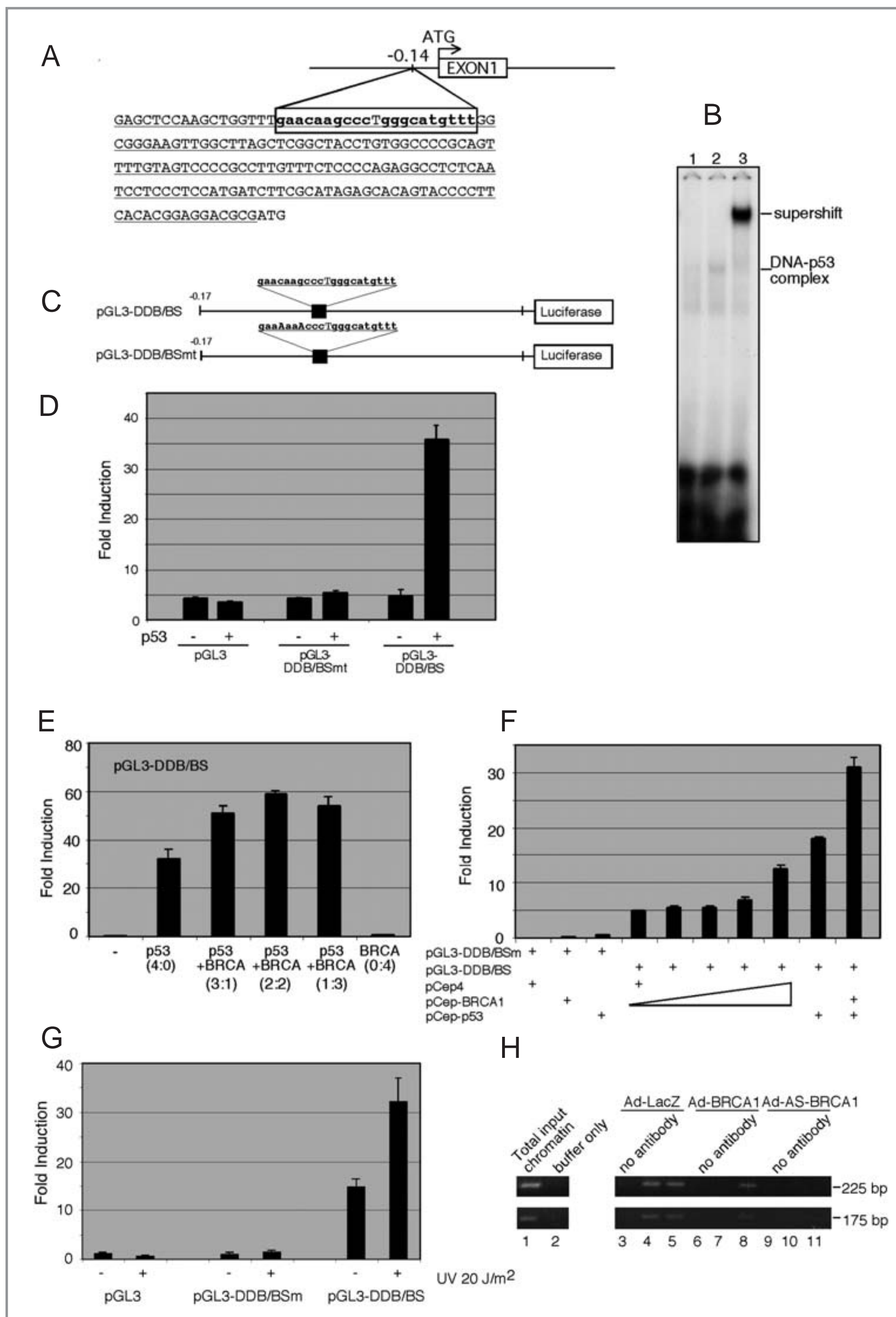

Figure 5. Identification of p53 DNA-binding site in human DDB2 promoter. A. Bold and boxed letters indicate the p53-DNA binding response element within the human DDB2 promoter. B. Electrophoretic mobility shift assay using wt DDB2 binding site probes (underlined in Figure 5A) in the presence of nuclear extracts from cells infected with Ad-LacZ (lane1), Ad-p53 (lane 2, 3). Lane 3 shows a p53-specific supershift. C. Schematic depiction of reporter plasmids used. D. SW480 cells were co-transfected with 1 µg of the indicated reporter plasmids and 500 ng of either pCEP4 control vector (-) or pCEP4-p53 (+). At 24 hrs after transfection, cells were harvested and luciferase activity was quantitated. E. BRCA1 enhances reporter activity from the DDB2 promoter in the presence of wt p53. One µg of pGL3-DDB/BS was cotransfected with pCEP4-BRCA1 (BRCA) and/or pCEP4-p53 (p53) into SW480 cells at the indicated ratios (total amount of the additional DNA was adjusted to one µg in each case), and cells were harvested at 24 hrs after transfection. F. Overexpression of BRCA1 transactivates DDB2 promoter-reporter luciferase activity in wt p53 expressing cells. One µg of pGL3-DDB/BS or pGL3 reporter constructs was co-transfected along with 0.5 µg of pCEP4-p53 and increasing amounts of pCEP4-BRCA1 (0–1.0 µg). Cells were harvested for the luciferase assay at 24 hrs after transfection. G. UVC exposure enhances DDB2 promoter-reporter luciferase activity in wt p53-expressing cells. U2OS cells were transfected with the indicated reporters and exposed to 20 J/m^2 of UV. Cells were harvested at 24 hrs after UV exposure for luciferase assay. H. BRCA1 stimulates p53 binding to the DDB2 and p21^{WAF1} promoters. U2OS cells were treated with (+) or without 10 µM CDDP for 12 hrs following infection by Ad-LacZ, Ad-BRCA1, or Ad-AS-BRCA1 (as indicated), and the harvested for the ChIP assay. As negative controls, lysis buffer alone was added to an anti-p53 IP (lane 2), or lysates were incubated without antibody (lanes 3, 6, 9) to demonstrate specificity of PCR products. For total input of chromatin, 5 µl of a 1: 300 dilution of the DNA was used for PCR.

