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Advances in Brief

The Tumor Suppressor Candidate p33^{ING1} Mediates Repair of UV-Damaged DNA[1]

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Abstract

The biological functions of the tumor suppressor, *ING1*, have been studied extensively in the last 5 years since it was cloned. It shares many biological functions with those of *p53* and has been reported to mediate growth arrest, senescence, apoptosis, anchorage-dependent growth, and chemosensitivity. Some of these functions, such as cell cycle arrest and apoptosis, have been shown to be dependent on the activity of both ING1 and p53 proteins. In this study, we report that p33^{ING1} (one of ING1 isoforms) is also involved in the modulation of DNA repair. We found that overexpression of p33^{ING1} enhances repair of UV-damaged DNA and that p53 is required for the repair process. Furthermore, binding between ING1 and GADD45 has been detected. These observations suggest that p33^{ING1} cooperates with p53 in nucleotide excision repair and that GADD45 may be one of its components.

Introduction

The tumor suppressor gene *ING1* has been shown to inhibit cell growth in the G_1 phase by transactivating the cyclin-dependent kinase inhibitor p21^{waf1} in the presence of *p53* (1, 2). Overexpression of *ING1* enhances serum starvation-induced cell death (3), and adenovirus-mediated transfer of both *ING1* and *p53* induces apoptosis in glioma cells (4). ING1 can also sensitize cells to stress agents, such as etoposide and γ-irradiation, in wild-type but not in *p53*-deficient cell lines (2). Furthermore, ING1 appears to play a role in senescence, because senescent cells express higher levels of ING1, and antisense ING1 can increase the replicative life of the cell (5). Higher expression and rearrangement of *ING1* have been reported in neuroblastoma cell lines (1). Decreased expression of *ING1* was seen in lymphoid tumor cell lines (6), breast cancer primaries and cell lines (7, 8), and gastric cancers (9). A few missense and silent mutations in *ING1* were also detected in head and neck squamous cell carcinomas (10). Findings from these studies strongly support the notion that *ING1* is a tumor suppressor gene and plays a significant role in the process of carcinogenesis. Four isoforms of the *ING1* gene, encoding M_r 46,751, 31,843, 27,000, and 23,656 proteins, have thus far been found (10–12). Their biological functions are being investigated intensively.

In light of the functional similarities between ING1 and p53 and the recent finding that the expression of the p33^{ING1} isoform is induced by UV irradiation in a dose-/time-dependent and tissue-specific manner (13), we investigated if p33^{ING1} plays a role in UV-stress response, such as repair of UV-damaged DNA. In this study, we show that overexpression of p33^{ING}G_1 confers enhanced repair efficiency of UV-damaged DNA in melanoma cells and that this repair capability of p33^{ING1} requires the participation of p53.

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Materials and Methods

Cell Lines and Culture. MMRU and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum and HCT116−/− cells in McCoy's 5A medium with 10% fetal bovine serum (Canadian Life Technologies, Inc., Mississauga, Ontario, Canada) at 37°C in a 5% CO_2 atmosphere. The *p53* mutational status of MMRU has been determined previously (14).

UV Irradiation. Medium was removed, and the cells were exposed to UVB (290–320 nm) using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ). Medium was replaced, and cells were incubated in a 5% CO_2 incubator at 37°C after UVB irradiation.

Western Analysis. Cells were harvested by scraping and solubilized by the triple detergent lysis buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP40, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. Concentrations of proteins were determined by the DC Protein Assay (Bio-Rad, Mississauga, Ontario, Canada). Fifty μg/lane of proteins were separated on 10% polyacrylamide/SDS gels and electroblotted onto polyvinylidene difluoride filters. Filters were incubated with primary antisera for 1 h, followed with 3× washes in PBS for 5 min, and then were incubated with horseradish peroxidase-conjugated secondary antisera for 1 h at room temperature. Signals were detected with SuperSignal enhanced chemiluminescence (Pierce, Rockford, IL). Antibodies used for Western blotting were anti-p33^{ING1} rabbit polyclonal antibody (PharMingen, Mississauga, Ontario, Canada), anti-p53 DO-1 mouse monoclonal, anti-GADD45 mouse monoclonal, anti-XPA rabbit polyclonal, and anti-XPB rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), secondary IgG (Calbiochem, San Diego, CA), and anti-β-actin goat monoclonal antibody (Santa Cruz Biotechnology).

