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Degradation of Transcription Repressor ZBRK1 through the Ubiquitin-Proteasome Pathway Relieves Repression of Gadd45a upon DNA Damage

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Induction of gene expression in response to DNA damage is important for repairing damaged DNA for cell survival. Previously, we identified a novel zinc finger protein, ZBRK1, which contains a KRAB domain at the N terminus, eight zinc fingers at the center, and a BRCA1-binding region at the C terminus. In a BRCA1-dependent manner, ZBRK1 represses Gadd45a transcription through binding to a specific sequence in intron 3. In addition, ZBRK1-binding sequences are located at the regulatory region of many DNA damage-inducible genes, suggesting that ZBRK1 may have a role in DNA damage response. However, it is unclear how transcription repression by ZBRK1 is relieved subsequent to DNA damage. Here we report that ZBRK1 is rapidly degraded upon treatment with the DNA-damaging agents UV and methyl methanesulfonate. Specific proteasome inhibitors block DNA damage-induced degradation of ZBRK1, and the polyubiquitinated form of ZBRK1 is detectable, suggesting that the ubiquitin-proteasome pathway mediates the degradation of ZBRK1. In both BRCA1-proficient and -deficient cells, ZBRK1 is degraded with similar efficiencies independent of BRCA1 E3 ligase activity. By analysis of a series of ZBRK1 mutants, a 44-amino-acid element located between the N-terminal KRAB domain and the eight zinc fingers was found to be sufficient for the DNA damage-induced degradation of ZBRK1. Cells expressing a ZBRK1 mutant lacking the 44-amino-acid element are hypersensitive to DNA damage and are compromised for Gadd45a derepression. These results indicate that ZBRK1 is a novel target for DNA damage-induced degradation and provide a mechanistic explanation of how ZBRK1 is regulated in response to DNA damage.

DNA damage triggers a complex signaling pathway that activates various cellular responses, including the arrest of cell cycle progression and the recruitment of the protein machinery to repair damaged DNA. Coordinated regulation of the expression of a subset of genes that play essential roles in these responses is a key step in maintaining genomic integrity. Failure to induce the expression of these genes often leads to cell death due to incomplete repair of the damaged DNA (19, 42). To achieve precise control of the expression of these genes, the stability and/or activity of transcription activators and repressors is tightly regulated through different mechanisms, including phosphorylation, acetylation, and ubiquitination. It has been demonstrated that the activity of many important transcription regulators, such as NF-κB (33), p53 (28), c-Jun (44), β-catenin (1), and E2F-1 (12), are regulated by the ubiquitin-proteasome pathway. Protein ubiquitination is a multistep process that requires three classes of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (35). Previous studies have shown that major control and selectivity are determined by ubiquitin E3 ligase at the substrate ubiquitination step. Polyubiquitination of a substrate protein leads to its recognition and degradation by the 26S proteasome (5). Aberrations in the ubiquitin-proteasome pathway have been implicated in the pathogenesis of several human diseases, including cancers (3, 27, 31).

It has been shown that BRCA1 regulates the transcription of several DNA damage response genes, including p21 and Gadd45a (9, 25, 26, 41, 59). However, BRCA1 must associate with sequence-specific binding transcription factors to execute its transcription regulation because BRCA1 lacks the ability to recognize these regulatory sequences. Zheng et al. previously identified a novel zinc finger protein, ZBRK1, which encodes a 532-amino-acid polypeptide containing an N-terminal KRAB (Krüppel-associated box) domain, a central eight-zinc-finger domain, and a BRCA1-binding region at the C terminus (59). Furthermore, it has been shown that ZBRK1 represses Gadd45a transcription through recognition of and binding to its cognate DNA element, GGGxxxCAGxxxTTT, within intron 3 in a BRCA1-dependent manner. It has been noted that additional potential ZBRK1-binding sites exist in the Gadd45a promoter region, suggesting that ZBRK1 represses Gadd45a expression through multiple binding sites.

ZBRK1 may serve as a transcription repressor either through its KRAB domain or by interacting with BRCA1. The KRAB domain, a highly conserved 75-amino-acid motif at the N terminus of zinc finger proteins (2), has a potent transcription repression activity (30, 46, 49). KRAB domain-mediated repression requires recruitment of a corepressor, KAP-1 (7), also named TIF1β (32) or KRIP-1 (20). KAP-1 acts as a tether to recruit various silencing molecules, such as the NuRD histone deacetylase complex, a methyltransferase, and members of the heterochromatin protein 1 (HP1) family (23, 38, 39).

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Similarly, BRCA1 has also been implicated in transcription repression. BRCA1 represses c-Myc-mediated transcriptional activation (47) and inhibits the transactivation activity of estrogen receptor (6, 57), perhaps through its association with the corepressor protein CtIP (25) and histone deacetylases (52). Thus, it is most likely that ZBRK1 executes its transcription repression through both the KRAB domain and the BRCA1-interacting region. Consistent with this prediction, it has been shown that ZBRK1 mutants lacking either the KRAB domain or the BRCA1-binding region fail to repress Gadd45a transcription (59).

In addition to Gadd45a, which plays a crucial role in mediating cell cycle arrest upon DNA damage, a large number of other DNA damage-inducible genes also contain the potential ZBRK1-binding sites in their regulatory regions (59). Thus, it is likely that ZBRK1 plays a key role in regulating DNA damage-inducible gene transcription. In this report, we show that ZBRK1 is degraded following UV and methyl methanesulfonate (MMS) treatment through the ubiquitin-proteasome pathway to relieve the targeted gene repression, thus providing a mechanistic view for the regulation of ZBRK1 function in response to DNA damage.

MATERIALS AND METHODS

Cell culture. U2OS, a human osteosarcoma cell line, was cultured in low-glucose Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS), and HCC1937, a human breast carcinoma cell line (43), was grown in RPMI medium containing 10% FCS. *p53*$^{-/-}$ and *p53*$^{-/-}$/*Brca1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) that were generated previously (59) were cultured in high-glucose Dulbecco modified Eagle medium containing 5% FCS.

Treatment with DNA damage agents and protease inhibitors. DNA damage treatment was performed with gamma irradiation (20 Gy) by using a ^{131}Cs source, UV (40 J/m^2) by using a germicidal UV lamp, or MMS (0.05%; Sigma). To measure the half-life of GFP-ZBRK1 protein, cells were treated with cycloheximide (50 μg/ml) alone or with either UV (40 J/m^2) or MMS (0.05%) and harvested at different time points for Western blot analysis. To examine proteasome pathway-dependent degradation, specific proteasome inhibitors (MG132 [20 μM; Sigma] and epoxomicin [10 μM; Calbiochem]), a calpain inhibitor (*N*-acetyl-Leu-Leu-Met-al [ALLM; 50 μM; Sigma]), or a trypsin-cysteine protease inhibitor (leupeptin [200 μg/ml; Roche Applied Science]) were used.