that DDB2 is required for DNA repair in vivo. In cases of high dose of UVC exposure there was less difference between the two cell lines, possibly due to severe DNA damage. To further investigate DDB2 function using the in vivo system, we examined the effect of overexpressed DDB2 in Saos-2 cells. We co-transfected both the UVC-irradiated β-galactosidase vector together with a V5-tagged DDB2 expression vector into Saos-2 cells, and measured b-galactosidase activity. We observed significantly more reporter activity in the V5-tagged DDB2-transfected cells than the mock-transfected cells. At a dose of 500 J/m^2 of UVC, the repair activity in the Saos2 cells transduced with the DDB2 gene was 2.5-fold higher than the mock-transfected cells (Fig. 6D). Together with the previous experiments, the results indicate that DDB2 may be involved in DNA repair as well as cell survival following UV-induced DNA damage.

Abrogation of DDB2 Sensitizes Cells to DNA Damage. To further understand the role of DDB2 in cell survival following DNA damage, we constructed an antisense-DDB2 expression construct (AS-DDB2) and examined cell survival upon UV or CDDP exposure. Inhibition of DDB2 expression slightly decreased colony formation upon UV exposure in the mutant BRCA1 expressing HCC1937 cells (Fig. 7A). Exogenous overexpression of DDB2 rescued colony formation, presumably due to enhanced repair of UV damage (Fig. 7A). As shown in Figure 7B, the subG1 phase apoptotic population was increased by CDDP treatment in control and AS-DDB2 transfected cells whereas in the case of sense cDNA S-DDB2 transfected cells there was protection from the cytotoxic effect of cisplatin. This observation supports the finding that DDB2 may be involved in DNA repair and cell survival.

