Aberrant Regulation and Function of Wild-Type p53 in Radioresistant Melanoma Cells[1]

Kapaettu Satyamoorthy, Nabil H. Chehab, Matthew J. F. Waterman, Marcus C. Lien, Wafik S. El-Deiry, Meenhard Herlyn, and Thanos D. Halazonetis[2]

The Wistar Institute [K. S., N. H. C., M. J. F. W., M. C. L., M. H., T. D. H.], and Departments of Genetics and Medicine, Howard Hughes Medical Institute, University of Pennsylvania [W. S. E.], Philadelphia, Pennsylvania 19104-4268

Abstract

Sporadic human tumors and the hereditary cancer predisposition syndrome Li-Fraumeni are frequently associated with mutations in the *p53* tumor suppressor gene that compromise its ability to function as a DNA damage checkpoint. A subset of Li-Fraumeni patients with wild-type *p53* alleles have mutations in *chk2/hcds1*, one of the genes signaling the presence of DNA damage to the p53 protein. This suggests that p53 may be kept inactive in human cancer by mutations targeting DNA damage signaling pathways. Melanoma cells are highly radioresistant, yet they express wild-type p53 protein, raising the possibility of defects in the pathways that activate p53 in response to DNA damage. We have described a *chk2/hcds1*-independent DNA damage signaling pathway that targets Ser-376 within the COOH terminus of p53 for dephosphorylation and leads to increased p53 functional activity. We now report that in several human melanoma cell lines that express wild-type p53, the phosphorylation state of Ser-376 was not regulated by DNA damage. In these cell lines, neither the endogenous wild-type p53 protein nor high levels of ectopic wild-type p53 led to cell cycle arrest or apoptosis. Thus, defective activation of p53 in response to DNA damage may underlie the radioresistance of human melanoma cells.

Introduction

Within the last few decades, there has been significant progress in the treatment of specific types of human cancer, such as lymphomas, whereas other human cancers, including melanomas, pose a formidable challenge and have a poor prognosis despite aggressive treatment. Most of the agents used to treat cancer are DNA-damaging agents. The rationale for their use has been that during DNA replication, cells may be more sensitive to DNA damage. Recently, however, the increased sensitivity of cancer cells to DNA damage has been shown to involve the p53 tumor suppressor protein (1, 2). p53 is a sequence-specific DNA-binding transcription factor (3). In response to DNA damage, p53 induces cell cycle arrest or apoptosis (4–7) and thereby influences the outcome of therapy (1, 2). Many human cancers have developed genetic mutations that allow them to resist p53-dependent apoptosis. The most common mutations target the *p53* gene itself, leading to expression of inactive p53 protein, which cannot induce apoptosis in response to DNA damage (3).

Melanoma cells typically express wild-type p53 protein (8–15) and would be expected to be sensitive to the DNA-damaging agents used for cancer therapy; however, these cells are extremely radioresistant (16, 17). There are several ways by which the function of wild-type p53 could be inhibited in melanoma. The defect could be downstream of p53. For example, high levels of Mdm2 protein could inhibit the ability of p53 to activate transcription, as occurs in sarcomas (18). Alternatively, the defect could be upstream, in the signaling pathways that activate p53 in response to DNA damage. Consistent with this hypothesis, mutations in *chk2/hcds1*, a gene that encodes a DNA damage signaling kinase upstream of p53 (19–22), have been identified in a subset of families with the hereditary Li-Fraumeni cancer predisposition syndrome (23).

The hypothesis that defective activation of wild-type p53 in response to DNA damage accounts for melanoma radioresistance can now be pursued because of the recent progress in our understanding of the molecular mechanisms by which DNA damage activates p53. Specifically, DNA damage leads to an increase in p53 protein levels (4, 24) and to an increase in the functional activity of the p53 protein (25, 26). The molecular mechanism for increased p53 protein levels in response to exposure to IR[3] involves a signaling pathway that includes the ATM and Chk kinases; ATM activates Chk1 and Chk2/hCds1, which in turn phosphorylate p53 on Ser-20 (19–22, 27). The latter phosphorylation leads to dissociation of p53 from Mdm2 (21, 28–30), an intracellular protein whose normal function is to target p53 for degradation (31, 32). The mechanism for the increase in p53 functional activity in response to IR involves dephosphorylation of Ser-376 within the COOH terminus of p53. In the absence of DNA damage, Ser-376 and Ser-378 of p53 are

Received 5/1/00; revised 7/13/00; accepted 7/17/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by USPHS Grants CA25874 (to M. H. and T. D. H.) and CA09171 (Wistar Institute Training Grant) awarded by the National Cancer Institute, Department of Health and Human Services.
[2] To whom requests for reprints should be addressed, at the The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104-4268. E-mail: halazonetis@wistar.upenn.edu.

[3] The abbreviations used are: IR, ionizing radiation; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; ARF, alternate reading frame; IB, immunoblotting; MOI, multiplicity of infection.

phosphorylated; after exposure to IR, Ser-376 becomes dephosphorylated, creating a binding site for 14-3-3 proteins (33). Interaction of p53 with 14-3-3 proteins allows p53 to transactivate target genes, whose protein products then induce cell cycle arrest or apoptosis.[4] Unlike p53 stabilization, which is dependent on both the ATM and Chk kinases, Ser-376 dephosphorylation is ATM dependent, but Chk2/hCds1 independent (21).

In an effort to better understand the molecular basis of melanoma radioresistance, we examined the regulation of p53 by DNA damage in human melanoma cell lines. Our analysis revealed an aberrant response, which may explain why melanoma cells are radioresistant.

Results

Constitutive Dephosphorylation of Ser-376 of p53 and Lack of Interaction of p53 with 14-3-3 Proteins in Melanoma Cells. The normal response of p53 to IR includes an increase in p53 protein levels, dephosphorylation of Ser-376, and association of p53 with 14-3-3 proteins. As far as we could determine, normal melanocytes exhibited a typical p53 response. IB of nuclear extracts with antibody DO1 indicated that p53 protein levels increased about 3-fold 1 h after irradiation. Furthermore, Ser-376 became dephosphorylated, as indicated by increased immunoreactivity with PAb421, an antibody specific for p53 that is not phosphorylated on Ser-376 (Fig. 1*A*). The association of p53 with 14-3-3 proteins after exposure to IR could not be studied because of the overall low levels of endogenous p53 in normal melanocytes.