Western blot analysis. Cells harvested after treatment were lysed in Lysis250 buffer for immunoprecipitation and Western blot analysis as described previously (59) by using anti-green fluorescent protein (anti-GFP) antibodies (Roche Applied Science), antibodies for actin (C-2) and Gadd45a (H-165) (Santa Cruz, Inc.), antiubiquitin antibodies (P4G7) (Covance, Richmond, Calif.), anti-BARD1 antibodies (EE6) (provided by R. Baer) (16), antihemagglutinin (anti-HA) antibodies (12CA5), and anti-BRCA1 antibodies (6B4) (59). Polyclonal and monoclonal antibodies for ZBRK1 were used for detection of endogenous ZBRK1 (59). The ECL system (Amersham/Pharmacia) was used for detection of immune complexes, and the intensity of bands was quantified by densitometry using NucleoTech GelExpert 3.5 software. The relative values of each band intensity were calculated relative to the band intensity of untreated sample after normalization with the actin signal.

Plasmid constructions. Plasmids for expressing GFP-ZBRK1 (pCHPL-GFP-ZBRK1) and mutants with a deletion of either the KRAB domain (pCHPL-GFP-ZBRK1-ΔK) or C-terminal region (pCHPL-GFP-ZBRK1-ΔC) were generated as previously described (59). The series of GFP-ZBRK1 deletion constructs shown in Fig. 4A were generated using a QuikChange site-directed mutagenesis kit (Stratagene). The plasmid pCHPL-BRCA1 was generated for expression of BRCA1 by inserting a full-length BRCA1 cDNA into the pCHPL vector (24). A HA-ubiquitin expression vector, HA-pCS2-FA-Ub, was generated by fusing a full-length ubiquitin cDNA in frame with the HA vector, HA-pCS2-FA (provided by R. Yew).

Transfection and establishment of stable cell lines. To generate cell lines stably expressing GFP-ZBRK1, *p53*$^{-/-}$ and *p53*$^{-/-}$/*Brca1*$^{-/-}$ MEFs were transfected with pCHPL-GFP-ZBRK1 plasmid by using Lipofectin reagent (Invitrogen) and subjected to selection with 200 μg of hygromycin/ml for 2 weeks. The resulting colonies were isolated and analyzed for GFP-ZBRK1 expression by immunoblotting. U2OS cell lines stably expressing the GFP-ZBRK1-ΔN3 mutant were generated after transfection of pCHPL-GFP-ZBRK1-ΔN3 plasmid as described above.

Luciferase reporter assay. U2OS cells (2×10^5) were transfected with 50 ng of pSV40 β-galactosidase and pGL3p-IN3AB reporter plasmid together with 200 ng of one of the pCHPL vectors, pCHPL-GFP-ZBRK1 or pCHPL-GFP-ZBRK1-ΔN3 plasmid. Cells were harvested 48 h after transfection, and the luciferase activity was assayed by using the Promega luciferase assay system and a luminometer (LB953; Berthold).

Ex vivo ubiquitination assay. U2OS (2×10^6) cells expressing GFP-ZBRK1 were transfected with 10 μg of the HA-ubiquitin expression plasmid by using Lipofectin reagent (Invitrogen). After 48 h, the cells were treated with either the proteasome inhibitor MG132 (20 μM in dimethyl sulfoxide [DMSO]) or vehicle together with either UV (40 J/m^2) or MMS (0.05%) for 2 h, lysed with sodium dodecyl sulfate (SDS) buffer (50 mM Tris [pH 7.5], 0.5 mM EDTA, 1 mM dithiothreitol, 1% SDS), and boiled for 5 min. The extracts were diluted 10-fold with buffer A (50 mM HEPES [pH 7.5], 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors and 10 mM *N*-ethylmaleimide (Sigma) and then immunoprecipitated with anti-GFP antibodies. The precipitates were immunoblotted with anti-GFP antibodies and reprobed with anti-ubiquitin antibodies.

Colony formation assay. U2OS and its derivative cell lines stably expressing GFP-ZBRK1 or GFP-ZBRK1-ΔN3 (1.5×10^3) were seeded in a 100-mm-diameter plate and treated with UV (20 J/m^2) or MMS (200 μg/ml) for 2 h. The cells were grown for 12 days to form colonies. The number of colonies was counted after staining with methylene blue and was expressed as a percentage of untreated cells. The colony formation assay was repeated twice, and the data presented are mean values and standard deviations.

RESULTS

Degradation of ZBRK1 was induced by DNA damage. To understand the derepression mechanism of ZBRK1 upon DNA damage, we first examined endogenous ZBRK1 levels after treatment with several DNA-damaging agents including gamma irradiation, UV, and MMS. Because cells have only a small amount of ZBRK1, its detection by straight Western blot analysis was quite a challenge. To circumvent this difficulty, ZBRK1 was first immunoprecipitated by anti-ZBRK1 polyclonal antibodies from 3 mg of total protein lysates and then analyzed by Western blotting using anti-ZBRK1 monoclonal antibodies. As shown in Fig. 1A, an immunoreactive band was detected at the position with the same mobility as an in vitro-translated product (compare lanes 1 and 6). Immunoprecipitation by anti-Myc antibodies (Fig. 1A, lane 2) revealed no detectable immunoreactive bands with the same mobility, suggesting that the detected ZBRK1 band represents the authentic endogenous ZBRK1. Interestingly, the amount of ZBRK1 was decreased significantly upon UV and MMS treatment (Fig. 1A, lanes 4 and 5, respectively) but not after gamma irradiation (Fig. 1A, compare lanes 3 and 6).

