

Analysis of a Mutant p53 Protein Arising in a Medulloblastoma from a Mouse Transgenic for the JC Virus Early Region

MARTYN K. WHITE¹, ANNA SKOWRONSKA¹, JENNIFER GORDON¹, LUIS DEL VALLE¹,
SATISH L. DESHMANE¹, ANTONIO GIORDANO² and KAMEL KHALILI¹

¹Center for Neurovirology, Department of Neuroscience,

Temple University School of Medicine, Philadelphia, PA 19122;

²Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology,
Temple University, Philadelphia, PA 19122, U.S.A.

Abstract. *Background:* JC virus (JCV) is a polyomavirus that causes progressive multifocal leukoencephalopathy (PML) in humans and is highly oncogenic in experimental animals. Transgenic mice with JCV T-antigen develop cerebellar tumors, which resemble human medulloblastomas, containing two distinct cell subpopulations, T-antigen positive and negative. In T-negative clones, a novel mutant p53 was detected (p53^{mt}). *Materials and Methods:* We have compared p53^{mt} to wild-type p53 (p53^{wt}) in p53-null cells. *Results:* p53^{mt} had lost the transcriptional transactivation activity of p53^{wt}, and unlike p53^{wt}, partially localized to the cytoplasm. Unlike mutant p53 from many human cancers, p53^{mt} did not show a gain of function or a dominant negative phenotype. Adenovirus expressing p53^{wt} but not p53^{mt} inhibited cell growth and induced apoptosis of p53-null cells. *Conclusion:* During the course of tumor evolution of the JCV T-antigen mouse medulloblastoma, a mutation occurred that inactivated p53 allowing tumor progression even in the absence of continued T-antigen expression.

JC virus (JCV) is a human neurotropic polyomavirus and is the proven etiologic agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the CNS occurring mainly in immunocompromised patients, especially people with HIV/AIDS (reviewed in 1). Brain tumors have been found in patients with concomitant PML and JCV has also been reported to be associated with some

non-PML brain tumors as determined by analysis for the presence of viral DNA sequences by PCR and expression of viral proteins by immunohistochemistry (2-4). This suggests a possible role for JCV in the genesis of some brain tumors. It is important to note that there is no firm evidence that this virus has a causal role in human neoplasia as some laboratories have reported negative findings (5, 6), which may be due to different methodology (7). However, it is clearly established that JCV is highly oncogenic when injected into the brain of experimental animals including hamsters (8) and monkeys (9, 10). Also direct evidence for the involvement of JCV T-antigen in tumor formation is presented by transgenic mouse studies where mice bearing the JCV early region developed a variety of neural tumors including adrenal neuroblastomas (11), neuroectodermal tumors (12), pituitary neoplasia (13), peripheral nerve sheath tumors (14) and primitive tumors originating from the cerebellum (15). The nature of the tumors formed in these transgenic mice is dependent upon the particular subtype of the JCV promoter used and the strain of mouse (reviewed in 16). In studies with the early genome of the archetypal form of the virus (JCV_{CY}), the transgenic mice developed cerebellar neuroectodermal origin tumors that histologically resembled human medulloblastoma (15). Medulloblastoma is a highly invasive tumor of the cerebellum and is one of the most common types of neoplasia of the central nervous system (CNS) in children (17, 18).

Interestingly, immunohistochemical analysis of the JCV-induced mouse medulloblastoma-like cerebellar neuroectodermal origin tumors showed that expression of T-antigen was not seen in all of the tumor cells, *i.e.*, the tumor comprised a heterogeneous population of T-positive and T-negative cells (19). This observation is reminiscent of JCV-associated human medulloblastoma where expression of T-antigen occurred in some, but not all, of the tumor cells containing JCV DNA (20, 21). Previously, we reported that, in the case of the JCV-

Correspondence to: Martyn K. White, Center for Neurovirology, Department of Neuroscience, Temple University School of Medicine, 1900 North 12th Street, 015-96, Room 203, Philadelphia, PA 19122, U.S.A. Tel: 215-204-0620, Fax: 215-204-0679, e-mail: martyn.white@temple.edu

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transgenic mouse medulloblastoma system, it was possible to isolate clonal cell lines from tumor tissue some of which were positive for T-antigen expression and some of which were negative (19). The T-positive cell lines were morphologically distinct and were characterized by scant cytoplasm and shorter processes. Both T-negative and T-positive cells expressed neuronal nuclear proteins, such as neurofilament and synaptophysin but not the astrocytic marker glial fibrillary acidic protein (GFAP) (19), suggesting that they have the same marker proteins characteristic of the original medulloblastoma tissue (15). The T-positive cell lines contained wild-type p53 (p53^{wt}) that co-immunoprecipitated with T-antigen, whereas p53^{wt} was not detected in the T-negative cell lines. Rather it was found that the T-negative cells expressed a smaller mutant form of p53 (p53^{mt}), the cDNA of which was amplified by RT-PCR from RNA, cloned and sequenced. The p53^{mt} protein is characterized by a specific deletion that removes amino acid sequences between residues 35 and 123. This deletion removes the entire exon 4 creating an in-frame smaller p53 species (37,000) and is due to a single G to C base substitution in the splice acceptor site of intron 3. Importantly, the T-negative cell lines which express p53^{mt} are tumorigenic in nude mice, although tumors are smaller and less frequent than those produced by the T-positive cell lines (19). We hypothesize that the mutation in p53 contributes to the tumorigenic potential of the mouse medulloblastoma cells that lack T-antigen while in T-positive cells wild-type p53 is present but inactivated by association with T-antigen. In the present study, the properties and functional activities of the p53^{mt} and p53^{wt} proteins were compared by expressing them in a p53-null cell line.

Materials and Methods

Cell culture and transfection. The two p53-null cell lines, Saos-2 (human osteosarcoma) and HCT116 p53^{-/-} (human colon carcinoma) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin and 10 µg/ml streptomycin) at 37°C in a humidified atmosphere containing 7% CO₂. Transfections were performed using Fugene 6 according to the manufacturer's instructions (Roche, Indianapolis, IN, USA).

Plasmids. pCMV-p53^{wt} and pCMV-p53^{mt} were made by subcloning cDNAs (19) (Krynska *et al.*, 2000) corresponding to p53^{wt} and the smaller p53^{mt}, respectively, into the EcoR1/BamH1 sites of pcDNA3.1 (-) expression vector (Invitrogen, Carlsbad, CA, USA) where they are under the control of the CMV promoter. The plasmid pcDNA3/zeo/JCVT expresses JCV T-antigen under the control of the CMV promoter as described previously (22). phdm2-CAT, which expresses chloramphenicol acetyltransferase under the

control of the human mdm2 promoter, and p21-LUC which expresses luciferase under the control of the human p21/Waf promoter were kind gifts from Dr. Bassel E. Sawaya (Center for Neurovirology, Department of Neuroscience, Temple University School of Medicine, PA, USA). pRad51-LUC was constructed by cloning the human Rad51 promoter (-722 to +37) into pGL3 (Promega, Madison, WI, USA) where it drives expression of the luciferase gene. pGST-p53^{wt}, pGST-p53^{mt} and pGST-Rad51 were constructed by cloning the respective cDNAs into the pGEX-4T1 prokaryotic expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) allowing them to be produced in bacteria as GST fusion proteins.

Antibodies. p53 – Ab1 (Oncogene Science, clone 421), JCV T-antigen – Pab2 (Oncogene Research Products, clone Pab 416), Grb2 (610111; BD Biosciences, San Jose, CA, USA), p21/Waf (F-5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α -tubulin (T6074; Sigma, St. Louis, MO, USA) and Histone H1 (AE-4; Santa Cruz Biotechnology, Inc.).

Co-immunoprecipitation and Western blots. Co-immunoprecipitation and Western blot assays were performed as previously described (23). T-antigen and/or p53 plasmids were transfected into Saos-2 cells and after 48 hours cells were lysed in lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4) and 0.25% Nonidet P-40 supplemented with a cocktail of proteinase inhibitors (Sigma). Five hundred micrograms of whole-cell extract in a total volume of 0.5 ml was incubated with anti-p53 antibody or control normal mouse serum for 2 hours at 4°C. Immunocomplexes were precipitated by the addition of protein A-Sepharose beads (Pharmacia, Peapack, NJ, USA) for an additional 45 min, washed extensively with lysis buffer, resolved by SDS-10% PAGE and analyzed by Western blotting. Western blotting was performed as previously described (23, 24). For Western blots with total cell protein, 50 µg of protein was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with primary antibody (1/1,000 dilution) and secondary antibody (1/10,000 dilution). Bound antibody was detected with an ECL detection kit (Amersham, Arlington Heights, IL, USA).