We subsequently examined the p53 response in three melanoma cell lines that express endogenous wild-type p53. In all three cell lines, the wild-type p53 protein was expressed at very high levels, at about 30 times higher than that in primary melanocytes (Fig. 1, *B* and *C*). After irradiation, p53 levels were unchanged in one cell line (WM1158) and increased about 3-fold in the other two cell lines (1205LU and WM115). These results suggest that the DNA damage pathways leading to increased p53 protein levels are mostly intact. Dephosphorylation of Ser-376 was monitored by IB with antibody PAb421. In all three cell lines, there was no change in the phosphorylation state of Ser-376 in response to IR (Fig. 1*C*). Comparison of the PAb421 immunoreactivities of wild-type p53 from melanocytes, melanoma cell lines, and non-melanoma cell lines (U2OS osteosarcoma and MCF7 breast adenocarcinoma cells) indicates that in melanoma cells, in the absence of DNA damage, p53 is not phosphorylated on Ser-376 (Fig. 1, *B—D*). Thus, whereas the normal response of p53 to IR is dephosphorylation of Ser-376, in melanoma cells, Ser-376 is constitutively dephosphorylated.

Ser-376 is part of a recognition site within the COOH terminus of p53 for 14-3-3 proteins. The phosphorylation states of Ser-376 and Ser-378 within this site determine whether 14-3-3 proteins will bind to p53 (33). Because Ser-376 is aberrantly dephosphorylated in melanoma cells, we

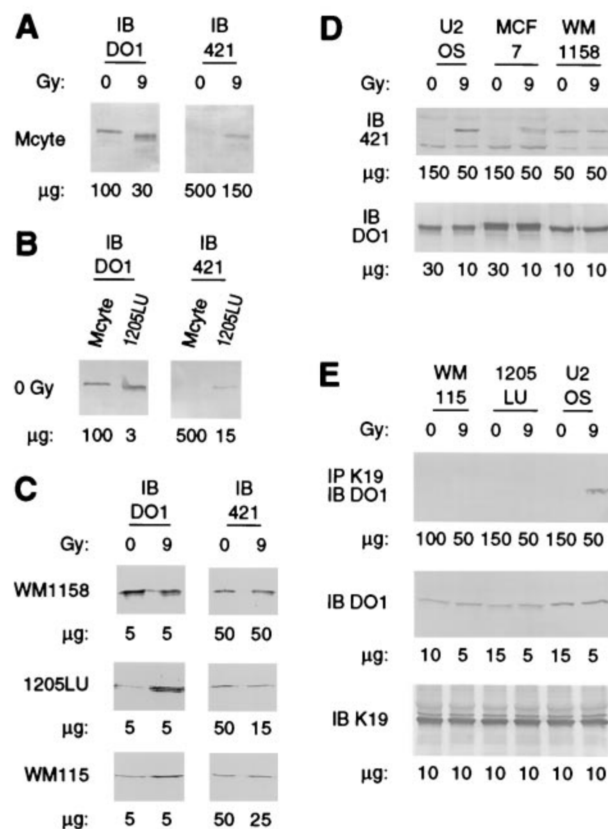

Fig. 1. Effect of IR on p53 protein levels, phosphorylation state of Ser-376, and interaction of p53 with 14-3-3 proteins in normal melanocytes and melanoma cells. *A—D*, p53 protein levels and phosphorylation state of Ser-376 in melanocytes (*Mcyte*), melanoma cell lines that express wild-type p53 (WM115, WM1158, and 1205LU), and non-melanoma cell lines that express wild-type p53 (U2OS osteosarcoma and MCF7 breast adenocarcinoma). The cells were nonirradiated or exposed to 9 Gy of IR. p53 protein levels were determined by IB with antibody DO1. The phosphorylation state of Ser-376 was determined by IB with antibody PAb421 (*421*), which recognizes p53 only when Ser-376 is dephosphorylated. The amount of extract loaded in each lane is indicated in micrograms. To facilitate the comparisons between nonirradiated and irradiated cells or between different cell lines, the amounts of extracts loaded in each lane were adjusted to have equal p53 protein levels. *E*, interaction of p53 with 14-3-3 proteins. Nuclear extracts from the indicated cell lines were immunoprecipitated (*IP*) with antibody K19, which recognizes 14-3-3, and the immunoprecipitates were blotted with antibody DO1. The amounts of p53 and 14-3-3 in the extracts were determined by IB.

examined whether p53 and 14-3-3 associate in these cells. By coimmunoprecipitation analysis, we could detect no interaction between the two proteins in both irradiated and nonirradiated cells, whereas, as a positive control, p53 and 14-3-3 associated in irradiated U2OS osteosarcoma cells (Fig. 1*E*). The absence of an interaction between p53 and 14-3-3 in melanoma cell lines could not be attributed to obvious differences in p53 or 14-3-3 protein levels as compared with non-melanoma cell lines (Fig. 1*E*) and implies that in melanoma cells, Ser-378, like Ser-376, is dephosphorylated because phosphorylated Ser-378, in conjunction with dephosphorylated Ser-376, would have created a high affinity site for 14-3-3 proteins (33).

[4] N. H. Chehab and T. D. Halazonetis, manuscript in preparation.

