RB plays an essential role in DNA damage-induced growth arrest and regulates the expression of several factors essential for DNA repair machinery. However, how RB coordinates DNA damage response through transcriptional regulation of genes involved in growth arrest remains largely unexplored. We examined whether RB can mediate the response to DNA damage through modulation of ZBRK1, a zinc-finger-containing transcriptional repressor that can modulate the expression of GADD45A, a DNA damage response gene, to induce cell cycle arrest in response to DNA damage. We found that the ZBRK1 promoter contains an authentic E2F-recognition sequence that specifically binds E2F1, but not E2F4 or E2F6, together with chromatin remodeling proteins CtIP and CtBP to form a repression complex that suppresses ZBRK1 transcription. Furthermore, loss of RB-mediated transcriptional repression led to an increase in ZBRK1 transcript levels, correlating with increased sensitivity to ultraviolet (UV) and methyl methanesulfonate-induced DNA damage. Taken together, these results suggest that RB-CtIP (CtBP interacting protein)/CtBP (C terminus-binding protein) /E2F1 complex plays a critical role in ZBRK1 transcriptional repression, and loss of this repression may contribute to cellular sensitivity of DNA damage, ultimately leading to carcinogenesis.

The DNA damage response that responds to genotoxic stress induced by radiation, chemicals, and endogenous reactive oxygen species is highly conserved in higher eukaryotes (1, 2). The cellular responses to DNA damage include activation of cell-cycle arrest, apoptosis, and DNA damage repair (3). In mammals, multiple partially overlapping DNA repair mechanisms, including base excision, recombination, and mismatch repair, are required for proper DNA damage repair. Defects in these repair pathways frequently lead to irreparable DNA damage, aging, and cancer.

RB, a prototypic tumor suppressor (4), is essential for regulating cell cycle progression through interaction with binding partners such as E2F (5). In quiescent and early G1 cells, RB associates with the E2F family of transcription factors to repress the expression of E2F-responsive genes involved in cell cycle progression (6). As cells progress toward S-phase, RB is phosphorylated by cyclin-dependent kinases, releasing E2F, which opens the DNA replication origin, and induces transcription of S-phase genes (7). When RB is lost or inactivated, DNA replication origins become readily accessible, resulting in uncontrolled transcription of S-phase genes and ultimately leading to premature S-phase progression (8). RB also plays an essential role in DNA damage-induced growth arrest (9) and transcriptional regulation of several DNA damage-repair factors involved in the ultraviolet (UV) damage repair process, including FEN1, XPC, RPA2-3, RFC4, and proliferating cell nuclear antigen (10). Although previous studies have established that phosphorylation of RB inactivates its binding activity with its interacting partners and promotes cell cycle progression (11); a recent study, however, indicated that the phosphorylation of RB at Ser-612 represents an activating event in response to DNA damage, suggesting a novel role of RB in DNA damage response (12).

Of all the E2F family members, E2F1 appears to be unique in its ability to induce both proliferation and apoptosis (13). In addition, it displays properties of both an oncogene and a tumor suppressor (14–18). Recent studies have revealed at least two mechanisms of E2F/RB-mediated gene repression: the recruitment of a histone deacetylase (19, 20) or the CtBP corepressor complex (21, 22). Although the expressions of many cell cycle genes are subject to RB/E2F repression (23), it remains unclear whether any of these genes participate in RB-mediated DNA damage response.

ZBRK1 was identified as a transcriptional repressor that recognizes a consensus DNA element, GGGXXCCAGXXTTT (24). Interestingly, ZBRK1 exhibits two different modes of repression: its C-terminal domain binds the BRCA1-CtIP (CtBP-interacting protein) co-repressor complex (25), whereas the N-terminal KRAB domain binds to the KAP1 co-repressor (26). ZBRK1 thus represents the first KRAB zinc finger protein (KRAB-ZFP) harboring two independent repression domains. ZBRK1 association with BRCA1 and CtIP repress transcription of Angiopoietin 1 (27) and high mobility group AT-hook 2 (28). The same repressor complex can also regulate the DNA damage response gene GADD45A for the maintenance of genomic integrity (29–31). ZBRK1 is rapidly degraded upon DNA damage through a ubiquitin-proteasome pathway (32), suggesting that the genes repressed by ZBRK1 may be activated during...
DNA damage. This further substantiates the role of ZBRK1 in DNA damage response.

In this communication, we show that the ZBRK1 promoter contains an authentic E2F1 binding site that serves as the platform for the RB-E2F1-CTBP (CTBP interacting protein)-CTBP (C terminus-binding protein) complex-mediated transcriptional repression of ZBRK1. This regulation is intimately correlated with cellular sensitivity toward UV- and methyl methanesulfonate (MMS)\(^3\)-induced DNA damage. These results provide a potential mechanism to elucidate how RB deficiency contributes to an elevated sensitivity to DNA damage.

