Loss of p21<sup>Waf1/Cip1/Sdi1</sup> enhances intestinal stem cell survival following radiation injury

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Am J Physiol Gastrointest Liver Physiol 296: G245–G254, 2009. First published December 4, 2008; doi:10.1152/ajpgi.00021.2008.—The microcolony assay following gamma irradiation (IR) is a functional assay of intestinal stem cell fate. The cyclin-dependent kinase (CDK) inhibitor p21<sup>Waf1/Cip1/Sdi1</sup> (p21) regulates cell cycle arrest following DNA damage. To explore the role of p21 on stem cell fate, we examined the effects of p21 deletion on intestinal crypt survival following IR and expression of the stem/progenitor cell marker Musashi-1 (Msi-1) and the antiapoptotic gene survivin. Intestinal stem cell survival in adult wild-type (WT) mice was measured using the microcolony assay. Msi-1, p21, and survivin mRNA were measured using real-time PCR and immunohistochemical analysis. Laser capture microdissection (LCM) was used to isolate mRNA from the crypt stem cell zone. No differences in radiation-induced apoptosis were observed between WT and p21<sup>−/−</sup> mice. However, increased crypt survival (3.0-fold) was observed in p21<sup>−/−</sup> compared with WT mice 3.5 days after 13 Gy. Msi-1 and survivin mRNA were elevated 12- and 7.5-fold, respectively, in LCM-dissected crypts of p21<sup>−/−</sup> compared with WT mice. In conclusion, deletion of p21 results in protection of crypt stem/progenitor cells from IR-induced cell death. Furthermore, the increase in crypt survival is associated with increased numbers of Msi-1- and survivin-expressing cells in regenerative crypts.

Musashi-1; gamma irradiation; survivin; crypt survival

Colorectal cancer is the second leading cause of mortality in the United States. In the United States, the cumulative lifetime risk of developing colorectal cancer for both men and women is 6%. Despite advances in the management of this disease, the 5-yr survival rate in the United States is only 62% (18). Colorectal tumors result from mutational activation of oncogenes combined with the inactivation of tumor suppressor genes. The transformation of a normal mucosal epithelial cell to an invasive colorectal carcinoma occurs via a well-coordinated accumulation of mutations in a series of critical genes (39). The time span between initiation and gross development of tumors creates an enormous challenge in dissecting the critical molecular mechanisms that regulate neoplastic change.

In the gut, tumorigenesis is thought to arise specifically in the stem cell (2) population located at or near the base of intestinal and colonic crypts. Transit cell populations originating from the stem cell zone become fully differentiated and are eventually sloughed into the lumen (4). Any deleterious effects of mutation in these cells are limited due to the short life span of these transitional zone cells in the intestinal or colonic crypt (2).

Identifying and assaying resident intestinal stem cells is quite difficult and contentious, because no definitive specific gut stem cell markers have been identified, although recently, two novel putative stem cell markers have been described (3, 34). However, the microcolony assay following gamma irradiation is by definition a functional assay of intestinal stem cell fate (52) and can provide a mechanism for examining the early events of tumorigenesis.

The intestinal epithelium is an exquisite model for the examination of self-renewing tissues (38–40). The microcolony clonogenic stem cell assay (52, 53) measures the number of intestinal stem cells surviving after exposure to severe radiation or cytotoxic injury. In this assay the number of regenerating crypts is measured in cross sections of mouse intestine 3.5 days following a cytotoxic exposure. By this time, crypts without functional stem cells have largely disappeared or are reproductively sterile. The number of clonogenic cells present within a crypt is dependent on the level of damage induced within the crypt (5, 36). Thus the microcolony assay following gamma irradiation can provide a potential mechanism for examining the early events that occur during the initiation (DNA damaging) phase of tumorigenesis in gut stem/progenitor cells.

The cyclin-dependent kinase (CDK) inhibitor p21<sup>Waf1/Cip1/Sdi1</sup> (p21) binds and inhibits CDK/cyclin complexes that regulate the G1-to-S-phase transition of the cell cycle and plays an essential role in p53-mediated growth arrest following DNA damage (6, 12). After DNA damage, expression of p21 is transcriptionally regulated by the tumor suppressor p53 and by other p53-independent mechanisms (15). Although the p21 gene encoding is rarely mutated in human cancers, a variety of studies suggest a potential tumor suppressor function for p21. Mice lacking p21 were found to be more prone to developing malignant skin tumors following carcinogen treatment (35, 48). In another study, aging p21-deficient mice (p21<sup>−/−</sup>) developed spontaneous tumors by 16 mo of age, compared with 20 mo for wild-type (WT) mice (33). Disruption of the p21 gene enhanced colon tumor formation in mice with mutations in...
adenomatous polyposis coli (APC) (54). Furthermore, following a single dose of gamma irradiation, not only did p21−/− mice develop more tumors, but these tumors had increased metastatic potential compared with those of WT mice (26). Together, these reports implicate p21 as a critical regulator of tumorigenesis.