To explore this potentially significant observation, we established cell lines ectopically expressing GFP-ZBRK1 that translationally fused ZBRK1 with GFP to investigate the basis of ZBRK1 stability. Consistent with the endogenous protein, exogenous GFP-ZBRK1 also decreased significantly after UV and MMS treatment but not after gamma irradiation (Fig. 1B). A kinetic study showed that GFP-ZBRK1 decreased to less than 10% of the untreated control after 2 h following UV and MMS treatment (Fig. 1C). To rule out the possibility that the decrease of GFP-ZBRK1 by UV and MMS treatment resulted from the inhibition of protein synthesis, we measured the GFP-ZBRK1 turnover rate in the presence of the protein synthesis inhibitor cycloheximide (Fig. 1D). In the absence of DNA

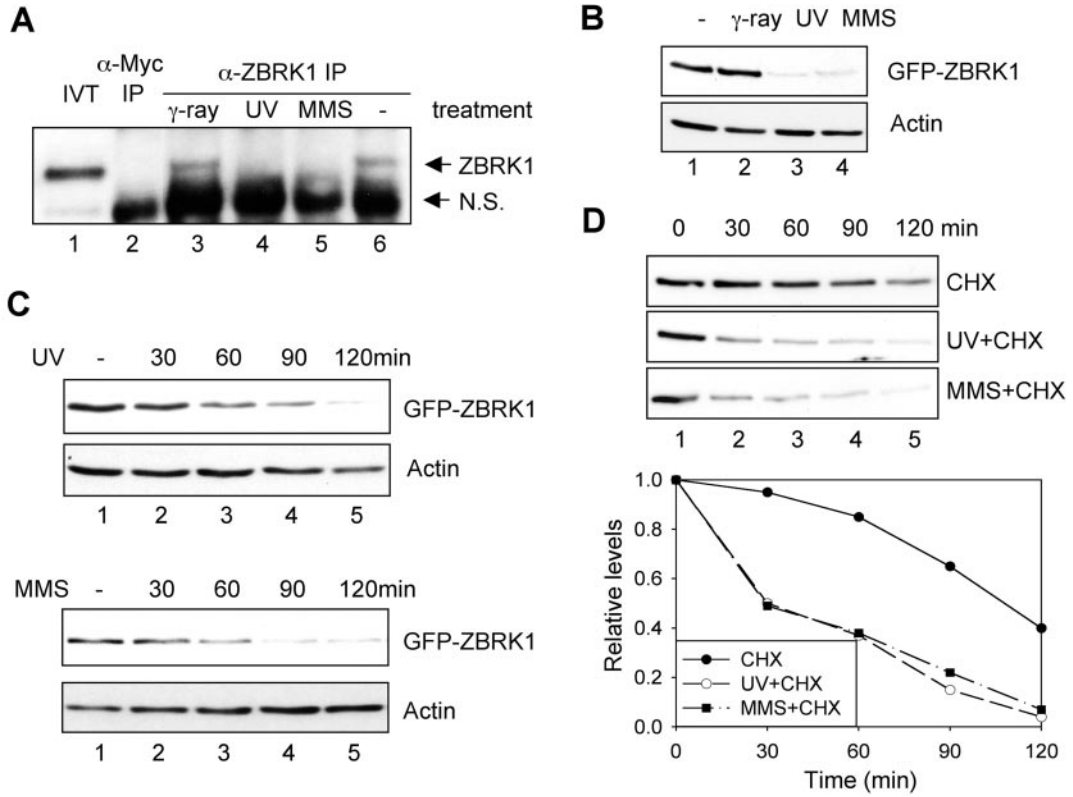

FIG. 1. DNA damage-induced degradation of ZBRK1. (A) U2OS cells were treated with gamma rays (20 Gy), UV (40 J/m^2), or MMS (0.05%) for 2 h before harvest and lysed for immunoprecipitation using anti-ZBRK1 polyclonal antibodies or anti-Myc antibodies. After extensive washing, the immunoprecipitated proteins were separated by SDS-PAGE and blotted with anti-ZBRK1 monoclonal antibodies. In vitro-translated ZBRK1 (IVT) (lane 1) served as the mobility marker. N.S., nonspecific protein. (B) U2OS cells stably expressing GFP-ZBRK1 (lane 1) were treated with gamma rays (20 Gy) (lane 2), UV (40 J/m^2) (lane 3), or MMS (0.05%) (lane 4) and harvested 2 h later. The lysates were separated by SDS-PAGE and subjected to Western blotting using anti-GFP antibodies or antiactin antibodies. (C) U2OS cells stably expressing GFP-ZBRK1 were treated with either UV (40 J/m^2) or MMS (0.05%) and harvested at the indicated time points. GFP-ZBRK1 and actin were detected with anti-GFP and anti-actin antibodies. (D) U2OS cells stably expressing GFP-ZBRK1 were treated with cycloheximide (CHX) (50 μg/ml) alone or together with either UV (40 J/m^2) or MMS (0.05%) and harvested at the indicated time points. GFP-ZBRK1 was detected with anti-GFP antibodies. The intensities of the bands were quantified by densitometry, and the relative intensities were calculated as described in Materials and Methods and plotted. The band intensity of GFP-ZBRK1 from untreated sample (0 min) was defined as 1.

damage, GFP-ZBRK1 has a half-life of approximately 115 min, but this was reduced to less than 30 min upon UV and MMS treatment. Taken together, these results suggest that ZBRK1 is rapidly degraded in response to UV and MMS treatment.

The ubiquitin-proteasome pathway mediates ZBRK1 degradation. Rapid degradation of regulatory proteins is one of the important functions of the ubiquitin-proteasome pathway (11, 34). To test whether this pathway is responsible for the DNA damage-induced degradation of ZBRK1, we examined the effect of specific proteasome inhibitors. As shown in Fig. 2, UV- and MMS-induced ZBRK1 degradations were completely abolished by the specific proteasome inhibitors MG132 and epoxomicin but not by the calpain inhibitor ALLM and the trypsin-cysteine protease inhibitor leupeptin. These results suggest that the degradation of ZBRK1 is mediated by the proteasome-dependent pathway in response to UV and MMS treatment.

Proteasome-dependent degradation usually requires ubiquitination of the protein substrate that is needed for recognition by the 26S proteasome (5). To examine whether ZBRK1 is ubiquitinated upon UV and MMS treatment, we performed an ex vivo ubiquitination assay for GFP-ZBRK1. As shown in Fig. 2C, immunoprecipitation with anti-GFP antibodies followed by immunoblot analysis with GFP antibodies detected a ladder of higher-molecular-weight forms of GFP-ZBRK1, indicative of its ubiquitination subsequent to UV and MMS treatment. Polyubiquitinated proteins are commonly detected as high-molecular-mass smears, which appear at the top in SDS-polyacrylamide gel electrophoresis (PAGE). After reprobing the blot with anti-ubiquitin antibodies, high-molecular-mass bands, which smeared downward, were also detected in UV and MMS treatment samples but not in untreated controls (Fig. 2C). These results suggest that the ZBRK1 protein is ubiquitinated subsequent to treatments with UV and MMS and then degraded by 26S proteasome.

