

## Small Molecular Weight Variants of p53 Are Expressed in Human Melanoma Cells and Are Induced by the DNA-Damaging Agent Cisplatin

Kelly A. Avery-Kiejda,<sup>1</sup> Xu Dong Zhang,<sup>1</sup> Luke J. Adams,<sup>1</sup> Rodney J. Scott,<sup>2</sup> Borivoj Vojtesek,<sup>3</sup> David P. Lane,<sup>4</sup> and Peter Hersey<sup>1</sup>

**Abstract** **Purpose:** Metastatic melanoma is largely unresponsive to DNA-damaging chemotherapy agents, although Wtp53 is frequently detected. Several isoforms of p53 have been discovered, some of which inhibit p53 function. We therefore examined whether p53 isoforms were present in melanoma and whether they may contribute to aberrant p53 function in melanoma. **Experimental Design:** We studied the expression and subcellular localization of p53 and its isoforms in a panel of human melanoma cell lines using Western blot, two-dimensional electrophoresis, and reverse transcription-PCR. We also characterized the relationship between the expression of p53, p53 isoforms, and p53 target genes following treatment with the DNA-damaging agent cisplatin. **Results:** We report that p53 $\beta$  and  $\Delta$ 40p53 were expressed in the majority of melanoma cell lines at the mRNA level, but were absent or expressed at low levels in fibroblasts and melanocytes, suggesting that their expression may play a role in melanoma development. Analysis by two-dimensional gel electrophoresis revealed that p53 $\beta$  was expressed at the protein level in melanoma cells. Both p53 and the small molecular weight forms of p53 were aberrantly expressed between the nuclear and cytosolic fractions of melanoma cell lines, compared with normal fibroblasts. Treatment with cisplatin had differential effects on Wtp53 and the small molecular weight form of p53 that were cell line dependent.  $\Delta$ 40p53 was shown to inhibit, whereas p53 $\beta$  was shown to enhance, p53-dependent transcription of p21 and PUMA. **Conclusions:** p53 $\beta$  and  $\Delta$ 40p53 are expressed in melanoma and this may have important implications for understanding resistance of melanoma to DNA-damaging chemotherapy.

The tumor suppressor p53 plays a vital role in the response to DNA damage and has been classed as a "guardian of the genome" due to its ability to coordinate multiple and diverse signaling pathways involved in this response (1–3). Mutation

of p53 is a common occurrence in many cancers and is associated with tumor progression, resistance to chemotherapy, and poor prognosis (4). Unlike other cancers, wild-type p53 is expressed in metastatic melanoma and its mutational inactivation is relatively rare (5–12). Nevertheless, as judged from the malignant nature of melanoma and its unresponsiveness to DNA-damaging chemotherapeutic agents (13), wild-type p53 in melanoma fails to function as a tumor suppressor.

Many mechanisms have been proposed to account for the functional inactivation of p53 in melanoma (13). Wild-type p53 may be aberrantly phosphorylated by the Chk2 kinase following ionizing radiation, and this has led to the suggestion that signaling pathways upstream of p53 may be deregulated in melanoma cells (14). In this regard, disruption of the p14<sup>ARF</sup> pathway is a common event in melanoma (15). However, endogenous wild-type p53 protein in melanoma accumulates after genotoxic stress and retains its transcriptional activity, at least in part, as shown by up-regulation of p21<sup>waf1</sup>, suggesting that signaling pathways upstream of p53 in the DNA damage response pathway remain intact (14, 16). Nevertheless, ionizing radiation was reported not to promote cell cycle arrest or apoptosis in melanoma cells expressing the wild-type protein (ectopically or endogenously), suggesting that signaling pathways downstream of p53 may be defective (14). In support of this, it has been reported that one of the key mediators of p53-dependent apoptosis, Apaf-1, was frequently lost or

**Authors' Affiliations:** <sup>1</sup>Oncology and Immunology Unit, Calvary Mater Newcastle Hospital and <sup>2</sup>Discipline of Medical Genetics, School of Biomedical Sciences, University of Newcastle, Newcastle, New South Wales, Australia; <sup>3</sup>Department of Clinical and Experimental Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic; and <sup>4</sup>Institute of Molecular and Cell Biology, Singapore, Singapore Received 6/8/07; revised 9/27/07; accepted 1/14/08.

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**Requests for reprints:** Peter Hersey, Oncology and Immunology Unit, Calvary Mater Newcastle Hospital, University of Newcastle, Room 443, David Maddison Clinical Sciences Building, Corner King and Watt Streets, Newcastle, NSW 2300, Australia. Phone: 61-2-49-236828; Fax: 61-2-49236184; E-mail: Peter.Hersey@newcastle.edu.au.

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reduced in melanoma (17). However, subsequent studies from our laboratory and others have shown that loss or reduction in Apaf-1 was a rare event and that the intrinsic apoptotic machinery in melanoma is operational (18, 19). It was also reported that ectopic expression of wild-type p53 in melanoma cells that harbor endogenous wild-type p53 did not induce apoptosis, whereas apoptosis was induced when wild-type p53 was ectopically expressed in melanoma cells that had an endogenous mutation in p53 (16). This suggests that inactivation of p53 in melanoma may be due to inhibition of the function of wild-type p53 itself.

Recently, several p53 isoforms have been identified. Moreover, their expression in human cancers suggests that they may play a role in tumor development or progression. p53 isoforms were described in breast cancer, acute myeloid leukemia, neuroblastoma, and in squamous cell carcinoma of the head and neck (20–25). Their expression in breast cancer has been correlated with poor prognosis, and in leukemia the expression of a small molecular weight (MW) form of p53 has been inversely correlated with the induction of responses to chemotherapy (21). The biological function of the p53 isoforms remains to be fully elucidated; however,  $\Delta 40p53$  (p47) and  $\Delta 133p53$  were shown to inhibit p53-mediated transcriptional activity and apoptosis when cotransfected with wild-type p53 (20, 26). The p53 $\beta$  isoform was also reported to enhance p53-dependent transcription from the Bax promoter but not the p21 promoter (20, 25).

In the present study, we examined the expression of small MW forms of p53 in melanoma and their possible effect on the function of wild-type p53. We report that small MW forms of p53 were commonly expressed in melanoma. In particular, the p53 $\beta$  and  $\Delta 40p53$  splice variants were found to be expressed in the majority of melanoma cell lines and primary isolates, whereas  $\Delta 133p53\beta$  was found to be expressed in only a small number of melanoma cell lines. Wild-type p53 and its protein isoforms were differentially localized between the nucleus and the cytosol of melanoma cells. The presence of p53 isoforms in melanoma cells seems to have functional significance in that they were responsive to the DNA-damaging agent Cisplatin (*cis*-diamminedichloroplatinum), and this was accompanied by differential regulation of p53-dependent proapoptotic target genes.