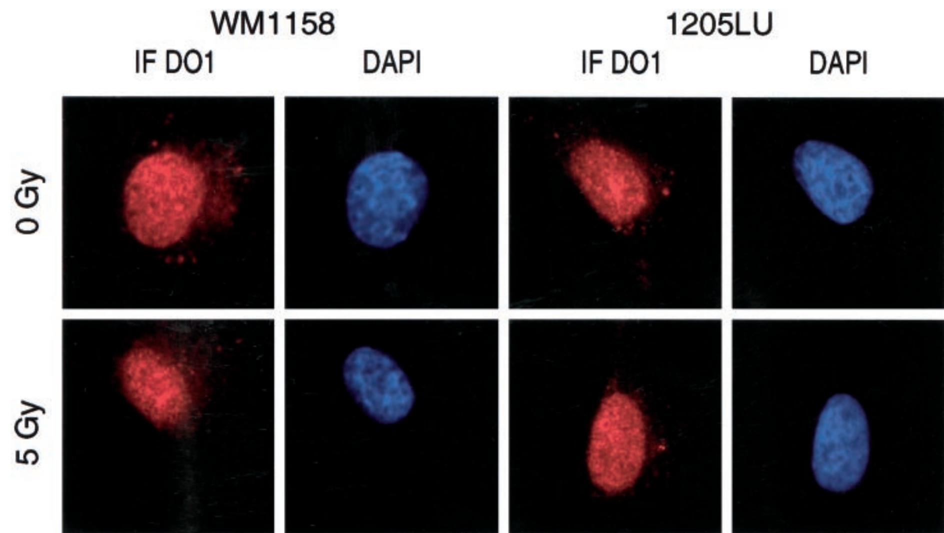

Fig. 2. Intracellular localization of p53 in nonirradiated (0 Gy) and irradiated (5 Gy) melanoma cells (WM1158 and 1205LU). Localization of the endogenous wild-type p53 was determined by immunofluorescence (*IF*) with antibody DO1, and the nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole.

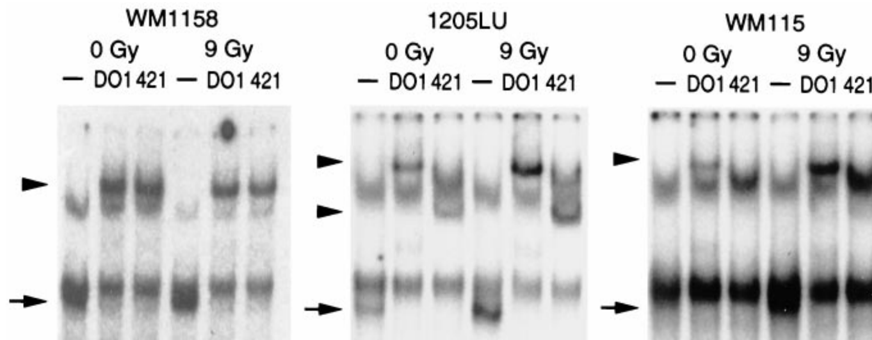

Fig. 3. Sequence-specific DNA binding activity of wild-type p53 from melanoma cells. Nuclear extracts were prepared from nonirradiated (0 Gy) or irradiated (9 Gy) melanoma cells and assayed for binding of p53 to its *p21/waf1/cip1* response element by native gel electrophoresis. p53-DNA complexes were identified by supershifting with the anti-p53 antibodies PAb421 (*421*) and DO1. *Arrowheads*, antibody-p53-DNA complexes; *arrows*, p53-DNA complexes.

Intracellular Localization, Sequence-specific DNA Binding, and Transcriptional Activities of Wild-Type p53 in Melanoma Cells. Using mutant p53 proteins that fail to interact with 14-3-3, we have recently established that the interaction between p53 and 14-3-3 does not affect the intracellular localization of p53 or its ability to bind sequence-specific oligonucleotides but is critical for its ability to activate transcription of endogenous genes and induce cell cycle arrest.[4] In melanoma cells, wild-type p53 does not bind to 14-3-3 proteins; therefore, we examined whether its functional properties would be similar to those of p53 proteins with mutant 14-3-3 recognition sites.

The intracellular localization of p53 was examined by immunofluorescence using antibody DO1. In two melanoma cell lines that express wild-type p53, WM1158 and 1205LU, the p53 protein was present in the nucleus, and its localization was not affected by exposure of the cells to IR (Fig. 2). Whereas some cells also showed cytoplasmic p53 localization (data not shown), there were no cells that showed exclusively cytoplasmic staining. Thus, the aberrant phosphorylation of p53 in melanoma and the lack of interaction with 14-3-3 proteins did not lead to exclusion of p53 from the nucleus.

To analyze the sequence-specific DNA binding activity of endogenous wild-type p53, we prepared nuclear extracts and incubated them with synthetic oligonucleotides containing the p53 response element of the *p21/cip1/waf1* gene (34). The p53-DNA complexes were resolved by native gel electrophoresis and distinguished from nonspecific complexes by supershifting with anti-p53 monoclonal antibodies. Consistent with the high levels of endogenous wild-type p53 in these extracts, p53-DNA complexes were readily evident (Fig. 3). In response to irradiation, p53 DNA binding increased, paralleling the increase in p53 protein levels (compare Figs. 1*C* and 3). As expected, no DNA binding was observed using nuclear extracts of melanoma cells that express mutant p53 (data not shown). From these results, we conclude that wild-type p53 from melanoma cells can bind avidly to sequence-specific oligonucleotides.

The transcriptional activity of endogenous wild-type p53 was monitored by examining the expression of known p53 target genes. The steady-state levels of these genes do not constitute a measure of p53 transcriptional activity because p53 is not the only transcription factor regulating their expression. Therefore, our strategy to assay p53 transcriptional activity was to infect melanoma cells with an adenoviral vector that expresses wild-type p53 and monitor changes in the expression of endogenous p53 target genes 24 h later. An adenoviral vector that induces expression of *lacZ* would serve as a control. In 1205LU melanoma cells, which have