**EXPERIMENTAL PROCEDURES**

*Materials*—Lipoferitoxin 2000, DMEM, Opti-MEM medium, FBS, TRIzol RNA extraction reagent, and SuperScript\(^{TM}\) III were purchased from Invitrogen. Super-Therm DNA polymerase was purchased from MBio Inc. (Taipei, Taiwan). Oligonucleotides for RT-PCR were synthesized by MDBio Inc. (Taipei, Taiwan). Oligonucleotides for DNA Affinity Precipitation Assay (DAPA) and siRNA assay were synthesized by Invitrogen. TaqDNA polymerases and the luciferase assay system were purchased from Promega (Madison, WI). The yTA cloning vector kit was purchased from Yeastern Biotech Co. (Taipei, Taiwan). The E2F1 expression plasmid pcDNA3/HA was a gift from Dr. Hsin-Fang Yang-Yen (IMB, Academia Sinica, Taiwan). The E2F6 cDNA was purchased from ATCC (number MGC-15924). The CCND1 reporter (CCND1-p) containing the 3.3-kb cyclin D1 promoter was a gift from Richard Pestell (Georgetown University) (33). Antibodies against E2F1 and CtBP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Rb (Rb245) and CtIP (19E8) were purchased from GeneTex (San Antonio, TX). The detection of endogenous ZBRK1 was performed as described in Zheng et al. (25). Antibody against β-actin was purchased from Sigma.

*Cell Culture and Treatment with DNA Damage Agents*—MCF7 and MDA-MB-468 (MB-468) breast cancer cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml of streptomycin, and 100 units/ml of penicillin. MCF10A cells were cultured as described in Debnath et al. (34). DNA damage induction was performed using the UV-Crosslinker (Spectroline) at 40 J/m2 or 0.05% MMS (Sigma). To measure the half-life of exogenous GFP-ZBRK1 and endogenous ZBRK1 protein, cells were treated with cycloheximide (50 μg/ml) alone or combined with either UV or MMS. Protein lysates were harvested at various time points for Western blot analysis.

*Plasmid Construction*—The ZBRK1 promoter fragments −1113 to +47 bp (ZBRK1-A) and −624 to +47 bp (ZBRK1-B) were obtained by PCR. The primers used were as follows: ZBRK1/KpnI-1113, 5’-GGGGTACCCATTTCCGCTCTTGGC-3’; ZBRK1/KpnI-624, 5’-GGGGTACCCGACA-GACGCAAGCTCGTTC-3’. The PCR fragments were cloned into the yTA vector and verified by sequencing. The KpnI/HindIII fragment was subcloned into the promoter-less pGL2-basic vector. Mutant reporter plasmids were derived from −1113/+47 WT and −624/+47 WT using site-directed mutagenesis in accordance to the instructions in QuikChange site-directed mutagenesis kit (Stratagene).

*Transfection and Reporter Gene Assay*—Promoter DNA constructs were transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions. For the reporter assay, lysates were harvested 16 h post-transfection, and luciferase activity was analyzed using the luciferase assay system. Luciferase activity was normalized to the level of relative luciferase activity per microgram of extracted protein. siRNA duplexes purchased from Invitrogen were: siE2F1, 5’-UGGAC-CACCUGAAGAAUATTT-3’, siRB-1, 5’-ACAGAAGAACU-UAGUUUATTT-3’, and siRB-2, 5’-GAUACCAGAUCAGATT-3’. Reverse transcription-PCR total RNA was isolated from cells using TRIzol RNA extraction reagent. Three micrograms of cellular RNA was subjected to reverse transcription with SuperScript III for cDNA synthesis. PCR was performed using primer pairs indicated as follows: human ZBRK1, 5’-GACATATGGAAGGTGTCGAAGTCT-3’ and 5’-ATTCTACTGCACCATGATGTTCCTCA-3’, E2F1, 5’-CATCCAGCTATGTCCGAGA-3’ and 5’-GATCCACCATTCCGTTCC-3’, GADD45A, 5’-GGTCACTAGTGAACATTTGGT-3’ and 5’-TTTTCTCTCTGTGATGTC-3’. Luciferase activity was analyzed using the luciferase assay system. The KpnI/HindIII fragment was subcloned into the promoter-less pGL2-basic vector. Mutant reporter plasmids were derived from −1113/+47 WT and −624/+47 WT using site-directed mutagenesis in accordance to the instructions in QuikChange site-directed mutagenesis kit (Stratagene).