## Materials and Methods

**Cell lines.** The derivation of the Mel-FH, Mel-MC, Mel-CV, Mel-RM, MM200, Me1007, Me4405, and Igr3 melanoma cell lines has been described previously (27–29). Sk-Mel-110 and Sk-Mel-28 melanoma cell lines were a kind gift from Dr. A. Albino (American Health Foundation, Valhalla, NY) and Dr. S. Ralph (Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia), respectively. MM186, Mel-KN, Mel-JD, MM283, Mel 4.1, Mel-FC, MM415, MM486, and MV3 cells were also used. FLOW2000 human fibroblasts were a kind gift from Dr. Rick Thorne (School of Biomedical Sciences, University of Newcastle, Newcastle, Australia). Melanocytes were purchased from Cascade Biologics. The primary melanoma cell lines described in Fig. 4 were isolated from fresh surgical biopsies from patients attending the Sydney and Newcastle Melanoma Units and were established in our laboratory. These cell lines had been passaged two to four times at the time of harvesting for RNA or protein. All human cell lines were cultured in DMEM containing 5% FCS

(Commonwealth Serum Laboratories) with the exception of primary melanoma cell lines and FLOW2000 human fibroblasts, which were cultured in DMEM containing 10% FCS (Commonwealth Serum Laboratories). Melanocytes were cultured in Medium 154 (Cascade Biologics). All cell lines were maintained in exponential growth at 37°C and 5% CO<sub>2</sub>.

**cDNA expression and reporter constructs.** The pRCMV-p53 expression construct containing full-length wild-type p53 was a kind gift from Dr. Helen Rizos (Westmead Institute for Cancer Research, University of Sydney, Sydney, Australia). The expression construct pCIN4.p47, containing full-length p47 (or  $\Delta 40p53$ ), was kindly supplied by Dr. Greg Matlashewski (Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada) and has been previously described (26). Expression constructs for the  $\beta$ ,  $\gamma$ , and  $\Delta 133$  isoforms of p53, pSV40-p53 $\beta$ , pSV40-p53 $\gamma$ , pSV40- $\Delta 133p53$ , pSV40- $\Delta 133p53\beta$ , pSV40- $\Delta 133p53\gamma$ , and the parental vector pSV40 were generously provided by Dr. Jean-Christophe Bourdon (Department of Surgery and Department of Molecular Oncology, University of Dundee, Dundee, Scotland, United Kingdom) and have been described previously (20). The p21 and PUMA luciferase constructs were a kind gift from Prof. Antony Braithwaite (Department of Pathology, University of Otago, Dunedin, New Zealand) and Prof. Bert Vogelstein (The Howard Hughes Medical Institute Research Laboratory and The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MA), respectively. The pRL-SV40 vector, which provides constitutive expression of *Renilla* luciferase, was purchased from Promega.

**Antibodies.** The mouse monoclonal antibody, Bp53-12, used for the detection of p53 and the mouse monoclonal antibody used for the detection of p21 were purchased from Upstate. The mouse monoclonal antibodies DO1, 1801, 421, and Bp53-10, used for the detection of p53, and the rabbit polyclonal antibody TLQ-40, used for the detection of p53 $\beta$ , were a kind gift from Dr. Borivoj Vojtesek (Department of Clinical and Experimental Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic) and Dr. Jean-Christophe Bourdon (Department of Surgery and Department of Molecular Oncology, University of Dundee, Dundee, Scotland, United Kingdom). The rabbit polyclonal sera TLQ-40 (recognizing p53 $\beta$  isoforms) was developed by Moravian Biotechnology by immunizing New Zealand white rabbit with the peptide TLQDQTSFQKEN coupled to carrier keyhole limpet hemocyanin 1 (Merck). The TLQ-40 antibody has not previously been characterized and was used at a concentration of 1:1,000 on immunoblots. The rabbit monoclonal antibodies used for the detection of PUMA and Bax were purchased from Cell Signaling Technology and Upstate, respectively. The mouse monoclonal antibody for the housekeeping gene *GAPDH* was purchased from Ambion.

**Western blot analysis.** Protein extraction, separation by SDS-PAGE, and Western blot analysis of cell lines was done as described previously (28, 30). Lysates of H1299 cells transfected with p53 $\beta$ , p53 $\gamma$ ,  $\Delta 133p53$ , or  $\Delta 133p53\beta$  were provided by Prof. David Lane (Institute of Molecular and Cellular Biology, Singapore, Singapore; ref. 20). The isolation of cytosolic and nuclear protein lysates from the same cell pellet was carried out using the Qproteome Nuclear Protein Kit (Qiagen) according to the manufacturers' instructions.

**p53 mutation analysis.** All 11 exons (including the intron/exon boundaries) of the *TP53* gene were analyzed by dideoxy sequencing of the respective PCR products. The analysis was done using a BIGDYE dideoxy sequencing ready reaction kit and analyzed on an ABI 3730 DNA analyzer (Perkin-Elmer).

**Two-dimensional gel electrophoresis.** Two-dimensional gel electrophoresis was done using the ZOOM IPGRRunner System according to the manufacturer's instructions (Invitrogen). Briefly, protein was extracted from cell pellets and the sample was prepared for two-dimensional gel electrophoresis using the ZOOM 2D Protein Solubilizer Kit (Invitrogen) according to the manufacturer's instructions. Isoelectric focusing of the sample was done on 7-cm (pH 3–10) ZOOM Strips (Invitrogen) at 200 V for 30 min, 450 V for 20 min, 750 V for 20 min, and 200 V for 1 hour. Following isoelectric focusing, the

samples were equilibrated in NuPAGE LDS Sample Buffer containing 100 mmol/L DTT for 15 min and then alkylated in NuPAGE LDS Sample Buffer containing 125 mmol/L iodoacetamide for a further 15 min. The second-dimension electrophoresis was done by aligning the ZOOM strip in the well of a NuPAGE Novex 4% to 12% Bis-Tris ZOOM Gel (Invitrogen) and electrophoresing the sample at 150 V for 90 min. Following electrophoresis, immunoblotting and Western analysis was done as described previously (28, 30).

**Reverse transcription-PCR.** Total RNA was extracted from human melanoma cell lines using the SV Total RNA Isolation System Kit (Promega). One microgram of total RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen) at 50°C according to the manufacturer's instructions. Control reactions were done with no template RNA and RNA minus Superscript III reverse transcriptase (Invitrogen) to check for contaminants in the reaction mix.

PCR reactions were done with cDNA generated from 100 ng of total RNA in a 25  $\mu$ L reaction volume, in duplicate for each sample. A control reaction was done with no template cDNA to check for contaminants in the reaction mix. PCR was done using primers specific for the p53 splice variants as detailed in the figure legends. Expression vectors containing full-length cDNA for each of the p53 splice variants were used as a positive control in each of the PCR reactions. The following primer combinations were used to specifically amplify p53 and its isoforms: wild-type p53, NE2 Fwd/E11 Rev; p53 $\beta$ , NE2 Fwd/ $\beta$ 1 Rev; p53 $\gamma$ , NE2 Fwd/ $\gamma$  Rev;  $\Delta$ 40p53,  $\Delta$ 40p53 Fwd/N-term Rev;  $\Delta$ 133p53, 4b2 Fwd/E9-10 Rev;  $\Delta$ 133p53 $\beta$ , 4b2 Fwd/ $\beta$ 2 Rev;  $\Delta$ 133p53 $\gamma$ , 4b Fwd/ $\gamma$  Rev. The primers used were as follows: NE2 Fwd, 5'-TGGAACTACTTCTCTGAAAACAAC-3'; E11 Rev, 5'-AGTCAGGCCCTTCTGTCTTG-3';  $\beta$ 1 Rev, 5'-AAGCTGGTCTGGTCTGAAG-3';  $\gamma$  Rev, 5'-CCATCGTAAGTCAAGTAGCATCTG-3';  $\Delta$ 40p53 Fwd, 5'-GATCCATTGGAAGGCCAGGCC-3'; NH<sub>2</sub>-terminal Rev, 5'-AAACCGTAGCTGCCCTGGTAGG-3'; 4b2 Fwd (20), 5'-TGGGTTGCAGGAGGCTTACAC-3'; E9-10 Rev, 5'-CGCC-CACGGATCTGAAG-3';  $\beta$ 2 Rev (20), 5'-TTGAAAGCTGGTCTGGTCTCTGA-3'; 4b Fwd (20), 5'-TAGACGCCAACTCTCTCTAG-3';  $\beta$ -actin Fwd, 5'-AGCCTTCTCTCTGGCATG-3' and  $\beta$ -actin Rev, 5'-CCGATCCACACGGAGTACTTG C-3'.

