The Novel Tumor Suppressor p33ING2 Enhances Nucleotide Excision Repair via Inducement of Histone H4 Acetylation and Chromatin Relaxation

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Abstract

p33ING2 is a novel candidate tumor suppressor, which has been shown to be involved in the regulation of gene transcription, cell cycle arrest, and apoptosis in a p53-dependent manner for maintaining the genomic stability. Previously, we showed that p33ING2 promoted UV-induced apoptosis in human melanoma cells. To further reveal the role of p33ING2 in cellular stress response to UV irradiation, we hypothesized that p33ING2 may enhance the repair of UV-damaged DNA, similarly to its homologue p33ING1b. Using the host-cell reactivation assay, we show that overexpression of p33ING2 significantly enhances nucleotide excision repair of UV-induced DNA damage in melanoma cells in a p53-dependent manner. Furthermore, DNA repair is completely abolished in cells treated with p33ING2 small interfering RNA, suggesting that a physiologic level of p33ING2 is required for nucleotide excision repair. In addition, we found that p33ING2 is an essential factor for UV-induced rapid histone H4 acetylation, chromatin relaxation, and the recruitment of damage recognition protein, xeroderma pigmentosum group A protein, to the photoloesions. These observations suggest that p33ING2 is required for the initial DNA damage sensing and chromatin remodeling in the nucleotide excision repair process. (Cancer Res 2006; 66(4): 1906-11)

Introduction

Nucleotide excision repair is a cellular stress response mechanism for the removal of bulky photoloesions or DNA adducts caused by UV light and a number of environmental carcinogens. Individuals with defects in nucleotide excision repair, such as xeroderma pigmentosum patients, are extremely sensitive to UV-induced skin cancer development with >1,000-fold higher incidence than normal population (1). Although over 30 factors have been identified to participate in different steps of the nucleotide excision repair process including DNA damage recognition, excision of the DNA adducts, and repair synthesis, little is known on the initial cellular response for the chromatin remodeling to allow the repair proteins to access the DNA damage sites.

The novel tumor suppressor candidate p33ING2 is a member of the inhibitor of growth (ING) family proteins. p33ING2 has been shown to negatively regulate cell growth in a p53-dependent manner through induction of G1-phase cell cycle arrest and apoptosis (2). Furthermore, p33ING2 modulates the activity of p53 by enhancing its stability through p53 acetylation at Lys382 and/or Lys373 (2). Recent studies indicated that the interaction of p33ING2 with phosphoinositides, PtdIns(3)P and PtdIns(5)P, through its plant homeodomain zinc finger motif is critical for its ability to enhance p53 acetylation and mediate p53-dependent apoptosis (3). We and others showed that the ING family tumor suppressors participate in cellular stress response to UV irradiation. Both p33ING1 and p33ING2 enhanced UV-induced apoptosis in human fibroblasts and melanoma cells (4-6). Our group also found that overexpression of p33ING1 significantly enhanced nucleotide excision repair of UV-damaged DNA in a p53-dependent manner (7). In light of the structural and functional similarities between p33ING2 and p33ING1b (8), we hypothesized that p33ING2 may also participate in nucleotide excision repair. Here we report that p33ING2 significantly enhances nucleotide excision repair in melanoma cells in a p53-dependent manner by rapidly inducing histone H4 acetylation, chromatin relaxation, and the recruitment of the damage recognition factor xeroderma pigmentosum group A protein (XPA) to the DNA photoloesions.

Materials and Methods

Cell lines and cell culture. Wild-type p53 MMRU and mutant p53 MeWo melanoma cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO2.

Small interfering RNA transfection. MMRU cells were transfected at 50% to 70% confluency with small interfering RNA (siRNA; 10 nmol/L) using SilentFect (Bio-Rad, Mississauga, ON, Canada). The sense sequence of the ING2 siRNA (Qiagen, Mississauga, ON, Canada) is r(GGGUUAA-UGC AUAAAGACU)dTdT and the antisense sequence is r(UAGUCUUA-U GCAUUUACC)dTdT. The p53 siRNA (Qiagen) sense sequence is r(GUGAUACCCGGAGGCCCAG)dTdT and the antisense sequence is r(AUGGGCC- CUCCGGGUUCAG)dTdT.

