Published in final edited form as:
Anticancer Res. 2009 October ; 29(10): 3741.

p53-mediated Down-regulation of the Human DNA Repair Gene *O*^{6}- Methylguanine-DNA Methyltransferase (*MGMT*) *via* Interaction with Sp1 Transcription Factor

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Abstract

O^6-Methylguanine-DNA methyltransferase (MGMT), a ubiquitous DNA repair protein, reverses mutagenic and cytotoxic effects of *O*^{6}-alkylguanine in DNA induced by chemotherapeutic *N*-alkyl *N*-nitrosourea and procarbazine type drugs by dealkylating the adduct. MGMT expression is down-regulated by wild-type p53 (WTp53) in human tumor cells. Here we report that p53 sequesters the Sp1 transcription factor to prevent its binding to the cognate *cis* elements in the MGMT promoter and thus inhibits MGMT expression. Sp1 overexpression abrogated the inhibitory effect of p53 on the MGMT promoter activity in a dose-dependent manner. Stable interaction of Sp1 with WTp53 was observed in HCT116 cells. Moreover, WTp53 overexpression reduced the binding of the nuclear extract to the Sp1 consensus sequence, even though recombinant p53 alone did not bind to the same sequence. Taken together, these results suggest that sequestration of Sp1 could be one of the mechanisms by which p53 negatively regulates MGMT expression, thus enhancing sensitivity of tumor cells to O^6-alkylguanine generating drugs.

Keywords

MGMT; p53; DNA repair; Sp1 transcription factor; drug resistance; alkylating agent

Alkylation of the *O*^{6}-position of guanine in DNA, induced by methylating (temozolomide, procarbazine) and chloroethylating [carmustine (BCNU); nimustine, (ACNU)], is considered to be the most mutagenic and carcinogenic lesion (1,2). Alkylating agents such as nitrogen mustards, procarbazine and nitrosoureas are among the most widely used chemotherapeutic agents. Mutagenic and cytotoxic adducts are removed from the *O*^{6} position of guanine by *O*^{6} methylguanine-DNA methyltransferase (MGMT) (3–6). MGMT is a ubiquitous DNA repair protein that acts in a stochiometric reaction in which an alkyl group attached to the *O*^{6} position of guanine is transferred to a specific cysteine residue in the protein's active site [reviewed in (7,8)]. This *in situ* dealkylation restores the original guanine in DNA while inactivating the protein MGMT (9). The MGMT level varies widely in both normal and tumor tissues and in tumor cell lines. Approximately 20% of *in vitro* transformed tumor cell lines have no detectable MGMT and are highly sensitive to methylating and chloroethylating agents; these are called Mer−/Mex− (10–14). Several studies showed that human MGMT down-regulation in human Mer−/Mex− cells was linked to the presence of methylated CpGs in the promoter region, while

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other studies indicated that regulation of MGMT expression was complex (15–18). Increased MGMT expression was observed in rat hepatoma cells after treatment with alkylating agents or UV light (19). One recent study showed that treatment with cysteine prodrugs and herbal antioxidant such as curcumin also increased the MGMT level in many cancer cells (20). Tumor cells with high levels of MGMT are known to have high resistance to BCNU, and depletion of MGMT activity by pseudosubstrates (such as O^6-benzylguanine, O^6-BG) reverses this resistance (4,21,22). Hence the level of MGMT expression could be a key determinant in tumor cell resistance to alkylating agents.

The *MGMT* promoter, like that of many housekeeping genes, does not have any TATA or CAAT boxes, but contain six putative Sp1-binding sequences, two glucocorticoid response-elements (GRE-1), and two activator protein-1 (AP-1) binding sequences (23–27). The involvement of Sp1, AP-1, GRE-1 in MGMT expression has been previously documented (26,27). Interestingly, in spite of the lack of p53-binding sites in the *MGMT* promoter, several studies have explored the impact of tumor suppressor p53 on MGMT expression. P53 serves as a transcription factor, and has a wide range of functions in cell cycle regulation, cell cycle arrest, DNA repair and apoptosis through transactivation of specific genes in response to DNA damage (ionizing radiation, IR and UV light, chemotherapeutic agents) and other cellular stress signals (28,29). P53 not only activates transcription of target genes (e.g. *p21, GADD45*) but also mediates repression of a wide range of viral and cellular gene promoters that do not contain consensus p53-binding sites (30–33). For example, p53 down-regulated basal expression of human MGMT and on the other hand, p53 was also shown to be required for MGMT up-regulation after ionizing radiation in rodent cells (34–36). Moreover, inverse correlation between MGMT expression and p53 in breast cancer and other tumor samples has been documented (37–39).

P53 is one of the most commonly mutated genes in human cancer and 50% or more of all human carcinomas have an inactive *p53* allele and these tumors respond poorly to chemotherapy (40,41). Interestingly, some mutant p53 proteins, which do not interact with p53 consensus binding sites, have been shown to up-regulate expression of certain genes (31). Therefore, altered gene expression resulting from p53 inactivation has significant consequences on cell cycle regulation and drug resistance. In spite of several studies showing the involvement of p53 as a negative regulator of MGMT in human tumor cells, the mechanism by which it regulates MGMT expression has not been elucidated. A clear understanding of this mechanism is essential for more effective use of alkylating agents in the treatment of tumors with wild-type (WT) or mutant p53.

Materials and Methods

Plasmids

The p53 expression vectors containing WT and mutant forms of p53 were kindly provided by Dr. A. J. Levine (University of Medicine and Dentistry of New Jersey, New Brunswick) and are described elsewhere (42). The Sp1 mammalian expression vector was a generous gift from Dr. R. Tijian (University of California, Berkeley). Generation of MGMT promoter-Luciferase reporter plasmids are described elsewhere (26).

Cell culture

The human colorectal adenocarcinoma line p53 null HCT116 P53−/− (a gift from Dr. B. Vogelstein, Johns Hopkins University School of Medicine) and human lung carcinoma Calu6 line (ATCC 2023CL) were grown in McCoy's 5A and DMEM (Gibco Life Technologies, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St.

Louis, Mo, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Life Technologies) in a 5% CO_2 incubator at 37°C.