*Chromatin Immunoprecipitation (ChIP) and Re-ChIP Assay*—ChIP assay was performed essentially as described by Wang et al. (36). Briefly, the sheared chromatin fragments were immunoprecipitated with antibodies specific to E2F1, RB, CtIP, CtBP, or control mouse IgG at 4°C overnight. The DNA was dissociated from the immunoprecipitated chromatin prior to PCR.
amplification. For the re-ChIP assay, the first immune complex was washed twice with wash buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% Nonidet P-40, 150 mM NaCl, and 2.5 mM EDTA), twice with low-salt buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA), resolved in 10 mM DTT at 37 °C, diluted in ChIP dilution buffer, and processed according to the same ChIP assay protocol using the indicated antibodies. The primers used for PCR amplification were as follows:

- **ZBRK1**
  - Forward: 5'-H11002
  - Reverse: 5'-H11032

- **ZBRK1**
  - Forward: 5'-H11002
  - Reverse: 5'-H11032

- **GL2 primer**: 5'-H11032

The amplified DNA products were resolved by agarose gel electrophoresis and confirmed by sequencing.

Comet Assay—Cells were embedded in agarose gel and spread on a frosted microscope slide before lysing in lysis buffer (2.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 100 mM EDTA, and 1% Triton-X-100) for 1 h at 4 °C. After lysis, cells were preincubated for 20 min at 4 °C in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH > 13) before alkaline gel electrophoresis at 24 V/300 mA for 15 min. Following electrophoresis, the slides were rinsed with neutralization buffer and allowed to dry before staining with ethidium bromide for visualization under fluorescence microscopy. The tail moment was quantified by a computerized image analysis program. The tail moment was calculated by multiplying the total intensity of the Comet tail by the migration distance from the center of the Comet head.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay—For the TUNEL assay, the Apoptosis Detection System Fluorescein (Promega) was used. Cells grown on coverslips were treated with UV or MMS and allowed to recover for 2 h at 37 °C. Cells were then washed with PBS and fixed in 3.7% formaldehyde for 10 min before storage at 20 °C in 70% ethanol for at least 30 min. The coverslips were then washed in PBS for 5 min at room temperature, treated with 0.2% Triton X-100 in PBS for 5 min, and rinsed twice with PBS for 5 min at room temperature. The percentage of red fluorescing cells (TUNEL positive) was determined using images captured by fluorescence microscope.

Lentiviral shRNA—The lentiviral expression vectors pLKO.1-shLuc, containing 5'-CTTCGAAATGTCCGTTCGGTT-3', and pLKO.1-shZBRK1, containing 5'-CGTAACCATGACGGCTTTC-3', were obtained from the National RNAi Core Facility located at the Genomic Research Center of Institute of Molecular Biology, Academia Sinica. Virus was harvested after co-transfecting pLKO.1-shLuc or pLKO.1-shZBRK1 vector with pMD2.G and psPAX2 into Phoenix cells using Lipofectamine 2000.
RESULTS

Identification of the E2F-recognition Sequences in the ZBRK1 Promoter as a Negative Transcriptional Response Element—RB plays a critical role in the response to DNA damage. However, the precise mechanism by which RB mediates the DNA damage response remains unclear. Because ZBRK1 serves as a repressor regulating DNA damage response genes (25), we hypothesized that RB may act as an up-stream regulator of ZBRK1 for this purpose. To address this possibility, we examined the correlation between the expression of RB protein and ZBRK1 transcript in RB-depleted cells. As shown in Fig. 1, when RB was depleted by siRNA, the ZBRK1 transcript level was increased, suggesting that RB acts as a negative regulator of ZBRK1 transcript. Because RB is known to serve as co-repressor with E2F family members, we hypothesized that RB may act as up-stream regulator of ZBRK1 for this purpose. To confirm whether this repression is promoter-mediated, a reporter construct containing the ZBRK1 promoter was co-transfected into cells with E2F1, E2F4, or E2F6, and examined the expression of ZBRK1 reporters by co-transfection with the E2F1 expression vectors, including E2F1, E2F4, and E2F6, and examined the expression of ZBRK1 by RT-PCR and Western blot. E2F1, but not E2F4 or E2F6, specifically repressed ZBRK1 reporter activity. The reporter assay was performed by co-transflecting the ZBRK1 reporter and the indicated expression vectors. D, inhibition of E2F1 expression reversed ZBRK1 repression. MCF7 cells were transfected with E2F1 siRNA (siE2F1) or a scrambled control (siC). RT-PCR (left panel) and Western blot (right panel) analyses were performed using specific primers and antibodies as indicated.