Northern Analysis. Total RNA was extracted by TriZol reagent, and the concentrations were determined by UV spectrophotometry. Samples were heated to 65°C and run on 1% agarose gels containing formaldehyde and 0.5 μg/ml ethidium bromide. After separation, capillary transfer to nitrocellulose was performed overnight at room temperature, and its efficiency was assessed by UV light. The blot was then baked for 2 h in a vacuum oven at 80°C. Prehybridization was carried out by incubating the blot with a mixture containing 6 × saline-sodium phosphate-EDTA, 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml yeast tRNA for 1 h at 65°C. Hybridization was done by incubating the blot with the labeled probe at 65°C for 16–24 h. Filters were washed with 2 × SSC/0.1% SDS once for 15 min at room temperature and three washes 20 min each at 65°C. Blots were visualized on X-ray films after an overnight exposure.

Transfection. Cells were grown to 50–60% confluency. The ratio of 1 μg of DNA:25 μl of Effectene reagent (Qiagen, Mississauga, Ontario, Canada) was used for transfection. Plasmids used for transfection included pCI-p33^{ING1B} and pCI-antisense-p33^{ING1B} (a kind gift from Dr. Karl Riabowol, University of Calgary, Calgary, Alberta, Canada), pED1 and pECH (a kind gift from Dr. Samuel Benchimol, Univ. of Toronto, Toronto, Ontario, Canada), and pCMV*cat* (a kind gift from Dr. Lawrence Grossman, Johns Hopkins University, Baltimore, MD).

Host-Cell-Reactivation Assay. The pCMV*cat* plasmid contains a gene encoding for *cat*, under the transcriptional control of the immediate early promoter of the human cytomegalovirus. The pCMV*cat* plasmid DNA was irradiated at 40, 80, and 480 mJ/cm^2 using an UV-cross-linker at 50 μg/ml final concentration and used for transfection. Forty h after transfection, cells were harvested, and the cell pellet was resuspended in a 50-μl solution of 0.25 M Tris-Cl (pH 8.0) and 5 mM EDTA. Cell-free extracts of the transfected cells were made by three repeated freeze-thawings (liquid nitrogen to freeze; 37°C to thaw), heated to 65°C for 10 min, and centrifuged at 12,000 × *g* for 10 min,

and the cleared supernatant was used for the CAT assay. The assay reaction mixture contained 7.5 μl of 5 mM chloramphenicol, 50 μl of cell-free extract, 1 μl of 2.5 mM [^{3}H]acetyl-CoA, and 16.5 μl of distilled water. The reaction mixture was incubated at 37°C for 90 min. After incubation, 200 μl of ice-cold ethyl acetate was added, and tubes were shaken and centrifuged at 12,000 $\times$ *g* for 5 min. After quick-freezing the aqueous phase in a dry ice/ethanol bath, the organic phase was removed and extracted with 200 μl of distilled water. The organic phase was dried to completion, and radioactivity was determined in a scintillation counter. Determinants were performed in triplicates. Controls included transfection with undamaged plasmid DNA and mock transfection without plasmid DNA.

RIA. Antisera were raised against DNA dissolved in 10% acetone and irradiated with UVB light under conditions that have been shown to produce CPDs[3] exclusively. Heat-denatured sample DNA (2–5 μg) was incubated with 5–10 pg of poly(deoxyadenylated TMP; labeled to $>5 \times 10^8$ cpm/μg by nick translation with [^{32}P] dideoxythymidine 5′-triphosphate) in a total volume of 1 ml of 10 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, and 0.15% gelatin (Sigma Chemical Co., St. Louis, MO). Antiserum was added at a dilution that yielded 30–60% binding to labeled ligand, and, after incubation overnight at 4°C, the immune complex was precipitated with goat antirabbit immunoglobulin (Calbiochem) and carrier serum from nonimmunized rabbits (UTMDACC; Science Park/Veterinary Division, Bastrop, TX). After centrifugation, the pellet was dissolved in tissue solubilizer (NCS; Amersham, Piscataway, NJ) and mixed with ScintiSafe (Fisher, Hampton, NH) containing 0.1% glacial acetic acid, and the ^{32}P was quantified by liquid scintillation spectrometry. Under these conditions, antibody binding to an unlabeled competitor inhibits antibody binding to the radiolabeled ligand. Sample inhibition is extrapolated through a standard (dose-response) curve to determine the number of photoproducts in 10^6 bases (*i.e.*, CPDs/mb). For the standard, we used double-stranded salmon testis DNA (Sigma Chemical Co.) irradiated with increasing doses of UVC light, heat-denatured, aliquoted, and kept frozen at −20°C. Rates of photoproduct induction were quantified using nonimmunological enzymatic and biochemical techniques and determined to be 0.81 CPDs/mb/mJ/cm^2.

