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Tissue-specific Induction of p53 Targets *in Vivo*[1]

Peiwen Fei, Eric J. Bernhard, and Wafik S. El-Deiry[2]

Laboratory of Molecular Oncology and Cell Cycle Regulation, Howard Hughes Medical Institute, Departments of Medicine, Pharmacology, and Genetics, University of Pennsylvania School of Medicine and Abramson Cancer Center, Philadelphia, Pennsylvania 19104 [P. F. and W. S. E-D.], and Department of Radiation Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 [E. J. B.]

ABSTRACT

The *in vivo* response to radiotherapy is not well understood but appears to involve the p53 tumor suppressor protein. We investigated the expression of apoptosis-inducing p53 target genes during γ-irradiation-induced cell death in $p53^{+/+}$ or $p53^{-/-}$ mouse tissues using *in situ* hybridization. Our results reveal striking tissue specificity with distinct regulation of target p53-induced genes in different cells and tissue compartments, as well as variations in dependence on p53 for basal expression. p53-dependent induction of *Puma* occurred in the splenic white pulp, whereas *Noxa* and *Bid* were induced in the red pulp. These patterns correlated with activation of caspase-3 in both compartments. All apoptotic targets of p53 studied here (*DR5, Bid, Puma, Noxa*) were induced in the jejunum and ileum, which appeared to be the tissues most sensitive to irradiation. We also observed unexpected differences in p53 target gene activation between the transverse and descending colon. Finally, in the liver where irradiation did not lead to caspase-3 activation, we primarily observed $p21^{WAF1}$ induction as the major p53-dependent target gene response. Our findings indicate that the selectivity of p53 in transactivation following DNA damage *in vivo* results in unique tissue and cell type specificity, which may correlate with growth arrest or variable sensitivity to γ-irradiation.

INTRODUCTION

wtp53[3] protein exerts inhibitory effects toward the growth of abnormal cells and has been considered a guardian of the genome in preventing cancer development (1–4). This concept is supported by the fact that the p53 gene is the most common target for mutation in human cancer (5), that p53 knockout mice show a high incidence of tumor development (6), and that germ-line mutation of one p53 allele in humans gives rise to the Li-Fraumeni cancer-susceptibility syndrome (6). wtp53 is a short-lived protein with a rapid turnover under normal unstressed conditions. The precise mechanism by which p53 is activated by cellular stress is not completely understood. Upon genotoxic insult, a rapid stabilization of the p53 protein and its activation leads to cell cycle arrest and/or apoptosis; the arrest allows cells to repair damaged DNA, whereas apoptosis removes damaged cells from the replicative pool to maintain genome integrity (1, 7). The biochemical function of p53 that best explains its effects is its sequence-specific transcriptional activity that transactivates target genes through binding a consensus motif in their genomic DNA sequences (8–11).

The ability of p53 to promote cell cycle arrest is well understood in terms of its ability to transactivate three critical target genes: *$p21^{WAF1}$*; *GADD45*; and *14-3-3σ* (12–14). $p21^{WAF1}$ protein binds to and inactivates cyclin-dependent kinases, arrests cells in G_1 and prevents S-phase entry. GADD45 and 14-3-3σ appear to be involved in control of the G_2-M transition (15, 16). A number of p53 target genes with proapoptotic activity have been identified. They fall into three groups based on their subcellular location (17). The first group of genes encode proteins that localize to the cell membrane (*e.g.*, *CD95*, *KILLER/DR5*, *PERP*). The KILLER/DR5 and CD95 (Fas/APO-1) proteins are two unique members of the tumor necrosis factor receptor superfamily that are induced by DNA damage in a p53-dependent manner and appear to be sufficient to induce apoptosis in some systems (18–21). PERP is a plasma membrane protein whose induction by doxorubicin is correlated with activation of the p53-dependent apoptotic pathway in transformed mouse embryonic fibroblasts (22). The second group of genes encode proteins that localize to the cytoplasm, including *PIDD* and *PIG*s. *PIDD* can be up-regulated by γ-irradiation through a transcriptional mechanism (23). *PIG*s (p53-induced genes) have been found to be involved in apoptosis by generating or responding to oxidative stress (24). The third group of genes encode proteins that localize to the mitochondria (*e.g.*, *Bax*, *Noxa*, *Puma*, *p53Aip1*). Bax, the best characterized mediator of p53-dependent apoptosis, translocates to the mitochondria in response to DNA damage and, in turn, induces cytochrome *c* release from the mitochondria (25). Both *Noxa* and *p53Aip1* are dependent on p53 for induction following DNA damage. Furthermore, *p53Aip1* induction in response to DNA damage correlates with the phosphorylation of p53 at serine 46 and apoptosis induction (26, 27). *Puma* expression inhibits cell growth and rapidly induces apoptosis through a pathway involving cytochrome *c* release and activation of caspases 9 and 3 (28, 29). *Bid* was very recently found to be a p53 target and may contribute to chemosensitivity (30).

$p53^{+/+}$ and $p53^{-/-}$ mice have been used to study the roles of p53 itself and its previously defined targets in radiosensitivity *in vivo* (31–37). p53 null-mice have been found to be resistant to apoptosis induced by γ-irradiation in the developing nervous system (35), spleen, thymus (36), and the small intestine (31, 36, 37). Additionally, p53-null mice have been found to be resistant to the apoptosis triggered by 5-fluorouracil in small intestine (38, 39) by 1-β-D-arabinofuranosylcytosine in sympathetic neurons (40) and by adriamycin in the thymus, spleen, and small intestine (36). The activity of the p53 apoptotic pathway varies widely between tissues. A systematic investigation p53 target gene induction *in vivo* is of interest because it may lead to strategies for possible interference with expression. In addition, understanding the patterns of gene induction *in vivo* may help with the elucidation of pathways of cross-talk between factors affecting cell fate after irradiation.