ZBRK1 degradation induced by DNA damage is BRCA1 independent. The recently demonstrated ubiquitin E3 ligase activity residing in the N-terminal RING finger domain of BRCA1 might be important for its tumor suppressor function (4, 10, 29, 37). However, the exact biological significance of BRCA1 E3 ligase activity remains unclear. The identification

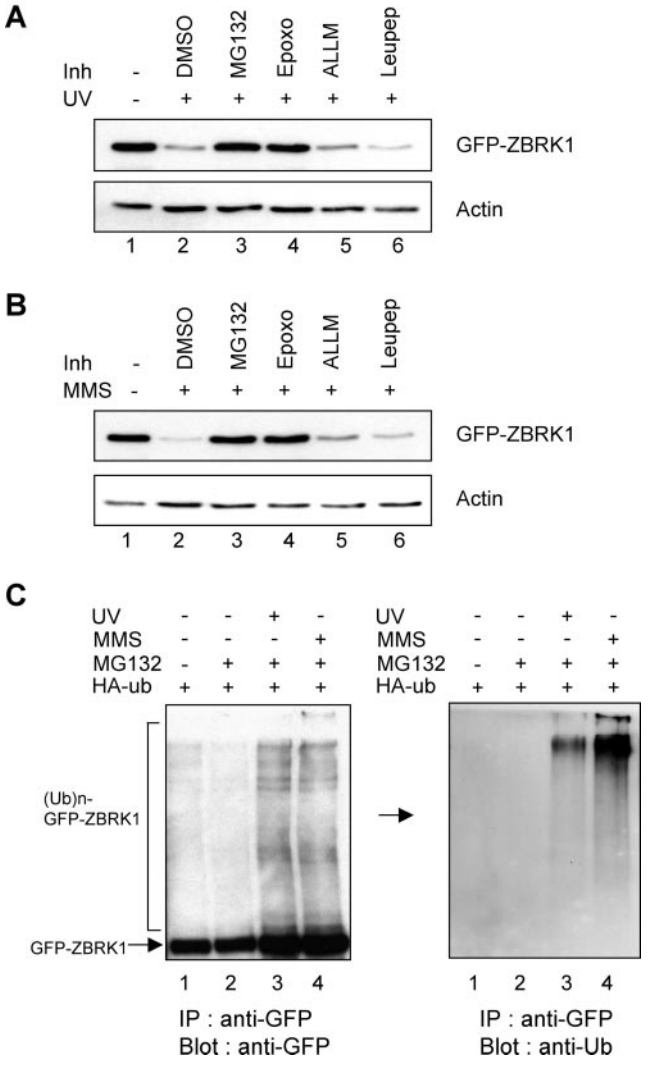

FIG. 2. Degradation of ZBRK1 is mediated by the ubiquitin-proteasome pathway. (A and B) U2OS cells expressing GFP-ZBRK1 were treated with either UV (40 J/m^2) (A) or MMS (0.05%) (B) and incubated with DMSO, the specific proteasome inhibitor MG132 (20 μM), epoxomicin (Epoxo, 10 μM), the calpain inhibitor ALLM (50 μM), or the trypsin-cysteine protease inhibitor leupeptin (Leupep, 200 μg/ml) for 2 h. Cell lysates were immunoblotted with anti-GFP antibodies to detect GFP-ZBRK1 or antiactin antibodies. (C) Cells stably expressing GFP-ZBRK1 were transfected with HA-ubiquitin-expressing plasmid, and an ex vivo ubiquitination assay was performed as described in Materials and Methods. Immunoprecipitated proteins were blotted with anti-GFP antibodies (left panel) and then stripped and reprobed with anti-ubiquitin antibodies (right panel).

of its physiological substrate would be an important step toward establishing its function. Since ZBRK1 is a BRCA1-interacting protein, which is degraded subsequent to treatment with DNA-damaging agents, it is possible that BRCA1 is involved in ZBRK1 degradation. As initial tests of this possibility, we used two different experimental systems. First, the breast cancer cell line HCC1937, which lacks functional BRCA1 protein (43), was used to examine the degradation of GFP-ZBRK1 upon MMS treatment. As shown in Fig. 3, GFP-ZBRK1 was degraded rapidly in HCC1937 cells upon DNA damage, and the ectopic expression of BRCA1 failed to change the kinetics of the degradation. Second, two isogenic BRCA1-deficient ($p53^{-/-}/Brca1^{-/-}$) and -proficient ($p53^{-/-}/Brca1^{+/+}$) MEF lines were used for comparison. Consistent with the observation in HCC1937 cells, the difference in the degradation kinetics of GFP-ZBRK1 in these two MEF lines was minimal (Fig. 3B). These results suggest that the in vivo degradation of BRCA1 induced by DNA damage is independent of the presence of BRCA1. Thus, it is likely that the E3 activity of BRCA1 defined in vitro is not involved in the degradation of ZBRK1. Furthermore, BARD1, a BRCA1-interacting protein that enhances BRCA1 E3 ubiquitin ligase activity and stability (29, 50), was expressed in these BRCA1-deficient or -proficient cells and its expression was not affected by DNA damage or the presence of BRCA1 (Fig. 3). These results suggest that BRCA1-independent degradation of ZBRK1 is not ascribed to the deficiency of BARD1 in these cells.

A 44-amino-acid motif located between the KRAB domain and zinc finger repeats of ZBRK1 is required for DNA damage-induced degradation. It has been shown previously that protein degradation mediated by the proteasome pathway requires specific amino acid elements, referred to as a degradation signal, of the substrates for efficient ubiquitination and destruction (11, 34). But ZBRK1 contains none of these known degradation signal motifs. Thus, to elucidate the mechanism of ZBRK1 degradation, identification of the signal motif for DNA damage-induced degradation is essential. Toward this end, we constructed a series of expression plasmids that encode either wild-type ZBRK1 or ZBRK1 with C-terminal or N-terminal deletions, all of which were translationally fused to GFP (Fig. 4A) and transfected into U2OS cells. Upon MMS treatment, the N-terminal deletion mutant (ΔK) was relatively resistant to degradation compared with the wild type or the other mutants (Fig. 4B). To precisely define the degradation signal motif at the N-terminal region that contains the conserved KRAB domain (between amino acids 6 and 67) and an additional 77 amino acids (Fig. 4A), three smaller deletion mutants, ΔN1, ΔN2, and ΔN3 (Fig. 4A), were generated and their stabilities upon MMS treatment were compared. Two of the KRAB domain deletion mutants, ΔN1 and ΔN2, were degraded similarly to the wild-type ZBRK1, while ΔN3, a mutant with a deletion of amino acids 80 to 122, was significantly resistant to MMS-induced degradation (Fig. 4C), suggesting that this region is necessary for the DNA damage-induced ZBRK1 destruction.