Transfection and luciferase assay

HCT116 p53−/− cells were cotransfected with 500 ng of each MGMT-reporter plasmid and expression plasmid for WT or mutant (V143A and L22G, T23S) p53 or Sp-1 expression plasmids or equivalent amounts of empty vector using LipofectAMINE 2000 (GIBCO Life Technologies), according to the manufacturer's instructions. At 48 h after transfection, cells were lysed with reporter lysis buffer (Promega, Madison, WI, USA) and the luciferase activity in the cell lysates was measured in a luminometer (AutoLumant LB593; Berthold, Oak Ridge, TN, USA) with a luciferase assay kit (Promega) according to the manufacturer's protocol. We observed that CMV promoter-dependent β-galactosidase and thymidine kinase-promoter-dependent renilla-luciferase (pRL-TK; Promega) levels were affected due to overexpression of WT p53 for unknown reasons. We therefore could not use these reporter plasmids for normalizing transfection efficiency. The luciferase activity was normalized using the amount of protein in the lysates.

Preparation of cell extracts and Western blot analysis

Whole cell extracts were prepared as described elsewhere (43). Nuclear and cytosolic extracts were obtained as follows: Cells washed with cold phosphate-buffered saline were resuspended in buffer A, consisting of 10 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)], 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethyleneglycol tetraacetic acid (EGTA), 1 mM dithiotheritol DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and protease cocktail inhibitor tablet (Complete Mini; Roche, Nutely, New Jersey, USA) and allowed to swell on ice for 15 min. A volume of 2.5 μL of a 10% solution of Nonidet P40 was added to 400 μL cell suspension and the samples were vortexed vigorously for 10^S. The homogenate was centrifuged for 30^S. (20,800 x *g* at 4° C), and the supernatant (cytosol fraction) was separated. The pellets (nuclear fraction) were resuspended on 50 μL ice-cold buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and Complete Mini-protease inhibitor tablets, and shaken vigorously at 4°C for 15 min and the supernatant (nuclear fraction) was collected. The samples were then centrifuged for 5 min (20,800 x *g* at 4°C). Extracts (nuclear and cytosolic fractions) were aliquoted and stored at −80°C. Cell extracts were subjected to SDS-PAGE (12.5% acrylamide) and transferred to a nitrocellulose membrane (Trans-Blot 0.2μm; Bio-Rad, Hercules, CA, USA). Western immunoblot analysis with mouse monoclonal anti-p53 antibody (Sc-DO-1 and PAb1801, (dilution 1:200) Santa Cruz Biotechnology, Santa Cruz, CA, USA); Pab421 (Biomol), Phospho Ser15-specific P53 antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-Sp1 (dilution 1:2,000), and rabbit monoclonal anti-β-actin (Sigma; 1:1000 dilution) antibodies was carried out using enhanced chemiluminescence assay (ECL kit, Amersham Pharmacia, Piscataway, NJ, CA, USA). Lung carcinoma Calu6 cells were transfected with WT p53 expression plasmids or empty vector and at 48 h after transfection, cells were lysed and Western blot analysis was performed with polyclonal MGMT antibody as described elsewhere (25).

Co-immunoprecipitation

The nuclear extracts were immunoprecipitated with anti-Sp1 antibodies (Santa Cruz Biotechnology) or anti-FLAG M2 mouse antibody-conjugated agarose beads (Sigma) for 3 h. Immunoprecipitates of control and Sp1-FLAG or p53-FlAG-transfected cells were washed four times with TBS (50 mM Tris-HCl, pH 7.5; 150 mM NaCl) and boiled with Laemmli buffer (2% SDS and 100 μM β-mercaptoethanol). The supernatants were subjected to SDS-PAGE

followed by Western blot analysis with mouse monoclonal anti-p53 (PAb1801) and anti-FLAG M2 antibodies.

Electrophoretic shift mobility analysis (EMSA)

EMSA was performed as described elsewhere (44) with some modifications.

The 5′ ^{32}P-labeled' oligo 5´-GCCCCGG**C**CCCGCCCCCGCGCG- 3´ (containing an Sp1-binding site present in the MGMT promoter) and 5´-GCTATG**C**GTTATTGAGCACGCG- 3´containing mutated (underlined) Sp-1 site were annealed with appropriate complementary stands to generate duplex oligo. The DNA (50 fmol) was then incubated with 10 ng p53 or nuclear extracts (5 μg) isolated from HCT116 p53$^{-/-}$ cells for 20 min at 25°C in a buffer containing 40 mM HEPES-KOH, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol and 1 μg of poly (dI-dC) (Sigma). After electrophoresis in nondenaturing 5% polyacrylamide gels in Tris-borate buffer at 4°C, the gel was dried and the radioactivity quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and analyzed using IMAGEQUANT software (Piscataway, NJ, USA).

Clonogenic survival assay

Colony-forming assays were performed as previously described (45). Briefly, exponentially growing glioblastoma p53-null LNZ308 and its derivative 2024 cells (46) seeded at a density of 250 to 300 cells/60-mm dish were allowed to attach. After 24 h, the cells were treated with 1 μg/ ml doxicycline for another 24 h and then exposed to increasing concentrations of temozolomide (TMZ) for 30 min, then transferred back to the normal medium. After 2 weeks, the colonies were fixed and stained with a crystal violet/formalin solution and counted for plotting the survival curve.

Results

Effect of overexpression of WTp53 on MGMT promoter activity

An earlier study showed that WT p53 induction down-regulated endogenous MGMT expression in several human tumor cell lines, including two isogenic *p53*-null cell lines (36). Consistent with this, we observed that overexpression of WT p53 down-regulated endogenous MGMT expression in the human pulmonary lung carcinoma Calu6 line (Figure 1A). To further investigate the effect of p53 on the *MGMT* promoter, we used a *p53*-null colon carcinoma cell line, HCT116 p53−/−. We transiently co-transfected HCT116 p53−/− cells with *MGMT* promoter-reporter (containing the 954 bp region upstream of the transcription initiation site) and WT p53 expression plasmids. Ectopic expression of WT p53 significantly reduced *MGMT* promoter activity (70% as compared to vector transfected control; Figure 1B). We also examined the effects of two *p53* point mutants on *MGMT* promoter activity namely, Val143Ala (V143A), a missense mutation which inactivates p53 sequence-specific DNA-binding, and a Leu22Gln Trp23Ser (p53LT) double mutant which retains the ability to bind p53-*cis* sequences but lacks transcriptional activation ability (42). While WT p53 overexpression significantly reduced *MGMT* promoter-dependent luciferase expression, ectopic expression of the *p53* mutants had no significant inhibitory effect (Figure 1B). Western analysis of p53−/− cell lysates, transiently transfected with p53 expression plasmids, revealed that the levels of mutant p53 were higher (2-fold; Figure 1C) than that of WT p53. These results indicate that in spite of the higher level, mutant-p53 could not inhibit the *MGMT* promoter.