To date, although a large number of p53 targets have been identified as candidate effectors of p53-dependent apoptosis, none of them appears to be a principal mediator of the p53 apoptotic signal. We hypothesized there may be uniqueness among p53 targets and that there may be tissue/cell type specificity in their regulation in response to a variety of stimuli that signal p53-dependent apoptosis. In addition, we hypothesized there may be coordinate regulation of apoptotic targets that may ultimately correlate with radiosensitivity. In the present studies, one representative target of p53 function in growth arrest, *$p21^{WAF1}$* (12) and four recently identified p53 target genes functioning in cell death/apoptosis (*KILLER/DR5*, *Bid*, *Noxa*, and *Puma*; Refs. 20, 26, 28–30) were systematically studied. We found that γ-irradiation-induced p53 transactivation leads to some apparent

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[2] To whom requests for reprints should be addressed, at Laboratory of Molecular Oncology and Cell Cycle Regulation, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, 415 Curie Boulevard, CRB 437A, Philadelphia, PA 19104. Phone: (215) 898-9015; Fax: (215) 573-9139; E-mail: wafik@mail.med.upenn.edu.

[3] The abbreviations used are: wtp53, wild-type p53; ABC, avidin-biotin complex; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

overlap as well as unique tissue/cell type specificity. The observed patterns may correlate with caspase-3 activation in the corresponding tissues and provide some insights into the variable sensitivity of tissues to γ-irradiation.

MATERIALS AND METHODS

Animals and γ-Irradiation. Five- to six-week old female $p53^{+/+}$ and $p53^{-/-}$ mice were obtained from Jackson Laboratories. Two $p53^{+/+}$ mice and two $p53^{-/-}$ mice received total body γ-irradiation using a dose of 5 Gy, whereas an additional two $p53^{+/+}$ mice and $p53^{-/-}$ mice were used as experimental controls with no treatment. The mice were euthanized 6 h later using an approved Institutional Animal Care and Use Committee Protocol, which followed recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Thymus, spleen, liver, duodenum, jejunum, ileum, transverse colon, and descending colon were harvested, fixed in 4% paraformaldehyde overnight at 4°C and paraffin embedded.

Probe Preparation and *in Situ* Hybridization. IMAGE clones of mouse *Puma* and *Noxa* purchased from ResGen Invitrogen Corporation were prepared to generate mouse *Puma* and *Noxa* cDNA fragments. Approximately 500-bp PCR products of mouse $p21^{WAF1}$ (12) mouse *KILLER/DR5* (21), mouse *Bid* (30), mouse *Puma*, and *Noxa* were cloned into the Topo-TA vector (Invitrogen) with both sp6 and T7 promoters in order to make either antisense or sense RNA probes with a Dig RNA labeling kit (SP6/T7; Roche). The subcloned cDNA fragments were sequenced to confirm the authenticity of the inserts before their use to generate *in situ* RNA probes. *In situ* hybridization was performed as described previously (41).

Immunohistochemistry. Routine 5-μm paraffin sections were prepared. Deparaffinized sections were heated for 15 min in 0.01 M citrate buffer (pH 6.2) in a microwave oven for antigen retrieval. The sections were then immunostained using the ABC peroxidase method followed by a weak hematoxylin counterstain. In some cases, 0.5% methyl green was used as the counterstain. The primary antibodies used were those that could detect active mouse Caspase-3 (Novacastra), mouse Ki67 (Novacastra), or mouse p53 (Santa Cruz Biotechnology).

TUNEL Assay. The TUNEL assay was performed by using the ApopTag Plus Peroxidase in Situ Apoptosis Detection Kit (Intergen, Purchase, NY) following the protocols provided by the manufacturer.

RESULTS

Variable Levels of p53-dependent Apoptotic Activity in γ-Irradiated Tissues. The p53 tumor suppressor pathway is a pivotal mediator of genotoxic stress responses. These responses ultimately protect the organism from accumulating genetically altered and potentially cancerous cells by inducing growth arrest and/or apoptosis in damaged cells (3, 4). To better define the relationship between the apoptotic response to γ-irradiation and p53 status/p53 target gene activation, we systematically measured caspase-3 activity in various tissues of control and irradiated (5 Gy) wild-type or p53-null mice. As expected from previous studies that have measured DNA fragmentation, poly(ADP-ribose)polymerase cleavage, or TUNEL staining in tissues, the induction of cell death after γ-irradiation as measured by the active caspase-3 assay appears to require the presence of wtp53. wtp53-containing thymus, spleen, duodenum, jejunum, ileum, transverse colon, and descending colon all induced caspase-3 activity to differing extents after irradiation (Fig. 1). The observed cell death induction was correlated with increased p53 protein expression after radiation, although there was no obvious correlation between the magnitude of p53 stabilization and the degree to which caspase-3 was activated (Figs. 1 and 2). $p53^{+/+}$ jejunum- and ileum-activated caspase-3 in response to γ-irradiation to extremely high levels as compared with other tissues. In contrast, the level of caspase-3 activity was too weak to be detected in liver tissue sections. To confirm these two extremes of possible response (ileum/jejunum *versus* liver), we performed TUNEL assay and analysis of the proliferation marker ki67 in irradiated jejunum, ileum, and liver. We detected a strong signal by TUNEL assay in irradiated $p53^{+/+}$ jejunum and ileum (Fig. 3*A*) coupled with decreased ki67 expression (Fig. 3*B*). In the liver, we detected a weak signal after irradiation by TUNEL assay and a strong signal for ki67 expression. Thus, the two extremes in caspase-3 activity between liver and small bowel have been confirmed by an independent measure of cell death *in vivo*. In summary, p53 is required for irradiation-induced apoptosis *in vivo*, and the level of p53-dependent apoptosis appears to vary widely in a tissue-specific manner with a minimum apoptosis observed in the liver, and a maximum death observed in the small bowel.