A characteristic feature of degradation signals is the ability to destabilize heterologous proteins (11). To test the above-identified ZBRK1 destruction motif for this property, a 44-amino-acid peptide (N3, from amino acids 79 to 122 in ZBRK1) was translationally fused to GFP (Fig. 5A) and expressed in cells. Upon UV or MMS treatment, the GFP-N3 fusion protein was decreased significantly compared to GFP alone (Fig. 5B). To confirm that GFP-N3 is degraded through the ubiquitin-proteasome pathway, we tested whether the specific proteasome inhibitor MG132 blocks the GFP-N3 degradation induced by UV and MMS. As shown in Fig. 5C, adding MG132 to the cultured cells reduced the degradation of GFP-N3 (Fig. 5C). Furthermore, the polyubiquitinated form of GFP-N3 was also detected after UV and MMS treatment (Fig. 5D). These results suggest that the N3 element is suffi-

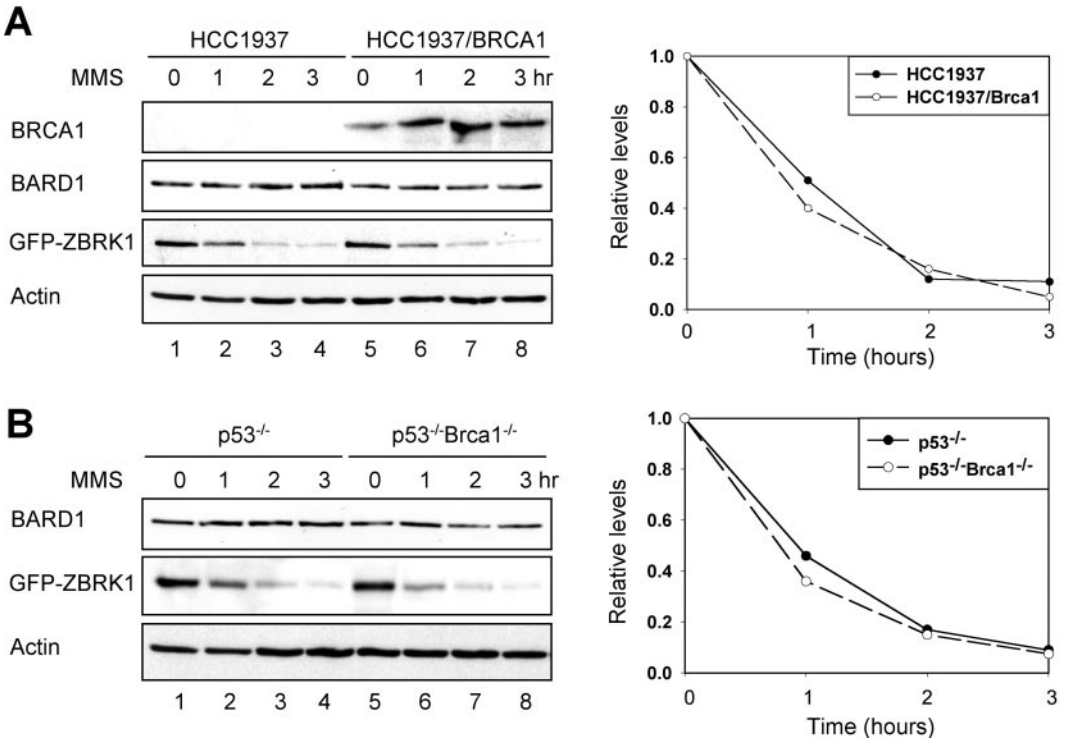

FIG. 3. ZBRK1 degradation is BRCA1 independent. (A) HCC1937 cells were transfected with GFP-ZBRK1-expressing plasmid with either BRCA1 expression plasmid (HCC1937/BRCA1) or vector alone (HCC1937), cultured for 24 h, and split into four plates. At 48 h after transfection, cells were treated with MMS (0.05%) for 2 h. Cell lysates were subjected to immunoblotting with anti-BRCA1 antibodies (6B4), antiactin antibodies, anti-BARD1 antibodies (EE6), or anti-GFP antibodies to detect GFP-ZBRK1. The intensities of the bands were quantified by densitometry. The relative intensities were calculated as described in Materials and Methods and plotted as shown on the right. The GFP-ZBRK1 band intensity of untreated sample (0 h) was defined as 1. (B) $p53^{-/-}$ and $p53^{-/-}/Brca1^{-/-}$ MEFs expressing GFP-ZBRK1 were treated with MMS (0.05%) and analyzed for BARD1, GFP-ZBRK1, and actin protein levels as described for panel A.

cient to provide signals for the DNA damage-induced degradation of ZBRK1.

Overexpression of ZBRK1ΔN3 abolishes cellular DNA damage response. The N-terminal KRAB domain, eight zinc fingers, and C-terminal BRCA1-binding region are necessary for transcriptional repression of ZBRK1 (59). It is expected that the ZBRK1Δ3 mutant retains the repression activity because deletion of the N3 sequence did not disturb other functional motifs. To test this possibility, we performed a luciferase reporter assay using the pGL3p-IN3AB reporter that contains a ZBRK1-binding site from the third intron of the Gadd45a gene (59). The ZBRK1Δ3 mutant, like wild-type ZBRK1, efficiently repressed luciferase activity driven by pGL3-IN3AB (Fig. 6A). Since N3 provides sufficient signal for DNA damage-induced degradation, it is likely that overexpression of the ZBRK1Δ3 mutant would inhibit the derepression of ZBRK1 target genes and the subsequent consequences in cell response upon DNA damage. To test these possibilities, several U2OS cell lines stably expressing either GFP-ZBRK1Δ3 or wild-type GFP-ZBRK1 at comparable levels were established. Using these cell lines, we examined the efficiency of Gadd45a induction upon DNA damage. As shown in Fig. 6B, Gadd45a induction was detected at 6 h after MMS treatment and peaked at 12 h in parental U2OS cells as well as in GFP-ZBRK1-expressing cells. However, even though induction of Gadd45a in GFP-ZBRK1-Δ3-expressing cells was comparable to that in wild-type GFP-ZBRK1-expressing cells at 6 h, further induction was not seen at 12 h (Fig. 6B). These results suggest that the degradation of ZBRK1 is essential for maximal induction of a target gene such as Gadd45a in response to DNA damage. Subsequently, it is expected that impairment of Gadd45a induction in cells expressing ZBRK1Δ3 would likely manifest itself as a reduced ability to respond effectively to genotoxic stress. Consistent with this notion, the results of colony-forming assays indicated that these ZBRK1Δ3-expressing cells were hypersensitive to UV and MMS (Fig. 6C) treatment compared to the parental U2OS or the wild-type ZBRK1-expressing cells.

