Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5742–5746, June 1993
Genetics

p53 mutations increase resistance to ionizing radiation

(γ radiation/DNA damage/transgenic mice/carcinogenesis)

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Communicated by Arnold J. Levine, March 11, 1993 (received for review January 5, 1993)

ABSTRACT **Mouse and human tumors of diverse origin frequently have somatically acquired mutations or rearrangements of the p53 gene, or they have lost one or both copies of the gene. Although wild-type p53 protein is believed to function as a tumor-suppressor gene, it is as yet unclear how p53 mutations lead to neoplastic development. Wild-type p53 has been postulated to play a role in DNA repair, suggesting that expression of mutant forms of p53 might alter cellular resistance to the DNA damage caused by γ radiation. Moreover, p53 is thought to function as a cell cycle checkpoint after irradiation, also suggesting that mutant p53 might change the cellular proliferative response to radiation. We have used transgenic mice expressing one of two mutant alleles of p53 to test this prediction. Our results show that expression of both mutant variants of the mouse p53 gene significantly increases the cellular resistance of a variety of hematopoietic cell lineages to γ radiation. These observations provide direct evidence that p53 mutations affect the cellular response to DNA damage, either by increasing DNA repair processes or, possibly, by increasing cellular tolerance to DNA damage. The association of p53 mutations with increased radioresistance suggests possible mechanisms through which alterations in the p53 gene might lead to oncogenic transformation.**

The p53 gene is thought to play an important role in neoplastic development (1, 2). The observation that Friend virus-induced leukemia is commonly associated with rearrangements of the p53 gene *in vivo* suggested that p53 is a tumor-suppressor gene (3). Consistent with this idea, the p53 gene is frequently mutated in mouse and human tumors of diverse origin (4). Moreover, wild-type p53 is known to exert antiproliferative (5–8) and antitransforming (9, 10) effects *in vitro*. Mutant p53 alleles, on the other hand, act like oncogenes and can immortalize primary cells *in vitro* (11) and cooperate with activated *ras* in primary cell transformation (11–13). The mechanism of mutant p53 action is not clear, but it has been suggested that p53 mutations act in a dominant-negative manner to suppress the activity of the wild-type p53 protein (14). Consistent with this view, mice overexpressing mutant p53 transgenes develop tumors at a much higher frequency than their wild-type littermates (15), as do mice homozygous for a p53 null mutation (16).

The increased tumor frequency in mice harboring dominant-negative or null alleles of the p53 gene is a phenotype that these mice share with Li–Fraumeni patients. Individuals with Li–Fraumeni disease carry a germ-line p53 mutation (17, 18) and are inherently susceptible to early-onset brain, lymphoid, and bone tumors (19). In addition to their neoplastic predisposition, cells from a Li–Fraumeni family exhibit increased resistance to γ radiation (20–22). However, it is unknown whether this resistance is causally related to the presence of a germ-line p53 mutation.

Wild-type p53 has been postulated to play a role in DNA repair (23), possibly by arresting cells in late G_1 phase of the cell cycle after irradiation (24, 25). Moreover, the levels of p53 protein increase after treatment of cells with either ionizing radiation (26) or ultraviolet light (27). Thus, the expression of mutant forms of p53 might alter cellular resistance to the DNA damage caused by γ radiation.

In this report we have used transgenic mice expressing mutant alleles of p53 to measure the resistance of hematopoietic cells to ionizing γ radiation. The data show that expression of either of two mutant variants of the mouse p53 gene significantly increases the resistance of a variety of hematopoietic cell lineages to γ radiation.

MATERIALS AND METHODS

Transgenic Mice. p53-2, p53-3, and pL53-2 mice are three different lines of transgenic mice, each containing a p53 genomic transgene under the control of its endogenous promoter and differing from wild-type p53 either by an arginine-to-proline mutation at residue 193 (lines p53-2 and p53-3) or an alanine-to-valine substitution at residue 135 (line pL53-2) (15). Unlike wild-type p53, both the $p53^{Pro193}$ and $p53^{Val135}$ alleles complement *ras* in *in vitro* transformation assays (28–30). Transcription of the mutant p53 transgenes in p53-2, p53-3, and pL53-2 mice is detectable at high levels in all tissues studied, including spleen, lung, thymus, liver, kidney, and skeletal muscle (15). Animals hemizygous for either of the transgenes are fertile and show no gross developmental abnormalities, but they have an abnormally high incidence of lung, bone, and lymphoid tumors (15).