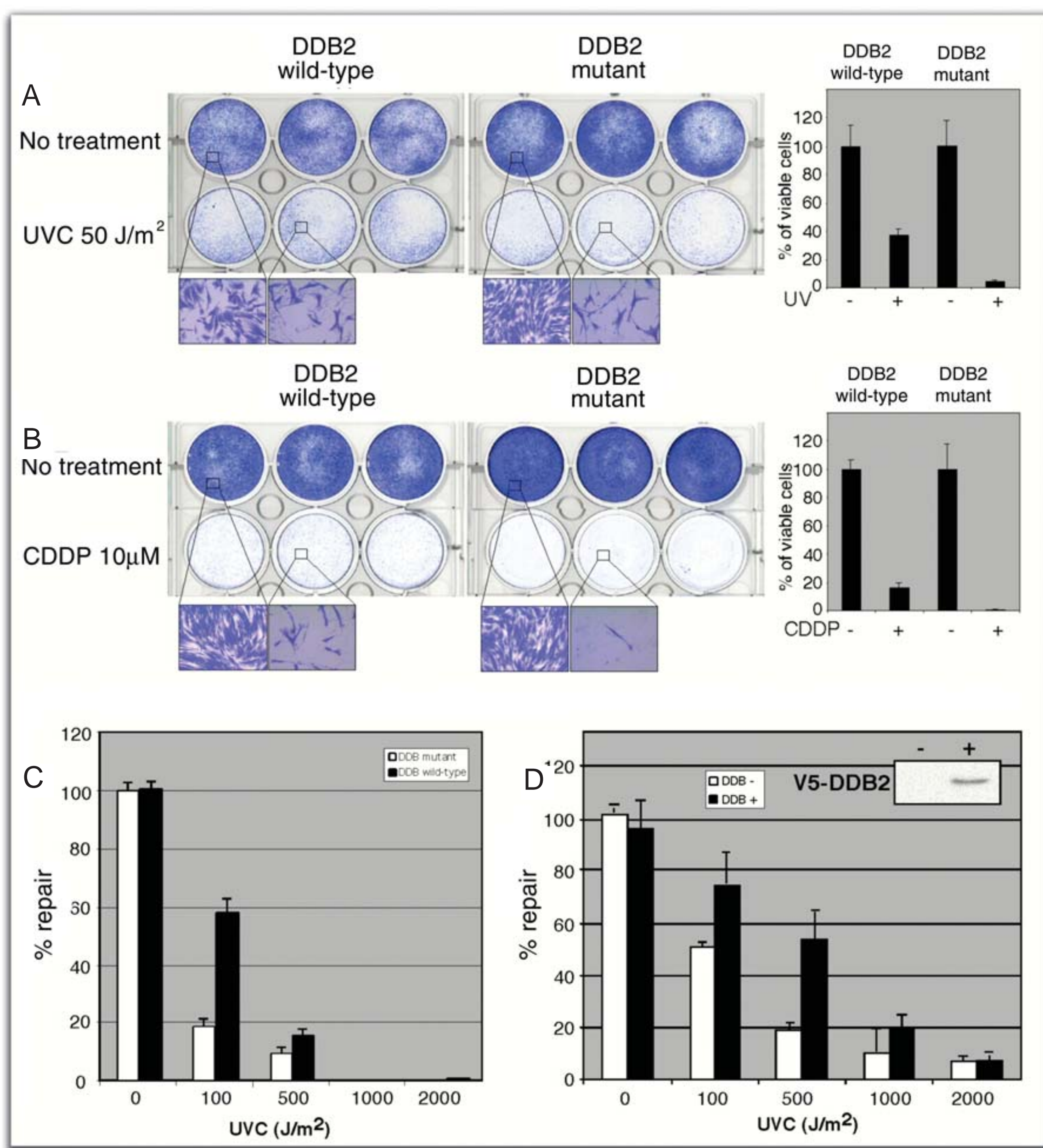

Figure 6. DDB2 is required for cell survival following UVC and CCDP exposure. A, B. Cell survival assay for wt and mutant-DDB2 expressing fibroblasts after 50 J/m^2 UVC (A) or 10 μM CDDP (B) exposure. Right panels show percent of viable cells as compared to each untreated control. C. GM01389 has impaired DNA-repair activity as assessed by a host-cell reactivation assay. GM01389 cells were transfected with a CMV-ß-galactosidase reporter following in-vitro exposure of the DNA to increasing doses (X-axis) of UVC light. ß-gal activity was quantitated at 24 hrs after transfection. D. Re-introduction of DDB2 stimulates DNA-repair activity in Saos2 cells. ß-galactosidase activity was measured at 24 hrs after transfection. The right upper inset shows DDB2 protein in cells untransfected or transduced with DDB2. Similar results were obtained from two experiments.

BRCA1 Cooperates with DDB2 in DNA Repair. In order to elucidate the role of BRCA1 in DDB2-mediated DNA repair, we conducted a host-cell reactivation assay using the mutant DDB2-expressing fibroblasts derived from XPE. Re-introduction of BRCA1 into wt DDB2 expressing cells restored β-galactosidase activity to similar levels observed with transduction of DDB2, and this restoration was enhanced by co-transfection of BRCA1 and