DISCUSSION

ZBRK1 is a sequence-specific DNA-binding transcription repressor which regulates a subset of genes, including Gadd45a, important for vital cellular processes including cell cycle control, apoptosis, and DNA damage response (59). Implied in this scenario is a mechanism by which ZBRK1-mediated repression of these genes is released in response to DNA damage. The evidence presented here indicates that the degradation of ZBRK1 by the ubiquitin-proteasome system is likely to be part of this regulatory mechanism. ZBRK1 degradation is completely abolished by the specific proteasome inhibitors MG132 and epoxomicin, whereas the calpain inhibitor ALLM

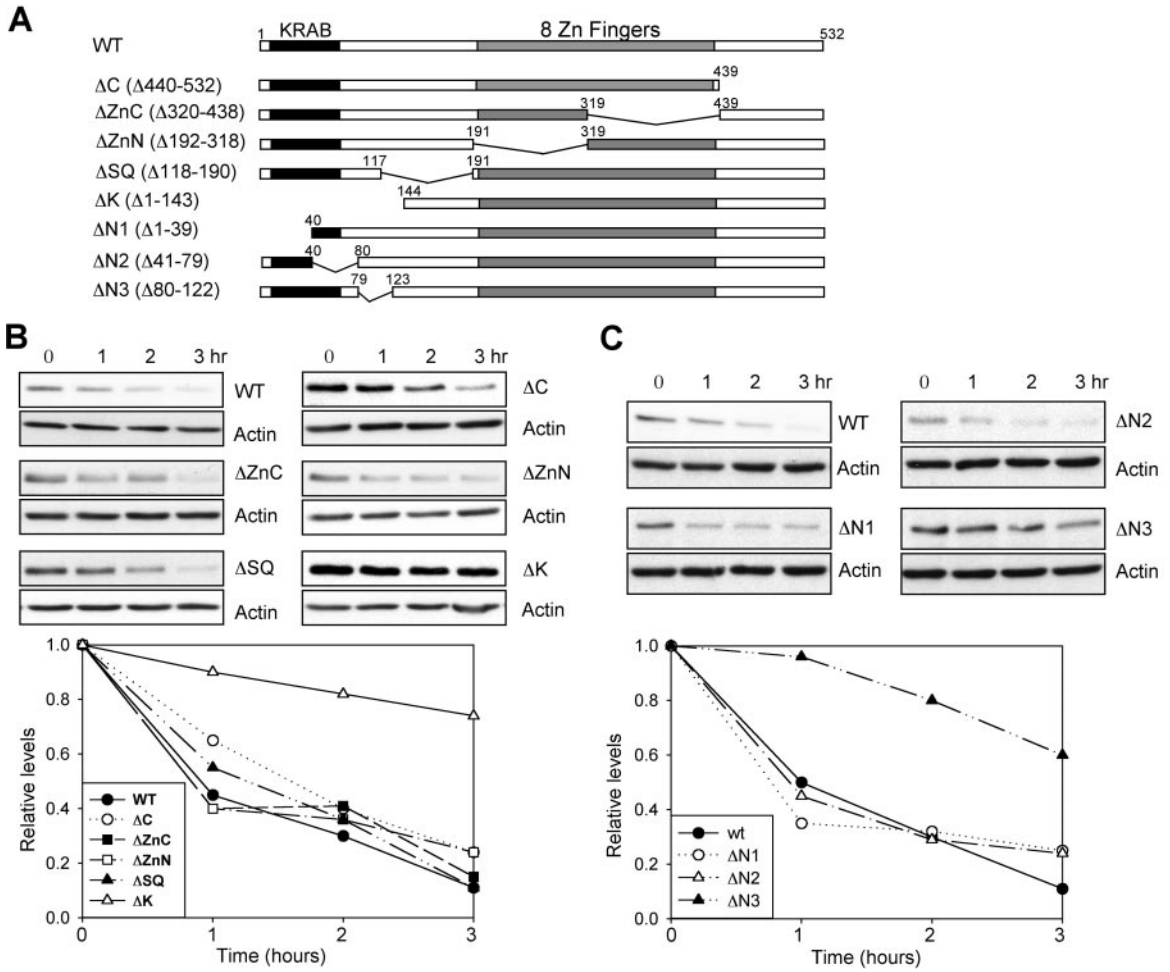

FIG. 4. Identification of a specific amino acid element responsible for ZBRK1 degradation. (A) Schematic diagrams of GFP-ZBRK1 deletion constructs used in this study. The amino acid coordinates encompassing the deletions are indicated in the names of the ZBRK1 mutants. (B and C) U2OS cells were transiently transfected with expression plasmids containing either the wild type (WT) or deletion mutants of GFP-ZBRK1. At 48 h after transfection, cells were treated with either MMS (0.05%) or DMSO (0 h) and harvested at the indicated time points. Cell lysates were analyzed by Western blotting with anti-GFP antibodies to detect wild-type or mutant GFP-ZBRK1 or with antiactin antibodies. The intensities of the bands were quantified by densitometry, and the relative intensities were calculated as described in Materials and Methods and plotted. The band intensity of the 0-h sample was defined as 1.

and the trypsin-cysteine protease inhibitor leupeptin have no effect. The polyubiquitinated form of ZBRK1 was also detected in an ex vivo ubiquitination assay. In spite of the fact that BRCA1 has E3 ligase activity, it has little influence in DNA damage-induced ZBRK1 degradation in vivo. Furthermore, a 44-amino-acid element located between the N-terminal KRAB domain and the central eight zinc fingers is sufficient for providing signals for the DNA damage-induced degradation of ZBRK1. Cells expressing a ZBRK1 mutant lacking these 44 amino acids are compromised for Gadd45a induction and are hypersensitive to DNA damage. This study provides a mechanistic view of how the repression of ZBRK1 is released in response to DNA damage.