Immunoprecipitation. Cells were grown to ~80% confluency in 100-mm tissue culture dishes. Their lysates were harvested and incubated with anti-p33^{ING1} antibody or a nonspecific control anti-interleukin-12B rabbit polyclonal antibody (Santa Cruz Biotechnology) at 4°C for 1 h and then with protein A-Sepharose at 4°C overnight. The beads were washed three times with solubilization buffer before boiling for 5 min. The precipitates were then resolved by electrophoresis, followed by Western analysis as described above.

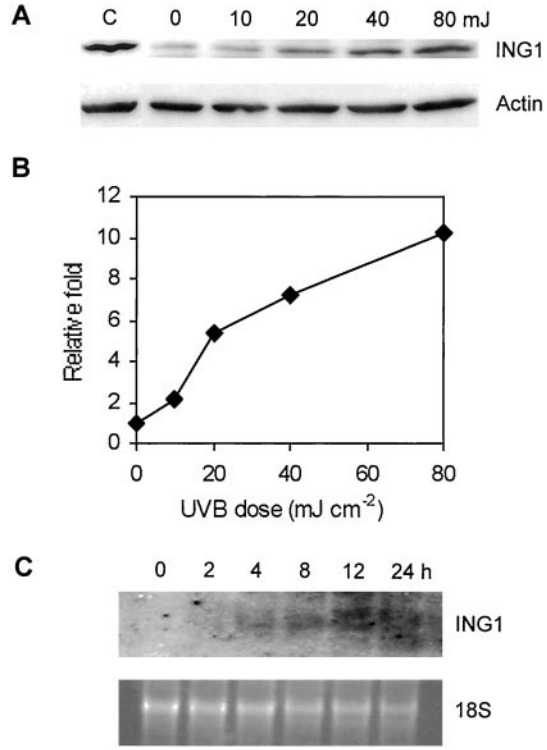

Fig. 1. p33^{ING1} is UV-inducible in a dose- and time-dependent manner. *A*, Western analysis of UV-induced p33^{ING1} protein expression in MMRU cells. Cells were UV-irradiated at 0, 10, 20, 40, and 80 mJ/cm^2 and harvested after 24-h incubation. An anti-p33^{ING1} antibody was used for primary antibody incubation, and β-actin was used as loading control. *Lane C*, lysate from MMRU cells overexpressing the pCI-p33^{ING1B} plasmid, confirming that the bands induced by UV irradiation were the p33^{ING1} protein. *B*, densitometry of p33^{ING1} induction in *A*. *C*, Northern analysis of UV-induced p33^{ING1} mRNA in MMRU cells. Cells were UV-irradiated at 40 mJ/cm^2 and harvested at 0-, 2-, 4-, 8-, 12-, and 24-h time points. The p33^{ING1} probe was first made by amplifying a 577-bp fragment by PCR using primer 1 (5′-GATCCTGAAGGAGCTAGACG-3′) and primer 2 (5′-AGAAGTGGAACCACTCGATG-3′) and then labeling it with [α-^{32}P]dCTP (10 mCi/ml) according to the manufactured protocol in the Random Primers DNA Labeling System (Canadian Life Technologies, Inc.). 18s rRNA was used as loading control.