Radiation Sensitivity of Bone Marrow Cells. Adult femur bone marrow cells were obtained from transgenic mice or their wild-type littermates and were resuspended in Iscove's modified Dulbecco's medium (IMDM)/10% fetal calf serum (HyClone) at 5×10^6 cells per ml. Then 2.5×10^5 cells were added to a 15-ml test tube containing 2 ml of IMDM supplemented with 1.1% methylcellulose, 41% fetal calf serum, erythropoietin (Boehringer Mannheim) at 1.7 units/ml, and 11% X63Ag8-653 conditioned medium containing interleukin 3 (31). The mixture was vortexed and subsequently given various doses of γ radiation in a ^{137}Cs γ-irradiator at a rate of 7300 rad/hr (1 rad = 0.01 Gy). One milliliter of the irradiated cell mixture was then plated in a 35-mm suspension cell culture dish (Nunc) and incubated at 37°C under 5% CO_2. Ten days later, colonies larger than 50 cells were counted. Survival (S) is expressed as a fraction of the nonirradiated culture, and a value of 1.0 corresponds to approximately 80–100 colonies. D_0 is the amount of radiation required to reduce fractional survival to approximately 0.37 ($\ln S = -1$).

Radiation and Mutagen Sensitivity of Spleen Cells. Spleen cells from transgenic mice or their wild-type littermates were removed and resuspended to 4×10^6 cells per ml in RPMI

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Abbreviations: LPS, lipopolysaccharide; EMS, ethyl methanesulfonate; BMC, bone marrow colony-forming cell(s).

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1640 medium/10% fetal calf serum. Suspensions were then γ-irradiated, and 4×10^5 of the irradiated spleen cells were placed in one well of a flat-bottomed 96-well plate (Corning). Salmonella typhosa lipopolysaccharide (LPS; Sigma catalog no. L 2387) or concanavalin A (Con A; Pharmacia) was added to a final concentration of 20 μg/ml or 0.5 μg/ml, respectively, in a total volume of 200 μl. For mutagen sensitivity, dilutions of ethyl methanesulfonate (EMS; Sigma) were added with the Con A. Seventy-two hours after irradiation or mutagen treatment, 1 μCi (37 kBq) of [*methyl*-^{3}H]thymidine (NEN) was added to each well. Twenty hours later, cells were harvested and thymidine incorporation was measured.

Programmed Thymocyte Death. Thymuses were removed from transgenic mice or their littermates and thymocytes were resuspended to 1×10^7 per ml in RPMI 1640 medium. Some thymocytes were then given 50 rad of γ radiation, and 1×10^6 irradiated or untreated thymocytes were then added to one well of a 96-well plate. Dexamethasone (Sigma) was added to some of the unirradiated thymocytes to a final concentration of 1 μg/ml. At 24-hr intervals, viable thymocytes were counted by using the vital dye trypan blue.

RESULTS

Radiation Sensitivity of Bone Marrow Cells. To assess the effects that mutant p53 expression might have on sensitivity to ionizing radiation, we investigated the ability of γ radiation to prevent colony formation by bone marrow cells derived from p53-2 ($p53^{Pro193}$) transgenic animals. Fig. 1 shows the effects of γ radiation on the survival of bone marrow colony-forming cells (BMC) derived from either a p53-2 mouse or its nontransgenic littermate. The bone marrow colonies, which include both myeloid and erythroid lineages, were similar in size and appearance between transgenic and nontransgenic bone marrows (data not shown). Fig. 1 shows that BMC from p53-2 mice exhibited markedly increased resistance to γ radiation compared with control littermates ($P < 0.002$). The D_0, which is the amount of radiation required to reduce the number of colonies by 63%, was calculated from Fig. 1 as 280 rad for p53-2 BMC, compared with a D_0 of 175 rad for littermate nontransgenic BMC.