Initially, we hypothesized that BRCA1 regulates ZBRK1 degradation on the basis of the following facts. First, the RING domain of BRCA1 has ubiquitin E3 ligase activity (4, 10, 29, 37). Second, ZBRK1 binds to BRCA1 (59) and the degradation of ZBRK1 upon DNA damage is mediated through the ubiquitin-proteasome pathway. However, our results suggest that ZBRK1 degradation is independent of the presence of BRCA1 in cells. Furthermore, the ZBRK1ΔC mutant, which cannot interact with BRCA1 (59), was degraded as efficiently as the wild-type ZBRK1, a result which failed to support the hypothesis. Thus, the in vivo substrate for BRCA1 E3 ligase activity remains to be shown and the relevant E3 ligase for ZBRK1 degradation induced by DNA damage has to be identified. Moreover, this ZBRK1 E3 ligase is unlikely to be a KRAB domain-binding protein because the presence of this domain has little influence on the degradation of ZBRK1.

It was known that a specific stretch of amino acids called a degradation signal is required for the regulated destruction of proteins (11). For instance, many proteins contain PEST sequences enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) for regulated proteolysis (36). Mitotic cyclins and other cell cycle-regulatory proteins require a partially conserved, 9-amino-acid sequence motif referred to as a destruction box for their regulated degradations (8, 22, 51). Because we were unable to identify any known degradation signal

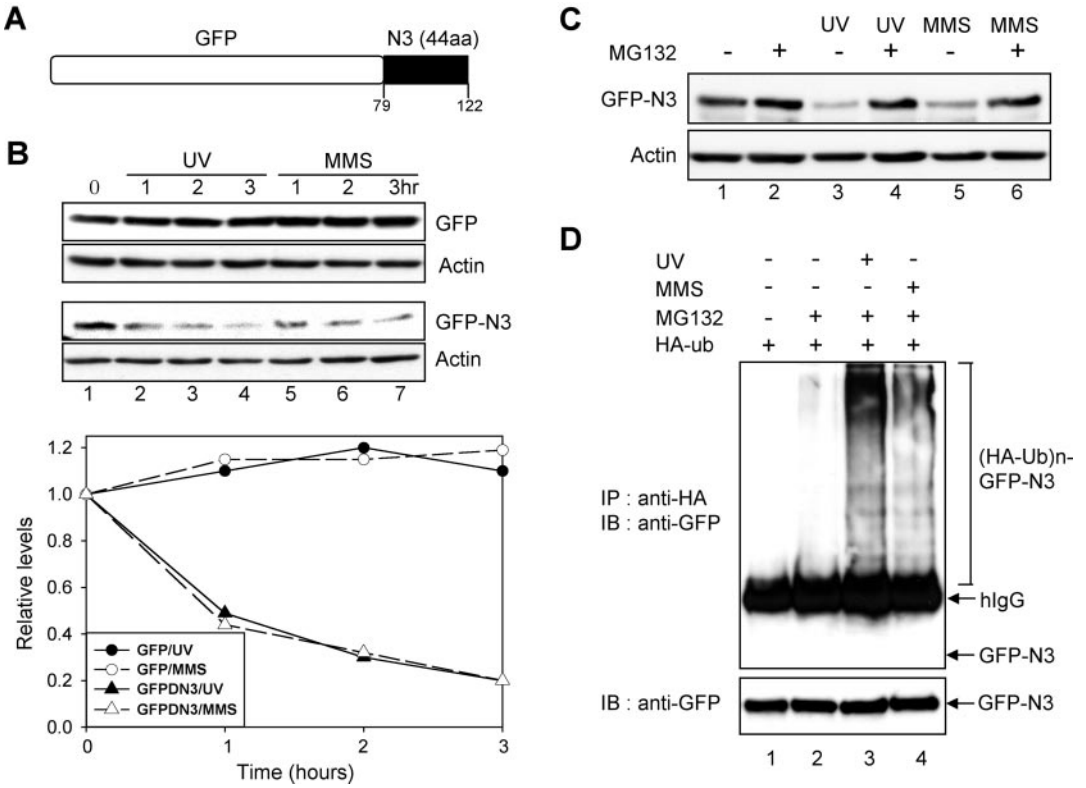

FIG. 5. Appending the 44-amino-acid element destabilizes a heterologous protein. (A) Schematic diagram of the GFP-N3 fusion protein. (B) U2OS cells were transfected with either GFP or GFP-N3 expression plasmid. After 48 h, cells were treated with UV (40 J/m^2) or MMS (0.05%) and harvested at the indicated time points. Cell lysates were analyzed by Western blotting as described in the legend to Fig. 4. Band intensities of GFP and GFP-N3 were measured by densitometry, and the relative intensities were calculated as described in Materials and Methods and plotted as shown. The band intensity of untreated sample (0 h) was plotted as 1 in the graph. (C) U2OS cells were transfected with GFP-N3 expression plasmid. After 48 h, cells were treated with either UV (40 J/m^2) or MMS (0.05%) and incubated with either DMSO (−) or MG132 (20 μM) (+) for 3 h. Cell lysates were analyzed by Western blotting using anti-GFP and antiactin antibodies. (D) U2OS cells were transfected with GFP-N3 and HA-ubiquitin-expressing plasmid, and an ex vivo ubiquitination assay was performed as described in Materials and Methods. Cell lysates were immunoprecipitated with anti-HA antibodies (12CA5) and immunoblotted with antiubiquitin antibodies. The migration position of unmodified GFP-N3 is indicated. hIgG, the immunoglobulin G heavy chain that reacts with the secondary antibody. GFP-N3 in each lysate (50 μg) was also detected by immunoblotting with anti-GFP antibodies.

in ZBRK1, the element important for DNA damage-induced ZBRK1 degradation was experimentally mapped to 44 amino acids located between the KRAB domain and the eight zinc fingers. Importantly, appending this 44-amino-acid element to a heterologous GFP protein destabilized it in response to UV and MMS treatment. Moreover, a proteasome inhibitor, MG132, specifically blocks the degradation of GFP-N3 that was ubiquitinated following DNA damage. These results suggest that this 44-amino-acid motif of ZBRK1 is sufficient for providing signals for proteasome-mediated degradation. Mechanistically, the DNA damage signal, which is frequently transmitted through phosphorylation, could be received by the 44-amino-acid element that is recognized by the relevant E3 ligase. The sequence of N3 contains three lysines at positions 83, 101, and 117 that are potentially involved in ubiquitination and four serines at positions 93, 95, 116, and 118 for phosphorylation. Importantly, an SQ site possibly recognized by ATR/ATM kinases (21) is also found at position 117-118. Thus, this motif in ZBRK1 is likely a novel degradation signal element and will be a useful tool for identifying the relevant E3 ligase.