Transient transfection assays. Saos-2 cells plated in 6-well plates were transfected with reporter constructs alone (0.5 µg) or in combination with T-antigen, p53^{wt} and p53^{mt} (0.5 µg). The total amount of DNA transfected into the cells was normalized with relevant empty vector DNA. Cells were harvested 48 hours after transfection in reporter lysis buffer (Promega). Chloramphenicol acetyl transferase (CAT) activity of samples was determined by utilizing 30-60 µg of protein for each sample as previously described (23). Luciferase activity of samples was determined by utilizing 5-10 µg of protein for each sample as previously described (25).

ChIP assay. Saos-2 cells were transfected with plasmid expressing p53^{wt} or p53^{mt} alone or in combination and ChIP was performed using the ChIP assay kit (Upstate Cell Signaling Solutions), as previously described (25). Briefly, cross-linking was performed with formaldehyde and the DNA sheared by sonication. The cells were lysed and immunoprecipitation was performed with rabbit polyclonal antibody IMG-583 against p53 (Imgenex, San Diego, CA, USA) or control normal rabbit serum. The following primers

spanning the human p21/Waf promoter upstream p53-binding site (GenBank: 50603) were used for PCR: *ggcactctgtgtccccaggctgag* and *accatcccttctcactgaaac*.

GST pull down assay. GST-fusion proteins were expressed and purified as described previously (26). Briefly, bacteria were grown, induced for 2 hours at 37°C with 0.5 mM isopropyl- β -D-thiogalactopyranoside, and lysed by sonication. The bacterial lysate was incubated with glutathione-sepharose beads (Amersham Pharmacia Biotech) and binding of the GST-fusion proteins was allowed to occur overnight at 4°C. Beads were pelleted and washed. The integrity and purity of the GST-fusion proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining. The GST pull down assays were performed as previously described (24). One hundred μ g of total cell extract was incubated with GST alone or GST fusion protein immobilized on Sepharose beads in 1 ml of lysis buffer, washed extensively in lysis buffer, boiled in Laemmli sample buffer, resolved by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted.

Adenovirus construction. Two adenovirus constructs were made that express p53^{wt} and p53^{mt} from the mouse CMV promoter as follows. Each p53 was excised from pcDNA3.1 with BamHI/NotI and subcloned into the BamHI/SalI sites of the pDC315 adenoviral shuttle plasmid (Microbix Inc., Ontario, Canada). Recombinant plasmids containing each p53 were co-transfected into HEK293IQ cells with pBHGfrt Δ E1E3FLP (Microbix Inc.) which contains adenovirus type 5 (Ad5) backbone deleted for E1 and E3 and allows insertion of the expression cassette in the E1 region of the Ad5 genome by frt-FLP-mediated site specific recombination. Adenoviruses generated in this fashion were cloned by limiting dilution and plaque isolation using agarose overlay. Virus was then grown and purified by cesium chloride density gradient centrifugation. Ad-null was generated in a similar fashion using empty shuttle plasmid with no transgene.

Adenovirus transduction. The medium was removed from cell cultures in 60 mm dishes and purified virus was added at an moi of 10 in 500 μ l of fresh medium. After 1 hour at 37°C, an additional 5 ml of fresh medium was added and the cultures were grown at 37°C until harvesting.

FACS analysis. Cells were harvested by trypsinization followed by addition of complete medium. Cells were pelleted by centrifugation, washed with PBS and fixed in ice-cold 70% ethanol. After incubation for 24 hours at -20°C, cells were washed with PBS containing 1% BSA, stained with propidium iodide 10 μ g/ml in PBS containing 250 μ g/ml RNase A and incubated at 37°C for 30 minutes in the dark before analysis by FACS. Flow cytometry was performed with a Becton Dickinson FACScan flow cytometer. Data were analyzed using the ModFitLT v2.0 (PMAC) software.

Cell synchronization and transduction experiments. HCT116 p53^{-/-} cells were plated in 60-mm dishes and after 24 hours fluid changed to DMEM without serum. After 4 days at 37°C, adenovirus transduction was performed as described above followed 24 hours later by the addition of fresh complete medium (time zero). Cells were harvested at 0, 4, 8, 16 and 24 hours for FACS analysis.

Cell fractionation. Saos-2 cells were transfected as described above and nuclear and cytoplasmic fractions were prepared after

48 hours using the NE-PER nuclear and cytoplasmic reagents according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL, USA).

Immunocytochemistry. Cells were plated in poly-L-lysine-treated glass chamber slides, grown at 37°C, washed with PBS and fixed with cold acetone. After washing with PBS and blocking with 10% horse serum, p53 was visualized by incubation with primary mouse monoclonal anti-p53 antibody (DACO, Glostrup, Denmark) at a dilution of 1:100 followed by fluorescein-conjugated horse anti-mouse antibody (Vector Labs, Burlingame, CA, USA) at a dilution of 1:250. Coverslips were mounted with Vectashield mounting medium containing propidium iodide allowing DNA to be visualized. Slides were examined by fluorescence microscopy.

Results

In an earlier report, T-antigen positive and negative clonal cell lines were prepared from a medulloblastoma tumor arising in a mouse transgenic for the JCV early region which expressed wild-type and mutant forms of p53, respectively (19). RT-PCR from RNA from the BS-1B8 and BS-1a cell lines was used to amplify cDNAs corresponding to p53^{wt} and the smaller p53^{mt}, respectively, and these cDNAs were cloned, sequenced and subcloned into the pcDNA3.1(-) expression vector where they are under control of the CMV promoter. These constructs were transiently transfected into the p53-null Saos-2 human osteosarcoma cell line, either alone or in combination with JCV T-antigen. Extracts were prepared and analyzed by Western blot (Figure 1). As shown in Figure 1A, antibodies to p53 and T-antigen demonstrated that all three proteins were expressed as expected. The positive control in this experiment was BS-1B8 cells which express both JCV T-antigen and p53^{wt} (lane 1) and the negative control was Saos-2 cells that had been transfected with vector plasmid (lane 2). Grb2 was used as a loading control. To investigate the transactivation of p21/Waf1, we analyzed the same cell extracts by Western blot with antibody to p21 (Figure 1B). p53^{wt} but not p53^{mt} induced expression of p21/Waf1 (compare lane 2 to lane 3). T-antigen expression alone did not induce p21/Waf1 expression nor did it inhibit the induction of p21/Waf1 by p53^{wt} (lanes 1 and 4). To further investigate the induction of p21/Waf1 by p53^{wt}, we transiently transfected p53^{wt} alone or in combination with T-antigen or p53^{mt} (Figure 1C). Transactivation of p21/Waf1 expression by p53^{wt} was not affected by co-expression of T-antigen (compare lanes 2 and 4) or by p53^{mt} (compare lanes 2 and 5). It is interesting that T-antigen is unable to inhibit p53^{wt}-induced p21/Waf1 expression. This suggests that there might be an excess pool of p53 present in cells that is unbound to T-antigen and which induces p21 expression. Alternatively, the wild-type p53 in complex with T-antigen may retain its ability to up-regulate p21 expression. A third possibility is that T-antigen activates a p53-independent pathway of p21 induction (27).