**Cisplatin and Adriamycin treatment.** The effect of the DNA-damaging agents Cisplatin and Adriamycin was examined in melanoma cells. Cells were treated with 10  $\mu$ g/mL Cisplatin (Pharmacia Upjohn) or 1  $\mu$ mol/L Adriamycin (Pharmacia Upjohn) for the indicated times, before being harvested for apoptosis, Western blot analysis, or luciferase assays.

**Apoptosis analysis.** Cells were seeded in 6- or 24-well culture plates at a density of  $4.8 \times 10^5$  or  $1 \times 10^5$  per well, respectively, and allowed to grow for 24 h before treatment with 10  $\mu$ g/mL Cisplatin (Pharmacia Upjohn). Quantitation of apoptotic cells by measurement of sub-G<sub>1</sub> DNA content using the propidium iodide method was carried out as described elsewhere (28, 31). Analysis was carried out using a Becton Dickinson FACScan flow cytometer.

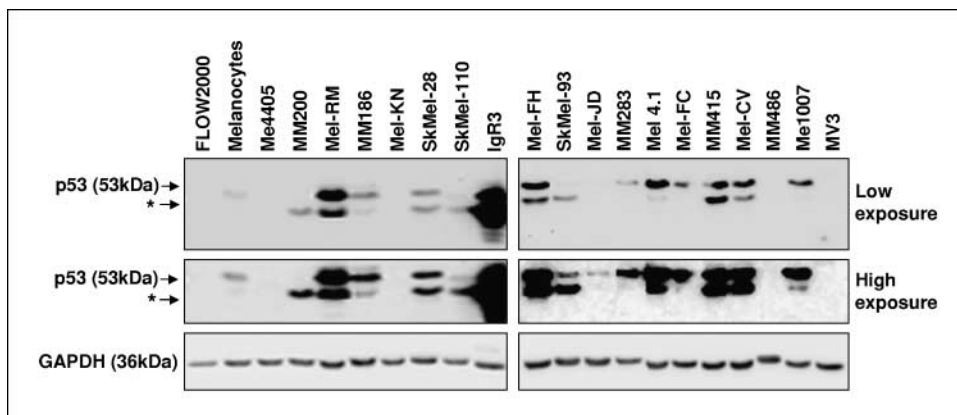
**Transfection and luciferase reporter assay.** The human melanoma cell line Mel-RM was seeded at a density of  $6.25 \times 10^4$  per well in a 24-well plate, 24 h before transfection. Cells were transfected in duplicate with 85 ng/well of the indicated expression vector, 105 ng/well of either the p21 or PUMA luciferase reporter construct, and 10 ng/well of the *Renilla* luciferase reporter pRL-SV40, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours following transfection, cells were treated with 10  $\mu$ g/mL Cisplatin (Pharmacia Upjohn) or 1  $\mu$ mol/L Adriamycin (Pharmacia Upjohn) for 6 h, then harvested for Western blot analysis and luciferase assays. Luciferase assays were done using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Results were analyzed using a FLUOstar OPTIMA microplate reader (BMG Labtech).

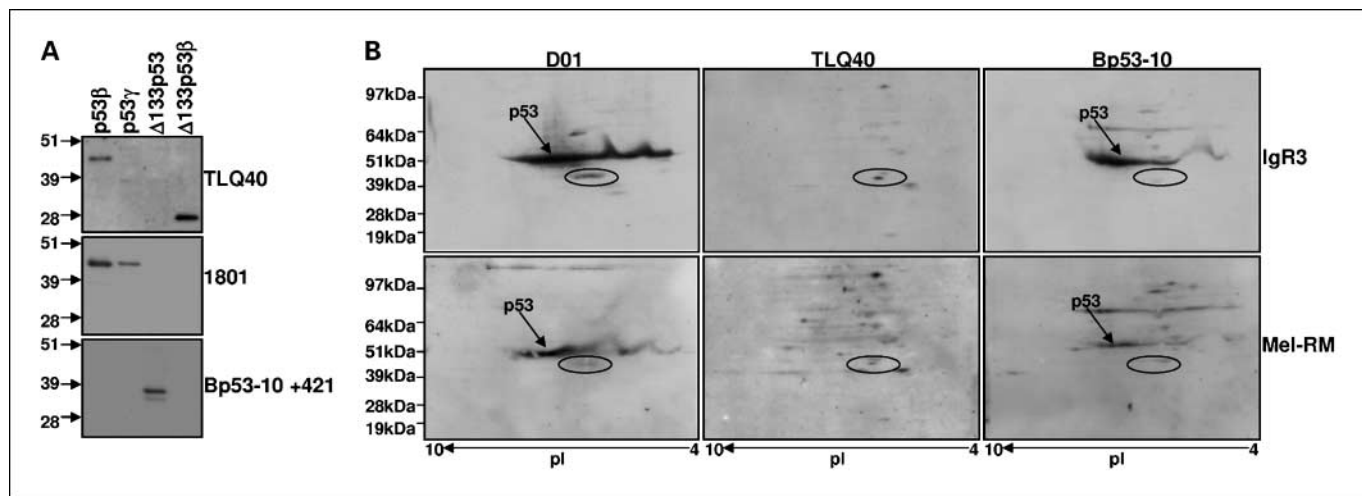
## Results

**Small MW forms of p53 are commonly expressed in human melanoma cells.** The expression of p53 in a panel of melanoma cell lines was examined by Western blot using an antibody against an NH<sub>2</sub>-terminal epitope of p53 (Bp53-12). This antibody recognizes a similar NH<sub>2</sub>-terminal epitope of p53 (epitope mapping amino acids 20-25) as the DO1 antibody (epitope mapping amino acids 21-25). Detection of a band consistent with the expected MW of wild-type p53 was found in the majority of melanoma cell lines [13 of 19 (68.4%); Fig. 1]. In addition, a band of smaller MW ( $\sim$ 44 kDa) was observed in 12 of 19 (63.2%) melanoma cell lines (Fig. 1). The expression of the small MW form of p53 was variable between the different cell lines and was often coexpressed with the wild-type protein. In most cases, the expression of the small MW form of p53 was less than wild-type p53 (Fig. 1). However, expression of the small MW form of p53 was more abundant than wild-type p53 in Sk-Mel-110 and Sk-Mel-93 (Fig. 1). FLOW2000 fibroblasts did not express wild-type p53 or the small MW p53 band, whereas melanocytes expressed wild-type p53 at very low levels and did not express detectable levels of the small MW p53 band (Fig. 1, *high-exposure image*). This is consistent with p53 being expressed at low levels in normal cells unless activated and stabilized by DNA damage (1).

Sequence analysis of p53 in nine melanoma cell lines revealed that mutations were uncommon (Supplementary Table S1). The rate of mutation detection [3 of 9 (33%)] is consistent with previous published studies on human melanoma (5-12). This suggests that in the majority of the cell lines, the small MW p53 band was not a truncated protein product caused by a mutation in p53.