Identification of the E2F-recognition Sequences in the ZBRK1 Promoter.

A, ZBRK1 mRNA and protein expression levels in MCF7 cells transfected with the indicated expression vectors. RT-PCR (upper panel) and Western blot analyses (lower panel) were performed with specific primers and antibodies as indicated. B, confirmation of the effect of E2F1 on the ZBRK1, CCND1, AURKA, and MCL1 reporters by co-transfection with the E2F1 expression vector in MCF7 cells. ***p < 0.001 versus control; NS, no significance compared with control. C, E2F1, but not E2F4 or E2F6, specifically represses ZBRK1 reporter activity. The reporter assay was performed by co-transfecting the ZBRK1 reporter and the indicated expression vectors. D, inhibition of E2F1 expression reversed ZBRK1 repression. MCF7 cells were transfected with E2F1 siRNA (siE2F1) or a scrambled control (siC). RT-PCR (left panel) and Western blot (right panel) analyses were performed using specific primers and antibodies as indicated.

Identification of the E2F-recognition Sequences in the ZBRK1 Promoter as a Negative Transcriptional Response Element—RB plays a critical role in the response to DNA damage. However, the precise mechanism by which RB mediates the DNA damage response remains unclear. Because ZBRK1 serves as a repressor regulating DNA damage response genes (25), we hypothesized that RB may act as an up-stream regulator of ZBRK1 for this purpose. To address this possibility, we examined the correlation between the expression of RB protein and ZBRK1 transcript in RB-depleted cells. As shown in Fig. 1, when RB was depleted by siRNA, the ZBRK1 transcript level was increased, suggesting that RB acts as a negative regulator of ZBRK1 transcript. Because RB is known to serve as co-repressor with E2F family members, we hypothesized that RB may act as up-stream regulator of ZBRK1 for this purpose. To confirm whether this repression is promoter-mediated, a reporter construct containing the ZBRK1 promoter was co-transfected into cells with E2F1, E2F4, or E2F6 expression vectors. We found that only E2F1 specifically repressed the ZBRK1 promoter activity (Fig. 3B). To confirm the authenticity of E2F1-mediated ZBRK1 repression, we examined ZBRK1 promoter activity in comparison to MCL-1, CCND1, and AURKA promoters. MCL-1 was previously reported to be negatively regulated by E2F1 (38), whereas CCND1 and AURKA were reported to be positively regulated by E2F1 (39, 40). Consistent with previous reports, we also found MCL-1 to be repressed, whereas CCND1 and AURKA were activated upon E2F1 expression. Using these controls, ZBRK1 promoter activity was demonstrated to be repressed upon E2F1 expression (Fig. 3C). Consistently, depletion of E2F1 by siRNA resulted in an increase in ZBRK1 expression at both the RNA and protein levels (Fig. 3D). Taken together, these results support the notion that E2F1 specifically represses ZBRK1 transcription.
RB Acts as a Negative Regulator of the ZBRK1 Promoter through the E2F Motif—RB plays an important role in E2F-mediated transcriptional repression of several genes, such as cdc25A (41) and cyclin E (42). To investigate whether RB coordinates with E2F1 in repressing ZBRK1 transcription, we performed a ZBRK1 reporter assay in cells with RB depleted by siRNA. As shown in Fig. 4A, depletion of RB enhanced ZBRK1 promoter activity (compare lanes 2 and 3 with lane 1). This was also the case in cells transfected with E2F1 (Fig. 4A, compare lanes 5 and 6 with lane 4), suggesting that depletion of RB reverses the E2F1-mediated repression of ZBRK1. On the contrary, promoter activity of the ZBmE reporter containing the mutant E2F motif was unaffected by either RB depletion or E2F1 overexpression (Fig. 4A, compare lane 7 with lane 12). To confirm this finding, we transfected the ZB and ZBmE reporters into RB-negative Saos-2 and RB-reconstituted SR5 cells (43) and found that the presence of RB in SR5 cells negatively affected ZB reporter activity, but not the activity of ZBmE (Fig. 4B). These results suggest that RB coordinates with E2F1 to regulate the ZBRK1 promoter through the E2F motif.