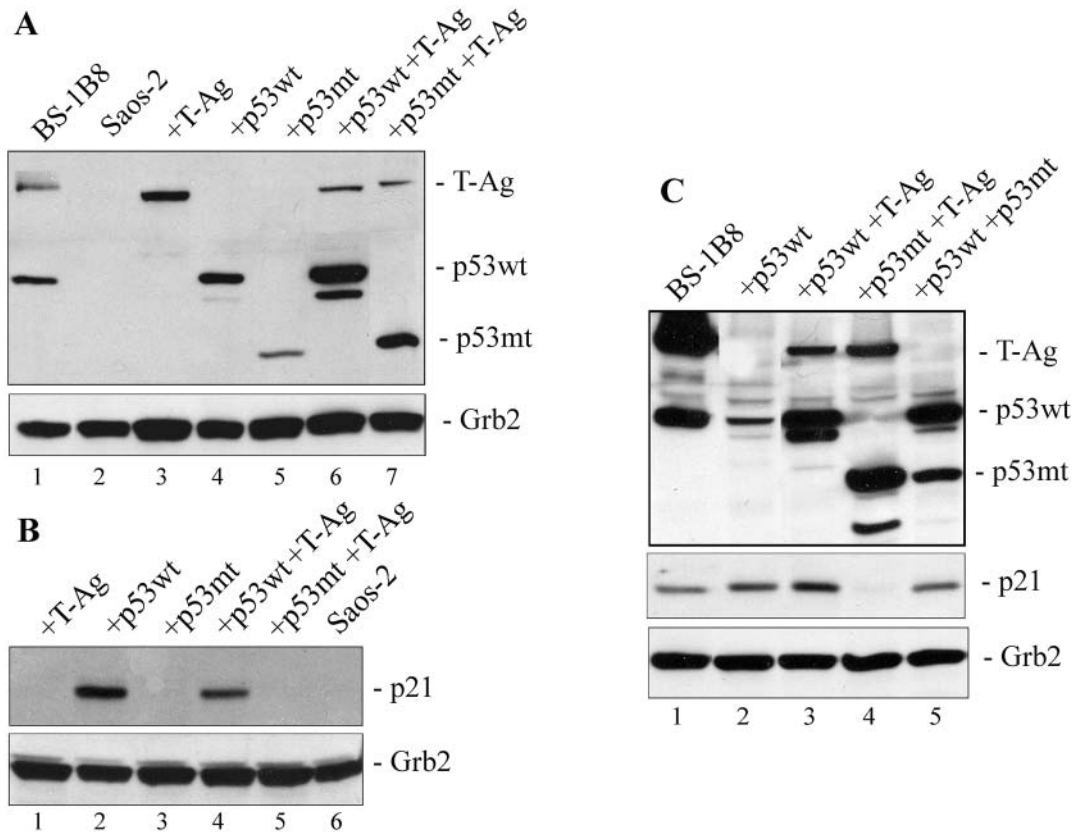


Figure 1. Transfection of p53-null Saos-2 cells with p53^{wt}, p53^{mt} and JCV T-antigen. A. p53 and T-antigen expression. Saos-2 cells (lanes 2-7) were transfected with p53^{wt}, p53^{mt} and JCV T-antigen alone and in various combinations as indicated above each lane and total cell protein analyzed for p53 and T-antigen expression by Western blot. Lane 1: positive control is BS-1B8 mouse medulloblastoma cell line which expresses p53^{wt} and JCV T-antigen. Lane 2: negative control is Saos-2 cells transfected with only plasmid vector. Western blot with antibody to Grb2 was used as a loading control. B. Effect of p53^{wt}, p53^{mt} and JCV T-antigen on p21/Waf1 expression. The Saos-2 cells that were transfected with p53^{wt}, p53^{mt} and JCV T-antigen alone and in various combinations (A) were analyzed for p21/Waf1 expression by Western blot. Lane 6: negative control is Saos-2 transfected with plasmid vector. Western blot with antibody to Grb2 was used as a loading control. C. Effect of p53^{mt} and JCV T-antigen on p53^{wt}-mediated induction of p21/Waf1 expression. In a separate transfection experiment, p53^{wt} was introduced into Saos-2 cells alone or in combination with p53^{mt} or JCV T-antigen. Total cell protein was analyzed for p53, T-antigen and p21/Waf1 expression by Western blot. Western blot with antibody to Grb2 was used as a loading control.

To further investigate transactivation by p53, we employed reporter constructs containing the p53-inducible promoters of the human p21/Waf1 and hdm2 genes (28, 29) coupled to the luciferase and CAT reporter genes, respectively, in co-transfection assays with the two form of p53 and with T-antigen. We also examined the human Rad51 promoter which is repressed by p53 (30). This was coupled to the luciferase reporter gene. The data obtained with these three reporter constructs is shown in Figure 2.

As shown in Figure 2A, expression from the p21 promoter (lane 1) was activated about 10-fold by coexpression of p53^{wt} (lane 3) but not p53^{mt} (lane 4). T-antigen alone gave a slight induction (2-fold, lane 2) and co-expression of T-antigen also increased p53^{wt}-induced expression about 2-fold (lane 5). Expression of p53^{mt} only slightly enhanced p53^{wt}-induced expression (lane 6).

Similarly, the data in Figure 2B indicate that the activity of the hdm2 promoter (lane 1) was induced about 20-fold by p53^{wt} (lane 3). p53^{mt} and T-antigen did not affect basal or p53^{wt}-stimulated hdm2 promoter activity (lanes 4-8). Finally, in the case of the human Rad51 promoter (Figure 2C), the basal promoter had a significant activity in the control cultures (lane 1). p53^{wt} repressed expression about 10-fold (lane 3) but p53^{mt} did not (lane 4). p53^{mt} did not abrogate the p53^{wt} repression of promoter expression (lane 7). Interestingly, there was a p53-independent stimulation of this promoter by T-antigen (~4-fold, lane 2).

To assess binding of p53^{mt} and p53^{wt} to its DNA recognition site *in vivo*, we performed ChIP assays using PCR primers flanking the upstream p53-binding site of the human p21/Waf1 promoter. Saos-2 cells were transfected with p53^{mt} and p53^{wt}, alone or in combination, and

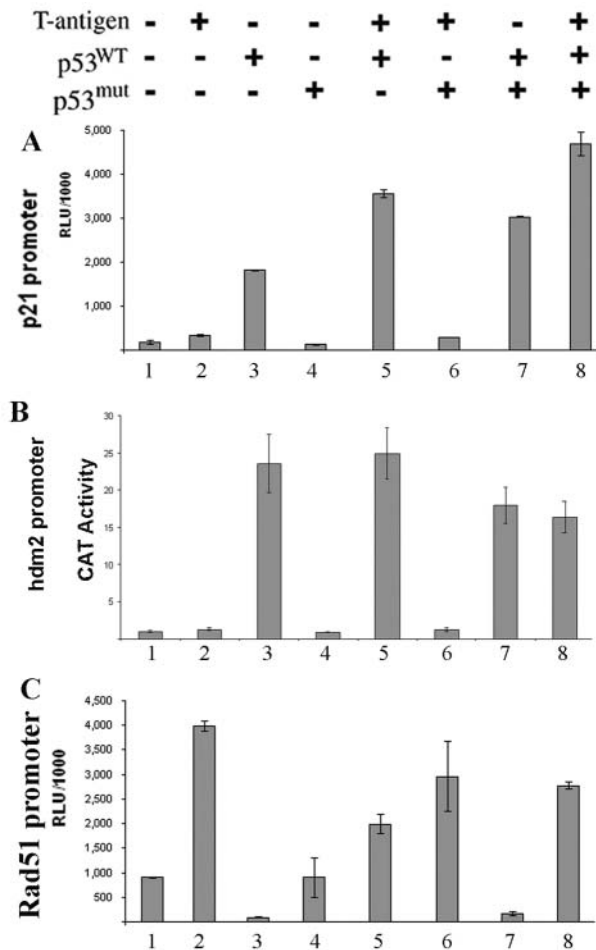


Figure 2. Effect of p53^{WT}, p53^{mut} and JCV T-antigen on p53-responsive promoters. Saos-2 cells were transfected with reporter constructs in the presence and absence of p53^{WT}, p53^{mut} and JCV T-antigen alone and in various combinations as indicated at the top of the Figure. Luciferase activity (A and C) or CAT activity (B) was measured as described in the Materials and Methods. A: p21-LUC expresses luciferase under the control of the human p21/Waf promoter. B: p53^{mut}-CAT expresses CAT under the control of the human mdm2 promoter. C: pRad51-LUC expresses luciferase under the control of the human Rad51 promoter.

harvested after 48 hours. After cross-linking, p53 was immunoprecipitated with antibody to p53 and DNA amplified by PCR. As shown in Figure 3A, cells expressing p53^{WT} (lane 4) gave a band at the same position as the positive control (lane 2) and this is indicated by the arrow; non-specific signal (primer-dimer) is indicated by the asterisk. Cells that were mock-transfected (lane 3) or transfected with p53^{mut} (lane 5) gave no signal nor did IPs using a control antibody (lanes 7-10). Expression of p53^{WT} and p53^{mut} in the transfected cells was confirmed by Western blot (Figure 3B). We conclude that p53^{WT} but not p53^{mut} is

able to bind to its site in the p21/Waf promoter *in vivo*. Thus, the loss of ability of p53^{mut} to transactivate gene expression is likely due to the failure of p53^{mut} to bind to its recognition sites in gene promoters *in vivo*.