**Fig. 1.** p53 is expressed as small MW forms in human melanoma cell lines. Protein (25  $\mu$ g) was harvested from a panel of exponentially growing melanoma cell lines and analyzed for the endogenous expression of p53 by Western blotting, using the mouse monoclonal anti-human antibody Bp53-12. The expression of p53 in the melanoma cell lines was compared with the normal, noncancerous cell lines FLOW2000 fibroblasts and melanocytes. \*, small MW form of p53. The expression of GAPDH was used as a loading control.





**Fig. 2.** Examination of p53 isoforms by two-dimensional electrophoresis. *A*, the specificity of the TLQ-40 antibody was examined by Western blotting of H1299 cell lysates transfected with either p53 $\beta$ , p53 $\gamma$ ,  $\Delta$ 133p53, or  $\Delta$ 133p53 $\beta$ . Western blots were probed with TLQ-40 ( $\beta$  specific), 1801 (NH<sub>2</sub>-terminal epitope), or Bp53-10 + 421 (COOH-terminal epitope). *B*, two-dimensional electrophoresis was done on IgR3 and Mel-RM cells as described in Materials and Methods, and the endogenous expression of p53 and p53 $\beta$  was examined by immunoblotting. An NH<sub>2</sub>-terminal antibody (DO1) and a COOH-terminal antibody (Bp53-10) were used to detect p53, whereas the TLQ40 antibody was used to specifically detect the p53 $\beta$  isoform. The circled spot is consistent with the MW and pI of the p53 $\beta$  isoform and was not detected by the COOH-terminal p53 antibody (Bp53-10).

**p53 $\beta$  is expressed in human melanoma.** Given that the antibody used to detect the small MW form of p53 in the melanoma cell lines by Western blot in the current study (Bp53-12) was specific for an epitope within the NH<sub>2</sub> terminus of p53, it is likely that the small MW form of p53 expressed in the melanoma cell lines may be caused by a COOH-terminal truncation of p53. Two alternatively spliced forms of p53 have been identified that cause truncation of the p53 protein at the COOH terminus, p53 $\beta$  and p53 $\gamma$  (20). Because the MW of the  $\beta$  and  $\gamma$  isoforms are the same, and the fact that a specific antibody has only been developed for the  $\beta$  isoform, it is not possible to distinguish the expression of these isoforms by immunoblots in one-dimensional electrophoresis. To further characterize the expression of the small MW form of p53 in IgR3 and Mel-RM melanoma cells, which showed high expression of the small MW p53 band, two-dimensional electrophoresis and immunoblotting for p53 was done. This method allows separation of proteins based on their MW and isoelectric point (pI) and has recently been used to distinguish the  $\beta$  and  $\gamma$  isoforms (21).

Before performing two-dimensional electrophoresis and immunoblotting for p53 $\beta$ , it was necessary to assess the specificity of the TLQ-40 antibody for the p53 $\beta$  and  $\Delta$ 133p53 $\beta$  isoforms, as it has not previously been characterized. Western blots were done on lysates from H1299 cells (p53 negative) that had been transfected with p53 $\beta$ , p53 $\gamma$ ,  $\Delta$ 133p53, and  $\Delta$ 133p53 $\beta$  (Fig. 2A). Only p53 $\beta$  and  $\Delta$ 133p53 $\beta$  were detected with the TLQ-40 antibody, whereas p53 $\gamma$  and  $\Delta$ 133p53 were not, thus showing its specificity for the  $\beta$  isoform of p53 (Fig. 2A).

Using two-dimensional gel electrophoresis, two immunoreactive spots were revealed by an antibody that detects an NH<sub>2</sub>-terminal epitope of p53 (DO1): a large intense spot, corresponding to wild-type p53 (53 kDa), and a smaller less intense spot, migrating at 44 to 46 kDa with a pI of  $\sim$  5 to 6 (Fig. 2B). The 44 to 46 kDa p53 spot was not detected by the COOH-terminal antibody Bp53-10 (Fig. 2B, circled). This spot was readily detected by an antibody specific to p53 $\beta$ , TLQ-40, confirming that one of the small MW forms of p53 was the

p53 $\beta$  isoform. This analysis was unable to distinguish a separate spot for p53 $\gamma$  with the DO1 antibody, although given the pI range of the small MW spot it is possible that this spot may also contain p53 $\gamma$ .

**Human melanoma cell lines express multiple p53 isoforms.** We characterized the expression of wild-type p53 and the expression of six previously identified isoforms: p53 $\beta$ , p53 $\gamma$ ,  $\Delta$ 40p53,  $\Delta$ 133p53,  $\Delta$ 133p53 $\beta$ , and  $\Delta$ 133p53 $\gamma$  in a panel of 16 melanoma cell lines and 9 primary isolates by reverse transcription-PCR (Figs. 3 and 4). The specific region of p53 in which each of the primers anneal to is shown in Fig. 3A. The specificity of the primer pairs used in this study to detect each of the isoforms is shown in Fig. 3B, where PCR was done on cDNA constructs of the p53 isoforms. MCF-7 breast cancer cells have previously been reported to express the p53 $\beta$  and  $\Delta$ 40p53 isoforms and were also used as a positive control in this study (20, 26). p53 was expressed in all melanoma cell lines with the exception of the Me4405 cell line (Fig. 4A), which does not express the p53 gene (data not shown). p53 $\beta$  was expressed in the majority of cell lines [15 of 16 (94%)] but was not detected in FLOW2000 fibroblasts and showed very low expression in melanocytes (Fig. 4A). The primers used in the amplification of  $\Delta$ 40p53 did not cross intron-exon boundaries, but allowed a clear distinction between genomic DNA contamination and the cDNA amplicon that was 93 bp larger (453 bp) than the latter, due to the inclusion of intron 3 (Fig. 4A, compare genomic DNA sample with the melanoma cell line cDNA samples). Expression of  $\Delta$ 40p53 at the mRNA level was detected in the majority of cell lines, with low expression in FLOW2000 and melanocytes (Fig. 4A).

The  $\Delta$ 133p53 isoform was not detected at the mRNA level in any of the cell lines analyzed; however,  $\Delta$ 133p53 $\beta$  was detected at very low levels in half of the melanoma cell lines (Fig. 4A). The expression of p53 $\gamma$  and  $\Delta$ 133p53 $\gamma$  was not detected in any of the melanoma cell lines in replicate analyses and is not shown. Although this analysis was not quantitative, the relative expression profiles of wild-type p53, p53 $\beta$ ,  $\Delta$ 40p53, and