Chloramphenicol acetyltransferase assay. pCMVCAT plasmid DNA was irradiated with UVC at 200 J/m2 using an UV cross-linker (Ultralum, Claremont, CA) and used to cotransfect melanoma cells with gene of interest using lipofectamine 2000 (Invitrogen). Forty hours after transfection, chloramphenicol acetyltransferase (CAT) assay was done as previously described (7).

Renilla luciferase assay. pBL-CMV plasmid DNA was irradiated with UVC at 200 J/m2 and cotransfected with gene of interest using Lipofectamine 2000 (Invitrogen). Forty hours after transfection, reporter enzyme level was analyzed with a lucferase assay kit (Promega, Madison, WI).

Immunofluorescence. MMRU cells were seeded on coverslips in six-well plates and transfected with p3XFLAG-ING2 at 50% confluency. After 24 hours, the cells were irradiated with UVC through a polycarbonate filter with 5-μm pores (Millipore, Nepean, ON, Canada) and...
cultured for a desired period of time. The cells were fixed with 2 mL of ethanol, precipitated with ethanol, and analyzed on 1% agarose gel. The aqueous phase was collected. DNA was extracted twice with phenol/chloroform.
expression of these plasmids, suggesting successful transfection (Fig. 1A). The activity of this reporter gene was used as an indicator of the extent of repair. Our data indicated that MMRU cells overexpressing p33ING2 had a significantly higher repair rate of UV-damaged plasmid (59.1%) compared with the vector (24.1%; P < 0.01) or antisense control (26.0%; P < 0.01, t test; Fig. 1B). In addition, we did a luciferase reporter assay to confirm whether the endogenous p33ING2 is required for DNA repair in a physiologic condition by gene silencing using p33ING2 siRNA. Cells treated with p33ING2 siRNA for 48 hours had an 80% reduction in p33ING2 protein expression (Fig. 1C). Similar to the CAT assay, we found that MMRU cells overexpressing p33ING2 had a significantly increased DNA repair rate (32.5%) compared with the vector control (11.2%; P < 0.01, t test). On the other hand, treatment with p33ING2 siRNA, which inhibited 80% p33ING2 protein expression, completely abolished nucleotide excision repair (Fig. 1D), indicating that a certain threshold of p33ING2 is required for the repair of UV-damaged DNA in melanoma cells. To further measure DNA repair rate in global genomic repair, we detected the levels of UV-induced CPDs and 6-4PPs in p33ING2-transfected MMRU cells compared with vector control at various time points after UV irradiation. We found that cells transfected with p33ING2 had a much faster removal rate of 6-4PPs and CPDs compared with the vector controls (Fig. 1E).

**p33ING2-enhanced DNA repair requires wild-type p53.** Because p33ING2 homologue p33ING1b requires functional p53 to enhance nucleotide excision repair (7), we investigated whether p33ING2-mediated DNA repair also requires the presence of wild-type p53. We disrupted the endogenous wild-type p53 in MMRU cells by introducing the p53 siRNA or a dominant-negative mutant p53 pED-1 plasmid (7, 10). Western blot analysis, using an anti-p53 antibody, confirmed that p53 siRNA silenced the expression of endogenous p53 by 90% (Fig. 1C). We found that p33ING2 overexpression could not enhance DNA repair in p53 siRNA–treated cells (3.2%; Fig. 1D) compared with the control cells (32.5%; P < 0.01, t test). Similarly, reduced DNA repair efficiency was observed in cells cotransfected with p33ING2 and pED1 compared with cells transfected with p33ING2 alone (see Supplementary Fig. S5A).

To further verify the p53 dependency of p33ING2-enhanced DNA repair, we carried out a CAT assay using a mutant p53 melanoma cell line, MeWo, and found that p33ING2 overexpression failed to enhance DNA repair in MeWo cells (see Supplementary Fig. S5B). These data indicate that p33ING2-enhanced repair of UV-damaged DNA does require functional p53.