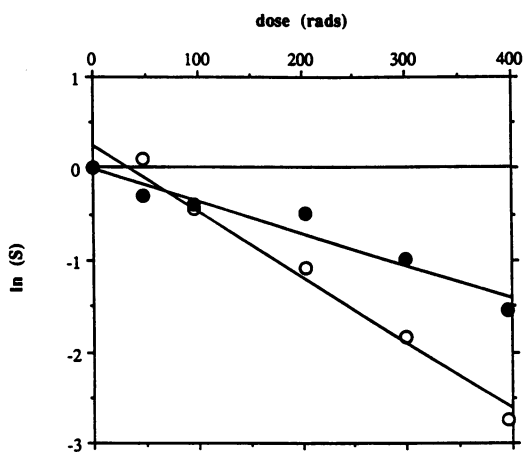

FIG. 1. Effect of γ radiation on BMC survival. Adult femur bone marrow cells were extracted from a p53-2 mouse (●) and its nontransgenic littermate (○), irradiated, and plated. Ten days later, colonies were counted, and the natural logarithm of survival (S) is plotted as a function of radiation dose. S is expressed as a fraction of the nonirradiated culture, and a value of 1.0 corresponds to approximately 80–100 colonies. Each point of the graph is the mean of duplicate cultures. The line of best fit was determined by Cricket Graph (Cricket Software, Malvern, PA), and the D_0 was determined from the equation of the line of best fit.

To confirm that the increased cellular resistance to γ radiation in p53-2 transgenic mice was a general property of mice expressing high levels of mutant p53 protein and was not the result of the integration event itself, we measured the BMC D_0 in p53-3 (also $p53^{Pro135}$) and in pL53-2 ($p53^{Val135}$) lines. As shown in Fig. 2, the mean D_0 values for BMC from p53-2, p53-3, and pL53-2 mice were 266, 268, and 248 rad, respectively, significantly ($P < 0.001$) higher than the 171-rad average D_0 of their nontransgenic littermates. The animals used in this study varied in age from 8 weeks to 11 months, with similar D_0 values between animals of all age groups (data not shown).

Radiation Sensitivity of Spleen Cells. To determine whether increased resistance to radiation could be observed in other cell types from p53 transgenic mice, we measured the effect of mutant p53 expression on the ability of splenic lymphocytes to proliferate after γ radiation. As shown in Fig. 3, splenocytes from p53-2 mice synthesized more DNA in response to LPS (Fig. 3*A*) or Con A (Fig. 3*B*) after γ irradiation than splenocytes from nontransgenic littermates. LPS preferentially stimulates the proliferation of mature, surface immunoglobulin-expressing B cells, while Con A stimulates T-cell growth. Similar results were observed with splenocytes from pL53-2 (data not shown) transgenic mice. Taken together, Figs. 1–3 suggest that expression of p53 mutant alleles confers increased resistance to γ irradiation in a number of hematopoietic cell lineages.

Mutagen Sensitivity. To test whether p53 mutations increase resistance to chemical, as well as physical, DNA-damaging agents, splenic T cells from p53-2 transgenic mice were tested for their ability to grow in the presence of EMS. EMS is an alkylating agent that causes mutations through chemical modification of DNA. As shown in Fig. 4, the proliferative response to EMS treatment was similar between T cells from p53-2 transgenic mice and their control littermates.

Programmed Cell Death. Wild-type p53 can induce a pathway of apoptotic cell death in myeloid leukemic cells (32). Increased resistance to γ radiation may result from the inability of cells expressing p53 mutant transgenes to undergo a program of cell death in response to γ radiation. One way of investigating possible alterations in pathways of programmed cell death is to look for dysfunction in thymocyte development. During T-cell development, most immature thymocytes undergo apoptotic programmed cell death (33, 34) *in vivo* and die quickly by apoptosis *in vitro* (35). In addition, *in vitro* or *in vivo* administration of the steroid hormone dexamethasone results in rapid thymocyte death (36, 37). Overexpression of the protooncogene *bcl-2*, which

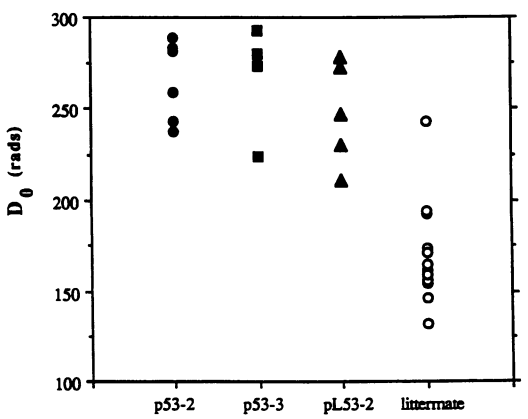

FIG. 2. Summary of the D_0 values for BMC from p53-2 (●), p53-3 (■), pL53-2 (▲), and nontransgenic littermates (○). Each point represents the D_0 calculated from a radiation survival curve of BMC derived from a single mouse as described in Fig. 1.