Effect of p53 on the minimal MGMT promoter

A 72 bp region upstream of the *MGMT* gene transcription initiation site, containing three Sp1-binding sites, was previously shown to be the minimal sequence required for reporter

expression (26)(Figure 2A). To test whether WT p53 is also able to inhibit the minimal *MGMT* promoter, we transiently co-transfected HCT116 p53−/− cells with minimal *MGMT* promoter-luciferase reporter and WT- or mutant-p53 expression plasmids. The minimal *MGMT* promoter was down-regulated by WT p53 in a dose-dependent manner (up to 80% compared to vector transfected controls). While overexpression of the p53LT mutant did not significantly down-regulate *MGMT* promoter activity compared to WT p53 (although moderate down-regulation was observed at a higher dose), V143A mutant had no inhibitory effect on *MGMT* promoter activity, but rather slight activation of the *MGMT* promoter was observed (Figure 2B). These suggest that WT p53-mediated inhibition of MGMT occurs *via* the Sp1 transcription factor.

P53-mediated inhibition of MGMT is relieved by overexpression of Sp1

To investigate whether p53-induced down-regulation of MGMT could be overcome by ectopic overexpression of Sp1, we co-transfected HCT116 p53−/− cells with WT p53 and Sp1 expression plasmids together with *MGMT* promoter-luciferase plasmid. We observed that Sp1 overexpression relieved WT p53-induced down-regulation of *MGMT* promoter activity in a dose-dependent manner (Figure 3A). To further confirm that p53-induced down-regulation of *MGMT* was achieved by regulation of Sp1-binding to the *MGMT* promoter, we used the minimal *MGMT* promoter plasmid containing only three Sp1-binding sites proximal to the transcription initiation site. Sp1 overexpression abrogated p53-mediated inhibition of the minimal MGMT promoter suggesting possible interference by p53 of endogenous Sp1 binding to the *MGMT* promoter (Figure 3B). Western analysis using Sp1-specific antibody in HCT116 p53−/− cells, showed that overexpression of WT p53 or p53 mutants did not affect endogenous or ectopic Sp1 expression (Figure 3C).

Effect of p53 expression on Sp1 binding to its consensus sequence on the MGMT promoter

The observation that Sp1 overexpression overcame the p53-mediated down-regulation of MGMT raised two possibilities: namely, either p53 competes with Sp1 for binding to one or more of the Sp1 consensus binding sites in the *MGMT* promoter, or p53 sequesters Sp1 thereby reducing its availability for binding to the *MGMT* promoter. To test whether p53 interferes with Sp1 binding to its consensus site in the MGMT promoter, we performed EMSA using a consensus Sp1-binding sequence present in the MGMT promoter. Significant reduction in the binding of the nuclear extract of HCT116 p53−/− cells with ectopic overexpression of WT p53 was observed (Figure 4; compare lane 1 *versus* 8). In contrast, overexpression of the p53LT mutant had no effect on the Sp1 binding (compare lane 1 *versus* 10). Interestingly, overexpression of the V143A mutant also showed reduced Sp1 binding (lane 9). As expected, ectopic overexpression of Sp1 increased the binding of nuclear extract (compare lane 1 *versus* 3), however coexpression of Sp1 with WT p53 significantly reduced the binding (compare lane 3 *versus* 4) while the p53LT mutant had no significant effect (lane 6). It is worth noting that the recombinant p53 polypeptide alone did not bind to the Sp1 consensus oligo (data not shown). However, addition of recombinant His-tagged p53 protein to the nuclear extract inhibited the binding to the Sp1 oligo (compare lane 3 *versus* 7). The specificity of the banding was tested by competition with cold oligonucleotides containing the Sp1 consensus binding sequence (lanes 11–13).

Stable interaction of WT p53, but not mutant with Sp1 in HCT 116 cells

WT p53 and some of its mutants are known to form heterocomplexes with Sp1 (47). Because direct interaction between p53 and Sp1 has been reported for the WT and some mutant forms of p53, we decided to investigate whether the differential effects of WT and mutant p53 on *MGMT* promoter activity were due to variable affinity for interactions with Sp1. To test this, we performed co-immunoprecipitation analysis of p53 in extracts of HCT116 p53−/− cells

transfected with WT or mutant p53 and FLAG-Sp1 expression plasmids. Western analysis of the immunoprecipitate with p53 antibody showed the presence of WT p53 but not the p53LT mutant in the Sp1 immunoprecipitate (Figure 5A). Comparison of the p53 level in the starting nuclear extracts (Figure 5B) indicated that V143A p53 mutant also interacted with Sp1, although not with similar affinity to the WT p53. Together, these results indicate that Sp1 interacts strongly with WTp53 but not the p53LT mutant. To further confirm these results, we performed reciprocal co-immunoprecipitation with Sp1 antibody and showed the presence of FLAG-tagged WT p53 in the Sp1 immunoprecipitate (Figure 5C). Taken together, these results strongly suggest that WT p53 but not its p53LT mutant interacts with Sp1 *in vivo*.

Induction of WT p53 enhances sensitivity of glioblastoma cells to the alkylating agent temozolomide

Because MGMT-mediated repair of O^6-alkylguanine prevents the formation of cytotoxic lesion induced by many alkylating agents, we examined the effect of p53-mediated down-regulation of MGMT level on cell sensitivity to temozolomide (TMZ) which produces O^6-methylguanine adduct. We have used human glioblastoma *p53*-null LNZ308 cell line and its derivatives isogenic 2024 cell line carrying a doxicycline (Dox)-inducible p53 expression system (46). Induction of *p53* in 2024 cells with Dox enhances phosphorylated p53 level which concomitant with p21 expression (Figure 6B). We determined the cologenic survival of these cells after exposure of increasing doses of TMZ. A four-fold reduction of IC_{50} (20 μM) for TMZ was observed after induction of *p53* with Dox in 2024 cells compared to noninduced 2024 (IC_{50}, 80 μM) or parental *p53*-null LNZ308 cells (Figure 6A). These data indicate that changes in the p53 level alter the sensitivity to alkylating agent.