DDB2 (Fig. 8A). We observed a small increase in repair activity in DDB2 mutant fibroblasts after transduction with BRCA1. This may be due to the upregulation of GADD45 or other DNA repair genes induced by BRCA1, or other transcription-independent effects of BRCA1 on DNA repair.

Our results reveal that BRCA1 stimulates DNA repair as measured by the host cell reactivation assay to a greater extent in wt DDB2-expressing cells as compared to mutant DDB2-expressing cells (Fig. 8A). It is not unexpected that BRCA1 would stimulate DNA repair even when DDB2 is mutated because BRCA1 clearly can influence DNA repair through other mechanisms. It is not entirely clear why the combination of BRCA1 + DDB2 does not rescue repair to the same extent in mutant DDB2-expressing cells as in wt DDB2-expressing cells (Fig. 8A). It is clear in Figure 6C and 8A that wt DDB2 re-expression can partially rescue repair of DDB2-mutant expressing cells. It is also clear that in the DDB2-mutant p48-expressing cells the combination of BRCA1 and wt DDB2 provides an additive partial rescue of repair in the host cell reactivation assay.

Finally, we investigated the effect of abrogation of BRCA1 on the removal of photoproducts induced by UV using photoproduct-specific antibodies.[49] DDB2 has been shown to play a key role in removing CPDs during GGR