RB Acts as a Negative Regulator of the ZBRK1 Promoter—Because both RB and ZBRK1 participate in DNA damage response, we next examined the relationship between ZBRK1 and RB expression profiles upon treatment with DNA damage inducers. We compared the mRNA and protein levels of ZBRK1 and its downstream target GADD45A in RB-proficient MCF7 and RB-deficient MDA-MB-468 (MB-468) cells. ZBRK1 expression was found to be higher in MB-468 cells than MCF7 cells, and GADD45A expression was inversely correlated with ZBRK1 expression patterns. When MCF7 and MB-468 cells were treated with DNA damage inducers (UV and MMS), expression of γH2AX and GADD45A correlated with a reduction in ZBRK1 expression (Fig. 6, A and B). These results support the notion that upon UV and MMS treatment, RB forms a complex with E2F1 to repress ZBRK1 expression, in turn to derepress the expression of GADD45A. In the absence of RB, the repression of ZBRK1 was lost and the expression of GADD45A was compromised upon DNA damage, likely leading to improper resolution of DNA damage. To confirm that DNA damage inducers lead to E2F1/RB-mediated repression of ZBRK1 via binding to the ZBRK1 promoter, we performed a ChIP assay to examine the presence of the complex on the ZBRK1 promoter and found that increased binding can be detected upon E2F1 increase (Fig. 5D). To further validate that RB, CtIP, CtBP, and E2F1 exist as one multiprotein repressor complex on the ZBRK1 promoter, a Re-ChIP assay was performed. Immunoprecipitation was first performed with E2F1 antibodies followed by a second immunoprecipitation with RB, CtIP, or CtBP antibodies. The results showed that RB, CtIP, CtBP, and E2F1 formed a complex on the ZBRK1 promoter and binding was enhanced in cells with E2F1 overexpression, suggesting that E2F1 may participate in the recruitment of the RB-CtIP-CtBP complex to the ZBRK1 promoter (Fig. 5E). To demonstrate the importance of the E2F motif as the platform for the recruitment of RB-CtIP-CtBP complex, we performed a ChIP assay to examine the presence of the complex on exogenously expressed wild-type and ZBmE promoters. Only the promoter containing the wild-type E2F1 site bound the RB-CtIP-CtBP complex (Fig. 5F). Similar results were obtained in U2OS cells (supplemental Fig. S3). Taken together, these results suggest that E2F1 may participate in the recruitment of the RB-CtIP-CtBP complex to the ZBRK1 promoter.
promoter, a luciferase reporter assay was performed. As shown in Fig. 6C, a decrease in ZBRK1 promoter activity was observed upon UV and MMS treatments in an E2F1- and RB-dependent manner. In addition, a ChIP assay was performed to detect the binding of the E2F1-Rb-CtIP-CtBP complex at the ZBRK1 promoter upon DNA damage. As shown in Fig. 6D, UV and MMS treatments induced binding of the E2F1-Rb-CtIP-CtBP complex at the ZBRK1 promoter. Together, these results suggest that the E2F1-Rb-CtIP-CtBP complex negatively regulates ZBRK1 expression in response to DNA damage.

Depletion of ZBRK1 Protects Cells from UV- and MMS-induced DNA Damage—Based on the observations that the loss of RB results in an accumulation of DNA damage (45) and the induction of ZBRK1 expression (this study), we proceeded to examine whether accumulation of DNA damage responses is a consequence of ZBRK1 expression. The Comet assay was performed to detect the level of DNA damage as a function of ZBRK1 expression in MB-468 cells. Before treatment with DNA damage inducers, cells were pretreated with lentiviral shZBRK1 for 24 h. Inhibition of ZBRK1 expression attenuated the level of DNA damage after treatment with UV or MMS (Fig. 7A). In addition, to verify whether inactivation of ZBRK1 reduces UV- and MMS-induced apoptosis, a TUNEL assay was performed using the described cell line. As shown in Fig. 7B, inactivation of ZBRK1 protects cells from UV- and MMS-induced apoptosis. These results suggest that ZBRK1 depletion can protect cells against UV- and MMS-induced DNA damage and cell death.

DISCUSSION

In this communication, we addressed how RB coordinates the DNA damage response through transcriptional regulation of genes involved in growth arrest. We found that the ZBRK1 promoter contains an authentic E2F-recognition sequence, which specifically binds E2F1, but not E2F4 or E2F6 (Fig. 3). RB, together with E2F1 and chromatin modifiers CtIP and CtBP, was recruited to this binding site to form a repression complex for ZBRK1 transcription. Furthermore, we found that loss of RB-mediated transcriptional repression led to an increase in ZBRK1 transcript levels, which correlated with increased sensitivity to UV- and MMS-induced DNA damage. These results revealed a potential mechanism whereby the RB-E2F1 complex represses ZBRK1 transcription, and loss of this repression contributes to cellular sensitivity to DNA damage, ultimately leading to accumulation of the genomic mutation and carcinogenesis.
In this study, we used the Comet and TUNEL assays to examine the response of ZBRK1 upon DNA damage in cell lines with varying RB expression. MCF7 and MB468 cell lines were chosen to be representatives of RB-proficient and RB-deficient cells, respectively. It is important to note that MCF7 and MB468 cells are also well known to be estrogen receptor positive and negative, respectively (46). Estrogens induce RB phosphorylation and thereby disrupt the formation of the RB/E2F1 complex, leading to cell cycle progression (47). Interestingly, our preliminary result demonstrated that the ZBRK1 transcript level decreased following estrogen-induced E2F1 expression in MCF7 but not in MB468. Therefore, how the RB-E2F1 complex-mediated ZBRK1 repression responses to estrogen is an interesting question that warrants further investigation.