Subsequently, whether p53^{mut} was able to bind to JCV T-antigen was investigated by performing a transfection/co-immunoprecipitation experiments in Saos-2 cells. As shown in Figure 4, antibody to p53 immunoprecipitated equivalent amounts of p53 from cells transfected with T-antigen plus p53^{WT} or p53^{mut} (compare lanes 7 and 8, lower panel). However, much more T-antigen was co-immunoprecipitated in the cells cotransfected with p53^{WT} than with p53^{mut} (compare lanes 7 and 8, upper panel). A band corresponding to T-antigen is clearly visible in lane 8 (p53^{mut}) but is much fainter than in lane 7 (p53^{WT}), showing that p53^{mut} still binds T-antigen but more weakly than p53^{WT}.

In order to investigate the interaction of p53^{WT} and p53^{mut} with Rad51, GST fusion proteins were constructed with these three proteins and pull-down experiments were conducted with glutathione-conjugated agarose beads. As shown in Figure 5A, Rad51 was able to bind equally well to both p53^{WT} and p53^{mut}. The integrity of the proteins used in these experiments is shown by the Coomassie Blue-stained gel. GST, GST-p53^{WT} and p53-p53^{mut} are 26, 79 and 86 kDa in size, respectively (Figure 5B).

The functional consequences of ectopic expression of p53^{WT} and p53^{mut} in Saos-2 cells were then examined. Saos-2 cells were transfected alone or in combination with p53^{WT}, p53^{mut} and empty vector and selected in G418. Photographs of these cultures that were taken one week after transfection are shown in Figure 6. Cultures that were transfected with empty vector (Figure 6A) or with p53^{mut} (Figure 6B) produced multiple colonies that grew quickly and reached confluency in about a week. p53^{WT} produced only rare colonies that grew very slowly and contained cells that exhibited a flattened morphology and had extended processes (Figure 6C). Cells that were transfected with both p53^{WT} and p53^{mut} had an intermediate phenotype (Figure 6D). In the following two weeks, rapidly growing G418-resistant cells arose in the p53^{WT}-transfected culture. Western blot analysis of these cells revealed that they no longer expressed p53^{WT} (data not shown). Thus, under extended transfection/selection conditions, it is likely that rare p53-null variants had arisen that yielded cultures that no longer expressed p53^{WT}. Therefore, plasmid transfection is not a feasible method to produce cultures to compare functional consequences of p53^{WT} versus p53^{mut} expression. For this reason adenovirus vectors that expressed p53^{WT} and p53^{mut} were constructed.

In a series of transduction experiments, we examined the effects of adenoviral transduction on the growth properties of Saos-2 cells. Four experimental groups were used: Mock transduced, Ad-null-transduced (adenovirus control with

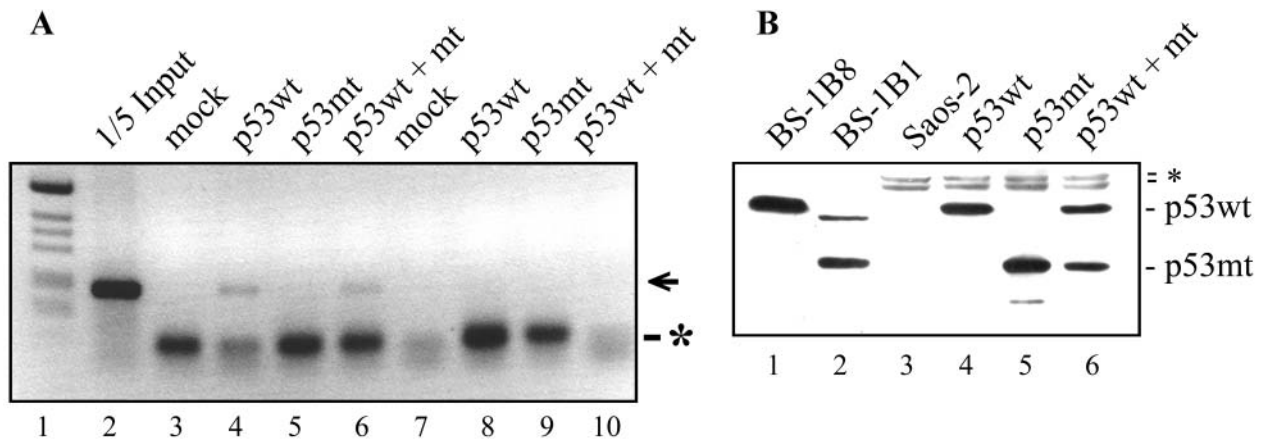


Figure 3. Binding of p53^{wt} and p53^{mt} to the p21/Waf promoter upstream p53-binding site in vivo. A: Saos-2 cells were transfected with p53^{wt} or p53^{mt} alone or in combination and cross-linked. Immunoprecipitation was performed with antibody to p53 (lanes 3-6) or normal rabbit serum (lanes 7-10) and ChIP assays were performed as described in the Materials and Methods. The PCR product is indicated by an arrow and primer-dimer by an asterisk. B: Expression of p53 in the transfected cells was confirmed by Western blot.

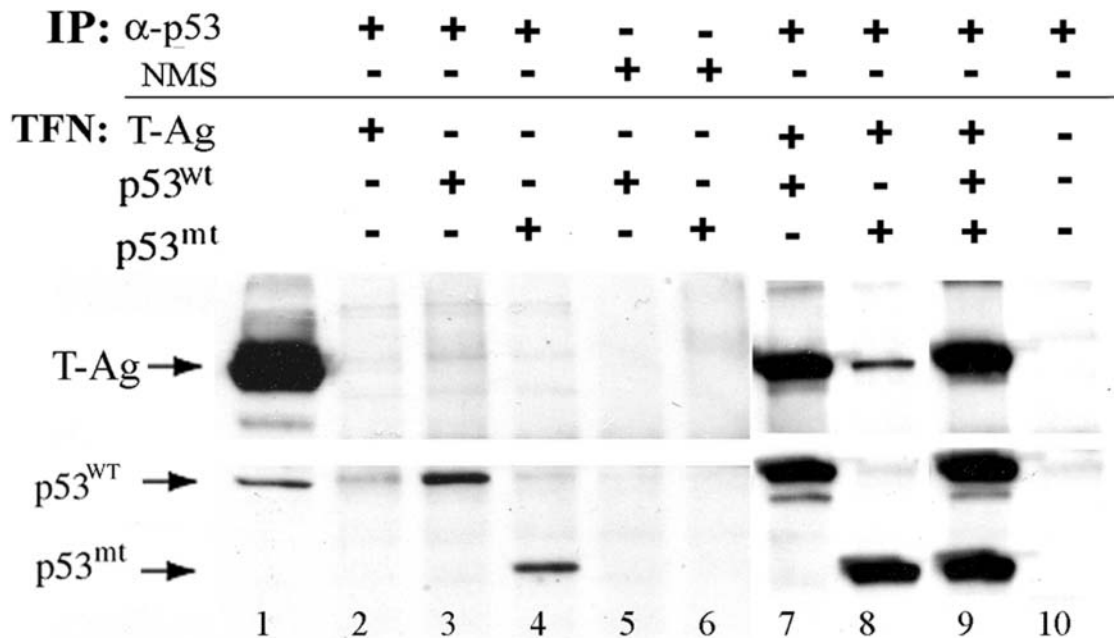


Figure 4. Co-immunoprecipitation of JCV T-antigen with p53^{wt} and p53^{mt}. Saos-2 cells (lanes 2-10) were transfected with p53^{wt}, p53^{mt} and JCV T-antigen alone and in various combinations and immunoprecipitation was performed with either antibody against p53 (α-p53) or non-immune mouse serum (NMS) as indicated above. Immune complexes were analyzed for p53 and T-antigen expression by Western blot. Lane 1 – positive control is total cell protein from BS-1B8 mouse medulloblastoma cell line which expresses p53^{wt} and JCV T-antigen. Lane 10 – negative control is p53 immunoprecipitation from Saos-2 cells transfected with plasmid vector.