To examine the in vivo effect of abrogating p21 on the regulation of crypt epithelial stem cells in response to DNA damage (ionizing radiation), we have conducted the following comprehensive study. WT and p21−/− mice were irradiated, and intestinal crypt stem cell survival was measured 3.5 days later using the modified microcolony assay (10). We report higher numbers of surviving crypts (3.5-fold) in p21−/− mice compared with WT littermates. These data suggest that functional p21 promotes programmed cell death in stem/colonogenic progenitor cells in response to lethal doses of whole body gamma irradiation. These data provide additional evidence for a potential tumor suppressor function of p21 either directly or indirectly in intestinal stem cells.

MATERIALS AND METHODS

Experimental animals. Genetically matched WT and p21−/− female littermates 10–12 wk of age on the C57BL/6;129/Sv background (6) were used for all experiments (Jackson Laboratory, Bar Harbor, ME). Genomic DNA isolation and PCR were used to confirm genotypes of the mice used. Animals were housed in the same room under controlled conditions, including a 12:12-h light-dark cycle, with ad libitum access to diet and water. For crypt survival experiments, animals were killed 3.5 days after irradiation (IR). In apoptosis and mitosis studies, animals were killed 6 h after IR. In all studies, bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) was injected intraperitoneally 2 h before death. All animal experiments were performed according to the animal protocols approved by the University’s Animal Studies Committee.

Irradiation procedure. Adult mice were exposed to whole body IR with air being pumped into the chamber during exposure. A Gamma-cell 40 137Cs gamma irradiator was used with a dose rate of 3.8 Gy IR per minute. All IR treatments were begun in the morning. For apoptosis studies, a dose of 6 Gy was used. In crypt survival studies, doses of 0, 8, 12, 13, or 15 Gy IR were utilized. Two hours before death, each mouse was intraperitoneally injected with BrdU/fluorodeoxyuridine (FrdU) (10).

Tissue preparation and histological analysis. After death, the entire small intestine was removed and fixed in 10% formalin overnight. After processing, the segments were sectioned transversely at 3–5 μm. These sections were stained with hematoxylin and eosin (H&E).

Immunohistochemistry and autoradiography. Fixed sections were immunostained with the specific antibodies rabbit polyclonal anti-p21 (Calbiochem, Gibbstown, NJ), rabbit polyclonal anti-caspase-3, Musashi (Msi)-1, rabbit polyclonal anti-survivin (Cell Signaling, Danvers, MA), and rabbit monoclonal anti-phosphorylated histone H3 (Thr11) (Millipore, Billerica, MA). Immunohistochemistry was performed using biotin-conjugated appropriate secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), horseradish peroxidase-linked avidin-biotin complex reagents (Dako, Glostrup, Denmark), and 3,3'-diaminobenzidine (Sigma-Aldrich) as immunodetection substrate, as previously described (34), and then counterstained with thionine blue.

Scoring procedure. Apoptotic and mitotic cells were scored on a cell-positional basis within the crypts of the small intestine according to the method of Ijiri and Potten (24). For every staining procedure, 50 half-crypts were counted from each individual mouse in every group. Apoptosis was assessed on the evidence of morphological characteristics, such as cell shrinkage, chromat condensation, and cellular fragmentation (28) in H&E staining and in cells positive for caspase-3. Mitotic cells were identified by virtue of chromatin con-
densation in the absence of cytoplasmic and nuclear shrinkage. In many mitotic cells, discrete chromosomal structure can be observed, and in addition, mitotic cells appear horizontally displaced from the other epithelial-lining cells, toward the lumen of the intestine (25). These can be found in routine in H&E staining, and mitotic cells are positive for phosphorylated histone H3.