Although E2F1 was characterized as a transcriptional activator (48), several reports demonstrated that E2F1 can also act as a transcriptional repressor by inhibiting promoter activity (38, 49–51). RB can form a complex with E2F1 to shut off an otherwise active promoter (52). Binding of RB to E2F1 was observed to increase after UV treatment (53). This is consistent with the finding that phosphorylation of RB at Ser-612 is induced by UV or ionizing radiation through the ATM-Chk1/2 pathway, leading to elevated formation of the complex between RB and E2F1 (12). The increased binding of RB and E2F1 was not only triggered by phosphorylation of RB, but also enhanced by stabilization of the E2F1 in response to DNA damage (12). These findings suggest that phosphorylation of RB plays an important role in the DNA damage response through enhancement of E2F1/RB complex formation. The identification of ZBRK1 as the in vivo downstream target controlled by the RB/E2F1 complex in response to DNA damage further supports this notion.

This mode of action of RB is opposite to the role of RB in the cell cycle control, where RB dissociates from E2F1 binding upon phosphorylation. The issue of how different kinases specifically phosphorylate different residues of RB to modulate E2F1 binding is quite interesting. Detailed analysis of this interaction is warranted. Furthermore, a recent study revealed that BRCA1 is autoregulated by a dynamic coregulatory complex containing BRCA1, E2F1, and RB at the BRCA1 promoter. This complex can be disrupted by DNA-damaging agents leading to up-regulation of its own transcription (53). Utilizing a reporter assay, our result showed that BRCA1 overexpression has no effect on ZBRK1 reporter activity, indicating that BRCA1 is not involved in RB-E2F1-mediated repression of ZBRK1.6

The cellular response to DNA damage is a complex process that involves a network of interacting signal transduction pathways (2, 54). Such genomewide transcriptional response is very complex and tightly regulated, thus allowing a coordinated global response to DNA damage. This process is initiated by as yet unidentified proteins that detect or sense DNA damage and

---

4 C.-C. Liao and J.-M. Wang, unpublished results.

---

FIGURE 6. RB-mediated ZBRK1 repression is involved in both UV- and MMS-induced DNA damage response. A and B, Western blot (upper panel) and RT-PCR (lower panel) showing ZBRK1 and γH2AX expressions in MCF7 and MB-468 cells upon treatment with UV or MMS. Lysates from MCF7 and MB-468 cells were harvested after treatment with UV (40 J/m²) and MMS (0.05%), C, depletion of E2F1 and RB reversed UV- and MMS-induced repression of ZBRK1 reporter activity. MCF7 cells were co-transfected with the ZBRK1-B reporter and various siRNAs as indicated, and treated with UV (40 J/m²) or MMS (0.05%) for 2 h. Lysates from transfectants were harvested for luciferase assay. *, p < 0.05 versus scrambled control (siC); **, p < 0.01 versus siC. D, increases in E2F1, RB, CtIP, and CtBP binding to the ZBRK1 promoter after treatment with DNA damage inducers. Extracts with chromatin-protein complexes harvested from MCF7 cells treated with or without DNA damage inducers were immunoprecipitated with indicated antibodies and PCR amplified with specific primers on the ZBRK1 promoter.
subsequently transmit a signal by activating a cascade of phosphorylation events. This results in a variety of cellular responses, including activation of cell cycle checkpoints, commencement of transcriptional programs, execution of DNA repair, and in some cases, induction of cell death. We demonstrate in this study that DNA damage caused by UV or MMS leads to the repression of ZBRK1 transcription through binding of the CtBP-CtIP-RB-E2F1 complex to the ZBRK1 promoter. This triggers an increase in expression of a cluster of DNA damage response genes, including GADD45A (Fig. 8). Importantly, previous studies have shown that ZBRK1 is degraded through the ubiquitin-proteosome pathway upon UV treatment (32). These two events are coordinately regulated to ensure that DNA damage response genes repressed by ZBRK1 can be activated in response to DNA damage.