no transgene), Ad-p53^{wt} and Ad-p53^{mt}. As shown in Figure 7, transduction of Saos-2 cells with Ad-p53^{wt} and Ad-p53^{mt} showed expression of the p53^{wt} and p53^{mt} proteins detectable by Western blot (Figure 7A). Cultures of Saos-2 cells transduced with p53^{wt}, but not p53^{mt}, showed a reduction in cell growth rate compared to mock as

determined by measuring total cell protein over time (Figure 7B). Ad-null cultures showed a comparable or higher cell growth rate compared to mock. Cell cycle analysis of transduced unsynchronized Saos-2 cells is shown in Figure 7C. The first box shows the cell cycle profile three days after transduction with p53^{wt} and the positions of the

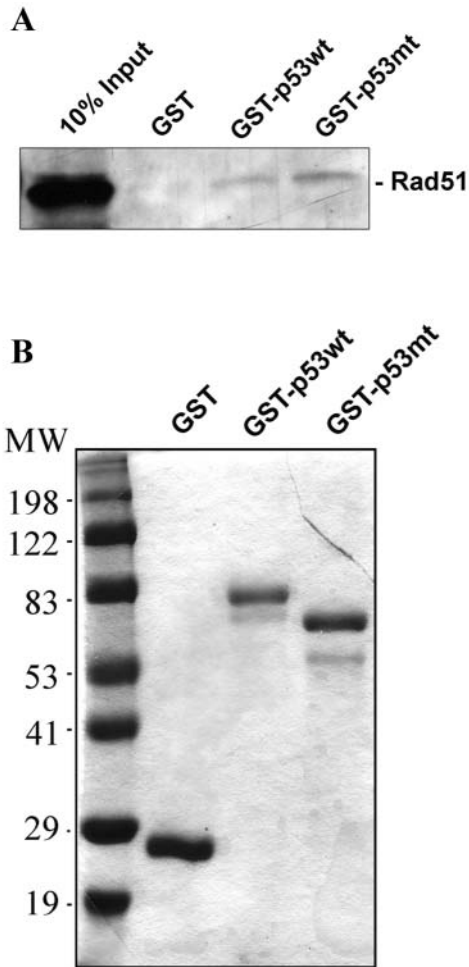


Figure 5. A: Binding of Rad51 to p53^{wt} and p53^{mt}. GST, GST-p53^{wt} and GST-p53^{mt} were incubated with cell lysates of mouse embryo fibroblasts that had been incubated for 24 hours with 1 mg/ml cisplatin to induce Rad51 expression. GST pull down with glutathione beads and washes were performed as described in the Materials and Methods followed by immunoblotting for Rad51. B: Fusion protein integrity. The purity and integrity of the GST, GST-p53^{wt} and GST-p53^{mt} that were used in Panel A were analyzed by SDS PAGE followed by Coomassie Blue staining.

windows used to measure each of the cell cycle phases are indicated. A second example is shown in the next box for the cell cycle profile three days after transduction with p53^{mt}. Subsequent boxes present the cell cycle data from the entire experiment as graphs of the percentage of cells in each phase of the cell cycle plotted against time. There are little differences between groups for the G1/G0 and G2 phases. However, for p53^{wt}, there is a marked suppression of cells in S-phase and increased accumulation of cell in the apoptotic and apoptotic/necrotic status. Thus, p53^{wt} but not p53^{mt} inhibits cell growth, blocks cells from S-phase and promotes cell death presumably by promoting apoptosis.

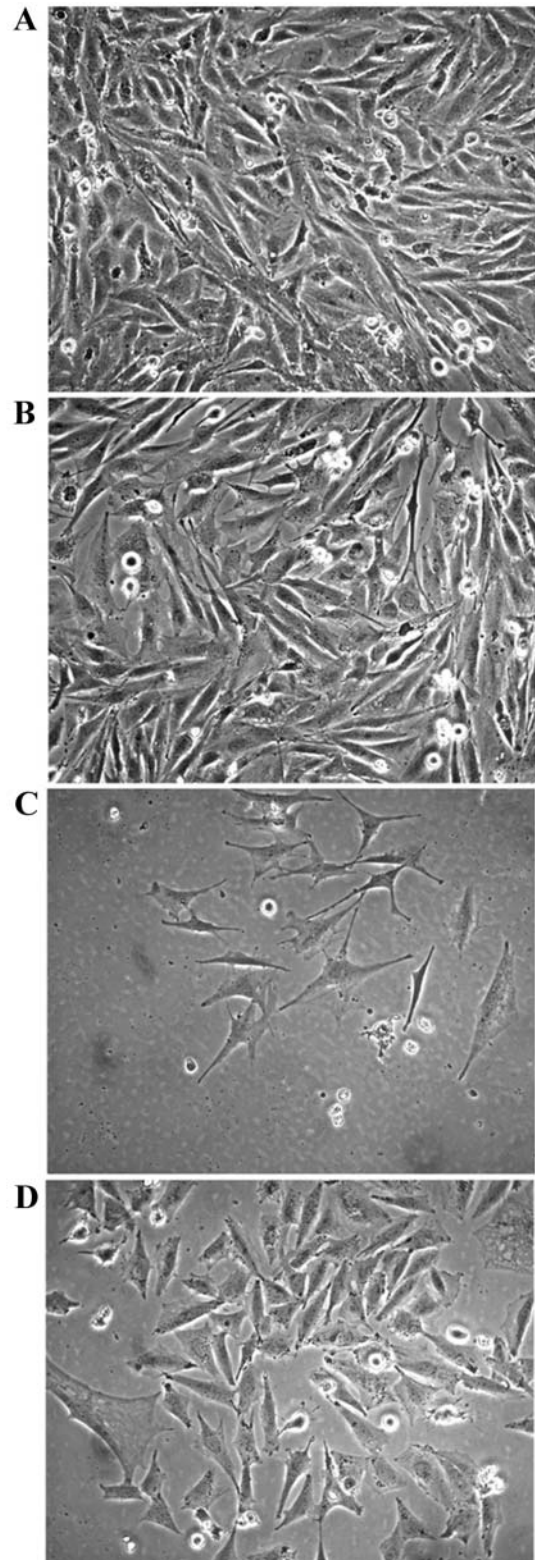


Figure 6. Effect of p53^{wt} and p53^{mt} on cell morphology. Saos-2 cells were transfected with vector plasmid (A), p53^{mt} (B), p53^{wt} (C) or with both p53^{mt} and p53^{wt} (D). Cells were selected in G418 for 7 days and phase contrast photomicrographs taken.