p53-dependent $p21^{WAF1}$ Induction by γ-Irradiation in Liver and Other Tissues Inversely Correlates with the Degree of p53-dependent Apoptosis *in Vivo*. The role of p53 protein in both cell proliferation and apoptosis is mainly mediated through its ability to transactivate target genes. $p21^{WAF1}$ (12, 42) is a well-known mediator of p53 function in cell cycle arrest in response to DNA damage. Moreover, numerous studies have observed that $p21^{WAF1}$ exerts a protective effect toward cell death and that deletion of $p21^{WAF1}$ confers sensitivity to a number of apoptotic stimuli. To systemically gain insight into the radiation response *in vivo* and possible influence of $p21^{WAF1}$ on death *versus* arrest responses, we examined $p21^{WAF1}$ expression by *in situ* hybridization in tissues from irradiated or control wild-type and p53-null mice. Fig. 4 and Table 1 show that $p21^{WAF1}$ was strongly induced by γ-irradiation in $p53^{+/+}$ liver (Fig. 4*A*) and descending colon (Fig. 4*C*) and slightly in $p53^{+/+}$ thymus (Fig. 4*B*), spleen (Fig. 4*B*), duodenum (Fig. 4*C*), ileum, and transverse colon (Fig. 4*A*), but there is no induction by γ-irradiation in $p53^{-/-}$ tissues studied here, except for a slight induction in $p53^{-/-}$ thymus and spleen (Fig. 4*B*) and no induction in $p53^{+/+}$ jejunum (Fig. 4*C*). $p21^{WAF1}$ basal expression was notably high in $p53^{-/-}$ jejunum, ileum, and descending colon (Fig. 4, *A* and *C*). This is consistent with previous observations regarding p53-independent $p21^{WAF1}$ expression in colonic epithelium (42). Interestingly, the level of $p21^{WAF1}$ expression was much higher in nonirradiated $p53^{-/-}$ descending colon as compared with $p53^{+/+}$ descending colon (Fig. 4*C*). As a control, no signal was detected from tissue sections after hybridization with the sense $p21^{WAF1}$ probe (data not shown). Taken together with the data shown in Fig. 1 and Table 1, the strongest $p21^{WAF1}$ induction after γ-irradiation occurred in tissues with the least or absent p53-dependent apoptotic activity. Thus, $p21^{WAF1}$ induction in tissues in response to γ-irradiation is generally p53-dependent and varies in a tissue-specific manner, which appears to inversely correlate with the level of p53-dependent apoptotic activity occurring in the corresponding tissues.

wtp53-dependent *KILLER/DR5* Induction in Response to γ-Irradiation in Thymus, Spleen, and Transverse Colon. *KILLER/DR5* was identified as a proapoptotic member of the tumor necrosis factor-related apoptosis-inducing ligand receptor family in a screen for p53 targets up-regulated in chemosensitive but not chemoresistant ovarian carcinoma cells (20). We therefore investigated *KILLER/DR5* expression by *in situ* hybridization using unirradiated and irradiated tissues from the wild-type and p53-null mice. We found (Fig. 5 and Table 1) that in $p53^{-/-}$ and $p53^{+/+}$ descending colon, $p53^{-/-}$ and $p53^{+/+}$ ileum, and $p53^{+/+}$ thymus, spleen, transverse colon, and descending colon, *KILLER/DR5* is slightly induced by γ-irradiation but not in other $p53^{-/-}$ or $p53^{+/+}$ tissues examined (liver, duodenum, jejunum). *KILLER/DR5* basal expression appears in all tissues but the thymus (Fig. 5*A*), with a wtp53-dependent induction in duodenum (Fig. 5*B*). In the thymus (Fig. 5*A*), there was a greater induction of DR5 in $p53^{+/+}$ tissue as compared with $p53^{-/-}$. In contrast, *KILLER/DR5* expression was increased by irradiation and did not correlate with wtp53 status in the ileum (Fig. 5*B*) and descending colon (Fig. 5*A*). These results indicate that *KILLER/DR5* can be expressed in a