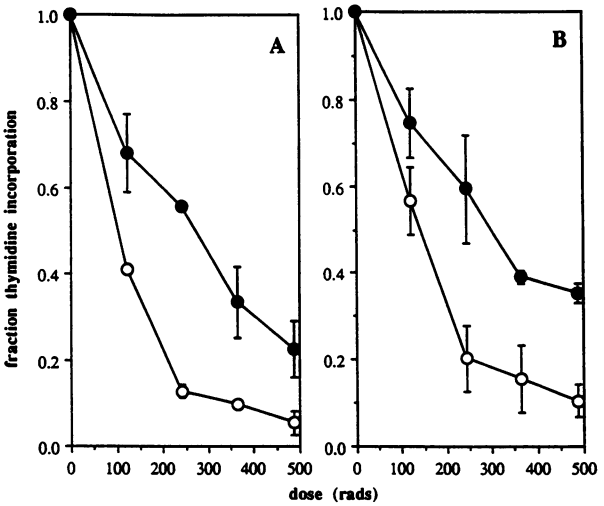

FIG. 3. Effect of radiation on the proliferation of splenic B cells (*A*) and splenic T cells (*B*). Spleen cells from p53-2 mice (●) or nontransgenic littermates (○) were removed, irradiated, and stimulated. Thymidine incorporation is expressed as a fraction of the unirradiated control and is plotted as a function of irradiation. A value of 1.0 in these experiments corresponds to absolute values in the range of 5×10^4 to 8×10^4 cpm (mean cpm: 6.1×10^4 and 6.6×10^4 for p53-2 and littermates, respectively). Each point of the graph is the mean and standard deviation of three independent experiments.

can rescue cells from programmed cell death, can slow thymic programmed death, reduce dexamethasone-induced death, and increase thymocyte resistance to γ radiation (38, 39). To determine whether mutant p53, like *bcl-2*, increases resistance to radiation through interference with programmed cell death, we investigated thymic death in p53-2 transgenic mice.

Thymocytes from p53 transgenic animals exhibit greater resistance to radiation than do thymocytes from their control littermates (Fig. 5*A*). Four days after irradiation, there were approximately twice as many surviving thymocytes from p53-2 mice compared with their wild-type littermates. However, the rate of death of untreated thymocytes *in vitro* (Fig. 5*B*) and the rate of death in response to *in vitro* dexamethasone treatment (Fig. 5*C*) were similar in both transgenic and control littermate animals. These observations suggest that mutant p53 expression results in increased resistance to γ radiation in a manner distinct from that of *bcl-2*.

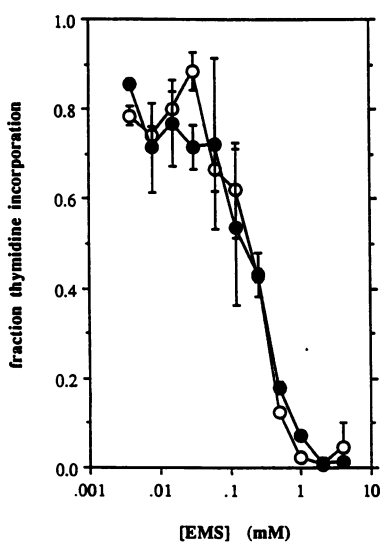

FIG. 4. Effect of EMS on the proliferation of splenic T cells. Spleen cells were removed from p53-2 transgenic mice (●) or wild-type littermates (○) and incubated with various concentrations of the alkylating agent EMS and Con A at 1 μg/ml to stimulate T-cell proliferation. Thymidine incorporation was measured 3 days later and is expressed as a fraction of an untreated control. Each point is the mean and standard deviation of three independent experiments.