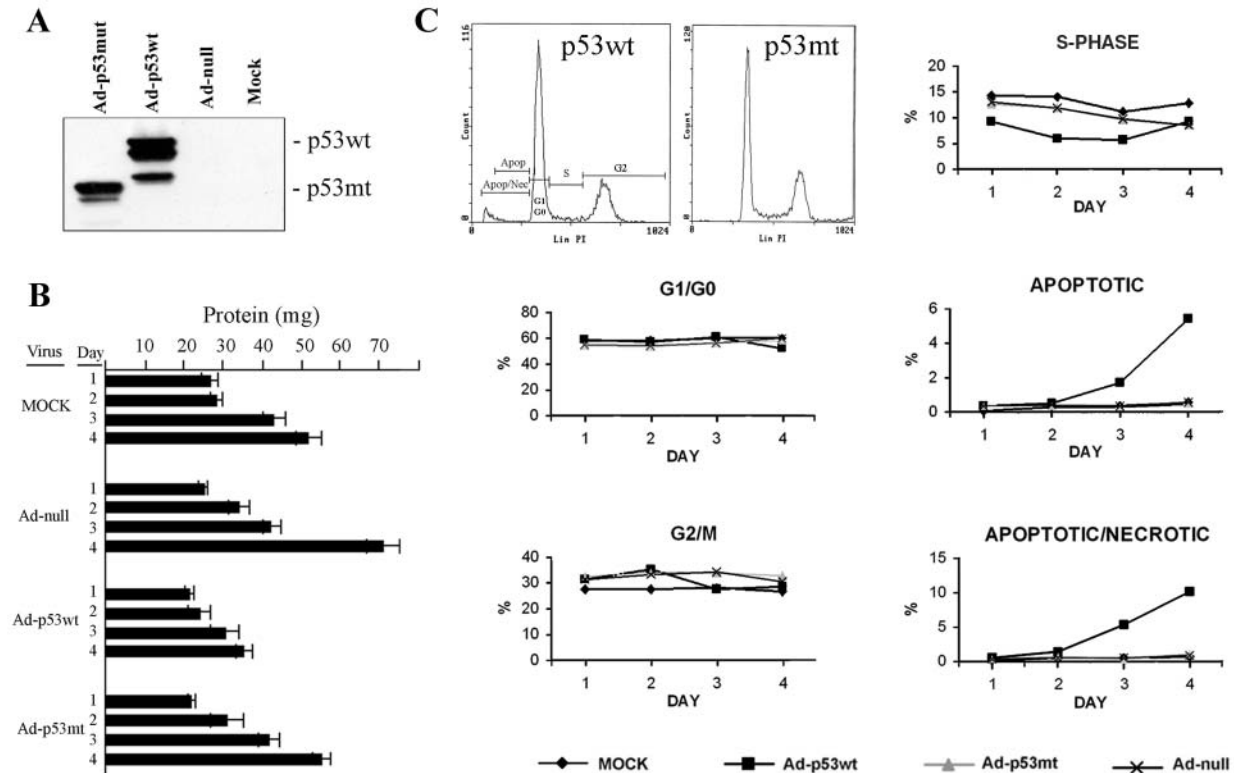


Figure 7. Effect of adenoviral transduction of $p53^{wt}$ and $p53^{mt}$ on cell growth. $p53^{-/-}$ human colon carcinoma cells were transduced with adenoviral vectors with the $p53^{wt}$ and $p53^{mt}$ transgenes or with an empty adenoviral vector (Ad-null) or were mock transduced. Expression of the transgenes was verified by Western blot (A). The effect of transduction on cell growth was determined by measuring the total amount of cell proteins harvested at 1, 2, 3 and 4 days after transduction (B). The effect of transduction was measured by FACS analysis at 1, 2, 3 and 4 days after transduction (C). The top left of this panel also shows examples of the cell cycle distributions ($p53^{wt}$ and $p53^{mt}$ at the 3 day time point) and indicates the windows that were used to determine the phases of the cell cycle.

In order to examine the effect of $p53^{wt}$ and $p53^{mt}$ on the cell cycle, cells of serum were starved to synchronize them in G0/G1, transduced with the adenovirus vectors, stimulated by adding fresh complete medium containing 10% serum and analyzed by FACS. Preliminary experiments showed that after 4 days of serum deprivation only 50-55% of Saos-2 cells were arrested in G0/G1 whereas for another $p53$ -null cell line, HCT116 $p53^{-/-}$, >70% of cells arrested in G0/G1 (data not shown). Therefore, HCT116 $p53^{-/-}$ cells were used for this experiment. A delay in the transit of cells through G1 by $p53^{wt}$ was expected. As shown in Figure 8, $p53^{wt}$ transduction triggered massive apoptosis at the 16 and 24 hour time points. More than 80% of cells were in the apoptotic/necrotic peak after 24 hours compared to around 13% for $p53^{mt}$ and 10% and 6% for the Ad-null and mock transduced cells, respectively.

Cellular localization of $p53^{wt}$ and $p53^{mt}$ using subcellular fractionation was then examined. Nuclear and cytoplasmic fractions were isolated from transduced Saos-2 cells and examined by Western blot for p53. As shown in Figure 9A,

$p53^{wt}$ was localized almost exclusively in the nucleus, whereas $p53^{mt}$ localized to both the nucleus and the cytoplasm. T-antigen was found mainly in the nucleus but with also a significant amount in the cytoplasm. The purity of the fractions was assessed by Western blot for α -tubulin (cytoplasm) and histone H1 (nucleus) as shown in the lower panel. Interestingly, when $p53^{wt}$ and $p53^{mt}$ were cotransfected together, the distribution of $p53^{wt}$ was altered and instead of being nuclear it adopted an approximately equal distribution between the nucleus and cytoplasm (Figure 9B).

These results were confirmed by immunocytochemistry (Figure 10). Cells transfected with $p53^{wt}$ showed an exclusively nuclear staining with antibody to p53, while cells transfected with $p53^{mt}$ or with $p53^{wt}$ and $p53^{mt}$ combined showed both nuclear and cytoplasmic staining.

Discussion

Medulloblastomas are malignant tumors that develop in the cerebellum and usually occur in children, comprising ~20%

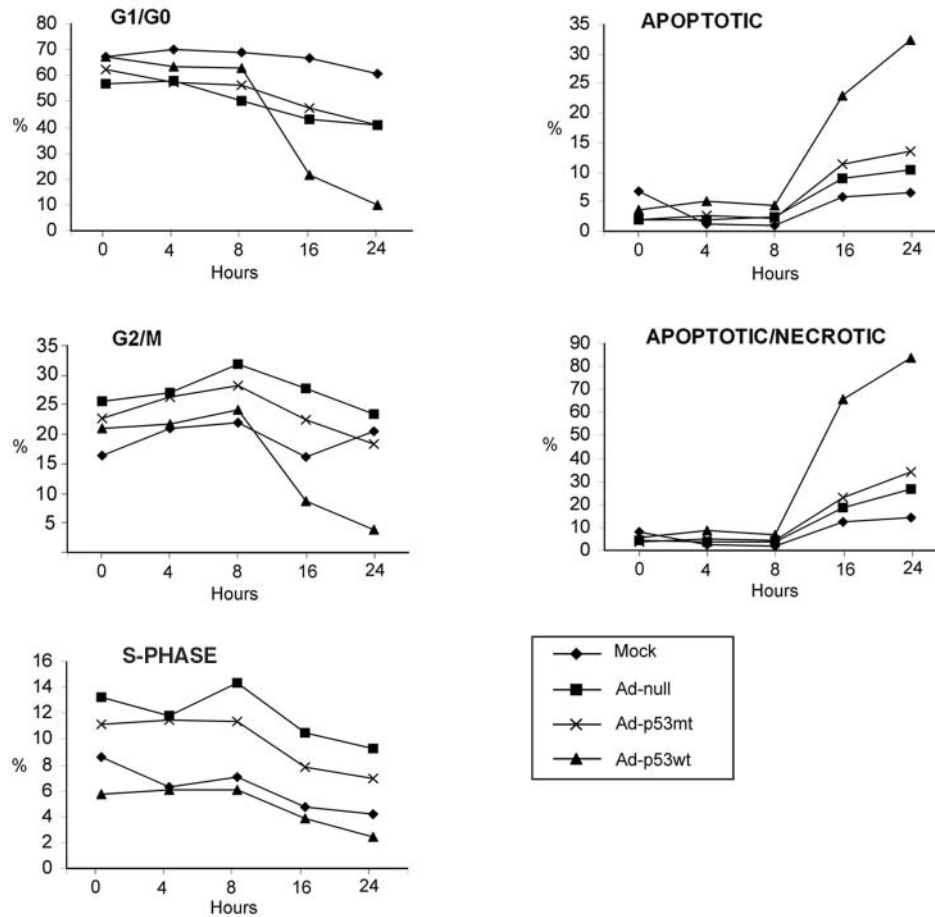


Figure 8. Effect of adenoviral transduction of $p53^{wt}$ and $p53^{mt}$ on cell cycle parameters of serum starved cells. HCT116 $p53^{-/-}$ cells were starved of serum for 4 days, transduced by adenovirus and fresh complete medium was added 24 hours later (time zero). Cells were harvested at 0, 4, 8, 16 and 24 hours for FACS analysis. The windows that were used to determine the phases of the cell cycle are the same as in Figure 7.

of childhood brain tumors (17, 18). Shortly after its discovery as the etiological agent of the demyelinating disease, progressive multifocal leukoencephalopathy (PML) (31), it was found that the human polyomavirus, JC (JCV), was highly neuro-oncogenic in newborn hamsters (8) and it was also able to induce brain tumors in monkeys (9, 10). In light of the neuro-oncogenicity of JCV in animals, a panel of human clinical medulloblastoma samples was examined by PCR for the presence of JCV genomic DNA and by immunohistochemistry for the expression of T-antigen (20, 21). It was found that 11 out of 23 samples were positive for JCV DNA, while 4 out of 23 were positive for T-antigen expression (20).