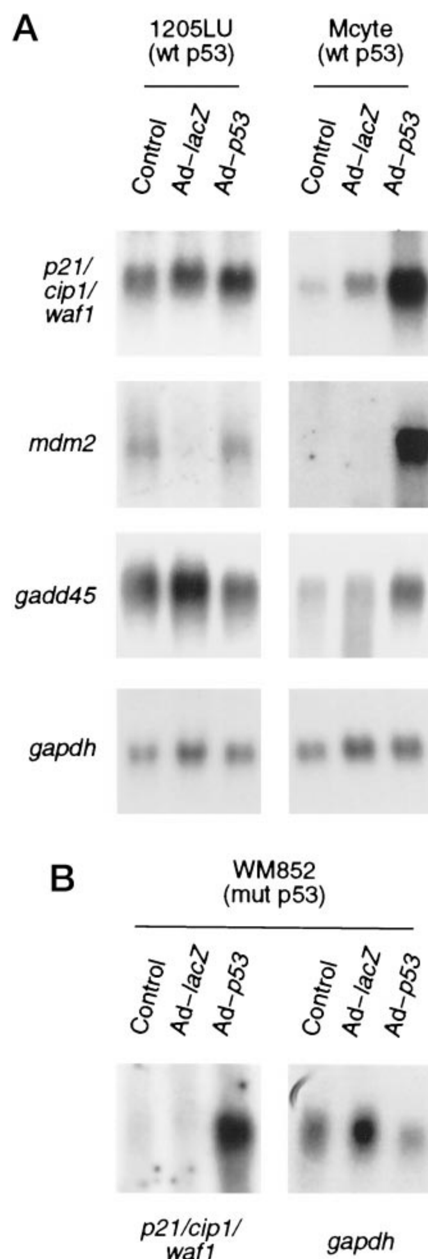

Fig. 4. Transcriptional activity of ectopically expressed wild-type p53 in normal melanocytes (*Mcyte*) and 1205LU melanoma cells that express endogenous wild-type p53 (*A*) or in WM852 melanoma cells that express endogenous mutant p53 (*B*). Expression of the p53 target genes *p21/waf1/cip1*, *mdm2,* and *gadd45* was assayed by Northern blot analysis using *gapdh* as a standard. The cells were examined 24 h after infection with adenoviruses that express *lacZ* (Ad-*lacZ*) or *p53* (Ad-*p53*) at a MOI of 10 or after mock infection (*Control*).

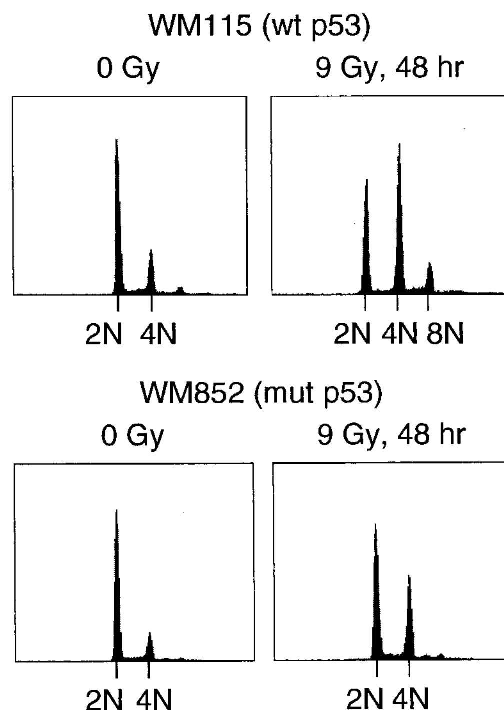

Fig. 5. Lack of apoptosis and cell cycle arrest in G_1 in melanoma cells after exposure to IR. WM115 and WM852 melanoma cells, which express wild-type and mutant p53, respectively, were either nonirradiated (0 Gy) or exposed to 9 Gy of IR. The cells were stained with propidium iodide, and their DNA content was determined by flow cytometry 48 h after irradiation.

endogenous wild-type p53, infection with the p53 adenoviral vector (Ad-*p53*) or the vector expressing lacZ (Ad-*lacZ*) either did not increase or only modestly increased expression of the p53 target genes *p21/cip1/waf1*, *mdm2,* and *gadd45* (Fig. 4*A*). Similar results were obtained with WM115 and WM1158 cells. In contrast, in melanocytes, expression of the p53 target genes was significantly induced after Ad-*p53* infection (Fig. 4*A*). Whereas it is formally possible that the inability of wild-type p53 to induce expression of several of its target genes in melanoma cells is due to independent mutations in the promoters of these genes, our favored interpretation of the results is that the transcriptional activity of wild-type p53 is compromised and that this defect developed during melanoma progression, possibly as a way to inactivate p53 function. Consistent with this interpretation, in melanoma cells, in which p53 function was inactivated by mutations in the *p53* gene itself, Ad-*p53* infection led to the induction of p53 target genes (Fig. 4*B*). Furthermore, the different responses among the cell lines could not be attributed to differences in p53 protein expression, which was monitored by IB (data not shown).

Cell Cycle Arrest and Apoptotic Activities of Wild-Type p53 in Melanoma Cells. The cell cycle arrest and apoptotic activities of endogenous wild-type p53 were examined by monitoring the cell cycle profile of WM115, WM1158, and 1205LU cells exposed to IR. All three cell lines exhibited very similar phenotypes. Untreated cells exhibited a cell cycle profile consistent with slow proliferation (Fig. 5; data not shown): most of the cells had a 2 N DNA content (G_1); a smaller but significant fraction of cells had a 4 N DNA content (G_2-M phase); and few cells had a DNA content greater than 2 N but less than 4 N (S phase). Forty-eight h after exposure to IR, the cell cycle profile suggested that the endogenous wild-type p53 protein was functionally inactive. First, the fraction of cells with a 2 N DNA content decreased, whereas

the fraction of cells with a 4 N DNA content increased, indicating cell cycle arrest in G_2-M phase, but not in G_1. Second, a significant number of cells had an 8 N DNA content. These are cells that exited mitosis and initiated a new round of DNA replication, although they failed to undergo cytokinesis. This indicates a defect in p53 function because cells with functional p53 do not undergo a new round of DNA replication in the absence of cytokinesis (35–38). Third, there were no cells with a sub-G_1 DNA content, which would be expected if the endogenous wild-type p53 had induced apoptosis. Fourth, the cell cycle profile was qualitatively identical to that of melanoma cells with endogenous mutant p53 (Fig. 5).

To further assess the functional activities of p53 in melanoma cells, we infected the cells with adenoviral vectors expressing wild-type *p53* or *lacZ* and monitored cell growth and apoptosis. The effect of p53 expression on the growth of the melanoma cell cultures was evaluated by thymidine incorporation. Briefly, melanoma cells were infected with the adenoviral vectors; at 60 h postinfection, the cells were incubated with tritiated thymidine, and incorporation of the tritiated label was determined 4 h later. The results demonstrate that inhibition of cell culture growth correlated with *p53* genotype (Fig. 6*A*); wild-type p53 overexpression did not inhibit the growth of cultures of melanoma cell lines that express wild-type p53 (WM115 and 1205LU) but did inhibit the growth of cultures that express mutant p53 (WM164 and WM852). To determine whether apoptosis underlaid the inhibition of cell culture growth, the infected cells were examined for signs of apoptosis using sub-G_1 DNA content and TUNEL assays. As observed with inhibition of culture growth, apoptosis following infection with the adenovirus that expresses wild-type *p53* also correlated with *p53* genotype (Fig. 6*B*). The resistance to apoptosis in melanoma cells with endogenous wild-type p53 could not be attributed to lack of p53 expression by the adenoviral vector because Western blot analysis revealed that infection of the melanoma cells with Ad-*p53* resulted in p53 protein levels that were significantly elevated compared with those of endogenous p53 (data not shown). Thus, both endogenous and ectopically expressed wild-type p53 are functionally defective in melanoma cells.