Real-time PCR analyses. Total RNA isolated from whole tissues or cells obtained from laser capture microdissection (LCM) were subjected to reverse transcription with SuperScript II RNase H- reverse transcriptase and random hexanucleotide primers (Invitrogen, Carlsbad, CA). The cDNA was subsequently used to perform real-time PCR by SYBR chemistry (SYBR green I; Molecular Probes/Invitrogen, Carlsbad, CA) for specific transcripts using gene-specific primers and Jumpstart Taq DNA polymerase (Sigma-Aldrich). The crossing threshold value assessed by real-time PCR was noted for the transcripts and normalized with β-actin mRNA. The changes in mRNA expression were expressed as the degree of change relative to control with values of means ± SE.

Crypt survival study. The parameters measured in the intestine were the number and width of surviving crypts. A surviving crypt was defined as containing five or more adjacent, BrdU-positive nuclei (10, 21). The circumference of a transverse section of intestine was used as a unit of length, and the number of crypts was scored in each circumference. Usually, six circumferences per mouse and four mice per experimental group were assessed. Crypt width was measured at the middle of each longitudinal crypt section using a Zeiss Axio-HOME computer-driven microscope system. Twenty cross sections were measured in this way for each mouse.

Data analysis for crypt survival. For generation of the crypt survival curve, all data were corrected for changes in crypt size following IR by using previously established procedures (19, 42), thus accounting for the fact that smaller surviving crypts would be less likely to be detected in a given tissue section.

Laser capture microdissection. Tissue collected from crypts using LCM was transferred onto CapSure HS LCM Caps (Molecular Devices, Sunnyvale, CA) using the supplied protocol. Total RNA was then isolated using the Pico Pure RNA isolation kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer’s protocol for use with CapSure HS LCM. DNA was removed from the samples by treating the purification columns with DNase and utilizing the RNase-Free DNase Set (Qiagen, Valencia, CA) according to the manufacturer’s protocol. After one round of amplification, antisense RNAs were reverse transcribed to cDNAs with SuperScript III, T7 Oligo(dT) primer, and Random Primer (Invitrogen). Reaction conditions were 25°C for 10 min, 50°C for 50 min, and 70°C for 15 min. The cDNA samples were then amplified using iQ SYBR Supermix (Bio-Rad Laboratories, Hercules, CA) at 95°C for 3 min, and then at 95°C for 15 s and 62°C for 15 s for a total of 40 cycles. All PCR reactions were performed in triplicate, or greater, in an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). Relative mRNA levels were assessed by standardization to β-actin.

RESULTS

CDK inhibitor p21 is expressed in intestinal crypts. To determine the cell-specific expression patterns of p21 in the

Fig. 2. Mitosis in uninjured WT and p21−/− mice. Mitotic figures were morphologically assessed following hematoxylin and eosin (H&E) staining in intestines of WT (A; arrows) and p21−/− mice (B; arrows) at baseline. Phosphorylated (p) histone H3 immunostaining was performed on the intestines of nonirradiated WT (C; arrows) and p21−/− mice (D; arrows). E: quantitation of mitotic figures counted in 10 high-power fields. Data are means ± SE; n = 4 mice per group. *P < 0.05 compared with WT mice.
intestinal crypt epithelial cells, we employed immunohistochemical techniques, using antibodies specific for p21 (51). Immunoreactive p21 is present primarily within the nucleus of several epithelial cells in uninjured adult WT mice (see Fig. 1A) at or near the stem cell zone (positions 4–6). We did not observe any immunoreactive p21 in p21<sup>-/-</sup> mice (see Fig. 1B).

p21 is expressed in the intestinal crypts following radiation injury. To investigate whether ionizing radiation altered p21 expression, we administered 6 Gy IR to adult WT mice. We chose this dose because it is sufficient to induce apoptosis in the crypt, yet below the threshold necessary to kill most of the stem cells in a particular crypt (10, 21, 22). We observed a modest (1.8-fold) increase in p21 mRNA expression in the WT mouse intestine 6 h post-IR (see Fig. 1D) compared with nonirradiated controls. Immunohistochemical analysis revealed an increased number of p21-expressing cells within the crypt with a gradient trending toward crypt villus junction (transit cells). Furthermore, we observed an overall increase in cytoplasmic expression of p21 (Fig. 1C).