In JCV positive medulloblastomas, only 5-20% of the cells within the tumor express T-antigen (21). In addition, the load of viral DNA is low and expression of VP1 in the tumor is not detectable indicating that the level of viral replication is either extremely low or that the virus is not

replicating at all within the tumor cells (20). These observations have led to the suggestion of a "hit and run" mechanism for JCV tumorigenesis (16). In this scenario, once dysregulation of one or more cellular pathways has occurred, genetic mutations in tumor suppressor genes may render T-antigen expression unnecessary for the continued pathological development of the tumor. Thus, JCV T-antigen may function at an earlier rather than later step in tumorigenesis and then its expression may be lost in the tumor cells. In support of this hypothesis, it has been reported that JCV T-antigen possesses mutagenic activity and may contribute to chromosomal instability observed in human B lymphocytes and colonic cells (32-35). The closely related SV40 large T-antigen rapidly induced numerical and structural chromosome aberrations in human fibroblasts that were evident before transformation occurred and continued throughout neoplastic progression (36, 37). In addition to chromosomal instability, there is evidence that

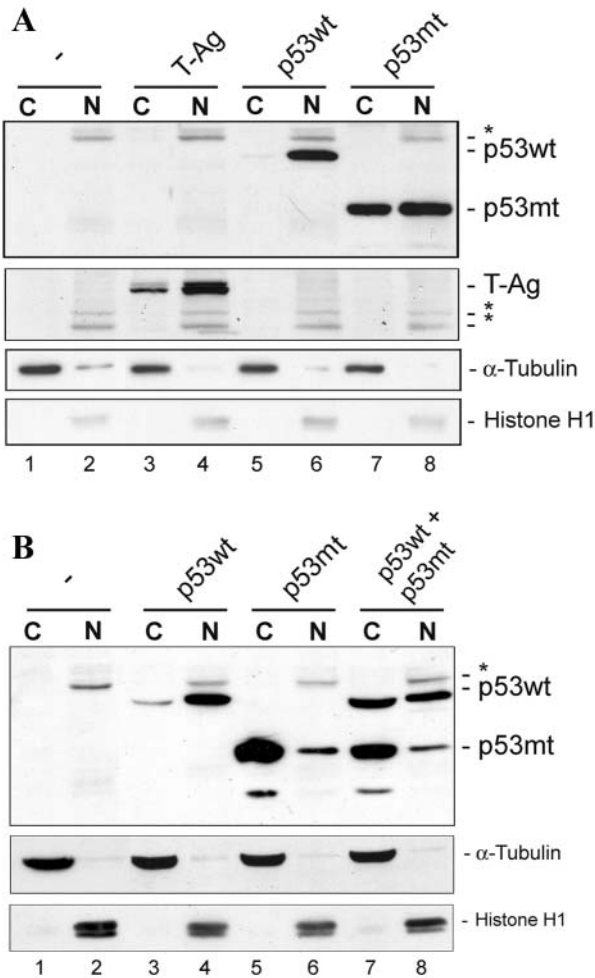


Figure 9. Subcellular fractionation. A. Subcellular distribution of p53^{wt}, p53^{mt} and JCV T-antigen. Saos-2 cells were transfected as indicated and nuclear and cytoplasmic fractions were prepared and analyzed for p53 and T-antigen as indicated. To assess the purity of the fraction, Western blotting was performed for α -tubulin (cytoplasmic marker) and for Histone H1 (nuclear marker). B. Subcellular distribution of co-transfected p53^{wt} and p53^{mt}. Saos-2 cells were transfected with p53^{wt} or p53^{mt} alone or in combination and analyzed as in A. N: nuclear; C: cytoplasmic.

DNA repair can be disrupted by both JCV T-antigen and JCV agnoprotein and this may provide another avenue for the induction of mutations by JCV (24, 38).

Transgenic mice expressing JCV T-antigen provide a model system for studying the processes involved in the induction of medulloblastoma. As described in the introduction, transgenic mice can develop a variety of neural tumors depending upon the strain of JCV promoter and the strain of the mouse (16). FVB/N transgenic mice with the archetypal form of the virus promoter (JCV_{CY}) developed cerebellar neuroectodermal origin tumors that histologically resembled human medulloblastoma (15). Interestingly,

immunohistochemical analysis of these revealed that, like JCV-associated human medulloblastomas, expression of T-antigen was seen in only a fraction of the cells (19). Further, it was possible to separate these populations using dilution cloning. Clonal cell lines were derived from the mouse tumor tissue that were either positive or negative for T-antigen expression (19). The T-positive clonal cell lines contained p53^{wt} that co-immunoprecipitated with T-antigen, whereas the T-negative cells expressed a smaller p53^{mt}. This immediately suggested that we had uncovered a "hit and run" event (as defined above) in this mouse model, *i.e.*, T-antigen transformation provided an early impetus for the induction of cellular transformation and genetic mutations but then was lost after genetic mutation(s) in one or more tumor suppressors (in this case p53) had occurred.

To further investigate this hypothesis, the biological properties of this mutant p53 have been tested in a series of experiments in which it has been compared to wild-type p53. The cDNA encoding for p53^{mt} protein was subcloned into a eukaryotic expression vector allowing it to be efficiently ectopically produced after transfection into target cells. Transfection of p53^{wt} but not p53^{mt} into p53-null Saos-2 cells resulted in the induction of the protein p21/Waf1 which is a major downstream effector of the inhibition of cell proliferation by p53. When both p53^{wt} and p53^{mt} were transfected, p21/Waf1 was still induced. Interestingly, T-antigen did not inhibit p21/Waf1 protein induction by p53^{wt}. These data are consistent with earlier reported data (13) where p53^{+/+} mouse medulloblastoma tumor cells expressing JCV T-antigen showed increased levels of expression of wild-type p53 and p21. One possibility is the presence in cells of excess p53 unbound to T-antigen. Free p53 would then be available to induce p21 expression. Alternatively, it may be the case that the wild-type p53 in complex with T-antigen retains its ability to up-regulate p21 expression. A third possibility is that T-antigen activates a p53-independent pathway of p21 induction (27). In any case, high level expression of p21 in T-antigen positive, wild-type p53 positive mouse medulloblastoma tumors did not appear to be sufficient to slow cellular proliferation (13).

Similar results to the p21/Waf Western blots were obtained with p53 transactivation experiments using reporter constructs containing p53-responsive promoters (Figure 2). p53^{wt} activated the p21/Waf1 promoter and the human mdm2 promoter, while p53^{mt} did not, and p53^{wt} activation was not abrogated by p53^{mt} co-expression. From these data, we conclude that p53^{mt} has lost its ability to transactivate p53-dependent transcription like the mutant p53s that are found in many human cancers. However, p53 mutations in human cancers often exhibit a gain of function or dominant negative phenotype, *i.e.*, expression of the mutant p53 interferes with the transactivation function of wild-type p53. This may be caused by the mutant p53