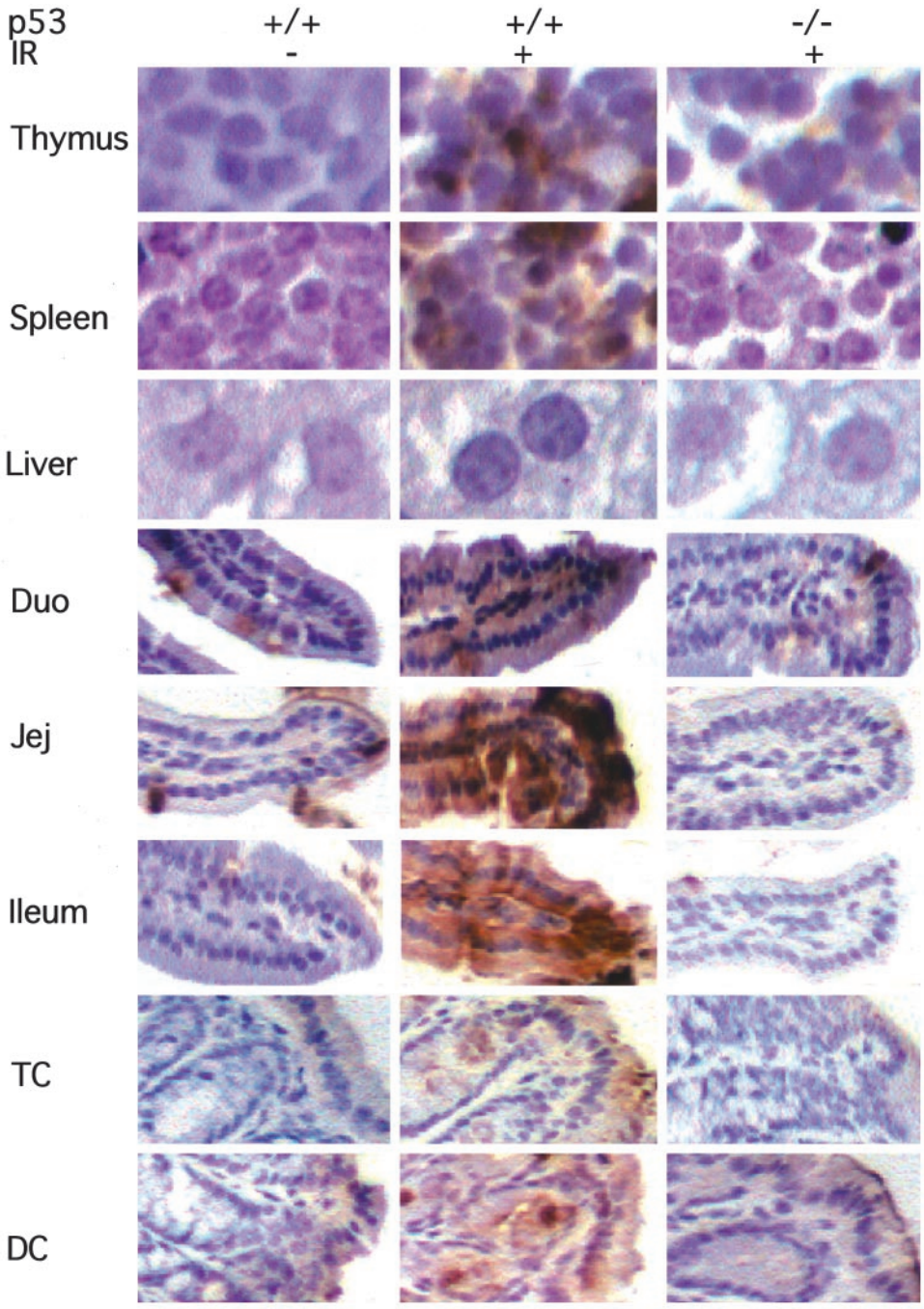

Fig. 1. γ-Irradiation-induced cell death *in vivo* is p53-dependent and varies in a tissue-specific manner. Caspase-3 activity in $p53^{+/+}$ or $p53^{-/-}$ mouse tissues. Immunohistochemistry was performed using the ABC peroxidase method by using an antibody against active caspase-3. The *brown color* located in cytoplasm represents the stained active caspase-3. The active caspase-3 appears in all irradiated $p53^{+/+}$ tissues except liver. Images of nonirradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues (Duo = duodenum, Jej = jejunum, TC = transverse colon, DC = descending colon.

p53-dependent or -independent manner and that the p53 dependence varies in a tissue-specific manner (*in situ* hybridization by using sense *DR5* probe was also performed, and no signal was detectable; data not shown). The mechanism of p53-independent *KILLER/DR5* induction *in vivo* in the thymus (Fig. 5*A*), duodenum (Fig. 5*B*), and descending colon (Fig. 5*A*) remains unclear. Possibilities include a potential role for p53 family members such as p63 or p73 or pathways that are independent of the p53 family. It should be noted, however, that the major induction of *KILLER/DR5* after γ-irradiation in the thymus and spleen (Fig. 5*A*) is p53-dependent, and in the transverse colon (Fig. 5*A*), *KILLER/DR5* induction by irradiation appears to occur exclusively in a p53-dependent manner.

***Bid* Expression Depends on wtp53 after γ-Irradiation in Spleen, Thymus, and Transverse Colon.** Bid is a BH3 homology domain-containing protein that acts as a bridge in apoptotic signaling between the extrinsic death receptor pathway and the mitochondrial pathway (43). *Bid* was recently identified as a p53 target gene (30) and was suggested to contribute to chemosensitivity. Thus, *Bid* was chosen as a target to further identify correlates between p53 target gene induction and irradiation-induced apoptosis *in vivo*. Fig. 6 and Table 1 show that among all p53-null tissues, *Bid* expression is detectable in irradiated jejunum and thymus. Among all $p53^{+/+}$ tissues, *Bid* induction by γ-irradiation shows a readily detectable increase in the transverse colon (Fig. 6*A*) and a more moderate increase in the thymus (Fig. 6*B*) and spleen (Fig. 6*A*). *Bid* basal expression, interestingly, is very strong in $p53^{+/+}$ jejunum with no detectable difference between nonirradiated and irradiated jejunum (Fig. 6*C*), indicating that *Bid* basal expression may depend on wtp53 but that induction by γ-irradiation may occur in the absence of p53 in this tissue. The signal from the sense probe was too weak to detect (data not shown). Thus, the dependence