Effect of p21 deletion on intestinal epithelial apoptosis and mitosis in nonirradiated mice. We assessed apoptosis and mitosis in intestinal epithelial cells of WT and p21<sup>-/-</sup> mice. Apoptosis was assessed by morphological characteristics, such as cell shrinkage, chromatin condensation, and cellular fragmentation on routine H&E-stained sections. In control animals (without IR), spontaneous apoptosis was rare with no significant differences noted in WT compared with p21<sup>-/-</sup> mice (data not shown). To investigate whether deletion of p21 affected crypt epithelial mitosis, we evaluated the number of intestinal crypt epithelial cell mitotic figures in WT and p21<sup>-/-</sup> mice. Mitotic figures were scored using morphologic appearance shown by H&E in WT (Fig. 2A and B) and p21<sup>-/-</sup> mice (Fig. 2C and D). There was a significant increase (5-fold) in the number of crypt epithelial mitotic figures in nonirradiated p21<sup>-/-</sup> compared with WT mice (Fig. 2E). These data together suggest that at baseline, there is no change in apoptosis, but there is increased mitosis in p21<sup>-/-</sup> compared with WT mice.

Effect of p21 deletion on intestinal epithelial apoptosis and mitosis following IR. Apoptosis and mitosis were assessed in intestinal epithelial cells of WT and p21<sup>-/-</sup> mice in response to low-dose (6 Gy) IR. In response to IR, a rapid increase in apoptotic cells was observed in WT and p21<sup>-/-</sup> mice. Apoptosis was assessed by H&E staining (Fig. 3, A and B) and cleaved caspase-3 immunoreactivity (Fig. 3, C and D). After >500 crypts were reviewed, no differences in apoptosis were found between WT (Fig. 3, A and C) and p21<sup>-/-</sup> mice (Fig. 3, B and D). No phosphorylated histone H3 staining was observed in WT or p21<sup>-/-</sup> mice.

Fig. 3. Apoptosis and mitosis in WT and p21<sup>-/-</sup> mice at 6 h post-IR (6 Gy). Apoptosis was assessed morphologically by H&E staining in WT (A; arrows) and p21<sup>-/-</sup> mice (B; arrows) and by cleaved caspase-3 immunostaining in WT (C; arrows) and p21<sup>-/-</sup> mice (D; arrows). No differences in apoptosis were found between WT and p21<sup>-/-</sup> mice. Mitosis was assessed by phosphorylated histone H3 immunostaining in WT (E) and p21<sup>-/-</sup> mice (F) post-IR. No phosphorylated histone H3 staining was observed in WT or p21<sup>-/-</sup> mice.
Deletion of p21 results in increased stem cell survival following radiation injury. Although no differences in radiation-induced apoptosis were noted in the stem cell zone in p21−/− mice, given the lack of definitive stem cell markers that can reliably identify the ultimate stem cell or functional clonogenic progenitor cells (2, 43), we sought to examine the effects of p21 deletion on crypt stem cell survival after radiation injury. Adult WT and p21−/− mice received 13 Gy whole body IR. Crypt stem cell/progenitor cell survival was measured via the modified microcolony assay (10, 23). Two hours before death, animals were injected with BrdU/FrdU to label S-phase cells. Disruption of the p21 gene resulted in increased crypt stem cell survival (see Fig. 4C, magnified in Fig. 4D) compared with WT (see Fig. 4A, magnified in Fig. 4B). When quantified, we found 7.83 surviving crypts per cross section in WT mice and 23.29 crypts per cross section in p21−/− mice. This represents a threefold increase (P < 0.01) in crypt survival (Fig. 4E). Thus p21−/− mice exhibit increased numbers of functional clonogenic stem cells following severe radiation injury (13 Gy). To investigate whether the observed enhancement of stem cell survival resulted from effects on clonogenic cells primarily, we treated WT and p21−/− mice with 8 Gy IR, a dose that does not usually result in crypt stem cell sterilization (22, 25), and scored the number of crypts 3.5 days after IR. We found no significant reduction in the number of surviving crypts following 8 Gy IR compared with baseline in WT or p21−/− mice (121 vs. 116 crypts, respectively; Fig. 4F). After high-dose IR (15 Gy), there were 4.7 surviving crypts per cross section in WT mice. In p21−/− mice, however, we observed 13.94 crypts per cross section after 15 Gy IR. This represents a 2.9-fold increase (P < 0.01) in the number of surviving crypts in p21−/− mice (Fig. 4F). Thus doses below 8 Gy do not result in significant loss of crypt stem/progenitor cells. This is consistent with earlier findings demonstrated in our laboratory and others (10, 21, 22, 25). Doses higher than 8 Gy resulted in a logarithmic decrease in the number of surviving stem cells per crypt unit in WT and p21−/− mice. Deletion of p21, however, resulted in a dose-dependent increase in stem cell survival compared with WT.
Increased expression of Msi-1 in intestinal crypt in p21 null mice. To investigate whether p21 deletion induces an increase in the number of potential clonogenic progenitor cells at baseline, we determined the expression pattern of Msi-1 RNA in the intestines of WT and p21/#^-/- mice by performing real-time RT PCR. Msi-1 is a putative intestinal stem cell marker and is expressed in the stem cell zone in the intestines of adult mice (11, 41). We observed a modest increase in the Msi-1 mRNA in p21/#^-/- compared with WT mice (Fig. 5A). Immunoreactive Msi-1 was detected in both WT and in p21/#^-/- mice in the lower one-third of intestinal crypts (Fig. 5, B and C). After radiation injury, we observed an increase in immunoreactive Msi-1 at 6 h; however, we did not observe any significant differences in cell-specific Msi-1 protein immunostaining between WT and p21/#^-/- mice (data not shown).