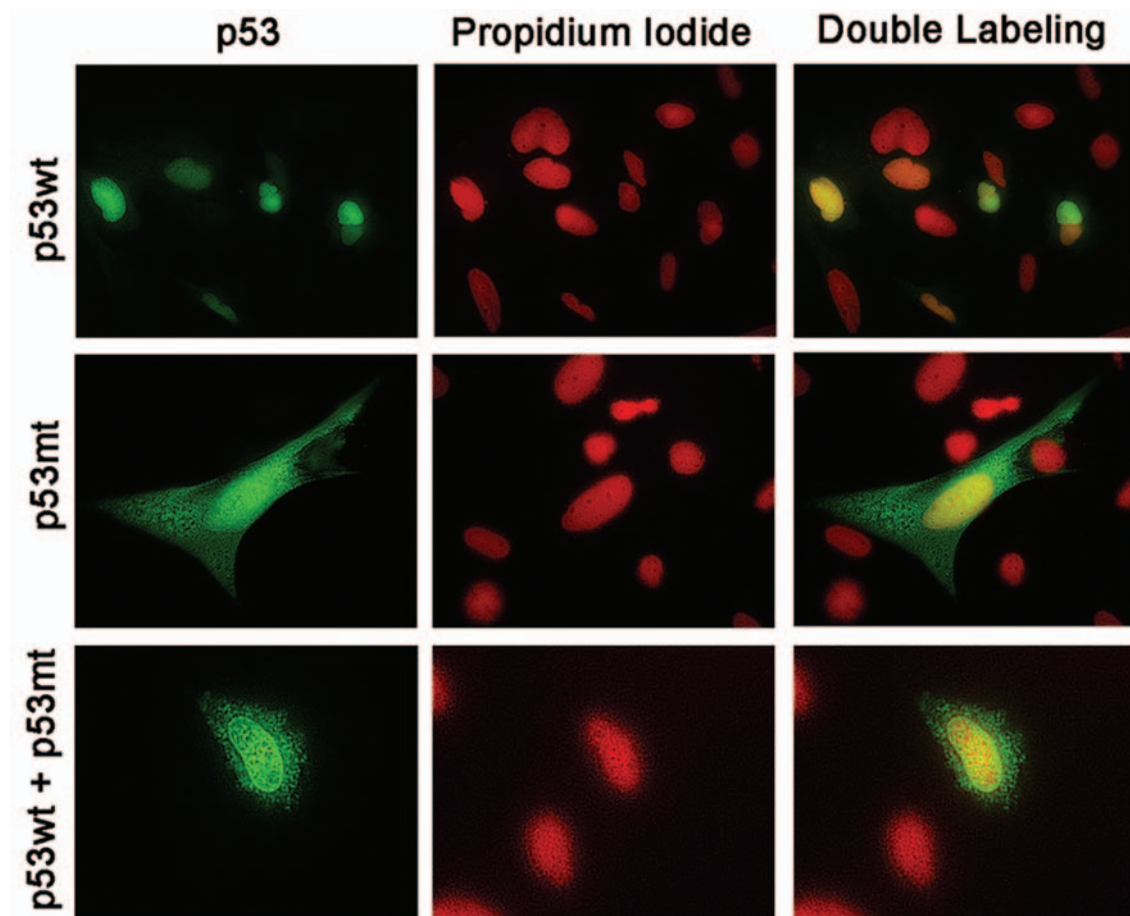


Figure 10. Immunocytochemistry of $p53^{wt}$ and $p53^{mt}$ -transfected Saos-2 cells. Slides of $p53^{wt}$ and $p53^{mt}$ -transfected Saos-2 cells were examined by fluorescence microscopy. p53 was visualized by immunostaining with fluorescein as described in the Materials and Methods (green fluorescence, left hand panels) and propidium iodide allowed nuclear DNA to be visualized (center panels). The right hand panels show the merged panels for each transfection where nuclear p53 appears yellow.

associating with wild-type p53 and forming inactive heteromers, or by the mutant p53 driving the wild type p53 into a mutant conformation or by the mutant p53 binding and sequestering ancillary transcription factors required by wild-type p53 (39, 40). Unlike many human mutant p53s, $p53^{mt}$ did not have any gain of function or dominant negative properties in these transactivation experiments.

As befits the molecule charged with the duty of guarding us against cancer, the regulation of p53 is extremely complex (41, 42). Not only can p53 transactivate certain genes, *e.g.* p21/Waf1, it can also repress transcription of other genes, *e.g.* Rad51 (30). The down-regulation of expression of this set of cellular genes is important in the induction of stress-induced apoptosis (42). In these experiments, we found that Rad51 promoter activity was strongly repressed by $p53^{wt}$ but again $p53^{mt}$ could not exert this activity nor could it reverse $p53^{wt}$ -mediated repression.

p53 is also able to bind to certain cellular and viral proteins and these interactions can modify its function. JCV T-antigen binds strongly to p53 (43). In this study, it has been shown by co-immunoprecipitation/Western blot experiments that the binding of the $p53^{mt}$ to JCV T-antigen is much reduced when compared to that of $p53^{wt}$. Since $p53^{mt}$ already has an impaired function and the mouse medulloblastoma cells from which $p53^{mt}$ was isolated lack T-antigen and $p53^{wt}$, p53-T-antigen interaction is presumably no longer required for tumorigenesis in these cells. p53 can also bind to Rad51 and this is thought to be a mechanism through which p53 exerts an antirecombinogenic activity (44). However, the pull-down experiments described here with GST- $p53^{wt}$ and $p53^{mt}$ illustrate that binding to Rad51 is not impaired in $p53^{mt}$. It should be noted that the Rad51 binding site lies near the C-terminus of p53 (44), while the deletion in $p53^{mt}$ lies near the N-terminus (19).

To investigate the functional consequences of p53^{wt} and p53^{mt} expression, we first transfected cells and then selected with G418. After one week, there were clear differences in cell morphology and cell number. Cultures that had been transfected with p53^{wt} contained small colonies with only a few cells that had a flattened morphology and had extended processes (Figure 6C), while for p53^{mt} and vector control, multiple rapidly growing G418-resistant colonies were formed that reached confluency and had a morphology that resembled the p53-null parental cells (Figure 6 A and B). Further comparisons were not possible, because the p53^{wt} cultures became overgrown at later time-points by aberrant variants that were resistant to G418 but no longer expressed p53. For this reason, adenovirus vectors to deliver p53 at high efficiency were constructed. Saos-2 cells transduced with adenovirus expressing p53^{wt} but not p53^{mt} showed an inhibition of cell growth and an induction of apoptosis (Figure 7). Remarkably, synchronized cultures of the HCT116 p53^{-/-} cells that had been transduced with p53^{wt} showed massive apoptosis (Figure 8) compared to p53^{mt} and controls.

It should be noted that p53^{mt} is not completely without activity in these functional assays. Saos-2 cells transfected with p53^{wt} and p53^{mt} grow faster than and are morphologically distinct from those transfected with p53^{wt} alone (Figure 6, compare Panels C and D). Similarly, while p53^{mt} did not induce massive apoptosis like p53^{wt}, p53^{mt}-transduced cells had a significantly higher proportion of apoptotic compared to the negative controls at the later time points (Figure 8). In this regard, there have been reports that p53 can function independently of its transcriptional transactivation by leaving the nucleus and interacting with cytoplasmic proteins of the Bcl2 superfamily to directly induce the mitochondrial pathway of apoptosis (reviewed in 41, 42). In this regard, we have found that, while p53^{wt} is found almost exclusively in the nucleus, p53^{mt} is found in both in the nucleus and cytoplasm as judged by cell fractionation (Figure 9) and immunocytochemistry (Figure 10). Thus, while it is clear that p53^{mt} has lost its capacity for transcriptional transactivation in the nucleus, it is possible that it has retained its activity with respect to cytoplasmic events.

In conclusion, we have characterized the biological activities of a mutant p53 protein that arose in a medulloblastoma from a mouse that was transgenic for JCV T-antigen. This protein was compared to wild-type mouse p53 in a number of assays. We found that the mutant p53 has lost its transcriptional transactivation capability. However, it does not exhibit a dominant negative or gain of function phenotype that is characteristic of many human p53 mutants. In this regard, it should be noted that the mouse medulloblastoma cell line from which it was derived does not express wild-type p53 and hence a gain of function ability would not be expected to be required to promote tumorigenicity in these cells. Other properties of this mutant p53 are a reduced affinity for T-

antigen, a retained ability to bind Rad51, reduced but detectable effects on cell growth, morphology and apoptosis and a cytoplasmic/nuclear rather than a nuclear subcellular localization. These data are in accord with the "hit and run" hypothesis for the genesis of medulloblastoma by JCV T-antigen. We think that JCV T-antigen is expressed at an early stage in tumorigenesis, causes mutations and then is lost. The resulting T-negative cells retain their tumorigenicity due to mutations induced by T-antigen in pathways that suppress cell growth. Here, we have demonstrated just such a mutation in p53 and that it impairs p53 function, most importantly the capacity for transcriptional transactivation.

In the mouse model of medulloblastoma, JCV T-antigen is supplied by the inserted viral transgene in the mouse genomic DNA. In JCV-associated human medulloblastoma, it is likely that JCV T-antigen arrives there due to the almost ubiquitous occurrence of JCV infections in the human population. Why T-antigen get lost during later stages of tumorigenesis? There are two possibilities. Firstly, T-antigen may be intrinsically toxic to cells due to its ability to continuously induce mutations and chromosomal instability. Secondly, T-antigen is a powerful immunogen and cells expressing it may be killed by a cell-mediated immune response. More experiments are warranted for the investigation of the possible roles and mechanisms of JCV T-antigen action in human cancer.

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