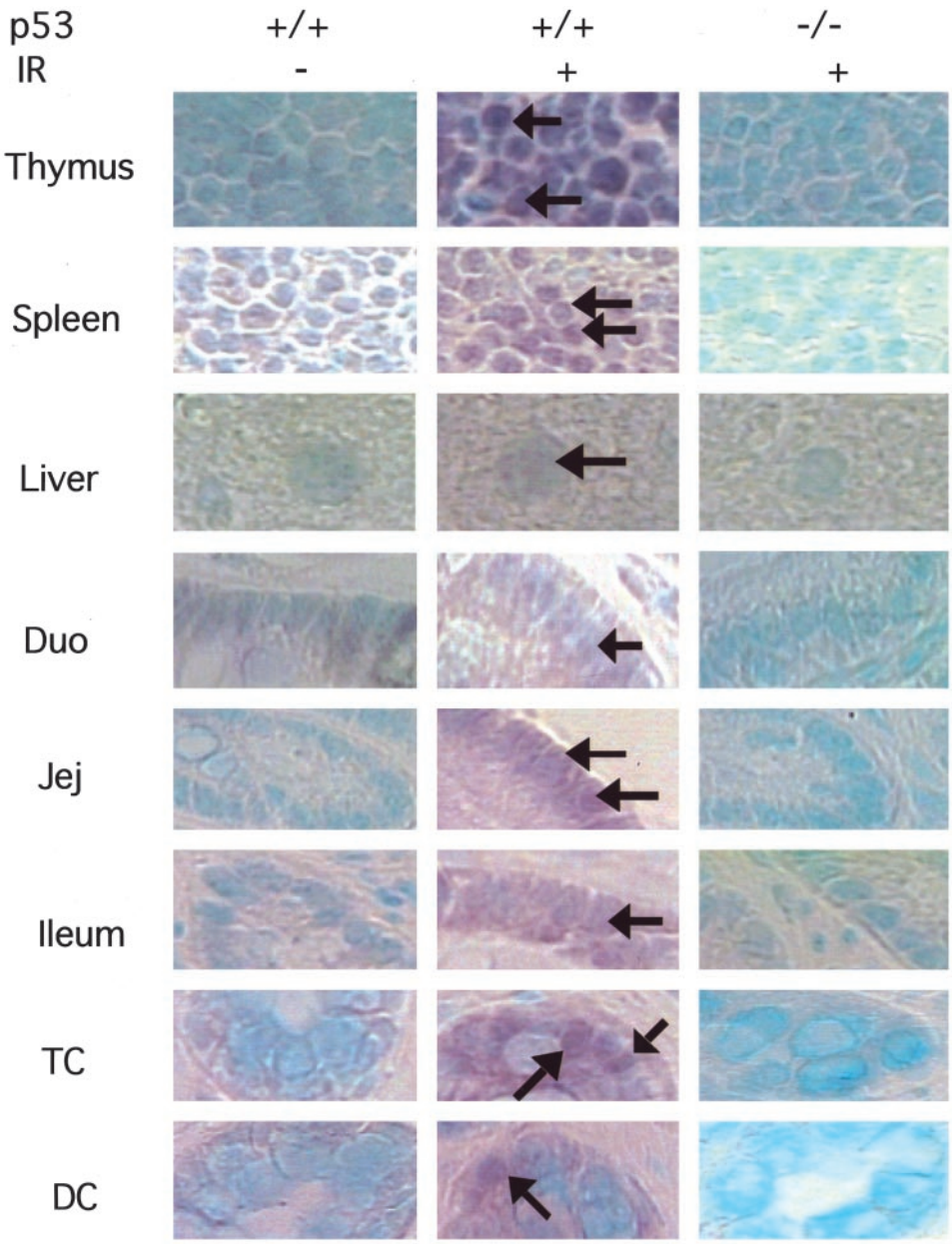

Fig. 2. Stabilization of p53 protein expression *in vivo* after γ-irradiation. *In situ* expression of p53 protein in irradiated and nonirradiated mouse tissues. Immunohistochemistry was performed on mouse tissue sections using the ABC peroxidase method by using an antibody against p53. The stained p53 protein appears as a *gray color* in the tissue sections (examples indicated by *arrows*) with a *green counterstaining.* In most of tissues presented here, the level of stained p53 is to some extent elevated in irradiated sections with $p53^{+/+}$ as compared with nonirradiated tissues. Images of nonirradiated $p53^{-/-}$ tissues (data not shown) are similar to the irradiated $p53^{-/-}$ tissues. Duo = duodenum, Jej = jejunum, TC = transverse colon, DC = descending colon.

of *Bid* expression on wtp53 and its induction after irradiation appears tissue specific.

***Noxa* Expression Is Strongly Induced in Thymus after γ-Irradiation.** *Noxa* (26) appears to be a candidate mediator of p53-dependent apoptosis as its induction after γ-irradiation in mouse cells depends on wtp53. To study *Noxa* regulation by p53 *in vivo*, we performed *in situ* hybridization to detect *Noxa* mRNA expression in tissues obtained from $p53^{+/+}$ or $p53^{-/-}$ mice with or without γ-irradiation by using the antisense and sense *Noxa* probes simultaneously. We found that *Noxa* was induced by γ-irradiation in p53-null (or $p53^{+/+}$) thymus, duodenum, jejunum, and transverse colon and in $p53^{+/+}$ (but not p53-null) spleen (Fig. 7 and Table 1). The induction of *Noxa* following γ-irradiation is very strong in $p53^{+/+}$ thymus (Fig. 7*B*), suggesting a possible role for *Noxa* induction in the cell death that occurs in the thymus. *Noxa* expression was found to be relatively high in unirradiated duodenum (Fig. 7*B*), jejunum (Fig. 7*B*), and ileum (Fig. 7*A*) from either $p53^{+/+}$ or $p53^{-/-}$ animals, as compared with the remainder of the untreated tissues that were examined. Data is not shown for the nondetectable *Noxa* sense signal. Therefore, *Noxa*, as a proapoptotic target of p53, is predicted to function mainly in the thymus, followed by spleen, presumably to mediate p53-dependent apoptosis after exposure to cellular stresses or DNA-damaging exposures such as γ-radiation. The basal expression of *Noxa* is relatively high in the small intestine and shows no obvious correlation with p53 status, suggesting possible p53-independent function in the small bowel.

Dependence of *Puma* Expression on wtp53 in Spleen and Descending Colon and Localized Induction in White Pulp but Not Red Pulp of Spleen after γ-Irradiation. *Puma* encodes a p53-up-regulated modulator of apoptosis, a member of the bcl-2 family, which may play a role in mediating p53-induced cell death through activation of mitochondrial cytochrome *c* release (28, 29). We investigated the basal expression of *Puma* as well as its induction after irradiation of wild-type and p53-null mice. We found no detectable induction of *Puma* expression by irradiation in any $p53^{-/-}$ tissue examined, except for duodenum and thymus (Fig. 8