Given the limited cell-specific expression differences of Msi-1 in the intestinal crypt, we decided to examine the relative mRNA expression of Msi-1, specifically in the putative stem cell zone. To investigate whether p21 deletion induces an increase in the number of epithelial cells expressing Msi-1 and is expressed in the stem cell zone in the intestines of WT and p21/#^-/- mice. Immunohistochemical staining (brown) for Msi-1 mRNA expression in WT mice (Fig. 5G) compared with WT mice (Fig. 6F). These data suggest that the lack of differences in Msi-1 mRNA expression in WT and p21/#^-/- mice at baseline may be the result of relative expression in whole intestinal sections compared with LCM-dissected epithelium confined to the putative stem cell zone. These data suggest that Msi-1 may have a functional role in the crypt regenerative process following radiation injury.

Interestingly, we observed distinct immunoreactive survivin protein in the crypts of p21/#^-/- (Fig. 7B) compared with WT mice (Fig. 7A), particularly in mitotic figures at baseline. These data suggest that loss of p21 results in the antiapoptotic protein expression, specifically in mitotic figures within the putative stem cell zone. Although we observed an increase in survivin protein in mitotic figures at baseline in p21/#^-/- mice, many of these mitotic figures were found at the upper portion of the intestinal crypts and outside of the putative stem cell zone. Analysis of LCM-dissected tissue from the putative stem cell zone revealed no differences in survivin mRNA between WT and p21/#^-/- mice (Fig. 7C). Thus p21 deletion may play a role in protecting mitotic cells from apoptosis via increases in survivin.

DISCUSSION

In this report we have demonstrated a marked increase in intestinal crypt stem cell/progenitor cell survival in animals with the p21 gene deleted. Moreover, the increase in survival is associated with increased expression of the putative stem cell marker Msi-1 and the antiapoptotic gene survivin. Increased expression of Msi-1 in regenerative crypts is suggestive of a functional role for Msi-1 in crypt epithelial progenitor cell proliferation. The increase in survivin expression is also consistent with its described role as an antiapoptotic protein. This

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Fig. 5. Expression of Musashi-1 (Msi-1) in WT and p21/#^-/- mice at baseline. Total RNA isolated from the intestine of WT and p21/#^-/- mice were subjected to real-time RT-PCR for Msi-1 (A). Data are means ± SE; n = 5 mice per group. *P < 0.01 compared with Msi-1 mRNA expression in WT mice. Immunohistochemical staining (brown) for Msi-1 is shown in WT (B) and p21/#^-/- mice (C).
is intriguing, because we have observed an increase in survivin staining in mitotic figures in p21\textsuperscript{−/−} mice. These data together strongly support an increased survival program in the regenerative crypts of p21\textsuperscript{−/−} mice.

Progression through the eukaryotic cell division cycle is in large part controlled by the activity of the CDKs. In the G1 phase of the cell cycle, CDK2, CDK4, and CDK6 become activated and phosphorylate the retinoblastoma protein (Rb), whose function is to sequester, and thereby inactivate, members of the E2F family of transcription factors. Phosphorylation of Rb releases E2F, allowing the transcription of a number of genes required for S-phase entry and DNA synthesis (47, 50).