Interestingly, the ZBRK1 protein is degraded upon UV and MMS treatment but not after gamma irradiation, suggesting that the repression by ZBRK1 may be relieved differently depending on the DNA damage signals. It has been shown that many different regulatory pathways participate in the transcription regulation of Gadd45a, a ZBRK1 target gene. For example, Gadd45a is induced after gamma irradiation in a p53-dependent manner through binding sites in the third intron (17, 54). In contrast, UV- and MMS-mediated induction of Gadd45a expression is independent of p53 function (55, 56); instead, OCT-1 and NF-YA transcription factors are important for this process (15). Our results described here further suggest that derepression through degradation of ZBRK1 also contributes, in part, to the p53-independent induction of Gadd45a expression in response UV and MMS. Consistently, overexpression of a ZBRK1 mutant resistant to DNA damage-induced degradation compromises the optimal induction of Gadd45a after MMS treatment. As was proposed previously, optimal induction of Gadd45a expression in response to DNA damage may be regulated by a concerted effect of both derepression and full activation (59). Expression of the ZBRK1ΔN3 mutant inhibits ZBRK1 derepression, but other factors required for transcription activation of Gadd45a may still be functional. Thus, the initial expression of Gadd45a at 6 h after

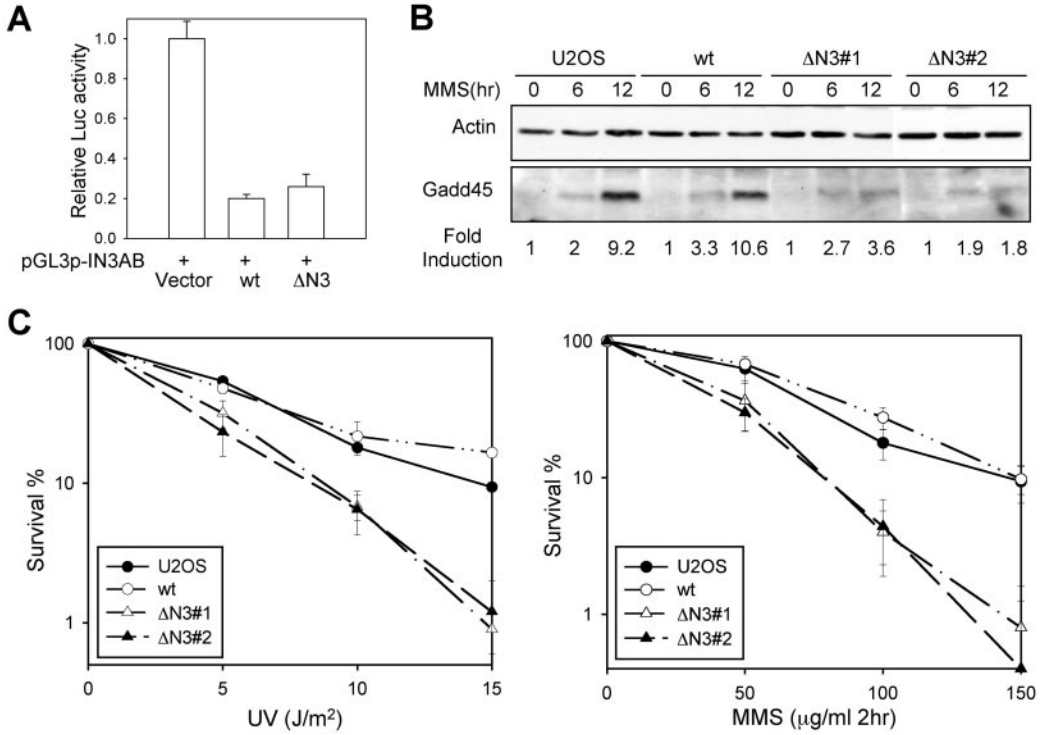

FIG. 6. Effect of ZBRK1ΔN3 mutant expression on Gadd45a induction and cell survival upon DNA damage. (A) U2OS cells were transfected with 50 ng of pSV β-galactosidase and pGL3p-IN3AB together with 200 ng of either GFP-ZBRK1 or GFP-ZBRK1ΔN3 expression plasmid. After normalization with β-galactosidase activity, luciferase activities were expressed relative to those obtained from pCHPL vector-transfected cells. (B) U2OS cell lines stably expressing wild-type (wt) GFP-ZBRK1 or the ΔN3 mutant were treated with MMS (200 μg/ml) for 2 h and harvested at the indicated time points. Cell lysates were analyzed by Western blotting using anti-Gadd45a and antiactin antibodies. (C) A colony of U2OS cells or U2OS cells stably expressing wild-type ZBRK1 or the ΔN3 mutant was quantified after treatment with UV (20 J/m^2) (left panel) or with MMS (200 μg/ml) for 2 h and growth for 12 days (right panel).

MMS treatment can be ascribed to the activation function of other factors including BRCA1. Failure to further increase Gadd45a induction at 12 h indicated that derepression of ZBRK1 by degradation is essential for the maximal induction of Gadd45a.

It is very likely that ZBRK1-mediated repression is released through another mechanism, such as phosphorylation, upon gamma irradiation. Alternatively, BRCA1 may regulate ZBRK1-mediated repression (59). Because BRCA1 is phosphorylated rapidly at different sites by different protein kinases depending on the type of DNA damage signal (reviewed in references 45 and 58), gamma irradiation-induced BRCA1 phosphorylation may lead to the dissociation of the BRCA1-ZBRK1 repression complex from its cognate binding site. It is also possible that ZBRK1 is an effector of the DNA damage signal pathway that results in derepression of target gene transcription. The precise regulatory consequences of gamma irradiation-induced phosphorylation on the BRCA1/ZBRK1 complex remain to be explored.

Gadd45a participates in several important cellular processes including cell cycle arrest, apoptosis, and DNA repair. Gadd45a inhibits Cdc2/cyclin B kinase activity through direct interaction with Cdc2 (14, 18, 40, 53). Overexpression of Gadd45a is associated with cell cycle arrest at the G_2/M phase, and suppression of Gadd45a expression leads to inhibition of the G_2 checkpoint triggered by DNA damage (48). Finally, mice deficient in Gadd45a show chromosome abnormalities signifying genomic instability and cells derived from the mice fail to activate checkpoint after UV and MMS treatment (13). These results suggest that, following DNA damage, Gadd45a expression is important for DNA repair and cell survival. Our finding that cells expressing ZBRK1ΔN3 are remarkably hypersensitive to UV and MMS treatment further corroborates the importance of the expression of Gadd45a for genotoxic stress. However, it is also likely that other, as-yet-unidentified target genes important for the cellular response to DNA damage are needed for the marked UV and MMS hypersensitivity observed here. There will be ample opportunities to find such target genes because many DNA damage-inducible genes contain ZBRK1-binding sequence in their regulatory regions (59).

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