Discussion

MGMT expression ranges from a high level in some tumor cells to an undetectable level in Mex− tumor cells (10,13,14). The level of MGMT expression plays a decisive role in protection of cells from toxicity to alkylating agents (4). Elucidating the molecular basis for this variation of expression is extremely important from both basic and clinical perspectives. In addition to its function in cell cycle regulation, p53 plays an important role in the induction of apoptosis after genotoxic damage. Because p53 plays a central role in sensing DNA damage and promoting DNA repair, the possibility that MGMT may be regulated by p53 had received much attention. Several previous studies showed negative regulation of MGMT expression by WT p53. This study confirms and further expands the initial observation by Harris *et al*. and others that WT p53 down-regulates human MGMT expression (34–36). Consistent with this, Srivenugopal *et al*. (36) conclusively showed that tetracycline regulated overexpression of p53 protein in a *p53*-null *MGMT*-proficient human lung tumor cells (H1299) curtails the transcription of the endogenous *MGMT* gene and alters their sensitivity to alkylating agents (36). Down-regulation of endogenous MGMT expression by WT p53 was further demonstrated in isogeneic p53 models. These included the H460 human lung cancer cell line, in which p53 function had been disrupted by the E6 papillomavirus oncoprotein, and GM47.23 glioblastoma cell line in which the stably integrated WT p53 gene could be activated by dexamethasone. Consistent with this, we have shown that overexpression of WT p53 also down-regulated endogenous MGMT level in lung cancer Calu6 cells. However, the mechanism by which p53 controls MGMT expression is not known. In this report we provide the first evidence that WT p53 sequesters Sp1 and abrogates MGMT expression and thus provides a molecular basis for WT p53-mediated MGMT regulation.

We have shown that ectopic expression of WT p53 but not mutant p53 in p53-null (HCT116 *p53*−/−) colon carcinoma cells suppresses basal *MGMT* promoter-dependent luciferase activity. This indicated that p53-mediated repression of promoter activity occurs at the

transcription level. The V143A *p53* mutant lacking DNA binding activity did not down-regulate the basal *MGMT* promoter which strongly suggests that the DNA-binding of p53 is a prerequisite for its repressor activity. However, the L22G T23S (p53LT) double mutant with normal DNA-binding activity, but lacking trans-acting activity, is also unable to repress the *MGMT* promoter as much as the WT p53 which suggests that both DNA-binding and transactivation abilities of p53 are required for the repression (42). It was shown earlier that some mutant p53 polypeptide do not degrade as fast as WT p53. Consistent with this, Western analysis showing that p53 mutants (48) are more stable than the WT p53 protein eliminated a possible reason that the former's inability to repress the *MGMT* promoter was due to reduced stability.

We showed that p53 represses the minimal *MGMT* promoter that does not contain any consensus p53-binding site. Therefore, p53-mediated repression of promoter activity appears to be executed without direct binding of p53 to the *MGMT* promoter. The observation that WT p53 can repress minimal *MGMT* promoter containing only three Sp1 transcription factor-binding sites raised the possibility that p53-mediated repression could be mediated *via* Sp1. Consistent with this Sp1 overexpression abrogated p53-mediated repression of *MGMT* promoter in a dose-dependent manner. The Sp1 sites are often found in the promoters of housekeeping genes such as *MGMT*, and needed for its basal promoter activity. The *MGMT* gene promoter lacking TATA and CAAT boxes has 10 Sp1-binding sites (24).

The observation that Sp1 overexpression relieved p53-mediated down-regulation of MGMT raises two possibilities: p53 may compete with Sp1 for binding to one or more of the Sp1 consensus binding sites in the *MGMT* promoter or it may sequester Sp1 and thus reduce its availability to bind the *MGMT* promoter. The fact that addition of recombinant p53 to the nuclear extracts reduced the binding while recombinant p53 alone could not bind to that sequence ruled out the possibility that p53 competes with Sp1 for binding to the same sequences present on the *MGMT* promoter. It thus appears that repression may be achieved through the physical interaction of WT p53 with Sp1 that prevents Sp1 from binding to the *MGMT* promoter. This is supported by the observation that WT p53 forms a stable complex with Sp1 *in vivo* and overexpression of Sp1 overcame the inhibitory effect of p53. However, we have also shown that V143A p53 interacted with and inhibited Sp1 binding, although it was unable to down-regulate *MGMT* promoter activity suggesting that p53 may down-regulate the *MGMT* promoter through multiple mechanisms including sequestration of Sp1. P53 has been shown to repress many promoters that lack p53 specific *cis* elements indicating that the mechanisms of p53-mediated repression are varied and complex (31,49–51). Such mechanisms could include interference with transcriptional activators, interference with the basal transcription machinery, compaction of the chromatin structure at the promoter site by recruitment of histone deacetylases and other histone-modifying enzymes (31). We have shown earlier that the basal *MGMT* promoter activity is strongly dependent on p300/CBP, a transcriptional co-activator with histone acetyltransferase activity and treatment with tricostatin A, a histone deacetylase inhibitor, activates MGMT expression through alteration of chromatin structure. Because p53 was shown to interact with p300/CBP and p53-mediated recruitment of mSin3A/HDAC complex was shown to be responsible for repression of many promoters (52), it is likely that p53 sequesters p300/CBP or recruits deacetylase complex to down-regulate the *MGMT* promoter. In any case, our study suggests that sequestration of Sp1 could be one of the mechanisms by which p53 negatively regulates MGMT expression and thus enhances the sensitivity of tumor cells to alkylating drugs.

Although reports on *p53* gene defects and MGMT expression have been inconsistent, the inverse correlation between the expression of MGMT and *p53* gene in breast cancer and other tumors has been documented (37–39). It is interesting to speculate that induction of WT p53 in tumors would cause drastic reduction of the MGMT level, which should increased

cytotoxicity of the alkylating drugs. Consistent with this, one recent study by Roos *et al* showed that TMZ-induced glioma cell death and apoptosis is greatly dependent on p53 (53). It was shown that WT p53 expressing glioma cells were more sensitive to TMZ than their p53 mutant-containing counterpart. It is important to note that radiation and O^6-methylating drugs administered concomitantly are being used in glioma therapy. Indeed one study indicated that the effect was additive (54). Therefore, combination of radiation and alkylating drugs should augment the apoptotic function of WT p53, resulting in significant potentiation of the cytotoxicity of alkylating drugs due to modulation of MGMT level.