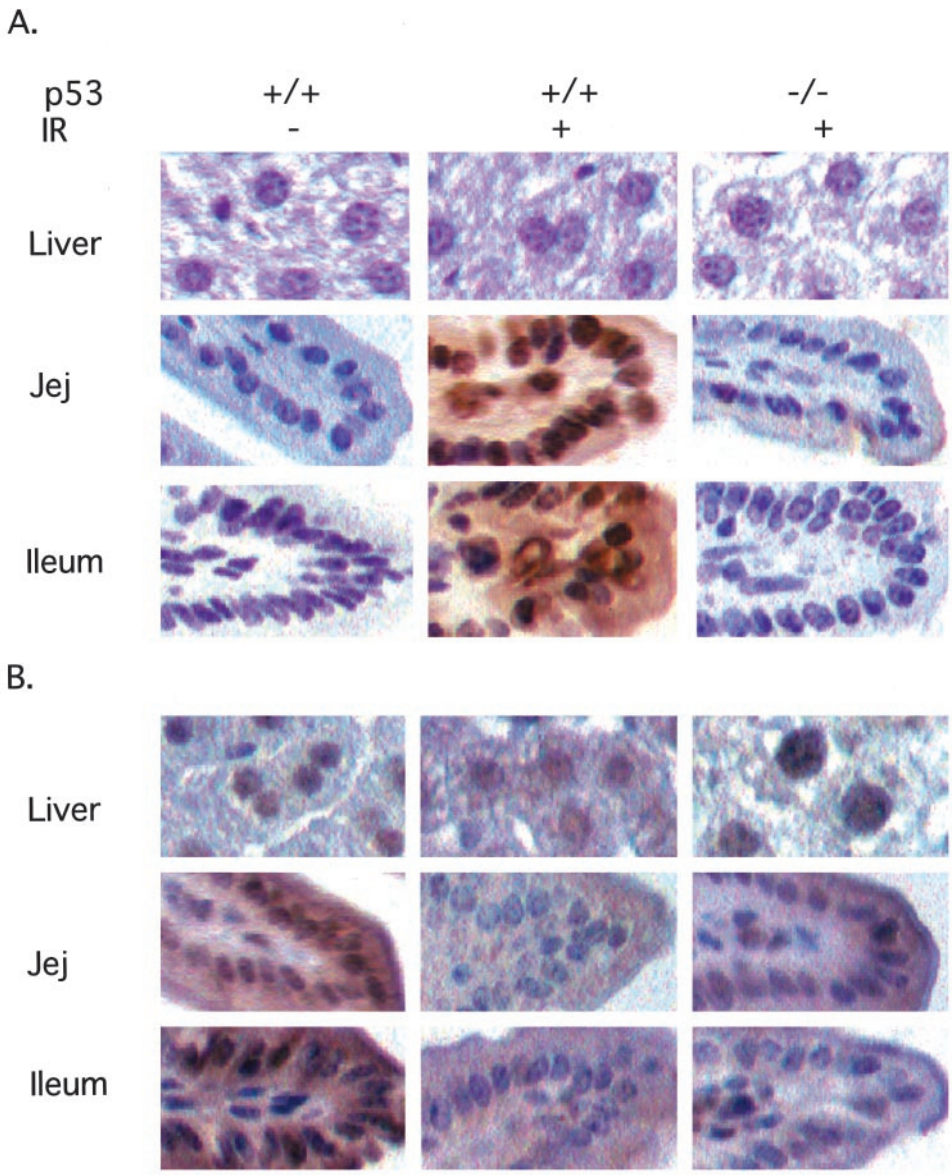

Fig. 3. Apoptotic death and growth arrest patterns in irradiated small bowel *versus* liver of wild-type and p53-null mice. *A*, TUNEL assay in mouse jejunum, ileum, and liver. ApopTag plus peroxidase kit was used. The apoptotic nuclei are stained *brown*. The *brown nuclei* appear only in $p53^{+/+}$-irradiated jejunum and ileum and not in liver. *B*, immunostaining for Ki67 expression in mouse jejunum, ileum, and liver. The expressed Ki67 protein stains nuclei *brown*. *Brown nuclei* are extensively noted in unirradiated tissues, as well as irradiated $p53^{+/+}$ liver but not in irradiated $p53^{+/+}$ jejunum and ileum. Images of non-irradiated $p53^{-/-}$ tissues are similar to irradiated $p53^{-/-}$ tissues. *Jej*, jejunum.

and Table 1). In contrast, in $p53^{+/+}$ spleen (Fig. 8*B*), *Puma* was highly induced by irradiation in the white pulp, as compared with *Noxa* and *Bid*, the induction of which occurred in the red pulp of the spleen (Figs. 6*A* and 7*A*). *Puma* was also highly induced in $p53^{+/+}$ thymus (Fig. 8*C*) and moderately induced in $p53^{+/+}$ descending colon (Fig. 8*B*) by γ-irradiation. In the $p53^{+/+}$ jejunum (Fig. 8*D*), the basal level of *Puma* expression was higher in $p53^{+/+}$ as compared with p53-null jejunum, and there was no induction observed after irradiation. Additionally, *Puma* was found to be expressed throughout the ileum (Fig. 8*A*) at similar levels between wild-type and

Table 1 *Patterns of p53 target gene expression in mouse tissues*

Tissue		Thymus		Spleen		Liver		Duodenum		Jejunum		Ileum		Transverse colon		Descending colon	
Gene	IR\p53	+[a]	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
p21waf1	−	−	−	−	−	+	+	+	+	+++	+++	++	++	−	−	+	+++
	+	++	+	++	+	++++	+	++	++	++	+++	++	++	++	−	+++	+++
DR5	−	−	−	+	+	+	+	++	+	++	++	++	++	+	+	+	+
	+	++	+	++	+	+	+	++	+	++	+	+++	+++	++	+	++	++
Bid	−	+	+	−	−	ND[c]	ND	++	+	+++	+	+	+	−	−	+	+
	+	+++	++	+[b]	−			+	+	+++	+++	+	+	+++	−	+	+
Noxa	−	+	+	−	−	+	+	++	++	++	++	++	++	−	−	+	+
	+	++++	++	+[b]	−	+	+	+++	+++	++++	++++	++	++	+++	+++	+	+
Puma	−	+	+	−	−	+	+	+	−	+++	++	++	++	−	−	++	++
	+	+++	++	++++[b]	−	+	+	+	+	+++	++	++	++	−	−	+++	++

[a] −, not detectable; +, weak expression; ++, moderate expression; +++, strong expression; ++++, stronger expression.
[b] Tissue compartment restriction (see Figs. 6, 7, and 8).
[c] ND, not determined.