ZBRK1 serves as a transcriptional repressor either by interacting with KAP1 through its N-terminal KRAB domain or by interaction with BRCA1 through its C-terminal domain (25). A group of genes have been identified to be tentatively regulated by ZBRK1 (25). Among these, GADD45A, Angiopoietin 1, and high mobility group AT-hook 2 have been shown to be regulated by ZBRK1 through interaction with the BRCA1-CtIP complex (27, 28, 32). In addition, our preliminary results showed that constitutive exogenous expression of ZBRK1 had no effect on the activation of cell cycle arrest and apoptosis in HeLa and U2OS cells, suggesting that transcriptional activation of genes essential for growth arrest or cell death are dependent on ZBRK1 repression. Moreover, our recent study demonstrates that ZBRK1 can suppress metastasis in cervical cancer (55). It is likely that ZBRK1, together with the KAP1 co-repressor, may repress other genes critical for the DNA repair process as well as other physiological functions, although solid evidence and examples remain to be demonstrated. It will be of interest to learn how ZBRK1 represses different genes through distinct co-repressor partners and how these complexes respond to different signals. This study is the first report to provide direct in vivo binding evidence demonstrating the E2F1-RB-CtIP-CtBP complex-mediated transcriptional repression of ZBRK1 and its role in the DNA damage response. RB-mediated gene repression was shown to have both HDAC-dependent and -independent pathways (21). CtIP was reported to participate in the HDAC-independent path (56). Interestingly, our preliminary DAPA data showed that HDAC1 can be recruited to the wild-type E2F motif of the ZBRK1 promoter, suggesting that formation of a CtIP-HDAC complex is in a
RB-E2F1 Mediates DNA Damage through ZBRK1

DNA damage inducer (UV, MMS) → DDR ↑ → Activation of DNA repair → Binding of repressive RB-E2F1 complex → ZBRK1 ↓ → GADD45A ↑ → DNA damage

FIGURE 8. Down-regulation of the ZBRK1 expression is involved in UV- and MMS-induced activation of DNA repair. DNA damage inducers, such as UV and MMS, trigger E2F1/RB-mediated transcriptional repression of ZBRK1, leading to activation of GADD45A. This loss of ZBRK1-mediated activation of DNA responsive genes can attenuate the effect of DNA damage response (DDR).

gene-specific manner. However, the detailed mechanism of HDAC1-regulated ZBRK1 transcription needs to be further investigated.

Acknowledgment—We thank Erin M. Goldblatt for critical reading the manuscript.