Acknowledgments

We thank Robert Tijian, University of California, Berkeley, for providing the Sp1 expression plasmid. This work was supported by R01 ES07572, R01 CA53791, P50 ES66076 (to SM), American Heart Association grant 0565008Y (to KB).

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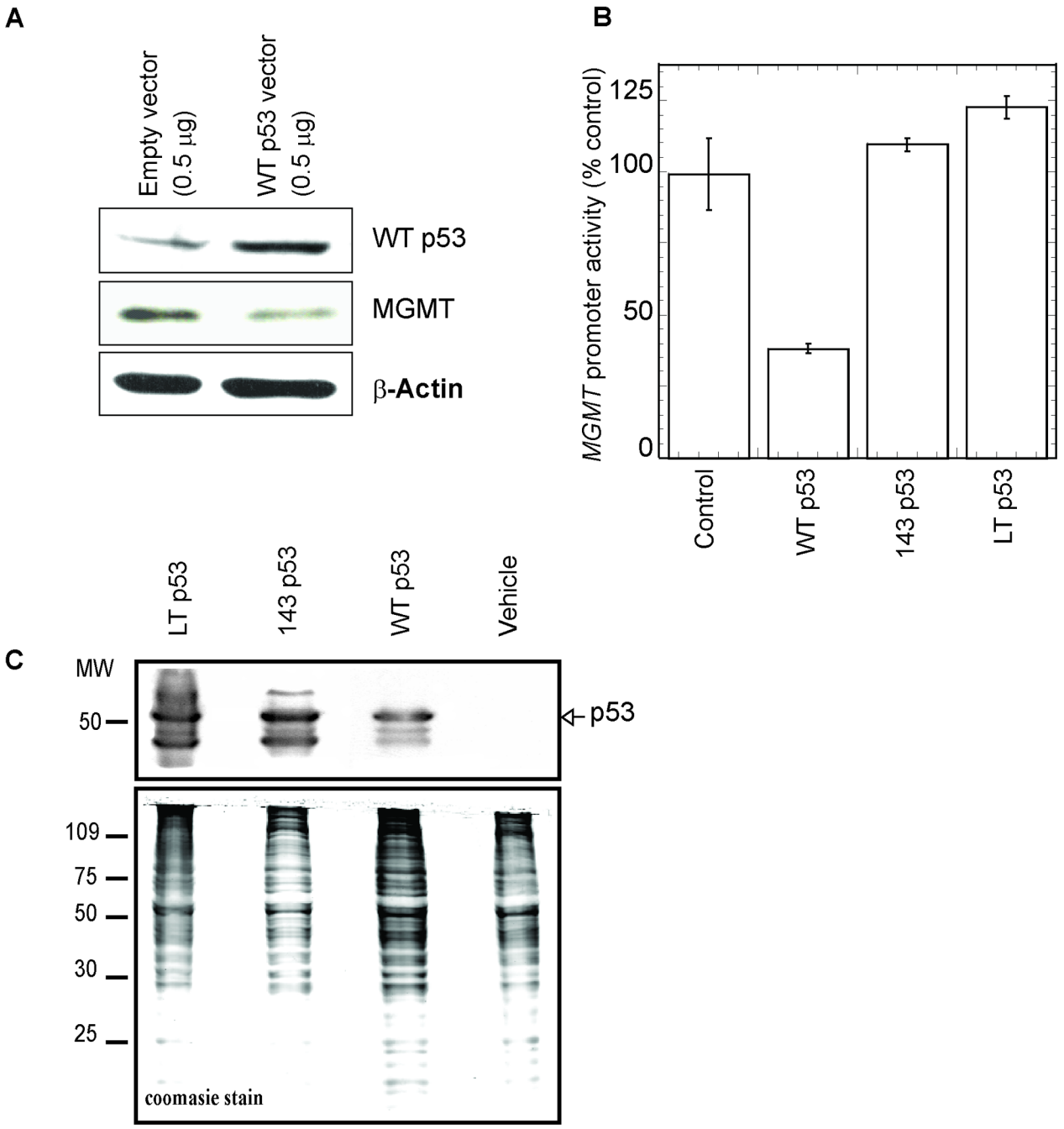

Figure 1.

A, Calu6 cells were transfected with expression plasmids for WT *p53* or empty vector. At 48 h after transfection, the MGMT level in the cell extracts was analyzed by Western blotting with MGMT antibody. **B,** Inhibition of *MGMT* promoter activity by WT but not mutant *p53*. HCT116 $p53^{-/-}$ cells were transiently transfected with (0.5 μg) of *MGMT* promoter-luciferase plasmid (−954/+24) and 0.01 μg of WT *p53* or mutant *p53*, or equivalent amount of empty vector. Luciferase activity was measured at 48 h after transfection and normalized for the amount of protein. The mean ± S.D. of five independent experiments performed in duplicate is shown. **C**, Western analysis of the p53 level in the transfected cell extracts used in (B).