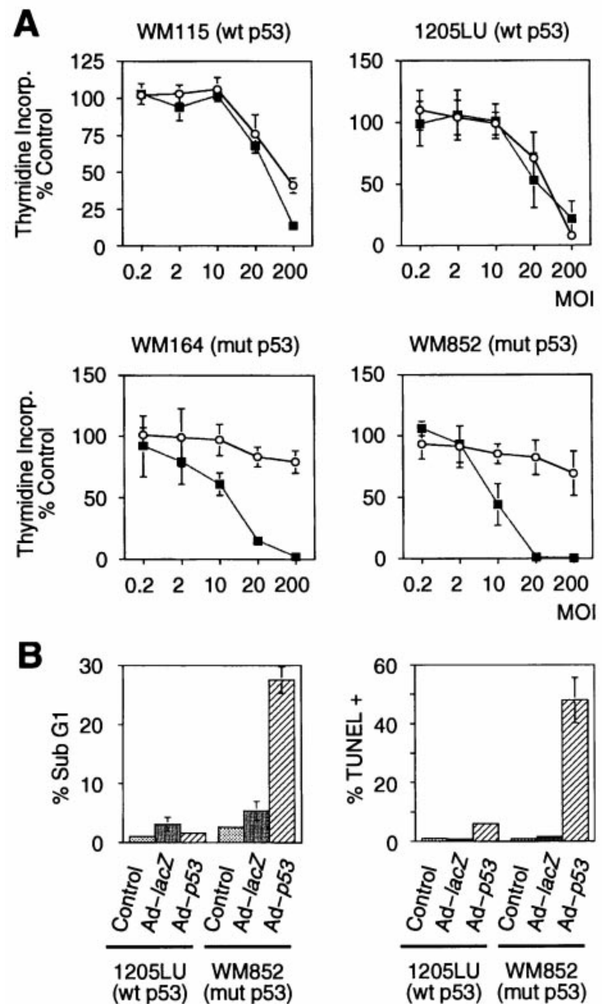

Fig. 6. Resistance of melanoma cells that express wild-type but not mutant endogenous p53 to apoptosis in response to p53 overexpression. *A*, inhibition of cell culture growth. Melanoma cells that express wild-type (WM115 amd 1205LU) or mutant (WM164 and WM852) endogenous p53 were infected with adenoviral vectors that express *lacZ* (○) or *p53* (■) at the indicated MOIs. After 60 h, the cells were incubated with tritiated thymidine, and the amount of incorporated label was determined 4 h later. *B*, apoptosis. Melanoma cells 1205LU (endogenous wild-type p53) and WM852 (mutant p53) were infected with adenoviruses expressing *lacZ* (Ad-*lacZ*) or wild-type *p53* (Ad-*p53*) at a MOI of 10, and the percentage of apoptotic cells was determined 24 h later by monitoring the fraction of cells with a sub-G_1 DNA content after propidium iodide staining or by the TUNEL assay.

Discussion

Most melanoma cells express high levels of the wild-type p53 tumor suppressor protein (39–42), which is a paradox because high levels of wild-type p53 are incompatible with tumor cell proliferation. We propose that this paradox can be resolved because the function of wild-type p53 protein is apparently compromised in melanoma cells. Specifically, in these cells, the endogenous wild-type p53 protein failed to induce cell cycle arrest or apoptosis in response to DNA damage, and ectopic wild-type p53 expressed by infection of melanoma cells with an adenoviral vector also failed to transactivate p53 target genes and promote apoptosis. These findings are consistent with a previous report showing decreased p53 transcriptional activity in melanoma cells (43). The p53 functional defect was specific to melanoma cells that have endogenous wild-type p53. Ectopic wild-type p53 activated transcription and induced apoptosis when expressed in melanoma cells that have endogenous mutant p53. Furthermore, ectopic wild-type p53 was functionally active in melanocytes, from which melanoma cells develop. Thus, inactivation of p53 occurred during melanoma progression and was due to either mutation of the *p53* gene or, more frequently, inhibition of the function of the wild-type p53 protein.

Wild-type p53 function involves two steps. First, upstream signaling pathways stabilize and activate the p53 protein in response to DNA damage or other forms of cellular stress. Activated p53 then binds to regulatory sequences of its target genes and activates their expression. Either step may

be defective in melanoma cells. Several proteins have been described that regulate p53 at the level of the second step. Mdm2 inhibits p53 transactivation when overexpressed and is clinically relevant in sarcomas (18, 44). However, melanoma cells, with few exceptions, do not express high levels of Mdm2 (45). Two other proteins, IFN regulatory factor 1 and p33ING1, physically associate with p53 and are required for p53-mediated transactivation (46, 47). However, mutations of the genes encoding these two proteins are not frequent in human cancer (48), and their role in melanoma development is unclear. Another protein that could account for the low transcriptional activity of p53 in human melanoma cells is p14ARF, one of the two protein products of the *INK4a* locus (49). p14ARF inhibits the Mdm2-dependent degradation of p53 as well as the ability of Mdm2 to suppress the transcriptional activity of p53 (50, 51). Thus, in melanoma cells, where *INK4a* mutations are exceedingly common (52–55), loss of p14ARF expression could very well explain the decreased p53 transcriptional activity. The only caveat of this model is that it predicts low level expression of p53 protein, whereas the inverse is typically true in melanoma cells (39–42).