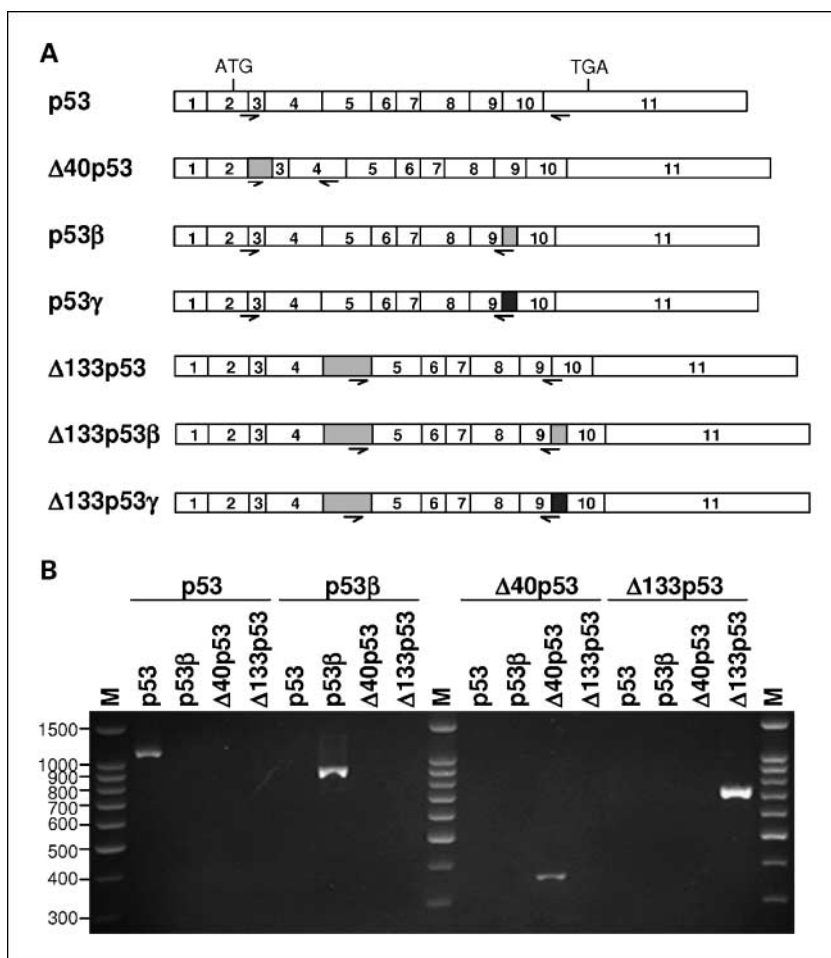
$\Delta 133p53\beta$  in each particular cell line was different (Fig. 4A), suggesting that the splicing mechanisms and/or transcription of these splice variants is regulated in melanoma. Furthermore, all melanoma cell lines showed a different profile of p53 and isoform expression compared with FLOW2000 fibroblasts and melanocytes, suggesting that the ratio of p53 to p53 isoforms may be important in melanoma.

To more closely determine whether p53 isoforms were expressed *in vivo*, we examined their expression in primary isolates of melanoma. p53 $\beta$  and  $\Delta 40p53$  were expressed in 5 of 9 (55%) and 9 of 9 primary isolates of metastatic melanoma, respectively (Fig. 4B). The  $\Delta 133p53$  and  $\Delta 133p53\beta$  isoforms were not detected at the mRNA level in any of the primary cell lines tested. Each of the primary isolates also expressed wild-type p53 (Fig. 4B). Of particular note, the expression of p53 $\beta$  was significantly increased in nonadherent sphere-forming cells (9b) of primary isolates from patient 9, compared with adherent monolayer cells from the same patient (9a), whereas the expression of the wild-type protein was dramatically reduced in cells that formed spheres (Fig. 4C). Melanoma cells that form spheres have been shown to have properties of stem cells (32). These cell lines have distinct coloration when pelleted, with sphere-forming cells forming a white pellet and adherent cells forming a brown pellet (Fig. 4D), consistent with the adherent cells being in a more differentiated state. This suggests that p53 $\beta$  may play a role in modulating melanoma growth and/or differentiation.

**Small MW forms of p53 are abnormally localized in melanoma.** p53 is a predominantly nuclear protein. The results of p53 screening in melanoma cells suggested that in most cell lines, the small MW form was of lower abundance than the wild-type protein in the whole-cell extract (Fig. 1). To determine whether there were differences in the subcellular expression of the small MW form of p53 compared with the wild-type protein, nuclear and cytosolic fractions were isolated from the same cell pellet and Western analysis was done. p53 expression was compared between three cell lines, Mel-RM, IgR3, and MM200, which were shown to have high expression of the small MW p53 band by Western blot analysis, and FLOW2000 normal human fibroblasts. p53 expression was analyzed using three different antibodies: Bp53-12, which detects an epitope in the NH<sub>2</sub> terminus and cannot recognize NH<sub>2</sub>-terminally deleted p53 isoforms; 1801, which detects an epitope within the NH<sub>2</sub> terminus but can recognize the  $\Delta 40p53$  isoform (26); and a combination of two antibodies (421 and Bp53-10), which detect epitopes within the COOH terminus and therefore cannot recognize p53 isoforms that are truncated at the carboxyl terminus (such as the  $\beta$  and  $\gamma$  isoforms).

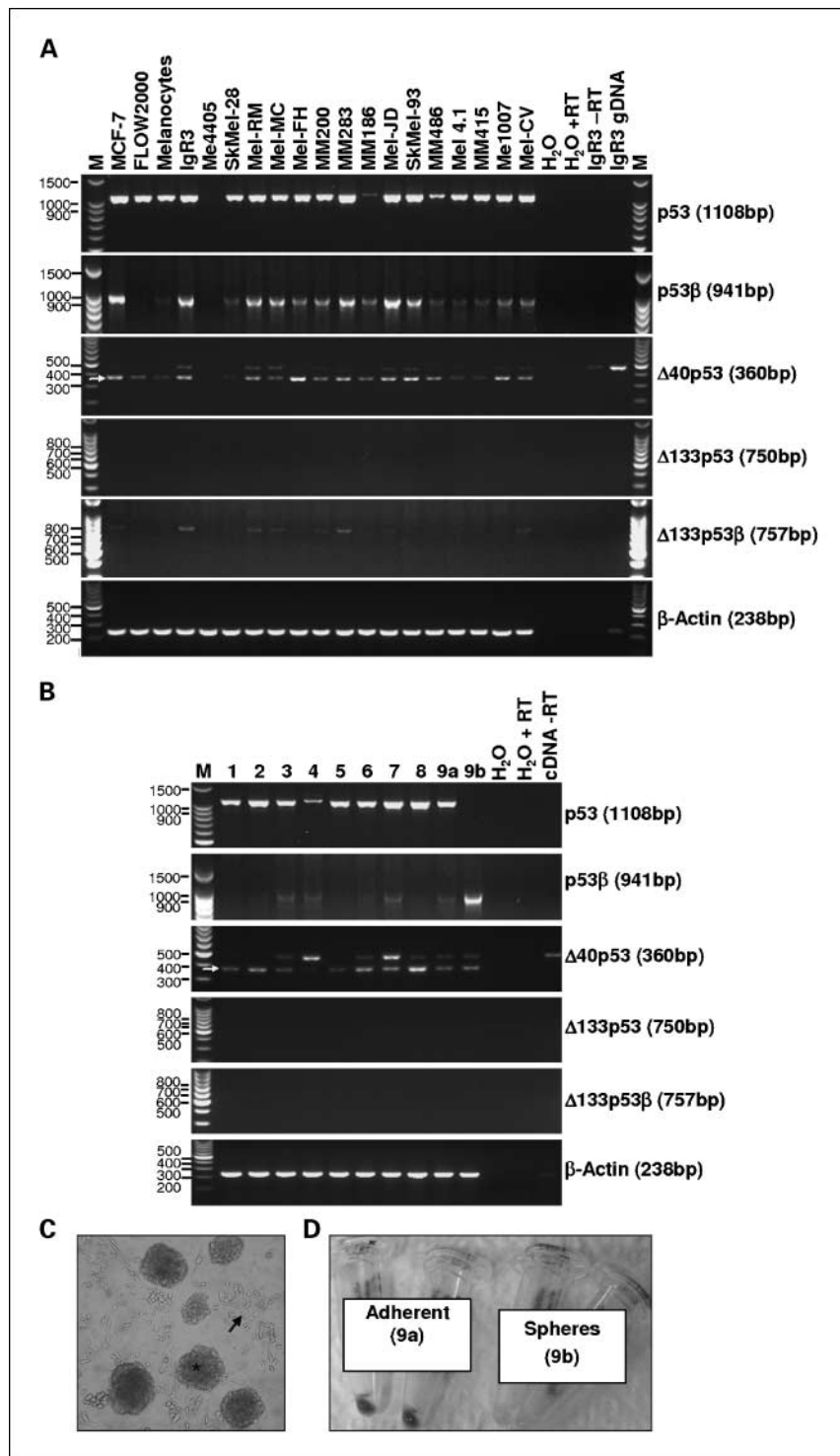
As expected, a single p53 band was detected only in the nuclear fraction of FLOW2000 fibroblasts with each of the p53 antibodies (Fig. 5). In contrast, Mel-RM and IgR3 cells expressed wild-type p53 in both the nuclear and cytosolic fractions, whereas MM200 cells expressed wild-type p53 only in the nuclear fraction. The small MW p53 band detected with the