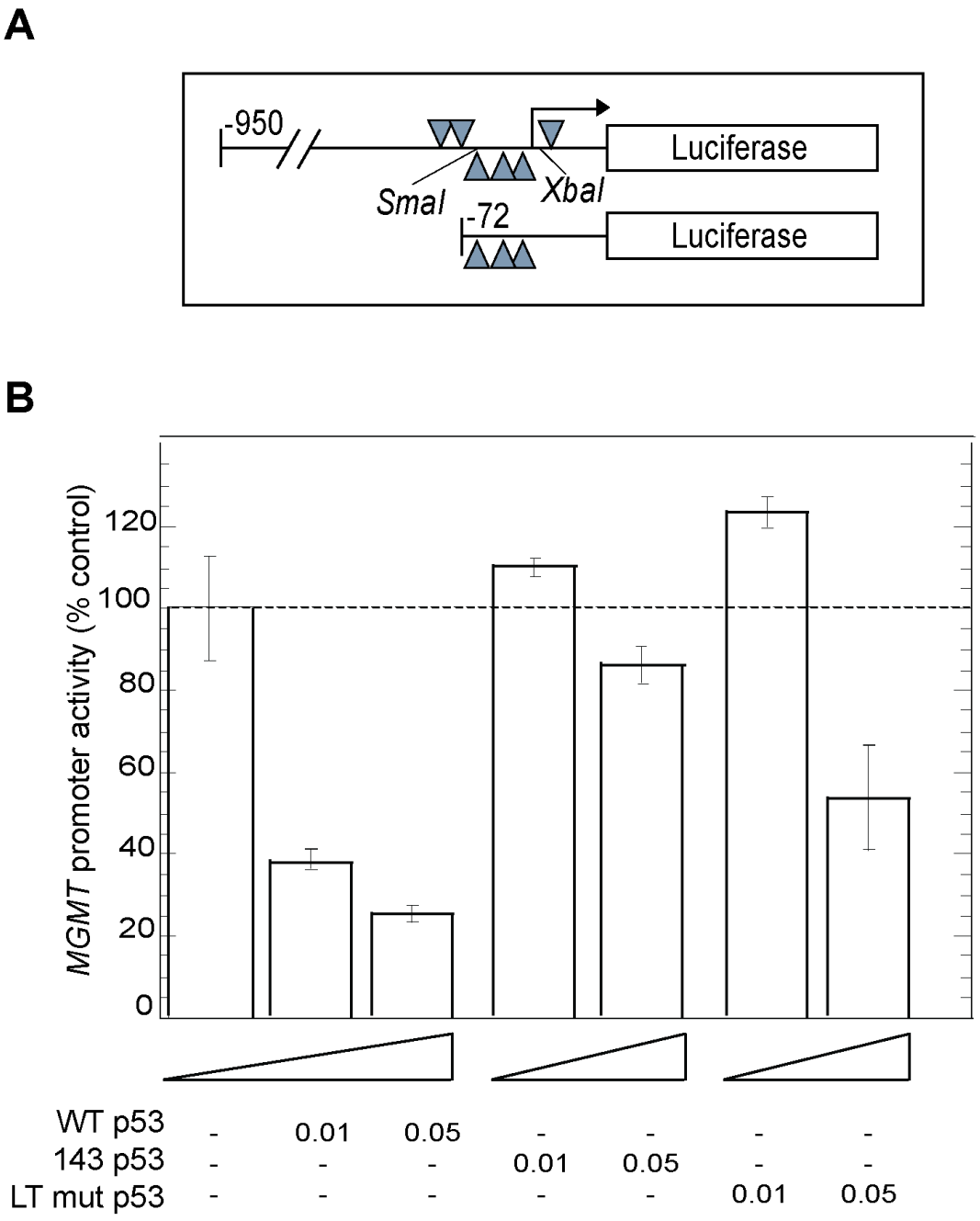

Figure 2. Effect of p53 on the minimal *MGMT* promoter

A, Schematic diagram of the MGMT promoter 5′ regulatory region showing the location of Sp1-binding sites cloned upstream of the luciferase coding region. **B,** HCT116 $p53^{-/-}$ cells were transiently transfected with (0.5 μg) of minimal MGMT promoter-luciferase plasmid (−72/+24) and various amounts of WT *p53* expression plasmid or mutant *p53*, or equivalent amount of empty vector. Luciferase activity was measured at 48 h after transfection and normalized for the amount of protein. The mean ± S.D. of five independent experiments performed in duplicate is shown.

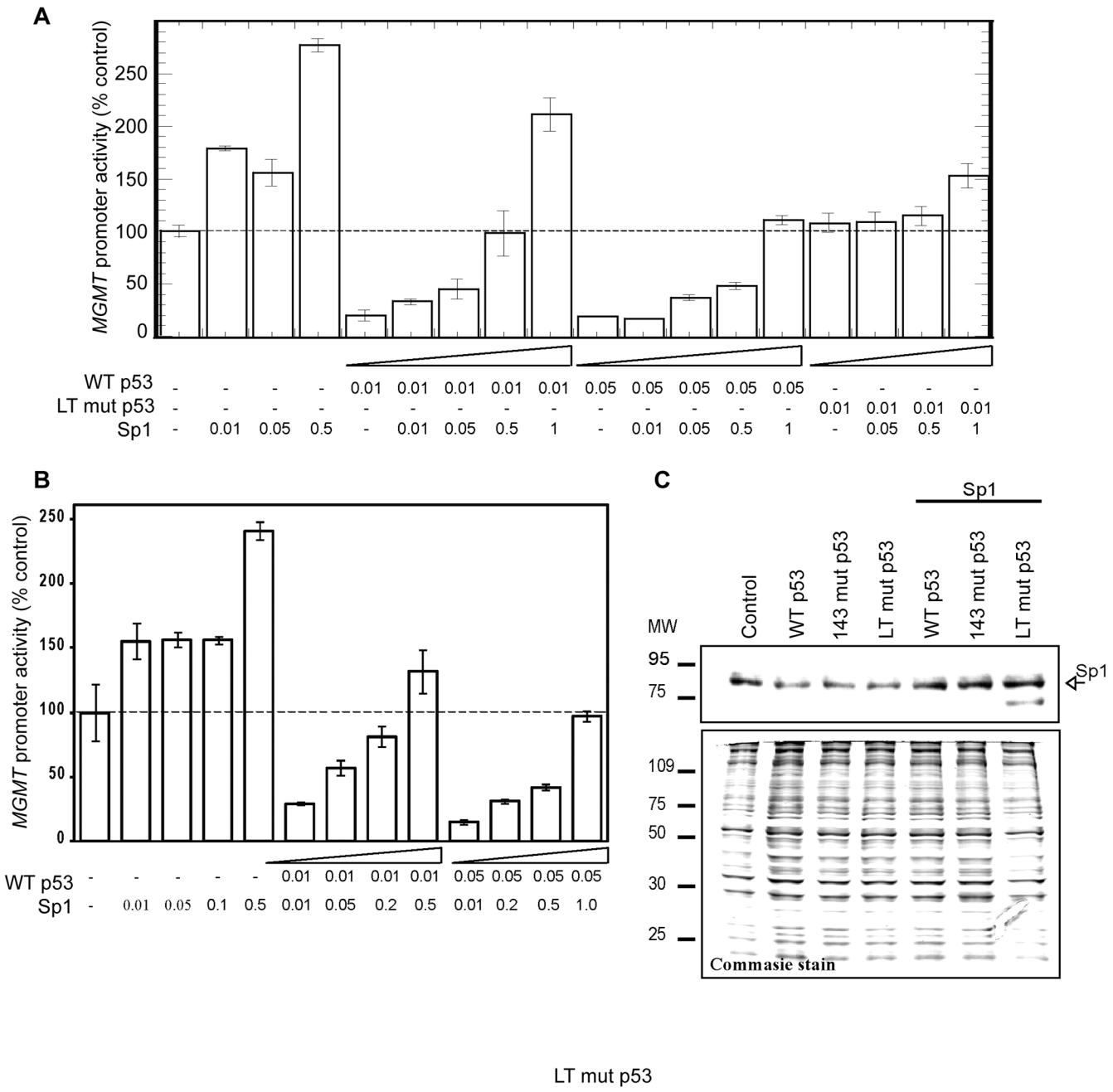

Figure 3. Effect of overexpression of Sp1 on P53-mediated inhibition of MGMT promoter