Results

UV Induces p33^{ING1} Expression in a Dose- and Time-dependent Manner. We first examined whether p33^{ING1} would respond to UV in a human melanoma cell line, MMRU, which contains wild-type *p53* (14). We found that there was a clear induction of p33^{ING1} protein with increasing UV doses (Fig. 1, *A* and *B*). To test the possibility that the induction was attributable to transcriptional regulation, we examined the RNA levels at various time points after UV irradiation. We found that UV-induced p33^{ING1} was indeed a result of transcriptional control (Fig. 1*C*). These results indicate that p33^{ING1} was induced in a dose- and time-dependent manner after UV irradiation.

p33^{ING1} Enhances the Repair of UV-Damaged DNA. To study if p33^{ING1} mediates DNA repair, we used the host-cell-reactivation assay where a UV-damaged plasmid containing the CAT reporter gene (pCMV*cat*) was cotransfected with either vector, p33^{ING1}, or antisense p33^{ING1} expression vector into MMRU cells. The activity of the reporter gene was used as an indicator of the extent of repair. Our data demonstrated that cells overexpressing the p33^{ING1} construct had 2–4-fold increase in the rate of repair of the UV-damaged plasmid compared with the vector and antisense controls (Fig. 2*A*). This enhancement in repair was maintained in conditions even when severely UV-damaged CAT plasmids (at 480 mJ/cm^2) were used (Fig. 2*A*). To confirm the results from the host-cell-reactivation assay, we performed RIA for global genomic repair. The levels of the major UV-induced photoproducts, CPDs, were monitored in MMRU cells overexpressing p33^{ING1}. The results showed that the repair rate of CPDs was nearly doubled in p33^{ING1}-transfected cells compared with the vector-transfected control cells 24 h after UV irradiation (Fig. 2*B*).

p53 Is Required for p33^{ING1}-Mediated DNA Repair. As expected, the p53 protein was induced in an UV dose-dependent manner (Fig. 3*A*). To examine the relationship between p33^{ING1} and *p53* in DNA repair, we disrupted the activity of endogenous wild-type p53 in MMRU cells by introducing the pED1 construct containing a dominant-negative mutant *p53* (15, 16). To confirm pED1 expression in the cells, an anti-p53 antibody, which recognizes both wild-type and mutant p53 proteins, was used. An elevated level of p53 was seen in pED1-transfected MMRU compared with the vector control, indicating successful transfection (Fig. 3*B*). Similar levels of p33^{ING1} between pED1-transfected and control cells were observed (Fig. 3*B*), eliminating the possibility that the overexpressed mutant p53 might block the expression of p33^{ING1}. Using the host-cell-reactivation assay, we noted that the repair enhancement of p33^{ING1} was dramatically suppressed in pED1-transfected cells but restored in wild-type p53 (pECH)-transfected cells (Fig. 3*C*), suggesting that p33^{ING1} requires the presence of p53 to repair damaged DNA.

[3] The abbreviations used are: CPD, cyclobutane pyrimidine dimer; CAT, chloramphenicol acetyltransferase.

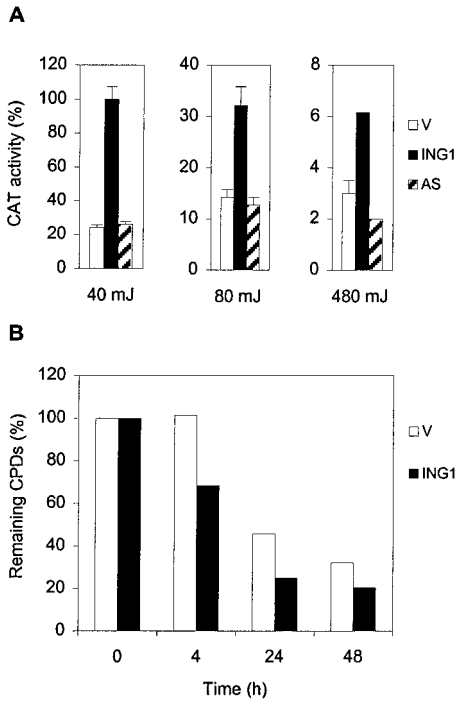

Fig. 2. p33^{ING1} enhances UV-damaged DNA repair. *A*, effect of p33^{ING1} on repair of UV-damaged plasmid DNA by host-cell-reactivation assay. Undamaged or UV-damaged pCMV*cat* plasmids were cotransfected with vector, pCI-p33^{ING1}, or pCI-antisense p33^{ING1} into MMRU cells and incubated at 37°C with 5% CO_2 for 40 h. CAT activity was determined by scintillation counting and expressed as: net dpm damage dose/net dpm zero dose. Experiments were performed in triplicates. Shown are representatives of two independent sets of experiments. *B*, effect of p33^{ING1} on repair of UV-damaged genomic DNA by RIA. MMRU cells transfected with vector or p33^{ING1} plasmids were UV-irradiated at 20 mJ/cm^2, and genomic DNA was harvested at 0, 4, 24, and 48 h. The percentage of remaining CPDs was then measured using antisera specific for CPDs (data presented as average of two independent experiments).