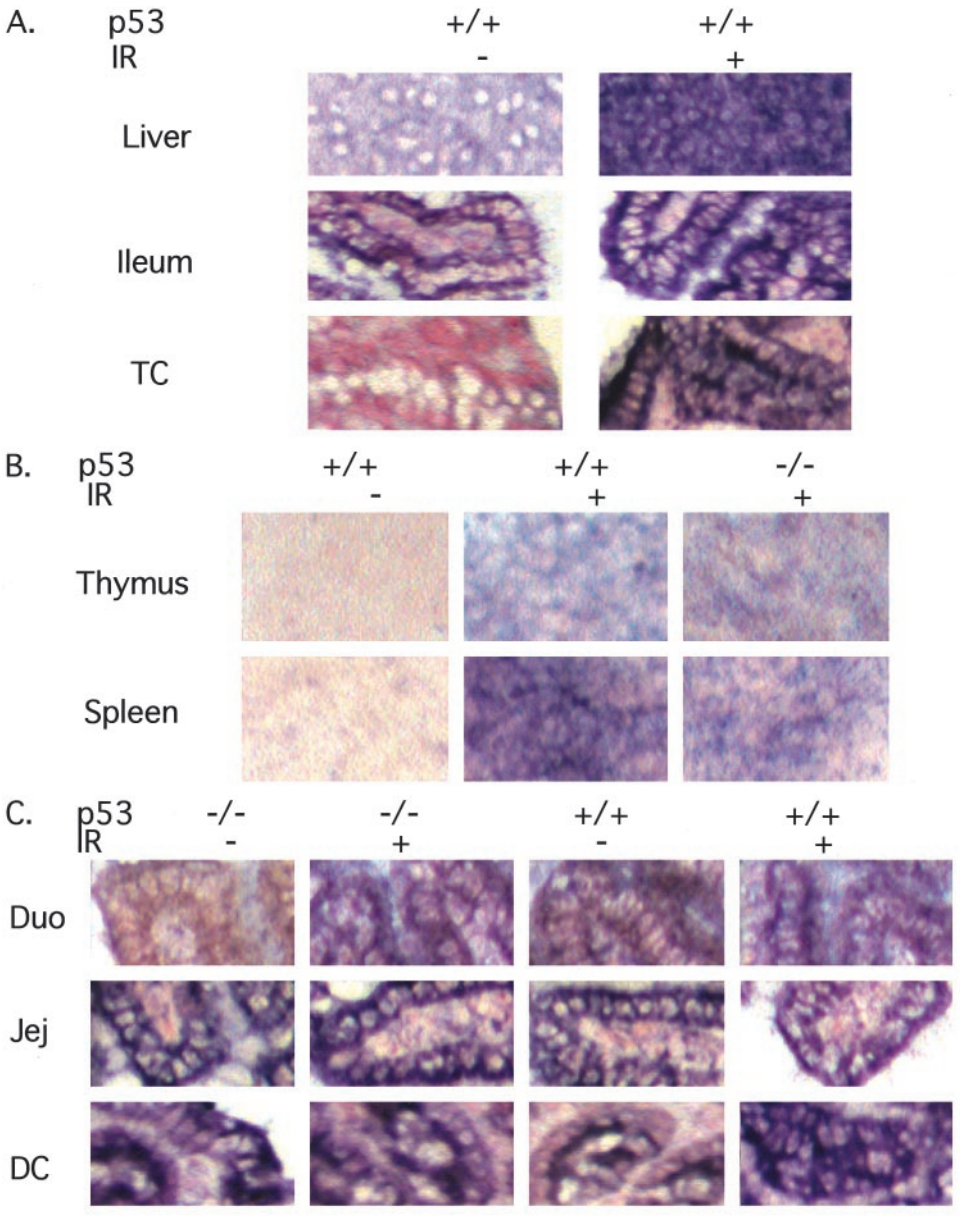

Fig. 4. p53-dependent and -independent expression of p21 *in vivo*; unique induction of p21 as a p53 target gene in liver following γ-irradiation. *A*, p21waf1 mRNA expression in mouse liver, ileum, and transverse colon. The signal appears as *blue/dark purple* with *pink/red color* counterstaining. The *blue color* is significantly noted in irradiated $p53^{+/+}$ liver and moderately noted in irradiated $p53^{+/+}$ ileum and transverse colon. The images of nonirradiated and irradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. TC = transverse colon. *B*, p21waf1 mRNA expression in mouse thymus and spleen. The signal appears as *blue/dark purple* with *pink/red color* counterstaining. The *blue color* is moderately noted in irradiated $p53^{+/+}$ thymus and spleen and is also detectable in irradiated $p53^{-/-}$ thymus and spleen but not in nonirradiated tissues. The images of nonirradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. *C*, p21waf1 mRNA expression in mouse duodenum, jejunum, and descending colon. The signal appears as *blue/dark purple* with *pink/red color* counterstaining. The *blue signal* is readily noted in all tissue sections but the nonirradiated $p53^{-/-}$ duodenum and nonirradiated $p53^{+/+}$ descending colon. Duo = duodenum, Jej = jejunum, DC = descending colon.

p53-null mice. There was no induction of *Puma* in the ileum after irradiation (Fig. 8*A*), and no detectable sense probe signal in all tissue sections presented here (data not shown). These results indicate that the basal expression of *Puma*, induction of its expression in response to γ-irradiation, and dependence of its expression on wtp53 vary in a tissue-specific manner. p53-dependent induction of *Puma* in the splenic white pulp provides a clear example where even within a given tissue, a p53-induced target can be restricted to a compartment.

DISCUSSION

A number of recently discovered p53 target genes proposed as candidate mediators of DNA damage-induced apoptosis has been examined here for their induction during cell death *in vivo*. Our results provide critical new insights into the complexity of the radiation response within tissues *in vivo*. We recognized several patterns of gene expression by *in situ* hybridization using probes for p53 target genes, including the cyclin-dependent kinase inhibitor $p21^{WAF1}$, as well as the proapoptotic genes *KILLER/DR5*, *Noxa*, *Bid*, and *Puma*. The observed patterns include the following (see Table 1): (*a*) no detectable basal expression in either wtp53-expressing or p53-null tissues but strong induction after exposure to 5 Gy γ-radiation; (*b*) detectable basal expression in $p53^{+/+}$ and $p53^{-/-}$ tissue and additional induction after irradiation; (*c*) detectable basal expression in $p53^{+/+}$ and $p53^{-/-}$ tissue but without additional induction of gene expression after irradiation; (*d*) no detectable expression in either $p53^{+/+}$ or $p53^{-/-}$ tissue and no induction after irradiation; and (*e*) induction of gene expression after irradiation in a p53-dependent manner that is restricted to a particular tissue compartment. In addition to the patterns of gene expression, we found evidence that the coordinated induction of certain p53 targets (or the lack thereof) may correlate well with whether apoptosis was observed and also with the degree to which a particular tissue underwent rapid or massive cell death *in vivo*. Finally, our study provides detailed comparisons showing remarkable differences in the patterns of gene expression before and after radiation at progressively distal locations within the gastrointestinal tract or within compartments of a given tissue such as the spleen.