HCT116 p53$^{-/-}$ cells co-transfected with WT *p53* (0.01 μg or 0.05 μg) and various amounts of Sp1 expression plasmids together with the longer (−954/+24; **A**) or the minimal (−72/+24; **B**) *MGMT* promoter-luciferase plasmid. Luciferase activity was measured at 48 h after transfection and normalized for the amount of protein. The mean ± S.D. of five independent experiments performed in duplicate is shown. C, Western analysis of the Sp1 level in the extracts of HCT116 p53$^{-/-}$ transfected with WT or mutant *p53* expression plasmids.

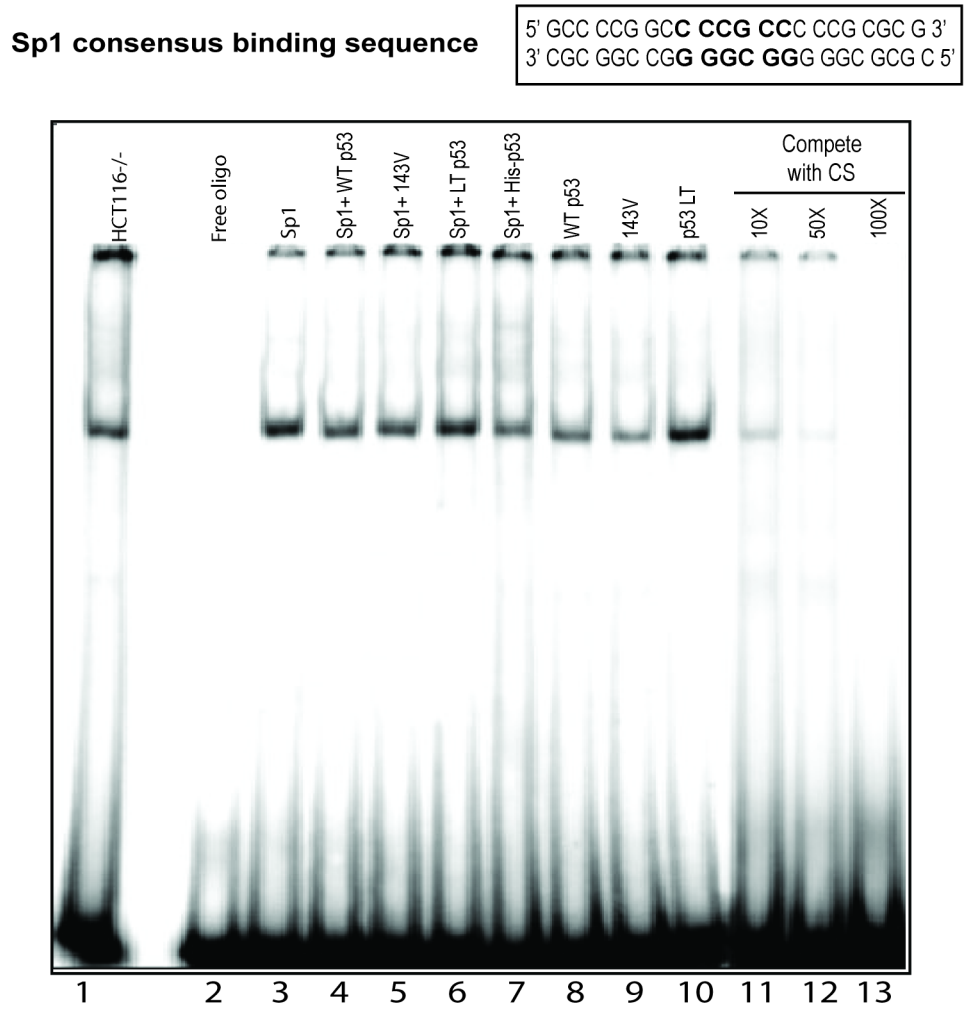

Figure 4. Effect of p53 expression on Sp1 binding

Representative EMSA with nuclear extracts of $p53^{-/-}$ cells transfected with empty vector or WT p53 expression plasmid using ^{32}P-labeled duplex oligo containing a SP1 sequence in the *MGMT* promoter.