DISCUSSION

In this paper we have shown that the expression of p53 mutant transgenes increases the resistance of a variety of hematopoietic cell lineages to γ radiation. This radioresistant phenotype has also been observed in fibroblasts from Li–Fraumeni patients heterozygous for a p53 mutation (20–22). The D_0 of noncancerous skin fibroblasts from members of one Li–Fraumeni family has been reported to be 20% higher than that of control fibroblasts (21). This family has a p53 protein with a glycine-to-aspartic acid mutation at residue 245 ($p53^{Asp245}$) (18). Our experiments show that overexpression of either $p53^{Pro193}$ or $p53^{Val135}$ increases the radiation resistance of mouse hematopoietic cell lineages by 45–57%. Thus, the increase in radioresistance of Li–Fraumeni cells may be directly related to their p53 mutation. The greater effects of the $p53^{Pro193}$ and $p53^{Val135}$ transgenes on radioresistance, compared with the effect of the germ-line $p53^{Asp245}$ mutation in Li–Fraumeni fibroblasts, may reflect the high levels of transgene expression, functional differences between different p53 alleles, or different cell-type-specific consequences of mutant p53 expression.

Although p53 transgenic animals show increased resistance to radiation, they do not possess increased resistance to EMS, another agent of DNA damage. Radiation causes single- and double-stranded breaks in chromosomal DNA, resulting in deletions and exchanges of DNA strands between broken chromosomes. EMS is an alkylating agent and is unlikely to cause chromosomal breakage. Therefore the effects of mutant p53 expression seem confined to increasing resistance to DNA damaging agents like ionizing radiation, which can cause DNA strand breakage.

There are several possible explanations for the increased radioresistance described here. The presence of mutant p53 may increase the activity of enzymes which repair DNA damage. If wild-type p53 normally attenuates the level or activity of DNA repair enzymes, then the loss of wild-type p53 activity brought about by the presence of a mutated p53 protein would serve to increase DNA repair. p53 has been shown to bind specifically to DNA (40, 41) and to act as either a transcriptional activator (42–44) or a repressor (45–47). It is not known whether DNA repair genes are downstream targets of the p53 protein. Alternatively, a mutant p53 protein, independent of any dominant-negative effects on wild-type p53 action, may have the capacity to increase the transcription or activity of DNA repair enzymes. The level of topoisomerase activity in a Li–Fraumeni family is increased, and it has been reported to correlate with resistance to radiation (48). Another possibility is that the p53 protein may itself be involved in DNA repair. Bain and Jenkins have recently reported[§] that a truncated wild-type p53 protein has DNA strand reassociation activity, an activity not observed in the full-length protein. Wild-type p53 may also have DNA repair activity that is under tight regulatory control. Truncation or mutation of p53 may alter the DNA repair capacity associated with the wild-type protein, resulting in increased repair activity.

In addition to p53 having a possible role in directly regulating the expression or the activity of genes involved in the

[§]Brain, R. & Jenkins, J. R., Sixth p53 Workshop, Tiberias, Israel, Nov. 1-5, 1992, p. 30 (abstr.).

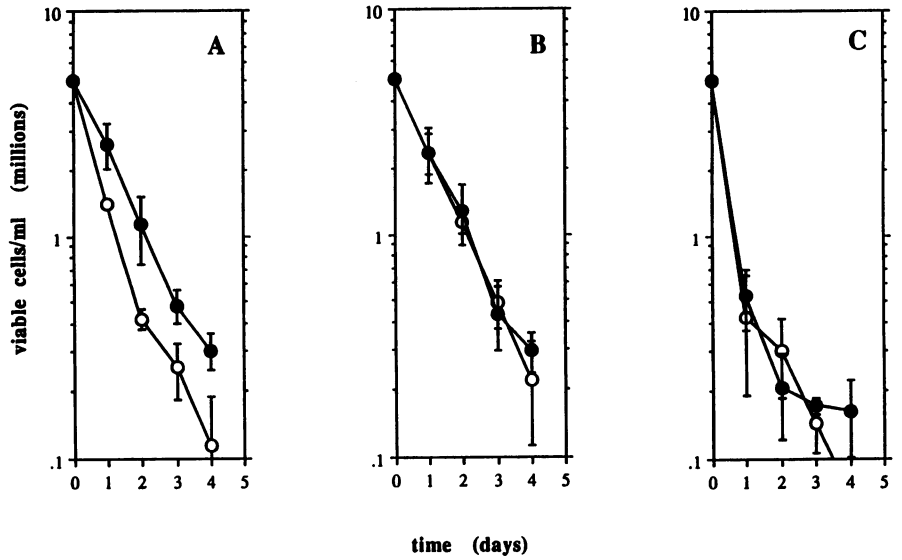

FIG. 5. Programmed cell death of thymocytes over time. Thymocytes from p53-2 (●) or wild-type littermates (○) were isolated and treated with 50 rad of γ radiation (*A*), untreated (*B*), or treated with dexamethasone at 1 μg/ml (*C*). At various times after treatment, viable cells were counted by trypan blue exclusion, and the number of surviving cells is expressed as a function of time. Each point on the curve is the mean and standard deviation of at least three independent experiments.