**Fig. 3.** The specificity of primers used in the amplification of p53 splice variants. **A**, diagrammatical representation of p53 mRNA and mRNA for the previously identified splice variants  $\Delta 40p53$ , p53 $\beta$ , p53 $\gamma$ ,  $\Delta 133p53$ ,  $\Delta 133p53\beta$ , and  $\Delta 133p53\gamma$ . White boxes, exons; gray boxes, intronic regions that are included in the splice variant mRNA. Black arrows, primers used to specifically amplify p53 and its splice variants. **B**, the specificity of the primer pairs (top of the figure above the horizontal line) used to amplify p53 and its splice variants was tested on expression constructs containing cDNA for p53 and the isoforms as detailed in Materials and Methods. The cDNA constructs used in the PCR are shown above each lane of the gel (below the horizontal line). The approximate sizes (bp) of the DNA ladder (M) are shown on the left of the gel.



Bp53-12 antibody (NH<sub>2</sub>-terminal epitope) was present in the nuclear and cytosolic fractions of both IgR3 and MM200 cells, but was only detected in the nuclear fraction of Mel-RM cells (Fig. 5, *white arrowhead*). This band was also detected by the 1801 antibody (NH<sub>2</sub>-terminal epitope that can recognize Δ40p53), although with much lower affinity, and was not detected by the COOH-terminal antibodies (421 + Bp53-10), further supporting that this small MW band may be the β

isoform of p53. Another small MW band was detected with high affinity in IgR3 cells by the NH<sub>2</sub>-terminal antibody 1801 and the COOH-terminal antibodies Bp53-10 and 421; however, it was not recognized by the NH<sub>2</sub>-terminal antibody Bp53-12 (Fig. 5, *black arrowhead*), suggesting that this band is the Δ40p53 isoform. This band was present in the cytosolic fraction and was detected at very low levels (almost undetectable) in the nucleus. Taken together, these results suggest that



**Fig. 4.** The mRNA for p53 splice variants is widely expressed in human melanoma cell lines and primary isolates. The expression of wild-type p53 and p53 splice variants was examined in a panel of melanoma cell lines (A) and primary melanoma isolates (B) by reverse transcription-PCR as described in Materials and Methods, and compared with the expression of the housekeeping gene *β-actin*. White arrow, the expected size of the Δ40p53 PCR product. The results shown are representative of two or more independent experiments. M, molecular weight marker; RT, reverse transcriptase; gDNA, genomic DNA. C, the morphology of primary isolates in B, which grew as an adherent monolayer (9a, *arrow*) or as nonadherent sphere-forming cells (9b, \*). Magnification, ×100. D, the coloration of cell pellets from adherent cells (9a) and from sphere-forming cells (9b).

the subcellular localization of p53 and its small MW isoforms is aberrant in melanoma compared with normal fibroblasts that only expressed wild-type p53 in the nucleus.

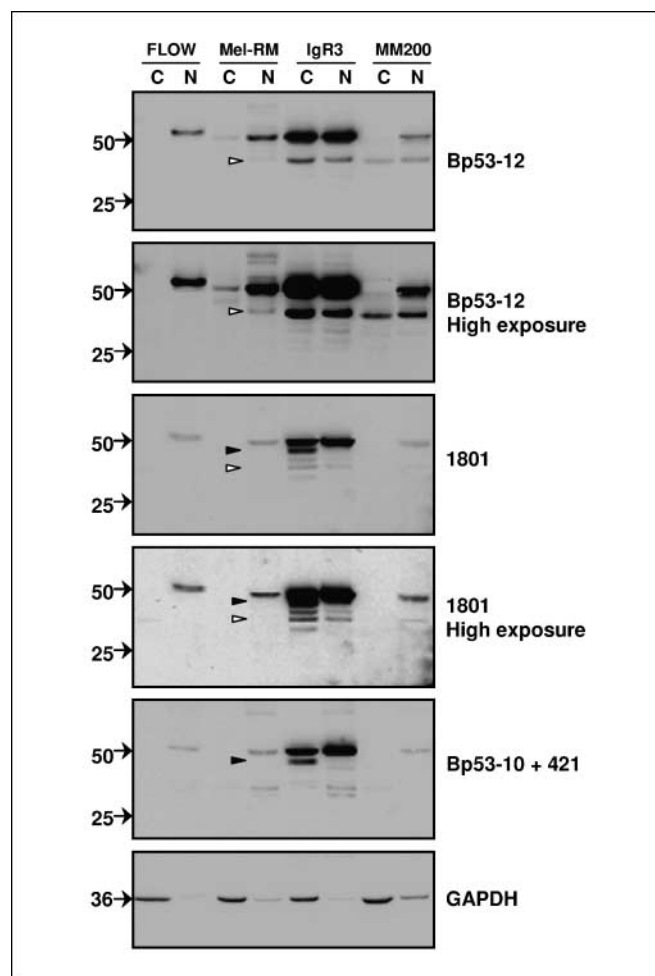
**The expression of the small MW form of p53 is aberrantly regulated in melanoma following treatment with a DNA-damaging agent.** Cisplatin is a DNA-damaging agent used widely in the treatment of cancer (33) and is known to up-regulate p53 and its downstream targets (33–37). To determine whether the expression of the small MW form of p53 could be induced by a DNA-damaging agent and whether this was associated with the induction of p53, melanocytes, IgR3, Mel-RM, and MM200 cells were treated with Cisplatin over a 48-hour period and its effects on the p53 pathway were studied by Western blotting.

Apoptosis was induced to varying degrees, in all cell lines analyzed, following 48 hours of Cisplatin treatment. Induction of apoptosis in melanocytes occurred at a later onset compared with the melanoma cell lines (Fig. 6A). The expression of wild-type p53 increased in a time-dependent manner, following Cisplatin treatment, in all cell lines analyzed (Fig. 6B). The expression of the small MW band of p53, which we have identified as p53 $\beta$ , was also increased in a time-dependent manner in MM200 and Mel-RM melanoma cell lines, but decreased at 36 and 48 hours in IgR3 cells (Fig. 6B). The endogenous expression of  $\Delta$ 40p53 in whole-cell lysates (using the 1801 antibody) was undetectable in this particular study (data not shown). These results suggest that the small MW forms of p53 in melanoma and normal cells can be induced by DNA-damaging agents, and in some cell lines, such as MM200, can exceed the induction of wild-type p53.