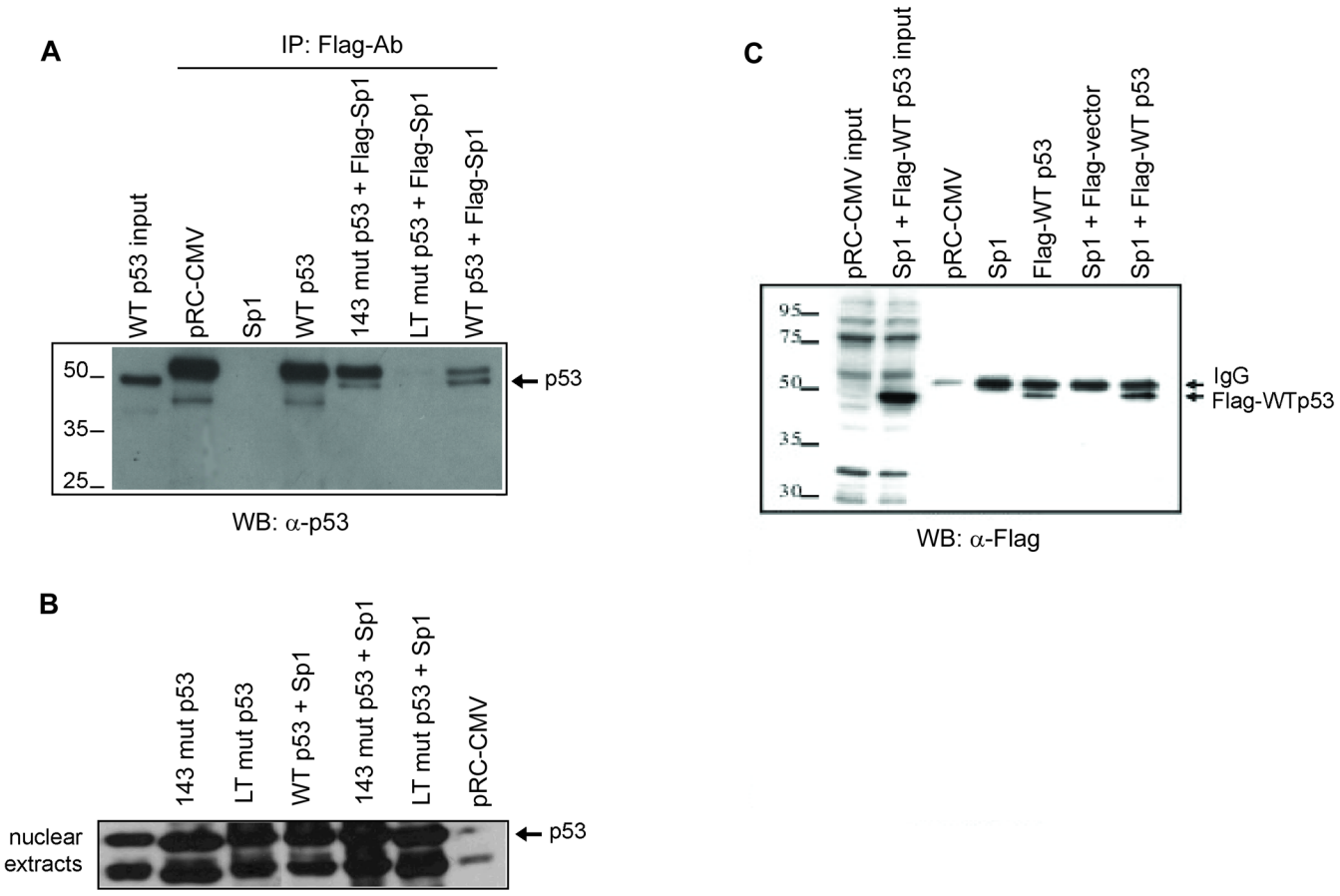

Figure 5. Stable interaction of WT p53, but not mutant p53 with Sp1

A, Extracts of HCT116 $p53^{-/-}$ cells co-transfected with Sp1-FLAG and WT or mutant *p53* expression plasmids or empty vector were immunoprecipitated with FLAG antibody and the immunoprecipitates were analyzed for the presence of p53 by Western analysis with a p53 antibody. B, Western analysis of the p53 levels in the nuclear extracts used for immunoprecipitation. C, Extracts of HCT116 $p53^{-/-}$ cells co-transfected with Sp1 expression plasmid and FLAG-tagged WT *p53* expression plasmids or empty vector were immunoprecipitated with Sp1 antibody and the immunoprecipitates were analyzed for the presence of p53 by Western analysis with a FLAG antibody.

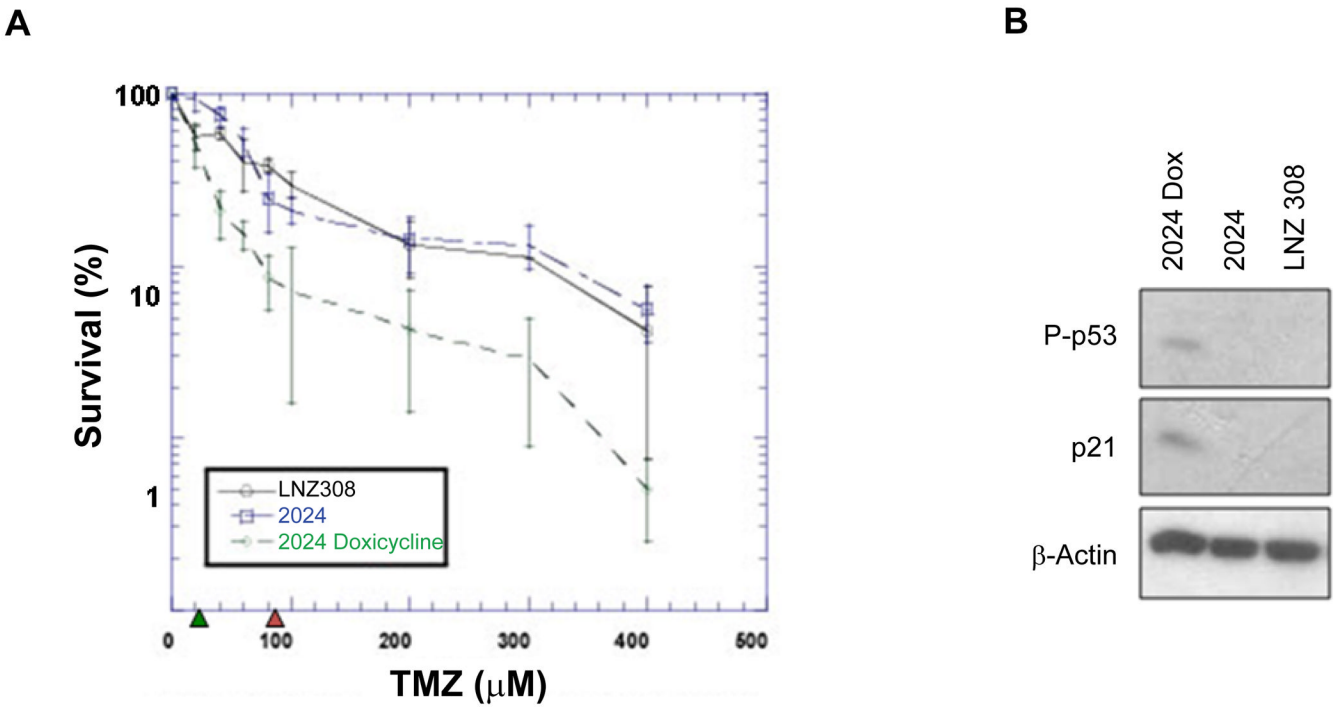

Figure 6. Induction of WT p53 sensitizes human tumor cells to the alkylating agent temozolomide

A, Clonogenic survival assay of the human glioblastoma LNZ308 *p53*-null cells and its derivates 2024 cells in the p53-induced (+DOX) and p53-uninduced (−DOX) states after exposure to different concentrations of temozolomide. The data points show the average ± SD values of three independent experiments performed in triplicates dishes. **B,** Western analysis of the phosphorylated (Ser15) p53, p21 levels in the extracts of cells used in A.