DNA repair process, the increased resistance to radiation brought about by mutant p53 genes might also reflect an involvement of p53 in cellular decisions to die or halt cell cycle progression after radiation damage. Loss of wild-type p53 function has been reported to perturb cell cycle control (49, 50), and recent experiments have suggested that wild-type p53 can act as a cell cycle checkpoint. Kuerbitz *et al.* (24) have recently reported that wild-type p53 can arrest the cell cycle at the G_1/S boundary after irradiation. Mutant p53 proteins interfere with this arrest, allowing cells which express mutant p53 genes to continue into S phase after irradiation. p53 may therefore function in a manner analogous to the yeast *RAD9* gene, which can arrest cells at the G_2/M boundary after irradiation, allowing damaged DNA to be repaired prior to the completion of mitosis (51, 52). The p53-mediated delay in the cell cycle after irradiation may also allow time for the repair of radiation-induced DNA damage. According to this model, a loss of p53 function would make cells more sensitive to ionizing radiation, as irradiated cells would enter into DNA synthesis prior to the repair of potentially lethal DNA lesions. Indeed, *Saccharomyces cerevisiae* strains with *RAD9* mutations are several orders of magnitude more sensitive to radiation than their wild-type counterparts (52). In the experiments reported here, however, the presence of a mutated p53 gene increased resistance to ionizing radiation. Thus, although p53 and RAD9 may both function as cell cycle checkpoints, the functional consequences of their mutations are quite distinct.

The wild-type p53 protein may normally be involved in scanning the genome for DNA damage and in signaling cell death if such damage is detected (23). Alternatively, p53 may act downstream of the detection of DNA damage, and it could be involved in the mechanics of cell death or apoptosis after DNA damage. Wild-type p53 could therefore serve as a "guardian of the genome" (23), preventing proliferation of a cell which has sustained genetic damage. If this is the case, then cells lacking wild-type p53 protein as the result of a dominant-negative action of mutant p53 might not undergo radiation-induced cell death, thereby increasing radiation resistance. Wild-type p53 is known to be able to signal apoptotic death in myeloid leukemia cells (32), and the protooncogene *bcl-2* is believed to increase resistance to γ radiation by interfering with programmed cell death (38, 39). This interference with programmed cell death results in a radiation-resistant phenotype and a perturbation of thymic death in transgenic mice overexpressing *bcl-2* (38, 39). Because p53 transgenic animals do not show dramatic evidence for attenuated thymic programmed cell death, we conclude that p53 mutations, unlike *bcl-2*, do not increase resistance to radiation by interfering with developmentally programmed cell death. However, wild-type p53 may still be acting to detect DNA damage caused by irradiation and to signal cell death.

Although cells have enzymatic machinery designed to repair DNA damage, not all DNA damage that is caused by radiation is repaired (53). Some unrepairable damage causes cell death by mitotic arrest, while other damage may not physically prevent continuation of the cell cycle. If p53 is important in controlling a radiation death pathway, then the loss of wild-type p53 function might lead to increased survival in response to radiation, but with a concomintant increase in mutation frequency and genomic instability. Loss of wild-type p53 is known to increase the frequency of gene amplification in Li–Fraumeni cells and fibroblasts derived from p53 null mice (49, 50). The accumulation of DNA damage induced by radiation or other mutagens in the presence of a mutant p53 protein might be a critical step in neoplastic development.

We thank Silvia Bacchetti, John Heddle, Xianhua Piao, and Yacob Weinstein for helpful discussion, Sandra Gardner and Jesse Luna for technical assistance, Jim Dennis for the use of his cell harvester, and Silvia Bacchetti, Richard Blouin, Lesley Forrester, Ken Kao, and Benny Motro for critical reading of the manuscript. This research is supported by a grant from the National Cancer Institute of Canada with funds from the Canadian Cancer Society. J.M.L. is a fellow of the National Cancer Institute of Canada, and A.B. is an International Research Scholar of the Howard Hughes Medical Institute.

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