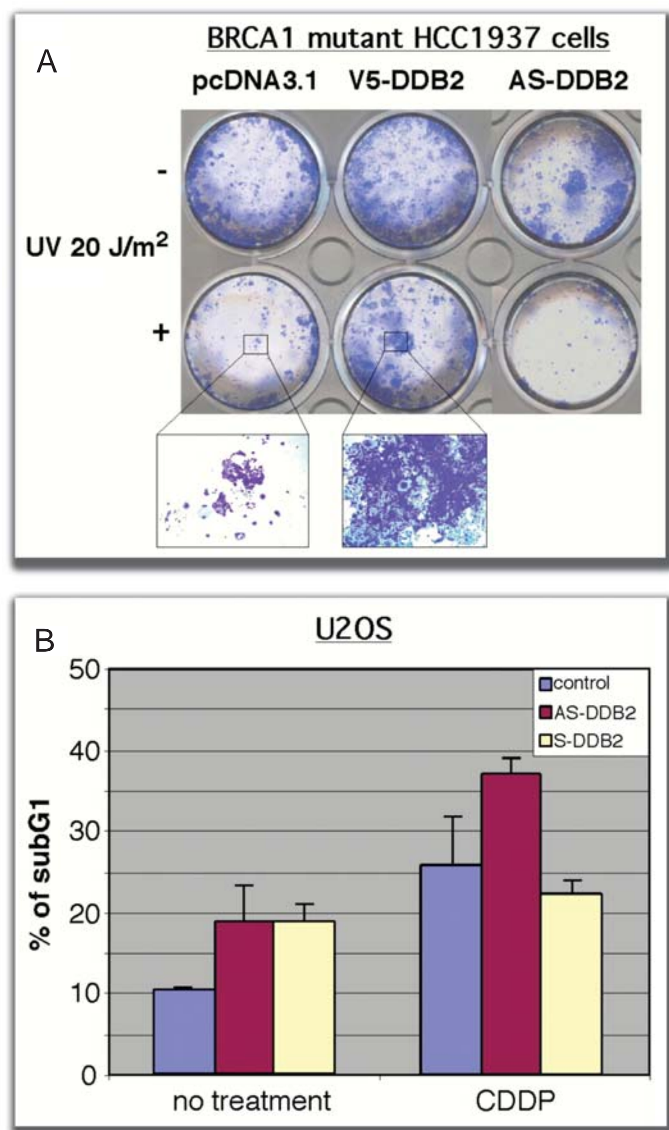

Figure 7. Introduction of AS-DDB2 sensitizes cells to DNA damage. A. HCC1937 mutant BRCA1-expressing breast cancer cells were transfected with mock (pcDNA3.1), V5-DDB2, or AS-DDB2 expression vectors for 24 hrs and exposed to 20 J/m^2 of UVC. Cells were stained with Coomassie Blue after 3 days. High magnification field of the well is shown in the bottom part of Figure 7A. B. U2OS cells were transfected with mock (pcDNA3.1), V5-DDB2, or AS-DDB2 expression vectors for 24 hrs and exposed to 10 μM CDDP for 48 hrs. Cells were subsequently analyzed by flow cytometry to quantitate the apoptotic population.

in vivo and it was proposed that the GGR of CPDs is initiated by the binding of DDB, leading to NER. We investigated the effect of antisense abrogation of BRCA1 on DNA repair of UV-induced photoproducts. As shown in Figure 7B, before exposure to UV none of the cells expressed any detectable CPD or 6-4PP photoproducts (panels a, f, k, and p), but immediately after UV both CPD and 6-4PP were detected (panels b, g, l, and q). Repair of both photoproducts was achieved within 24 hrs following UVC exposure in Ad-LacZ infected cells (CPD, panels b vs. e; 6-4PP, panels g vs. j). In the case of the 6-4 photoproduct repair in Ad-LacZ infected (control) cells was essentially complete as early as 6 hrs after UV exposure. In Ad-AS-BRCA1 infected wt p53-expressing U2OS cells, removal of photoproducts was delayed (Figure 8B, CPD, panels e vs. o; 6-4PP, panels j vs. t) following UV exposure. Our results suggest that the DNA repair function of BRCA1, in part, might be accomplished through a transcriptional induction of DDB2 required for DNA damage repair.

DISCUSSION

The important finding here is that the tumor suppressor BRCA1, through a transcriptional response involving the p53 protein, upregulates expression of a DNA repair protein, DDB2 or p48. While there is good evidence that BRCA1 influences DNA repair through association with repair proteins such as MRE11, RAD50, the BASC or BACH1,[2-4] our studies reveal a BRCA1-mediated transcriptional response leading directly to repair of DNA damage. This is also in contrast to a prevailing view that transcriptional responses by p53 or BRCA1 lead to cell cycle arrest[50-51] which allows time for repair of damage prior to continued DNA replication (i.e., a less direct connection between transcription and repair). Because DDB2 is mutated in XPE, a cancer predisposing syndrome, a link between DDB2 loss-of-function and tumor susceptibility in the case of BRCA1-associated breast and ovarian cancer may exist and is worth investigating. This link is further supported by recent reports that offspring of patients with bilateral breast cancers have an increased incidence of squamous skin cancers[52] and UV exposure can cause internal malignancy in p53 ± mice.[53]