Defective activation of wild-type p53 after DNA damage is our preferred mechanism for suppression of p53 function in melanoma cells. The response of p53 to DNA damage involves an increase in p53 protein levels due to stabilization of the protein and an increase in p53 functional activity (4, 24–26). Most likely there is no defect in regulation of p53 stabilization in response to DNA damage because p53 was stabilized in response to IR in two of the three melanoma cell lines examined, and the steady-state levels of p53 were very high. In contrast, regulation of the functional activity of p53 appears to be defective. In normal cells, regulation of p53 function by IR involves dephosphorylation of Ser-376 and association of p53 with 14-3-3 proteins (33). In melanoma cells, wild-type p53 had Ser-376 constitutively dephosphorylated and failed to interact with 14-3-3 proteins in response to DNA damage. The lack of interaction between p53 and 14-3-3 can lead to a p53 functional defect, as suggested by analysis of mutant p53 proteins that do not bind 14-3-3 in osteosarcoma cells.[4] Specifically, these mutant p53 proteins retain the ability to bind sequence-specific DNA *in vitro* and localize to the nucleus when expressed in cells, but they fail to activate transcription of endogenous p53 target genes and induce cell cycle arrest and apoptosis. These properties are very similar to the properties of wild-type p53 in melanoma cells.

The mechanism by which 14-3-3 modulates the activity of p53 is as yet unclear. 14-3-3 can affect the sequence-specific DNA binding activity of p53 (33). Thus, although wild-type p53 from melanoma cells bound DNA efficiently *in vitro*, its ability to bind to the promoters of its target genes *in vivo* may be compromised in the absence of 14-3-3. Alternatively, 14-3-3 proteins may modulate the transcriptional activity of p53. In plant cells, 14-3-3 proteins augment the transcriptional activity of several sequence-specific transcription factors. In some cases, the mechanism involves translocation of the transcription factor from the cytoplasm to the nucleus; in other cases, the mechanism involves recruitment of basal transcription factors (56–58). The latter mechanism may be more relevant for p53 because 14-3-3 proteins do not affect the intracellular localization of p53 in osteosarcoma cells,[4] and p53 was nuclear in the melanoma cell lines examined by us.

Whereas the most common mechanism leading to p53 inactivation in human cancer is mutations targeting the *p53* gene, it is becoming increasingly clear that in some tumors, the defect lies in the signaling pathways that regulate p53 in response to DNA damage. For example, the genes encoding the kinases ATM and Chk2/hCds1 are mutated in human cancer. *ATM* is required for stabilization and functional activation of p53 in response to IR and is mutated in patients with ataxia-telangiectasia, who have an increased incidence of cancer (59). *Chk2/hcds1*, which is required for p53 stabilization in response to IR, UV light, and replication blocks (20–22), is mutated in some families with the Li-Fraumeni cancer predisposition syndrome (23). According to this precedence, we propose that in melanoma cells, the regulation of p53 in response to DNA damage is defective. The defect leads to lack of interaction between p53 and 14-3-3 proteins, but its precise nature remains to be resolved. Because the 14-3-3 binding site on p53 includes Ser-376 and Ser-378 and binding occurs when Ser-376 is dephosphorylated and Ser-378 is phosphorylated (33), the defect may involve the kinase(s) and phosphatase(s) that target Ser-376 and Ser-378. Irrespective of its precise nature, a defect in p53 function could contribute to the high radioresistance of melanoma in the clinic (16, 17).

Materials and Methods

Cell Lines, Viral Vectors, and Antibodies. The melanoma cell lines used in this study were established from primary and metastatic lesions (60) and maintained in MCDB153/L15 medium containing insulin and 2% FCS. The *p53* genotype of these cell lines, which was known from the literature, was confirmed by sequencing cDNA amplified by PCR using *p53*-specific primers. Isolation and maintenance of normal melanocytes from the neonatal foreskin have been described previously (60). An adenoviral vector containing the *p53* gene under the control of the cytomegalovirus promoter (Ad-*p53*) has been described previously (61). This adenoviral vector and a vector containing the *lacZ* reporter gene (Ad-*lacZ*) were produced in human 293 cells, purified by double $CsCl_2$ centrifugation, and titrated according to standard procedures (62). Antibodies to p53 (PAb421 and DO1), p21 (EA10), and 14-3-3 (K19) were obtained from Calbiochem (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA).

p53 Protein Levels, Ser-376 Phosphorylation State, and DNA Binding Activity. Nuclear extracts were prepared as described previously (33). p53 protein levels were determined by IB of the extracts with antibody DO1. The phosphorylation state of Ser-376 was determined by IB with antibody PAb421, which recognizes p53 only when Ser-376 is dephosphorylated. For DNA binding, the nuclear extracts were incubated with ^{32}P-labeled oligonucleotide Ep21, which contains the p53 response element in the *p21/cip1/waf1* gene, and analyzed for p53 DNA binding by native PAGE (63).

Immunofluorescence. Melanoma cells were grown on glass coverslips, fixed in 1% paraformaldehyde, permeabilized with Triton X-100, and incubated with antibody DO1. After washing, the cells were incubated with a Texas red-conjugated secondary antibody and stained with 4′,6-diamidino-2-phenylindole.

p53 Transactivation. Melanoma cells infected with Ad-*p53* or Ad-*lacZ* at a MOI of 10 were harvested 24 h after infection, and total RNA was isolated using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). The RNA (15 μg/lane) was separated by electrophoresis on 1% agarose gels, transferred to nitron membranes, and probed for expression of p53 target genes.

Cell Growth and Apoptosis. Cell growth was assayed by [^{3}H]thymidine incorporation. Briefly, the melanoma cells were seeded in 96-well plates at 3×10^4 cells/well. Twenty-four h later, the cells were treated in quadruplicate with Ad-*lacZ* or Ad-*p53* at various levels of MOI for 3 h. Sixty h later, the cells were pulsed for 4 h with [^{3}H]thymidine, harvested on GF/C Whatman filter paper, and counted for radioactivity. The experiments were repeated twice. Apoptosis was examined in response to irradiation or in response to infection with Ad-*p53* or Ad-*lacZ* at a MOI of 10 using the TUNEL assay or propidium iodide staining (64, 65). For the TUNEL assay, the cells were harvested in 1% paraformaldehyde in PBS to immobilize the apoptotic DNA and then fixed with 80% ethanol to permeabilize the cells. The apoptotic DNA fragments were end-labeled with fluorescein-labeled dUTP using terminal deoxynucleotidyl transferase and analyzed by flow cytometry. For the propidium iodide assay, the cells were fixed in 80% ethanol, stained with propidium iodide in the presence of RNase A, and analyzed by flow cytometry.