**p33ING2 does not colocalize with UV-induced DNA lesions.** To study if p33ING2 is a component of the nucleotide excision repair complex, MMRU cells transiently overexpressing p33ING2 were irradiated with UVC at 200 J/m² with a filter containing 5-μm pores overlaid on the cells. Thus, only parts of the nucleus are UV irradiated through the filter whereas the remaining nucleus covered by the filter is blocked from the radiation. Therefore, this technique is useful in exploring protein colocalization with UV-induced DNA lesions. The intracellular colocalization of p33ING2 with two major UV-induced DNA lesions, CPDs and 6-4PPs, at different time points after UV irradiation were examined using immunofluorescent staining. Cells double labeled for XPA and CPDs were used as positive control as it was previously shown that XPA colocalized with UV-induced DNA lesions (11). Figure 2A shows that the majority of XPA (green) colocalized with CPDs (red) 1 hour after UV irradiation. Consistent with a previous report (3), the p33ING2 protein, labeled with green fluorescent, was primarily localized in the nucleus (Fig. 2B and C). The photolesions, 6-4PPs and CPDs, were labeled with red fluorescent. The fluorescent intensity of 6-4PPs faded at a faster rate than that of CPDs, which is consistent with the results from the slot-Western analysis (Fig. 1E). It became very weak 4 hours after UV irradiation and was hardly detectable after that point whereas the fluorescent intensity of CPDs remained strong in all time points examined. Our results showed that p33ING2 did not colocalize with DNA photolesions, 6-4PPs, or CPDs at all time points examined (Fig. 2B and C), suggesting that p33ING2 is not a component of the repair core complexes which are recruited to UV-induced DNA lesions.

**p33ING2 induces hyperacetylation of histone H4 after UV irradiation.** Only recently has the role of histone modification in complexes which are recruited to UV-induced DNA lesions. The intracellular colocalization of p33ING2 with two major UV-induced DNA lesions, CPDs and 6-4PPs, at different time points after UV irradiation were examined using immunofluorescent staining. Cells double labeled for XPA and CPDs were used as positive control as it was previously shown that XPA colocalized with UV-induced DNA lesions (11). Figure 2A shows that the majority of XPA (green) colocalized with CPDs (red) 1 hour after UV irradiation. Consistent with a previous report (3), the p33ING2 protein, labeled with green fluorescent, was primarily localized in the nucleus (Fig. 2B and C). The photolesions, 6-4PPs and CPDs, were labeled with red fluorescent. The fluorescent intensity of 6-4PPs faded at a faster rate than that of CPDs, which is consistent with the results from the slot-Western analysis (Fig. 1E). It became very weak 4 hours after UV irradiation and was hardly detectable after that point whereas the fluorescent intensity of CPDs remained strong in all time points examined. Our results showed that p33ING2 did not colocalize with DNA photolesions, 6-4PPs, or CPDs at all time points examined (Fig. 2B and C), suggesting that p33ING2 is not a component of the repair core complexes which are recruited to UV-induced DNA lesions.

![Figure 2](https://cancerres.aacrjournals.org/figure2.png)
following UV irradiation (13), we hypothesized that p33ING2 may be involved in histone acetylation and chromatin relaxation to mediate the repair of UV-damaged DNA. First, we examined if p33ING2 expression level is increased in MMRU cells after UV irradiation as a cellular stress response. Our data showed that the endogenous p33ING2 expression is not affected by UV irradiation (Fig. 3A). Then, we investigated if the acetylation level of histone H3 and H4 is changed in MMRU cells overexpressing p33ING2. Our results indicated that overexpression of p33ING2 can increase the acetylation of histone H4 by 2-fold compared with the vector-transfected control cells under normal nonstress condition (Fig. 3B). UV irradiation alone can also induce histone H4 acetylation in control MMRU cells by ~2-fold just 1 minute after irradiation (Fig. 3B). Interestingly, acetylation of histone H4 was increased even more (4-fold) in p33ING2-overexpressing cells 5 minutes after UV irradiation (Fig. 3B). We also examined the level of acetylated histone H3 in cells overexpressing p33ING2 and found that p33ING2 did not significantly enhance UV-induced acetylation of histone H3 (data not shown). To further investigate whether endogenous p33ING2 is required for histone acetylation, we treated MMRU cells with p33ING2 siRNA and examined histone H4 acetylation level. We found that in normal nonstress condition, knockdown of endogenous p33ING2 did not significantly affect histone H4 acetylation (Fig. 3C). However, p33ING2 siRNA completely abrogated UVC-induced histone H4 acetylation (Fig. 3C), suggesting that a physiologic level of p33ING2 is required for histone H4 hyperacetylation in cellular stress response to UVC irradiation. Due to the fact that p33ING2 cooperates with p53 in nucleotide excision repair, we next investigated if p33ING2 enhancement on histone acetylation requires p53 activity by siRNA.
gene silencing. As expected, p53 siRNA abolished the hyper-acetylation of histone H4 in p33ING2-overexpressing MMRU cells on exposure to UVC (Fig. 3D), which is consistent with p53 dependency of p33ING2-mediated nucleotide excision repair (Fig. 1D). To further verify that p33ING2 enhances histone H4 acetylation, we did immunofluorescence staining to visualize the expression level of acetylated histone H4 in single cells. We found that MMRU cells transfected with p33ING2 have a higher histone H4 acetylation level compared with control cells 5 minutes after UVC irradiation (Fig. 3E). We also noticed that p33ING2 induced global histone H4 acetylation, rather than localized histone H4 acetylation, at DNA damage sites (Fig. 3E). In addition, we found that p33ING2 enhanced histone H4 hyperacetylation when the cells were irradiated at a lower dose of UVC (20 J/m²; see Supplementary Fig. S6A). p33ING2 siRNA knockdown also reduced histone H4 acetylation after 20 J/m² UVC irradiation (Supplementary Fig. S6B), further confirming that p33ING2 is required for histone H4 acetylation at physiologic conditions.