Our results strengthen the association between BRCA1 and the p53 response. It is known that overexpression of p53 protein leads to apoptosis of most cancer cell lines, whereas overexpression of BRCA1 does not lead to apoptosis of most wt p53-expressing cell lines, despite p53 stabilization. Because BRCA1 overexpression leads to wt p53 stabilization,[20,51] we investigated the activation of target genes by BRCA1 versus p53. Our results reveal that BRCA1 directs a selective p53 transcriptional response that does not upregulate all p53 target genes non-specifically. It has recently been reported that p53 may selectively regulate its targets by recruiting specific cofactors to distinct DNA binding sites.[54] We are finding that BRCA1 overexpression in wt p53-expressing cells leads to BRCA1-dependent upregulation of p53 targets involved in cell cycle arrest (p21) or DNA repair (DDB2, p53R2) but not apoptosis (Bax, DR5, Fas, etc).

It is of interest that upregulation of DDB2 is abrogated by overexpression of anti-sense BRCA1 in wt p53-expressing cells exposed to UVC. It is clear that a p53 DNA-binding element exists in the DDB2 promoter and appears to mediate the BRCA1-dependent upregulation of DDB2. The result from the ChIP assay shows that abrogation of BRCA1 expression suppresses p53 binding to both the $p21^{WAF1}$ and the DDB2 promoter (Fig. 5H). While binding by p53 to the p21 and DDB2 loci was inhibited by antisense BRCA1 (Fig. 5H), there was apparently still some induction of p21 in response to UV (Fig. 4D). It is possible that increased $p21^{WAF1}$ expression may be mediated through p53-independent pathways. In the case of DDB2 it is possible that BRCA1 plays a more critical role in its upregulation in response to UV. This should be clarified in future work on how BRCA1 regulates p53 function, stabilization and the selectivity of p53 for target gene activation.

In summary, we identify the DDB2 gene mutated in the cancer prone XPE as a novel BRCA1 target gene, a member of a BRCA1-regulated p53 response involved in DNA repair. Future efforts can be directed at elucidating the contribution to tumor suppression by the transcriptional component leading to DDB2 induction versus the role of other BRCA1 interactions with repair proteins. By analogy to cell cycle checkpoint controlling mechanisms, it can be envisioned that rapid protein-protein interactions initiate the DNA repair response whereas a slower transcriptional response may maintain or enhance repair activity. Our results have some implications for the