Levels of p21 are regulated by transcriptional activation as well as by posttranscriptional (de)stabilization of mRNA and protein. Transcription factors that increase p21 mRNA levels are p53, Sp1, Sp3, E2Fs, STATs, and AP2, among others (15). Upregulation of p21 has been observed in a variety of cell types and in response to a number of stress and signaling...
molecules; however, the transcriptional activation of the p21 gene in response to DNA damage and oncogenic signals is primarily mediated by p53 (15).

Upon DNA damage, a cell will either activate a DNA damage checkpoint to slow down or arrest proliferation and allow for repair of the lesions or initiate apoptosis to remove cells with potentially deleterious mutations. The tumor suppressor p53 is a critical regulator of the G1 checkpoint, which acts as a mediator of the DNA damage response, sensing DNA damage and activating downstream effectors that stop cell cycle progression (45).

In response to DNA damage, the p53 level increases and its transcriptional activity is induced. Activated p53 then induces p21 expression, which results in the inhibition of CDK2 and CDK4 activity and ultimately induces cell cycle arrest.

Apoptosis provides an important protective mechanism for disposing of damaged cells that may escape growth control. Recently, in the azoxymethane (AOM) model, colonic apoptosis was greatly reduced in p21−/− mice in response to AOM treatment, compared with WT mice. These data suggest that p21 expression in the WT colon facilitates apoptosis of damaged cells. Although generally viewed as an inhibitor of apoptosis (reviewed in Ref. 14), targeted overexpression of p21 expression has been shown to enhance apoptosis in some systems (17, 27, 32, 46, 49). Disruption of p21 expression also has been shown to lead to decreased cell death (9, 20, 31). Thus, the contribution of p21 to cell death is likely dependent on the cell type and the nature of insult to which the cells are subjected.

The acute gut epithelial response to ionizing radiation has been well studied and represents an ideal model to study the cell-specific effects of p21 deletion in the crypt. Epithelial cells at the lower one-third of small intestinal crypts are the first to undergo apoptosis following low-dose IR (1 Gy) (44). It is postulated that these “true” or “ultimate” stem cells are programmed to undergo apoptosis rather than repair even relatively minor damage to their DNA. This may serve to reduce the risk of propagating a mutated clone within the crypt. If all of the so-called “ultimate stem cells” (39) are destroyed, then their more radioresistant daughter cells will assume stem cell functions and maintain the crypt. This assumption of stem cell capacity may contribute to the accumulation of mutations leading to tumorigenesis. The molecular mechanisms that regulate this transfer of clonogenic capacity, however, are poorly understood. Radiation experiments indicate that a single surviving clonogenic (daughter) cell can form a regenerative crypt containing all cell lineages (i.e., enterocytes, goblet cells, Paneth cells, and endocrine cells) and is therefore pluripotent (16).

The findings reported presently demonstrate an increase in the epithelial proliferative response in p21−/− mice or an adaptive protective response of regenerative crypt epithelial cells to further insult. Furthermore, these data support the hypothesis that p21 expression appears to confer an increased susceptibility to radiation-induced deletion of stem cell/clonogenic progenitor cells in the intestinal crypt. These data provide further evidence of a potential tumor suppressor function for p21 in gastrointestinal intestinal stem cells. This is in contrast to the reported antiapoptotic effects of p21 in other cell types and in other contexts (13). Moreover, this discrepancy in pro- or antiapoptotic function is revealed in our own data, which did not identify a difference in spontaneous or radiation-induced crypt apoptosis between WT and p21−/− mice. This is consistent with earlier findings reported by Brugarolas et al. (6). In that earlier report, however, clonogenic assays were not reported. There is an increasing amount of data suggesting a proapoptotic role for p21, however, in human clinical trials. Patients with colorectal tumors that expressed p21 had a
greater response to systemic chemotherapy (8), suggesting that p21 expression also may enhance apoptosis at some point during the course of cancer treatment. Determining the mechanisms underlying the ability of p21 to suppress or enhance apoptosis under different physiological conditions will clarify its role as a potential therapeutic target. This is quite important in that there are several CDK inhibitors that have been shown to cause apoptosis in a number of cancer cell lines, and several are in clinical trials. Our data, however, would support the view that the effects of CDK inhibition may be directly related to the cell type or tumor being investigated. There is the distinct possibility that increasing p21 expression particularly in intestinal or colonic stem cells or cancer stem cells, as opposed to inhibition, may be the optimal therapeutic strategy for cancer chemoprevention and/or chemotherapeutic strategies. It is clear that identification of the stem cell using reliable markers is essential to increase our understanding the molecular events that regulate stem cell fate.

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