**p33ING2 induces chromatin relaxation.** It has been shown that acetylation of histones on their lysine-rich amino-terminal tails, which protrude from nucleosomes, regulates gene expression through weakening histone-DNA contacts, leading to formation of transcriptionally competent chromatin domains (14). Thus, it is likely that histone acetylation plays an important role in chromatin remodeling to facilitate nucleotide excision repair (15). We next did a micrococcal nuclease digestion assay to look at chromatin de-condensation in p33ING2-overexpressing MMRU cells. We found that chromatin is more sensitive (79.3% of the DNA fragments <600 bp) to the nuclease (1 unit treatment) in p33ING2-overexpressing cells on UVC irradiation compared with MMRU control cells (15.9%; Fig. 4A). These results are consistent with the observation that p33ING2 enhances UV-induced histone H4 acetylation (Fig. 3), implying that p33ING2 may serve as a chromatin accessibility factor during the repair of UV-damaged DNA.

**p33ING2 facilitates XPA to the DNA damage sites.** As for every other process that requires access to DNA, efficient repair of UV-damaged DNA is dependent on the detection of bulky DNA photodisulfides and the recruitment of repair proteins, which require the unfolding of the chromatin into a more relaxed state (16). Recruitment of the photolesion-recognition protein XPA to the DNA damage site is considered to be a rate-limiting process for nucleotide excision repair (17). Thus, we investigated if p33ING2 is required for the recruitment of XPA by examining the colocalization of XPA and CPDs by immunofluorescence in control (Fig. 2A) and p33ING2 siRNA–treated cells (Fig. 4B). Punctate green staining of XPA at the same position with CPDs (Fig. 2A) is considered to be colocalized with DNA photodisulfides. Our results showed that p33ING2 siRNA treatment significantly reduced the recruitment of XPA protein to DNA damage site (81%) compared with control cells (23%) 30 minutes after UV irradiation ($P < 0.0001$; Fig. 4C), further confirming that p33ING2, in association with p53 and possibly other factors possessing intrinsic HAT activities, acts as a DNA damage sensor to induce histone acetylation and chromatin relaxation and, therefore, enhance the recruitment of damage recognition factors to the DNA damage sites to initiate the repair process.

Efficient nucleotide excision repair is crucial for cells to repair UV-induced DNA damage and prevent skin carcinogenesis. Previously, we and others showed that the p53 tumor suppressor plays an important role in nucleotide excision repair (18, 19). Recently, Rubbi and Milner (20) showed that the p53 is essential to induce histone H3 acetylation and chromatin relaxation after UV irradiation. However, it is not clear whether other factors cooperate with p53 to induce histone acetylation and chromatin relaxation during the nucleotide excision repair process and whether relaxed chromatin can lead to the recruitment of repair proteins to DNA.
damage sites. Our results for the first time showed that the novel tumor suppressor p33ING2 cooperates with p53 to induce histone H4 acetylation, chromatin relaxation, and recruitment of the indispensable damage-recognition XPA protein to photoslesion site. The fact that p33ING2 enhances histone H4 acetylation as early as 1 minute after UV irradiation (Fig. 3B) suggests that p33ING2 may act as a DNA damage sensor during the nucleotide excision repair process. Physiologic levels of p33ING2 are required for UV-induced rapid histone H4 acetylation, further supporting this notion (Fig. 3C). The essential role of p33ING2 in nucleotide excision repair, together with its role in cell cycle arrest and apoptosis, further indicates that p33ING2 is a tumor suppressor.

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References
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