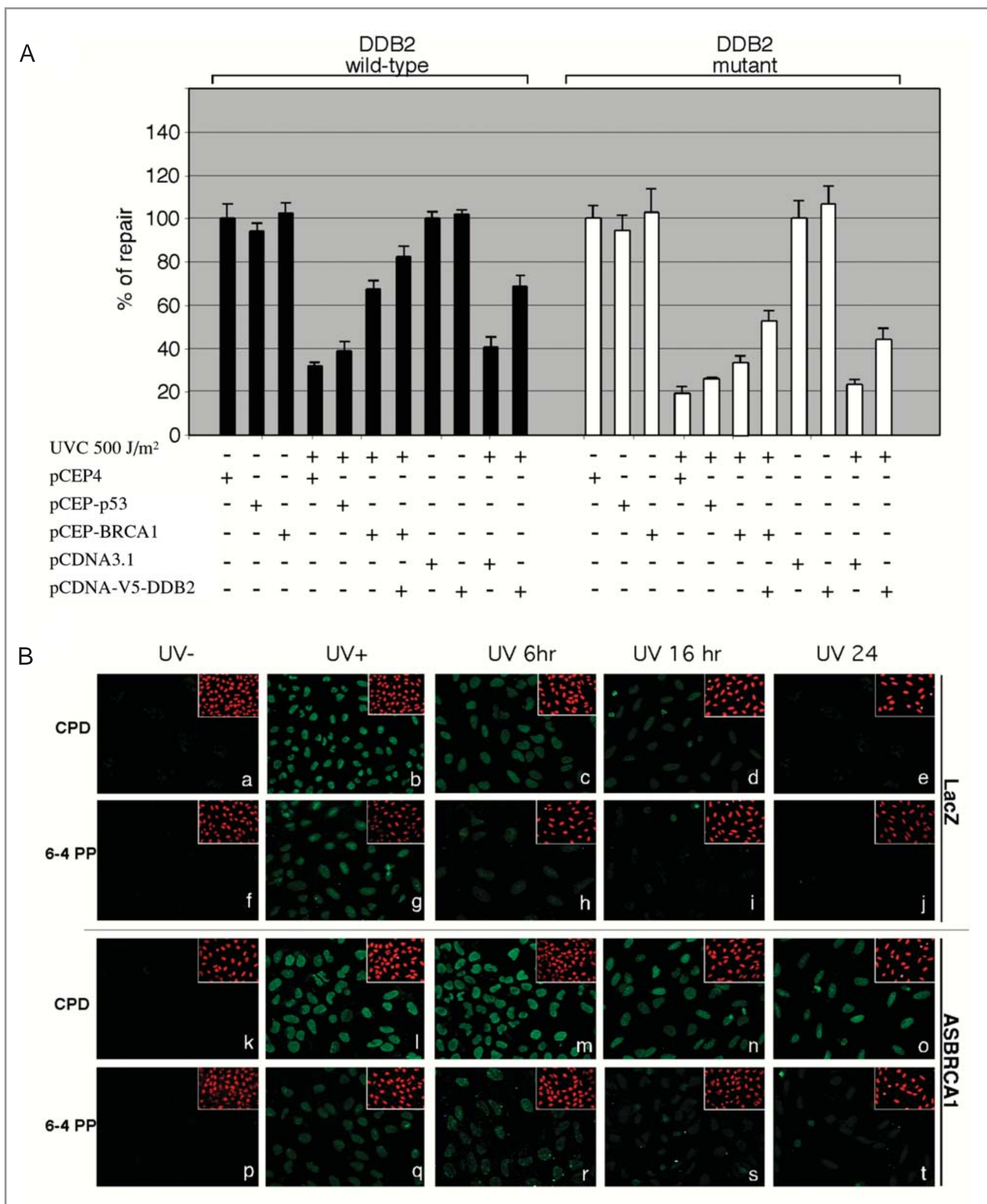

Figure 8. BRCA1 stimulates DNA-repair activity in DDB2-expressing fibroblasts as compared to mutant-DDB2 expressing cells. A. Cells were co-transfected with a CMV-β-galactosidase reporter plasmid following in-vitro exposure of the DNA to 500 J/m^2 of UV light along with either wt p53, BRCA1 and/or DDB2 expressing vectors (or combinations, as indicated), and then β-galactosidase activity was measured at 24 hrs after transfection. The results were obtained from three independent experiments. B. AS-BRCA1 delays the removal of damaged-DNA, CPD and 6-4PP, upon UVC exposure. U2OS cells were fixed immediately (UV +), at 6 hrs, 16 hrs, or 24 hrs after 20 J/m^2 UVC exposure following infection with Ad-LacZ (LacZ) or Ad-AS-BRCA1 (ASBRCA1) for 24 hrs previously. Cells were stained with photoproduct-specific antibody, CPD (panels a-e, and k-o), and 6-4PP (panels f-j, and p-t), and visualized. The right upper inset shows PI staining of the cells in the same fields.

mechanism of action of BRCA1. BRCA1 may normally be involved in surveying for DNA damage and signaling repair, in part through the p53 pathway. Mutation of BRCA1 would be expected to lead to ineffective repair, and accumulation of damage, thereby providing the opportunity for tumor development as well as loss of cell viability observed during development of BRCA1-null embryos. We suggest a testable hypothesis that reduced DDB2 function may contribute to tumorigenesis in breast and ovarian cancer.

Acknowledgements

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