ING1 Binds to GADD45. To study the pathways involved in p33^{ING1}-mediated DNA repair, we examined if p33^{ING1} is the upstream regulator of GADD45, XPA, and XPB, all of which have been shown to have significant involvement in DNA repair (17). We found that there was no change in expression in any of the aforementioned proteins in MMRU cells overexpressing p33^{ING1} (Fig. 4*A*), indicating that p33^{ING1} is not the upstream regulator of them. To test the possibility that ING1 may physically associate with GADD45, XPA, and XPB, we performed immunoprecipitation and found that there was a weak physical association, as indicated by the intensity of the signal, between ING1 and GADD45 (Fig. 4*B*). No binding was observed between ING1 and XPA/XPB (Fig. 4*B*).

Discussion

We have shown previously (18, 19) that the tumor suppressor p53 plays an essential role in cellular stress response to UV irradiation, such as enhancement of DNA repair and promotion of apoptosis. However, the exact molecular mechanisms of p53 enhancement in the repair of UV-damaged DNA are unclear. Recent findings that the tumor suppressor candidate ING1 shares similar biological functions with p53 (2, 4) and that the two proteins physically bind to each other (2) led us to hypothesize that ING1 may also participate in cellular stress response to UV irradiation. In this study, we show that p33^{ING1} is induced at both mRNA and protein levels in a melanoma cell line, MMRU, after UV irradiation (Fig. 1). Although the p53 protein is also accumulated after UV irradiation (Fig. 3), it is believed that p53 accumulation is attributable to prolonged half-life of the protein rather than transcriptional activation (20). Therefore, the mechanisms for UV-induction of p33^{ING1} appear different from that for p53. The upstream regulator of the p33^{ING1} gene has not yet been identified. Nevertheless, UV-induction of p33^{ING1} seems to be a common phenomenon in epidermal cells because we have shown recently (13) that p33^{ING1} is up-regulated at the transcriptional level in normal human keratinocytes and a keratinocyte cell line, HaCaT.

For the first time, we demonstrate that overexpression of p33^{ING1} enhances nucleotide excision repair of both UV-damaged genomic DNA and exogenous plasmid DNA (Fig. 2), further supporting the notion that p33^{ING1} is a tumor suppressor. Nucleotide excision repair is a crucial stress-response mechanism to maintain the genomic stability. UV radiation damages DNA primarily in the forms of CPDs and photoproducts (6–4). These photoproducts are repaired by nucleotide excision repair, which involves a complex series of proteins that orchestrate the identification and removal of damaged DNA, addition of nucleotides, and, finally, religation of the DNA strand (21). If UV-induced DNA photoproducts are not promptly removed, they will in turn lead to mutation and skin carcinogenesis; *e.g.*, xeroderma pigmentosum patients who have defects in nucleotide excision repair suffer a 1000-fold increase in skin cancer incidence (22).

Wild-type p53 binds to and modulates XPB and XPD (23), two components of the TFIIH transcription unit that possesses helicase, ATPase, and kinase activity (24). However, our results demonstrate that p33^{ING1} does not transcriptionally regulate or physically bind to XPA and XPB (Fig. 4). The physical association between p33^{ING1} and GADD45 (Fig. 4*B*) suggests that p33^{ING1} may be a crucial component in the GADD45-mediated nucleotide excision repair pathway. The fact that GADD45 is up-regulated by p53 and that p33^{ING1} requires the participation of functional p53 in DNA repair (Fig. 3) further supports the close association of p33^{ING1} and GADD45. Increasing