**Alternate downstream p53 target genes are activated in melanoma cells following Cisplatin treatment.** To determine whether the expression of small MW p53 isoforms was associated with altered regulation of p53 target genes following Cisplatin treatment in the MM200, IgR3, and Mel-RM cell lines, the induction of p53 downstream targets p21 and PUMA was studied and compared with the expression of these proteins in melanocytes. The expression of the cell cycle protein p21 was up-regulated in all cell lines following 3 to 6 hours of treatment; this preceded stabilization of p53 (Fig. 6B). At later time periods, expression of p21 in the extracts from the MM200 and IgR3 cells seemed to be inversely related to induction of the small MW isoforms, suggesting that they may inhibit the induction of p21. This was not evident in extracts of Mel-RM cells, in which the induction of wild-type p53 clearly exceeded that of the small MW isoform from 16 hours onward.

Expression of the proapoptotic protein PUMA was increased in IgR3 and Mel-RM cells in a time-dependent manner, followed by a decrease at the 36- and 48-hour time point, respectively, but was undetectable in MM200 cells and melanocytes (Fig. 6B). Taken together, these results suggest that different patterns of p53-dependent proapoptotic target genes are activated in melanoma, compared with melanocytes, by exposure to Cisplatin. Small MW isoforms of p53 may contribute to this pattern of expression, particularly that of p21.

**p53 isoforms differentially regulate the transcription of p53 target genes in melanoma.** To more specifically determine whether the p53 isoforms  $\Delta$ 40p53 and p53 $\beta$  could alter p53 function in melanoma, we analyzed the effect of the ectopically expressed proteins on the transcriptional activation of promoter constructs for the p53 target genes, *p21* and *PUMA*, following



**Fig. 5.** p53 isoforms are differentially localized in the nucleus and cytosol of melanoma in a cell line – dependent manner. Nuclear (N) and cytosolic (C) proteins (25  $\mu$ g) were isolated from the same cell pellet of FLOW2000, Mel-RM, IgR3, and MM200 cell lines as described in Materials and Methods. Endogenous expression of p53 and its isoforms was analyzed by Western blot using a combination of p53 antibodies. The NH<sub>2</sub>-terminal antibody Bp53-12 cannot detect the  $\Delta$ 40p53 isoform, whereas the 1801 antibody can detect this p53 isoform (26). The mouse monoclonal anti-human p53 antibodies, 421 and Bp53-10, detect epitopes within the COOH terminus and do not recognize COOH-terminal truncated forms of p53. White arrowhead, a small MW form of p53 present in Mel-RM, IgR3, and MM200 cell lines, which is detected by antibodies recognizing NH<sub>2</sub>-terminal epitopes of p53 but not those which detect COOH-terminal epitopes. Black arrowhead, a small MW form of p53 present in IgR3 cells that is detected by 1801 and Bp53-10 + 421, but is not detected by Bp53-12. The expression of GAPDH was used as a loading control and to show the purity of the nuclear and cytosolic fractions.

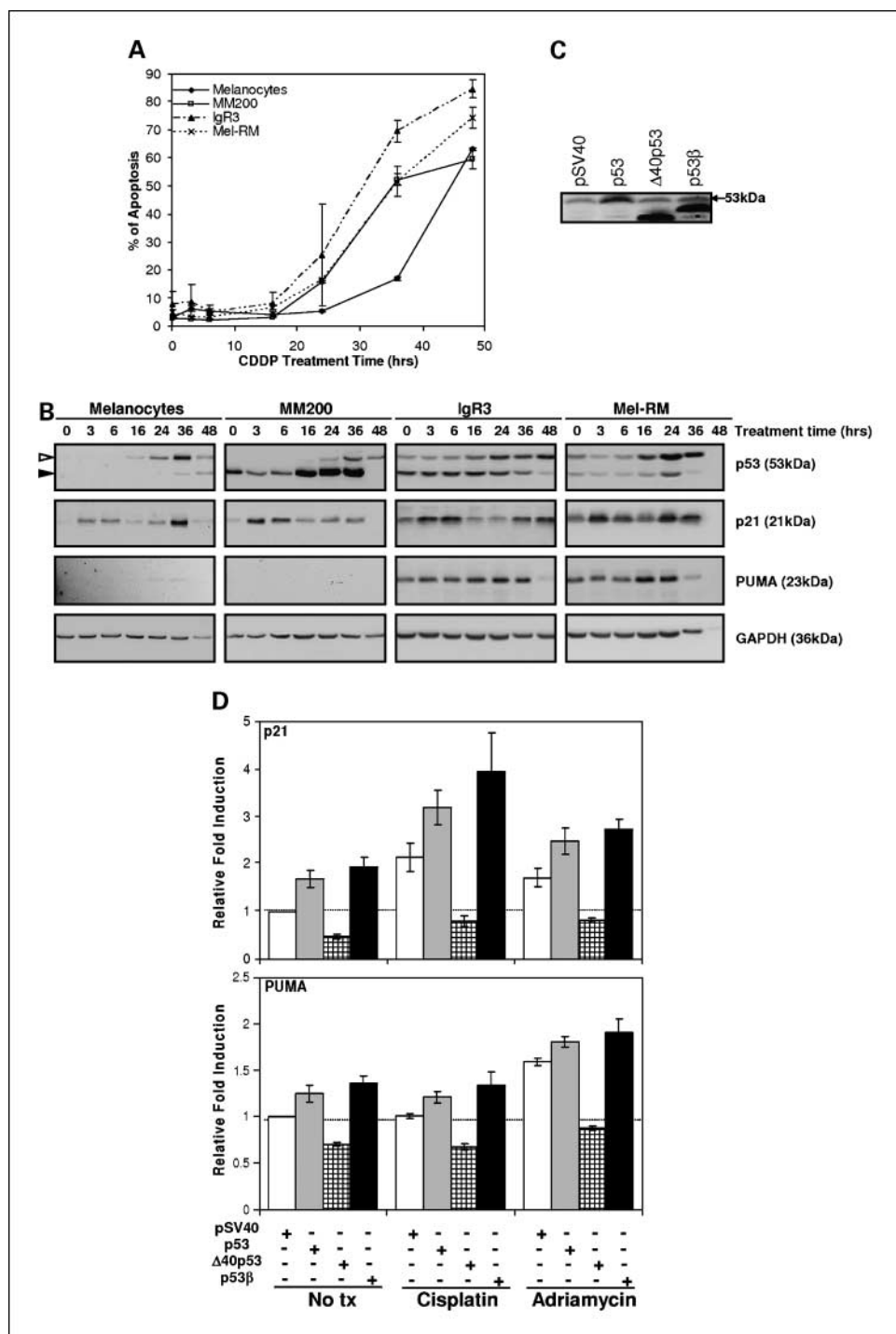
treatment with the DNA-damaging agents, Cisplatin and Adriamycin. In the p53-positive Mel-RM cell line, the p21 promoter was activated by endogenous p53 following treatment with Cisplatin and Adriamycin, whereas PUMA was activated following treatment with Adriamycin but not Cisplatin (Fig. 6D, compare *open columns*), in agreement with our Western blot analysis (Fig. 6B). Ectopically expressed p53 further enhanced transcriptional activation from the p21 and PUMA promoters in the presence and absence of DNA-damaging agents (Fig. 6D, *gray columns*).  $\Delta$ 40p53 inhibited the basal transcriptional activity of p53 on both the p21 and PUMA promoters and completely abrogated the transcriptional activation of these promoters following treatment with

Cisplatin and Adriamycin (Fig. 6D, compare *hatched columns* with *open columns*). In contrast, transfection of p53 $\beta$  enhanced both the basal and stress-induced transcriptional activation of the p21 and PUMA promoters (Fig. 6D, compare *black columns* with *open columns*). Transfection of  $\Delta$ 40p53 or p53 $\beta$  did not stimulate transcriptional activation of either the p21 or PUMA promoter in the p53-negative Me4405 cell line, in the presence or absence of DNA-damaging agents (data not shown). This suggests that  $\Delta$ 40p53 and p53 $\beta$  have no transcriptional activity on their own, as has been shown previously (20, 25, 26). Taken

together, these results suggest that  $\Delta$ 40p53 and p53 $\beta$  can differentially regulate the transcriptional activity of endogenous wild-type p53 on the p21 and PUMA promoters in melanoma cells, with  $\Delta$ 40p53 acting as an inhibitor and p53 $\beta$  acting as an enhancer.