Acknowledgments

We thank David Elder, Donna George, Dorothee Herlyn, Daniel Scolnick, and Elena Stavridi for helpful discussions and reagents.

References

1. Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold. Cell, *78:* 539–542, 1994.

2. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. p53 status and the efficacy of cancer therapy *in vivo*. Science (Washington DC), *266:* 807–810, 1994.

3. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell, *88:* 323–331, 1997.

4. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.

5. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA, *89:* 7491–7495, 1992.

6. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (Lond.), *362:* 847–849, 1993.

7. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature (Lond.), *362:* 849–852, 1993.

8. Volkenandt, M., Schlegel, U., Nanus, D. M., and Albino, A. P. Mutational analysis of the human *p53* gene in malignant melanoma. Pigment Cell Res., *4:* 35–40, 1991.

9. Castresana, J. S., Rubio, M. P., Vazquez, J. J., Idoate, M., Sober, A. J., Seizinger, B. R., and Barnhill, R. L. Lack of allelic deletion and point mutation as mechanisms of p53 activation in human malignant melanoma. Int. J. Cancer, *55:* 562–565, 1993.

10. Weiss, J., Schwechheimer, K., Cavenee, W. K., Herlyn, M., and Arden, K. C. Mutation and expression of the *p53* gene in malignant melanoma cell lines. Int. J. Cancer, *54:* 693–699, 1993.

11. Weiss, J., Heine, M., Arden, K. C., Korner, B., Pilch, H., Herbst, R. A., and Jung, E. G. Mutation and expression of TP53 in malignant melanomas. Recent Results Cancer Res., *139:* 137–154, 1995.

12. Albino, A. P., Vidal, M. J., McNutt, N. S., Shea, C. R., Prieto, V. G., Nanus, D. M., Palmer, J. M., and Hayward, N. K. Mutation and expression of the *p53* gene in human malignant melanoma. Melanoma Res., *4:* 35–45, 1994.

13. Florenes, V. A., Oyjord, T., Holm, R., Skrede, M., Borresen, A. L., Nesland, J. M., and Fodstad, O. TP53 allele loss, mutations and expression in malignant melanoma. Br. J. Cancer, *69:* 253–259, 1994.

14. Lubbe, J., Reichel, M., Burg, G., and Kleihues, P. Absence of *p53* gene mutations in cutaneous melanoma. J. Invest. Dermatol., *102:* 819–821, 1994.

15. Montano, X., Shamsher, M., Whitehead, P., Dawson, K., and Newton, J. Analysis of p53 in human cutaneous melanoma cell lines. Oncogene, *9:* 1455–1459, 1994.

16. Geara, F. B., and Ang, K. K. Radiation therapy for malignant melanoma. Surg. Clin. N. Am., *76:* 1383–1398, 1996.

17. Jenrette, J. M. Malignant melanoma: the role of radiation therapy revisited. Semin. Oncol., *23:* 759–762, 1996.

18. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. Oncoprotein Mdm2 conceals the activation domain of tumour suppressor p53. Nature (Lond.), *362:* 857–860, 1993.

19. Matsuoka, S., Huang, M., and Elledge, S. J. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science (Washington DC), *282:* 1893–1897, 1998.

20. Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev., *14:* 289–300, 2000.

21. Chehab, N. H., Malikzay, A., Appel, M., and Halazonetis, T. D. Chk2/hCds1 functions as a DNA damage checkpoint in G_1 by stabilizing p53. Genes Dev., *14:* 278–288, 2000.

22. Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. DNA damage-induced activation of p53 by the checkpoint kinase chk2. Science (Washington DC), *287:* 1824–1827, 2000.

23. Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., Birch, J. M., Li, F. P., Garber, J. E., and Haber, D. A. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science (Washington DC), *286:* 2528–2531, 1999.

24. Maltzman, W., and Czyzyk, L. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol., *4:* 1689–1694, 1984.

25. Chernov, M., and Stark, G. R. The p53 activation and apoptosis induced by DNA damage are reversibly inhibited by salicylate. Oncogene, *14:* 2503–2510, 1997.

26. Haapajarvi, T., Pitkanen, K., Tsubari, M., and Laiho, M. p53 transactivation and protein accumulation are independently regulated by UV light in different phases of the cell cycle. Mol. Cell. Biol., *17:* 3074–3080, 1997.

27. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell, *71:* 587–597, 1992.

28. Shieh, S-Y., Ikeda, M., Taya, Y., and Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by Mdm2. Cell, *91:* 325–334, 1997.

29. Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc. Natl. Acad. Sci. USA, *96:* 13777–13782, 1999.

30. Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M., and Haupt, Y. Critical role for Ser^{20} of human p53 in the negative regulation of p53 by Mdm2. EMBO J., *18:* 1805–1814, 1999.

31. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature (Lond.), *387:* 296–299, 1997.

32. Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by mdm2. Nature (Lond.), *387:* 299–303, 1997.

33. Waterman, M. J. F., Stavridi, E. S., Waterman, J. L. F., and Halazonetis, T. D. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nat. Genet., *19:* 175–178, 1998.

34. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, *75:* 817–825, 1993.

35. Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H., and Reid, B. J. A p53-dependent mouse spindle checkpoint. Science (Washington DC), *267:* 1353–1356, 1995.

36. Minn, A. J., Boise, L. H., and Thompson, C. B. Expression of Bcl-x_L and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. Genes Dev., *10:* 2621–2631, 1996.

37. Di Leonardo, A., Khan, S. H., Linke, S. P., Greco, V., Seidita, G., and Wahl, G. M. DNA rereplication in the presence of mitotic spindle inhibitors in human and mouse fibroblasts lacking either p53 or pRb function. Cancer Res., *57:* 1013–1019, 1997.

38. Lanni, J. S., and Jacks, T. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. Mol. Cell. Biol., *18:* 1055–1064, 1998.