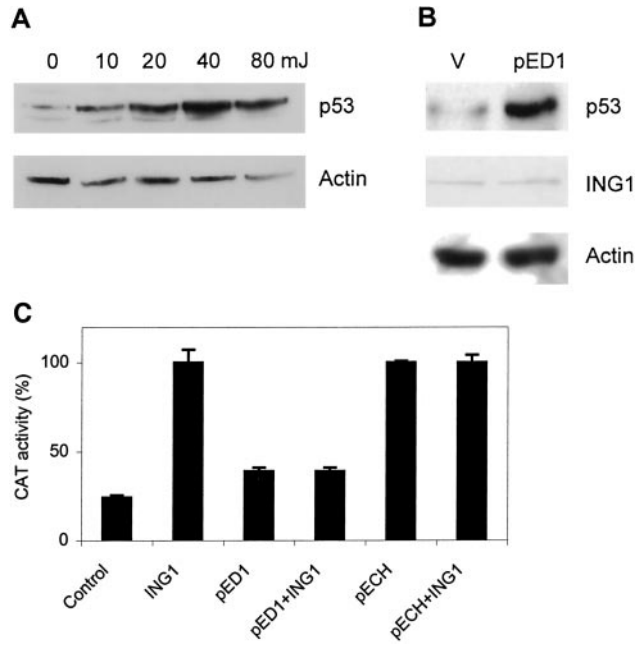

Fig. 3. p33^{ING1}-mediated DNA repair is *p53*-dependent. *A*, Western analysis of p53 protein expression in UV-irradiated MMRU cells. Cells were UV-irradiated at 0, 10, 20, 40, and 80 mJ/cm^2 and harvested after 24-h incubation. An anti-p53 antibody was used for primary antibody incubation, and β-actin was used as loading control. *B*, Western analysis of p53 and p33^{ING1} proteins in MMRU cells transfected with the dominant-negative mutant-p53 (pED1) expression vector. *C*, effect of p53 on p33^{ING1}-mediated DNA repair. Host-cell-reactivation assay was performed on MMRU cells transfected with UV-damaged (40 mJ/cm^2) pCMV*cat* plasmid and control vector, p33^{ING1}, pED1, p33^{ING1}/pED1, pECH, or p33^{ING1}/pECH. Forty h later, CAT activity was measured using the undamaged pCMV*cat* as control. Experiments were performed in triplicates. Shown is a representative of two independent sets of experiments.

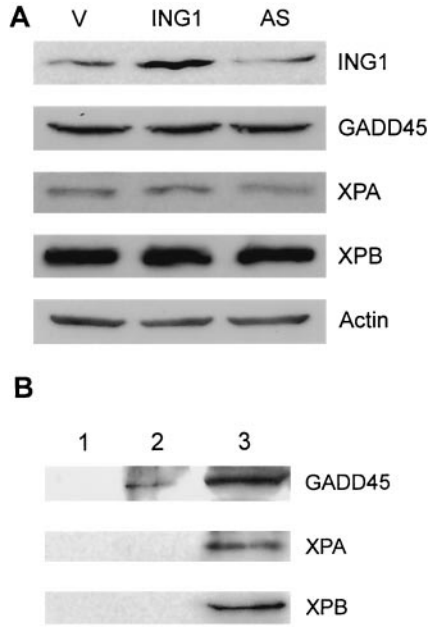

Fig. 4. ING1 physically interacts with GADD45 but does not transcriptionally upregulate GADD45, XPA, or XPB. *A*, effect of p33^{ING1} on the expression of GADD45, XPA, and XPB proteins. MMRU cells were transfected with vector alone, p33^{ING1}, or antisense p33^{ING1} expression vectors. Twenty-four h after transfection, cells were harvested, and their lysates were analyzed by Western blotting using anti-GADD45, anti-XPA, and anti-XPB antibodies. β-actin served as a loading control. *B*, coimmunoprecipitation of ING1 with XPA, XPB, and GADD45. MMRU total cell lysates were immunoprecipitated with a nonspecific control antibody (*Lane 1*), with the anti-ING1 antibody that recognizes different isoforms of ING1 (*Lane 2*), or without any antibody (*Lane 3*). Antibodies against XPA, XPB, and GADD45 were then used in Western analysis. The physical binding between ING1 and GADD45 was observed in three separate experiments.

evidence has indicated that GADD45 is essential in UV-damaged DNA repair and genome stability (17, 25, 26). Recently (27), an interesting report shows that GADD45 can recognize UV-altered chromatin state and modulate DNA accessibility to repair proteins such as DNase I and T4 endonuclease V. It would be of interest to exploit the mechanistic role of p33^{ING1} in this GADD45-mediated repair process.

Taken together, our results strongly demonstrate that p33^{ING1} enhances the nucleotide excision repair of UV-damaged DNA. Because there is a strong causal relationship between UV exposure and melanoma formation, loss or inactivation of p33^{ING1} can potentially contribute to neoplastic development.

Acknowledgments

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