### Discussion

The present study extends reports of the detection of small MW isoforms in other cancers, by showing that several isoforms



**Fig. 6.** p53 and its isoforms are differentially regulated in melanoma following treatment with the DNA-damaging chemotherapy drug Cisplatin. Cells were treated with Cisplatin (10  $\mu$ g/mL) for the indicated times and then harvested. **A**, quantitation of apoptotic cells by propidium iodide staining and analysis by flow cytometry, as described in Materials and Methods. Points, mean of two or three independent experiments; bars, range. **B**, the expression of p53 and its downstream targets, p21 and PUMA, were analyzed by Western blot. White arrowhead, wild-type p53; black arrowhead, the small MW form of p53. The expression of the housekeeping gene *GAPDH* was determined to ensure equal loading of the samples. Results are representative of at least two independent experiments. **C** and **D**, Mel-RM cells were cotransfected with either wild-type p53 (*gray columns*),  $\Delta$ 40p53 (*hatched columns*), p53 $\beta$  (*black columns*) or empty vector (pSV40, *open columns*), and a luciferase construct containing the natural promoter for p21 or PUMA, as described in Materials and Methods. The expression of p53 and its isoforms in protein lysates was analyzed by Western blotting using the 1801 antibody (**C**). Twenty-four hours following transfection, cells were treated with either Cisplatin (10  $\mu$ g/mL) or Adriamycin (1  $\mu$ mol/L) for 6 h and then harvested. Transcriptional activation from the p21 or PUMA luciferase reporter construct was analyzed by luciferase assay as described in Materials and Methods (**D**). The activity of *Firefly* luciferase from the inducible p21 and PUMA luciferase constructs was normalized to the activity of *Renilla* luciferase activity of pRL-SV40 to account for differences in transfection efficiency. Results are expressed as the normalized fold induction of luciferase activity relative to the pSV40 vector transfected cells (which have been set to 1). Columns, mean of three or four independent experiments done in duplicate; bars, SE.



of p53 are found in melanoma cell lines and fresh isolates of melanoma. Two of the isoforms, p53 $\beta$  and  $\Delta$ 40p53, were detected in practically all of the melanoma cell lines analyzed in this study, at the mRNA and protein level, whereas  $\Delta$ 133p53 $\beta$  was detected in only a small number of melanoma cell lines. These isoforms were not detected in normal melanocytes or fibroblasts. It was also notable that the protein levels of the isoforms exceeded that of wild-type protein in some cell lines. This contrasts with the detection of these isoforms only at low levels in other cancers (22, 25, 38).

An important finding of this study was that the endogenously expressed p53 isoforms may have differential localization between the nucleus and the cytosol of melanoma cells, compared with wild-type p53 (Fig. 5). The latter is normally located in the nucleus but may enter the cytosol during cellular stress (39–42). All of the isoforms identified to be expressed in melanoma in this study retain the nuclear localization sequence within p53, and studies done on ectopically expressed proteins have suggested that p53 $\beta$  and stably transfected  $\Delta$ 40p53 are predominantly nuclear with limited cytoplasmic staining (20, 26). However, in this study, the  $\Delta$ 40p53 isoform seemed to only localize to the cytosol (in IgR3 cells), whereas p53 $\beta$  was identified in both cell fractions even when p53 itself was exclusively nuclear, as in MM200 cells. This suggests that the subcellular localization of p53 isoforms may be cell line dependent. These results are of significance and may explain why the p53 isoforms can be difficult to detect in whole-cell lysates (22, 25, 38), but readily detected in nuclear and cytosolic fractions as shown in studies on MM200. Furthermore, the aberrant expression of the isoforms may have functional significance. Previous studies have shown that p53 localized in the cytosol can induce apoptosis either by binding to Bcl-xL and displacing Bax, freeing it to bind to mitochondria, or by direct interaction with the mitochondria (40–42). Hence, relatively high concentrations of the isoforms in the cytosol could interfere with this nontranscriptional role of p53.

Several findings of this study suggest that the expression of small MW isoforms of p53 in melanoma may be functionally significant. First, p53 $\beta$  was expressed at high levels in a nonadherent sphere-forming isolate from patient 9, whereas the adherent isolate from the same patient had low levels of p53 $\beta$  and normal levels of wild-type p53 (Fig. 4B–D). This may indicate that p53 $\beta$  plays a role in melanoma growth and

differentiation, although further studies on this aspect are required. Second, we found that exposure to the DNA-damaging agent Cisplatin resulted in up-regulation of the isoforms, which in some cell lines (such as MM200) exceeded the up-regulation of wild-type p53 (Fig. 6B). The small MW forms of p53 identified in this study were not due to cleavage of the wild-type protein by caspases, which has been shown to occur following treatment with Cisplatin (34, 35), as their generation was not prevented by pan caspase inhibitors (data not shown).

There was evidence from this study that the presence of the isoforms may differentially alter the transcription of p53 target genes in melanoma cells, following exposure to Cisplatin and Adriamycin. In particular, ectopic expression of  $\Delta$ 40p53 was shown to inhibit the basal and stress-induced p53-dependent transcriptional activation of the p21 and PUMA promoters, whereas p53 $\beta$  acted as an enhancer of p53-dependent transcription from these promoters. In this regard,  $\Delta$ 40p53 and p53 $\beta$  have been shown to form complexes with p53 (20, 26), and  $\Delta$ 40p53 has been shown to act as an inhibitor of p53-dependent transcriptional activation of p21 (26). Although p53 $\beta$  has been shown to bind to both the p21 and BAX promoters, it has only been shown to act as an enhancer on the BAX promoter and not that of p21 (20, 25). This suggests that enhancement of p53-dependent transcriptional activation from the p21 promoter by p53 $\beta$ , as observed in this study, may be cell line dependent or may require some as yet unidentified cofactors. Nevertheless, taken together, these results suggest that the ratio of p53 isoforms to the wild-type protein may play an important role in regulating p53-dependent transcription of p53 target genes in melanoma.

In summary, this study has shown that small MW isoforms of p53 are common in melanoma and can be induced by exposure to DNA-damaging agents, such as Cisplatin. Moreover, the expression of at least one of these isoforms,  $\Delta$ 40p53, was shown to inhibit p53-dependent transcriptional activation of the p21 and PUMA promoters, suggesting that the expression of these isoforms may account, in part, for the aberrant function of p53 in melanoma.

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