39. Lassam, N. J., From, L., and Kahn, H. J. Overexpression of p53 is a late event in the development of malignant melanoma. Cancer Res., *53* (Suppl. 10)*:* 2235–2238, 1993.

40. McGregor, J. M., Yu, C. C., Dublin, E. A., Barnes, D. M., Levison, D. A., and MacDonald, D. M. p53 immunoreactivity in human malignant melanoma and dysplastic naevi. Br. J. Dermatol., *128:* 606–611, 1993.

41. Platz, A., Ringborg, U., Grafstrom, E., Hoog, A., and Lagerlof, B. Immunohistochemical analysis of the N-ras p21 and the p53 proteins in naevi, primary tumours and metastases of human cutaneous malignant melanoma: increased immunopositivity in hereditary melanoma. Melanoma Res., *5:* 101–106, 1995.

42. Ross, D. A., and Wilson, G. D. Flow cytometric analysis of p53 oncoprotein expression in cutaneous melanoma. Br. J. Surg., *84:* 803–807, 1997.

43. Bae, I., Smith, M. L., Sheikh, M. S., Zhan, Q., Scudiero, D. A., Friend, S. H., O'Connor, P. M., and Fornace, A. J., Jr. An abnormality in the p53 pathway following γ-irradiation in many wild-type p53 human melanoma lines. Cancer Res., *56:* 840–847, 1996.

44. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. The p53-mdm2 autoregulatory feedback loop. Genes Dev., *7:* 1126–1132, 1993.

45. Gelsleichter, L., Gown, A. M., Zarbo, R. J., Wang, E., and Coltrera, M. D. p53 and mdm-2 expression in malignant melanoma: an immunocytochemical study of expression of p53, mdm-2, and markers of cell proliferation in primary *versus* metastatic tumors. Mod. Pathol., *8:* 530–535, 1995.

46. Tanaka, N., Ishihara, M., Lamphier, M. S., Nozawa, H., Matsuyama, T., Mak, T. W., Aizawz, S., Tokino, T., Oren, M., and Taniguchi, T. Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. Nature (Lond.), *382:* 816–818, 1996.

47. Garkavtsev, I., Grigorian, I. A., Ossovskaya, V. S., Chernov, M. V., Chumakov, P. M., and Gudkov, A. V. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. Nature (Lond.), *391:* 295–298, 1998.

48. Garkavtsev, I., Kazarov, A., Gudkov, A. V., and Riabowol, K. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat. Genet., *14:* 415–420, 1996.

49. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, *83:* 993–1000, 1995.

50. Pomerantz, J., Schreiber-Agus, N., Ligeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H-W., Cordon-Cardo, C., and DePinho, R. The Ink4a tumor suppressor gene product, p19ARF, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell, *92:* 713–723, 1998.

51. Zhang, Y., Xiong, Y., and Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppressor pathways. Cell, *92:* 725–734, 1998.

52. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A., Ally, D. S., Sheahan, M. D., Clark, W. H., Jr., Tucker, M. A., and Dracopoli, N. C. Germline p16 mutations in familial melanoma. Nat. Genet., *8:* 15–21, 1994.

53. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. Science (Washington, DC), *264:* 436–440, 1994.

54. Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., McClure, M., Aitken, J. F., Anderson, D. E., Bergman, W., Frants, R., Goldgar, D. E., Green, A., MacLennan, R., Martin, N. G., Meyer, L. J., Youl, P., Zone, J. J., Skolnick, M. H., and Cannon-Albright, L. A. Analysis of the *p16* gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat. Genet., *8:* 22–26, 1994.

55. Gruis, N. A., van der Velden, P. A., Sandkuijl, L. A., Prins, D. E., Weaver-Feldhaus, J., Kamb, A., Bergman, W., and Frants, R. R. Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. Nat. Genet., *10:* 351–353, 1995.

56. Schultz, T. F., Medina, J., Hill, A., and Quatrano, R. S. 14-3-3 proteins are part of an abscisic acid-VIVIPAROUS1 (VP1) response complex in the Em promoter and interact with VP1 and EmBP1. Plant Cell, *10:* 837–847, 1998.

57. Tang, S. J., Suen, T. C., McInnes, R. R., and Buchwald, M. Association of the TLX-2 homeodomain and 14-3-3 ϵ signaling proteins. J. Biol. Chem., *273:* 25356–25363, 1998.

58. Pan, S., Sehnke, P. C., Ferl, R. J., and Gurley, W. B. Specific interactions with TBP and TFIIB *in vitro* suggest that 14-3-3 proteins may participate in the regulation of transcription when part of a DNA binding complex. Plant Cell, *11:* 1591–1602, 1999.

59. Halazonetis, T. D., and Shiloh, Y. Many faces of ATM: Eighth International Workshop on Ataxia-Telangiectasia. Biochim. Biophys. Acta, *1424:* R45—R55, 1999.

60. Herlyn, M., Thurin, J., Balaban, G., Bennicelli, J. L., Herlyn, D., Elder, D. E., Bondi, E., Guerry, D., Nowell, P., Clark, W. H., Jr., and Koprowski, H. Characteristics of cultured human melanocytes isolated from different stages of tumor progression. Cancer Res., *45:* 5670–5676, 1985.

61. Blagosklonny, M. V., and El-Deiry, W. S. *In vitro* evaluation of a p53-expressing adenovirus as an anti-cancer drug. Int. J. Cancer, *67:* 386–392, 1996.

62. Satyamoorthy, K., Soballe, P. W., Soans, F., and Herlyn, M. Adenovirus infection enhances killing of melanoma cells by a mitotoxin. Cancer Res., *57:* 1873–1876, 1997.

63. Waterman, M. J. F., Waterman, J. L. F., and Halazonetis, T. D. An engineered four-stranded coiled coil substitutes for the tetramerization domain of wild-type p53 and alleviates transdominant inhibition by tumor-derived p53 mutants. Cancer Res., *56:* 158–163, 1996.

64. Gorczyca, W., Bigman, K., Mittelman, A., Ahmed, T., Gong, J., Melamed, M. R., and Darzynkiewicz, Z. Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. Leukemia (Baltimore), *7:* 659–670, 1993.

65. Schmid, I., Uittenbogaart, C. H., and Giorgi, J. V. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. Cytometry, *15:* 12–20, 1994.