REFERENCES

1. Feuerhahn, S., and Egly, J. M. (2008) Trends Genet. 24, 467–474
2. Zhou, B. B., and Elledge, S. J. (2000) Nature 408, 433–439
3. Elledge, S. J. (1996) Science 274, 1664–1672
4. Huang, H. J., Yee, J. K., Shew, J. Y., Chen, P. L., Bookstein, R., Friedmann, T., Lee, E. Y., and Lee, W. H. (1998) Science 242, 1563–1566
5. Chellappan, S. P., Hiebert, S. M., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991) Cell 65, 1053–1061
6. Hurford, R. K., Jr., Cobrinik, D., Lee, M. H., and Dyson, N. (1997) Genes & Dev. 11, 1447–1463
7. Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y., and Lee, W. H. (1989) Cell 58, 1193–1198
8. Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994) Nature 371, 72–74
9. Harrington, E. A., Bruce, J. L., Harlow, E., and Dyson, N. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 11495–11500
10. Bosco, E. E., Mayhew, C. N., Hennigan, R. F., Sage, J., Jacks, T., and Knudsen, E. S. (2004) Nucleic Acids Res. 32, 25–34
11. Kitagawa, M., Higashi, H., Jung, H. K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. (1996) EMBO J. 15, 7060–7069
12. Inoue, Y., Kitagawa, M., and Taya, Y. (2007) EMBO J. 26, 2083–2093
13. Di Gregorio, J., Levine, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 7245–7250
14. Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993) Nature 365, 349–352
15. Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. (1996) Cell 85, 549–561
16. Yamasaki, L., Jacks, T., Bronson, R., Guillot, E., Harlow, E., and Dyson, N. J. (1996) Cell 85, 537–548
17. Pan, H., Yin, C., Dyson, N. J., Harlow, E., Yamasaki, L., and Van Dyke, T. (1998) Mol. Cell 2, 283–292
18. Wu, L., Timmers, C., Maiti, B., Saavedra, H. I., Sang, L., Chong, G. T., Nuckolls, F., Giangrande, P., Wright, F. A., Field, S. J., Greenberg, M. E., Orkin, S., Nevins, J. R., Robinson, M. L., and Leone, G. (2001) Nature 414, 457–462
19. Shi, Y., Sawada, J., Sui, G., Affar, E. B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P., Nakatani, Y., and Shi, Y. (2003) Nature 422, 735–738
20. Subramanian, T., and Chinnadurai, G. (2003) FEBS Lett. 540, 255–258
21. Meloni, A. R., Smith, E. J., and Nevins, J. R. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 9574–9579
22. Sewalt, R. G., Gunster, M. J., van der Vlag, J., Satijn, D. P., and Otte, A. P. (1999) Mol. Cell. Biol. 19, 777–787
23. Froluv, M. V., and Dyson, N. I. (2004) J. Cell Sci. 117, 2173–2181
24. Chen, C. F., Li, S., Chen, Y. P., Shu, S. Z., and Lee, W. H. (1996) J. Biol. Chem. 271, 32863–32868
25. Zheng, L., Pan, H., Li, S., Flesken-Nikitin, A., Chen, P. L., Boyer, T. G., and Lee, W. H. (2000) Mol. Cell 6, 757–768
26. Friedlander, J. R., Frederickson, W. J., Jensen, D. E., Speicher, D. W., Huang, X. P., Neison, E. G., and Rauscher, F. J., 3rd (1996) Genes Dev. 10, 2067–2078
27. Furuta, S., Wang, J. M., Wei, S., Jeng, Y. M., Ji, X., Gu, B., Chen, P. L., Lee, E. Y., and Lee, W. H. (2006) Cancer Cell 10, 13–24
28. Ahmed, K. M., Tsai, C. Y., and Lee, W. H. (2010) J. Biol. Chem. 285, 4464–4471
29. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O’Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 3706–3711
30. Garcia, V., Garcia, J. M., Peña, C., Silva, J., Dominguez, G., Rodriguez, R., Maximiano, C., Espinosa, R., España, P., and Bonilla, F. (2005) J. Pathol. 206, 92–99
31. Zhan, Q. (2005) Mutat. Res. 569, 133–143
32. Yun, J., and Lee, W. H. (2003) Mol. Cell. Biol. 23, 7305–7314
33. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R. G. (1995) J. Biol. Chem. 270, 23589–23597
34. Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003) Methods 30, 256–268
35. Zhu, Y., Saunders, M. A., Yeh, H., Deng, W. G., and Wu, K. K. (2002) J. Biol. Chem. 277, 6923–6928
36. Wang, J. M., Tseng, J. T., and Chang, W. C. (2005) Mol. Biol. Cell 16, 3365–3376
37. Atwood, C., Lasserini Denchi, E., and Helin, K. (2004) EMBO J. 23, 4709–4716
38. Croxton, R., Ma, Y., Song, L., Haura, E. B., and Cress, W. D. (2002) Oncogene 21, 1359–1369
39. Ohtani-Fujita, N., Fujita, T., Takahashi, R., Robbins, P. D., Dryja, T. P., and Sakai, T. (1994) Oncogene 9, 1703–1711
40. Jun, D. Y., Park, H. S., Lee, J. Y., and Kim, Y. H. (2008) Biochim. Biophys. Acta 1783, 49–62
41. Doineo-Sixou, S. F., Serger, C. M., Carroll, J. S., Hui, R., Musgrove, E. A., and Sutherland, R. L. (2003) Endocr. Relat. Cancer 10, 179–186
42. Müller, H., Bracken, A. P., Vernet, R., Moroni, M. C., Christians, F., Grasslili, E., Prosperini, E., Helin, K., and Oliner, J. D., and Helin, K. (2001) Genes Dev. 15, 267–285
43. Ohtani-Fujita, N., Fujita, T., Takahashi, R., Robbins, P. D., Dryja, T. P., and Sakai, T. (1994) Oncogene 9, 1703–1711
51. Khan, M. Z., Brandimarti, R., Shimizu, S., Nicolai, J., Crowe, E., and Meucci, O. (2008) Cell Death & Differ. 15, 1163–1172
52. Weintraub, S. J., Prater, C. A., and Dean, D. C. (1992) Nature 358, 259–261
53. Höfferer, M., Wirbelauer, C., Humar, B., and Krek, W. (1999) Nucleic Acids Res. 27, 491–495
54. Dasika, G. K., Lin, S. C., Zhao, S., Sung, P., Tomkinson, A., and Lee, E. Y. (1999) Oncogene 18, 7883–7899
55. Lin, L. F., Chuang, C. H., Li, C. F., Liao, C. C., Cheng, C. P., Cheng, T. L., Shen, M. R., Tseng, J. T., Chang, W. C., Lee, W. H., and Wang, J. M. (2010) Cancer Res. 70, 192–201
56. Koipally, J., and Georgopoulos, K. (2002) J. Biol. Chem. 277, 23143–23149