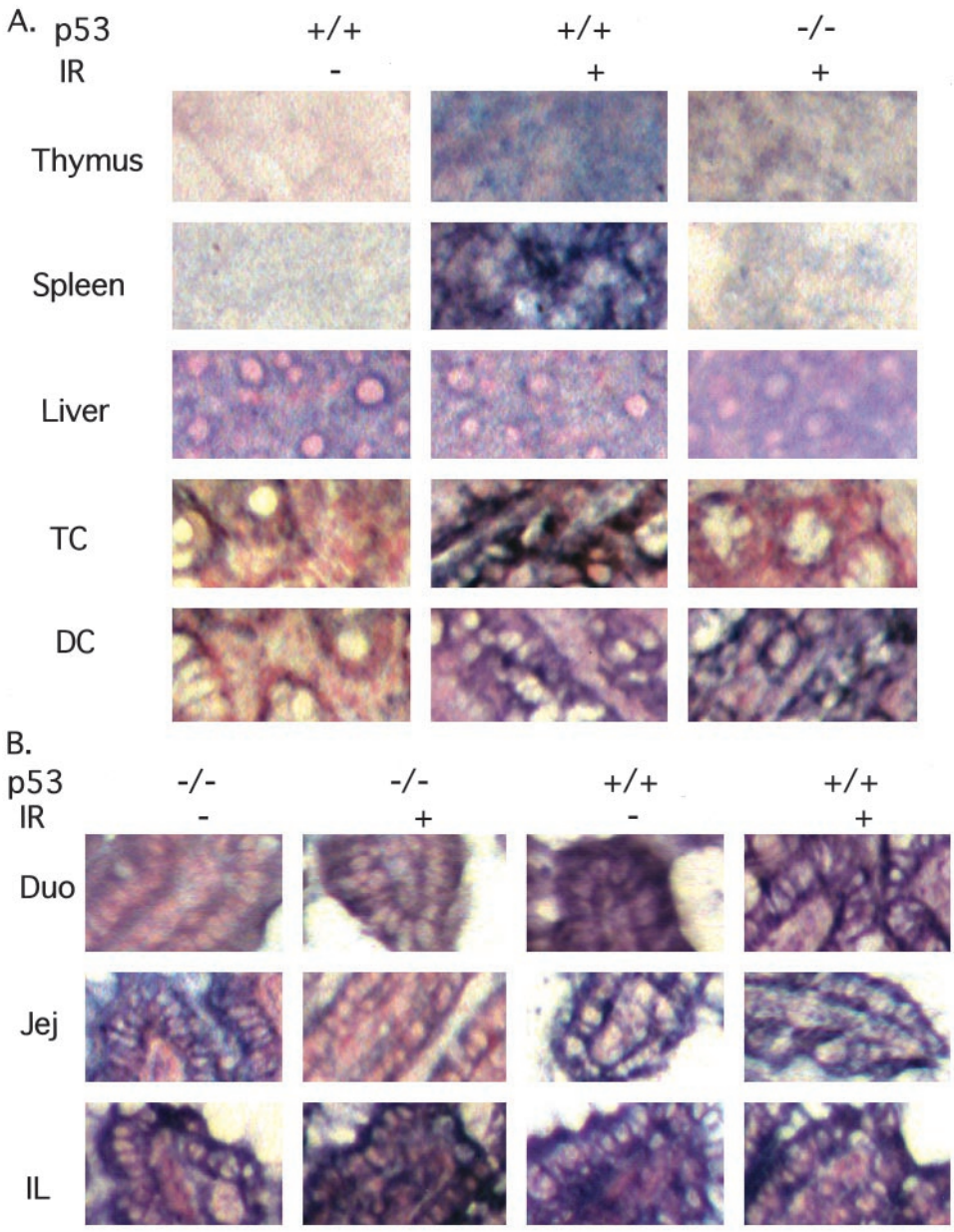

Fig. 5. Basal and p53-dependent γ-irradiation-induced KILLER/DR5 expression *in vivo*. *A*, *In situ DR5* mRNA expression in mouse thymus, spleen, liver, transverse colon, and descending colon. The signal appears as *blue/dark purple color* with *pink/red color* as the counterstaining. The elevated *blue color* is noted in irradiated $p53^{+/+}$ thymus, spleen, transverse, and descending colon and in irradiated $p53^{-/-}$ thymus, spleen, and descending colon as compared with nonirradiated tissues. The images of nonirradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. TC = transverse colon, DC = descending colon. *B*, *In situ DR5* mRNA expression in mouse duodenum, jejunum, and ileum. The signal appears as *blue/dark purple color* with *pink/red color* as the counterstain. There is no elevation of *blue color* in response to γ-irradiation. The *blue signal* in the duodenum appears to correlate with $p53^{+/+}$ status. Duo = duodenum, Jej = jejunum, Il = ileum.

In situ hybridization was used to reveal the cellular localization and relative level of expression of specific p53 target genes in tissue sections. The information thus derived about temporal and spatial expression and induction of the genes after irradiation suggests distinct *in vivo* situations where certain p53 targets may mediate apoptosis. Although Northern analysis and quantitative reverse transcription-PCR can identify the presence of a specific mRNA, as well as its level of induction, they do not provide information about the localization of the signal to specific cell populations in relation to tissue morphology (41). One of the major findings here is the specific compartmentalization of expression of *Bid*, *Noxa*, and *Puma* in irradiated spleen (Table 1, Figs. 6, 7, and 8) observed by *in situ* hybridization. In our study, *in situ* hybridization was performed among many kinds of tissues with five different probes. We optimized the hybridization conditions to maximize the signal with recognizable tissue structure before each probe was actually hybridized systematically. The level of gene expression in different tissues remains comparable because the tissue-specific conditions performed here were generated from the same standard (maximal signal with recognizable tissue architecture) in each pre-*in situ* hybridization.

This is, to our knowledge, the first detailed systematic examination of expression of a group of p53 target genes, including those recently discovered by *in situ* methodology in the *in vivo* response to γ-irradiation. One of the remarkable findings of this study is the apparent tissue specificity with which p53 selects targets for activation. In response to γ-irradiation in wtp53-containing tissue, *Noxa* was mainly induced in the thymus, *Puma* in white pulp of the spleen, $p21^{WAF1}$ in the liver, and *Bid* in the transverse colon. Different parts of small and large intestines also showed different patterns of target gene expression and activation. Interestingly, only *KILLER/DR5* and *Bid* were induced in the transverse colon, and a low level of *Puma* induction in the wtp53-containing descending colon was observed in response to γ-irradiation. We note that the basal expression of several genes appears to depend on wtp53, and those genes are not significantly induced by γ-irradiation. Examination of the tissue death response after irradiation revealed increased caspase-3 activity in all tissues but

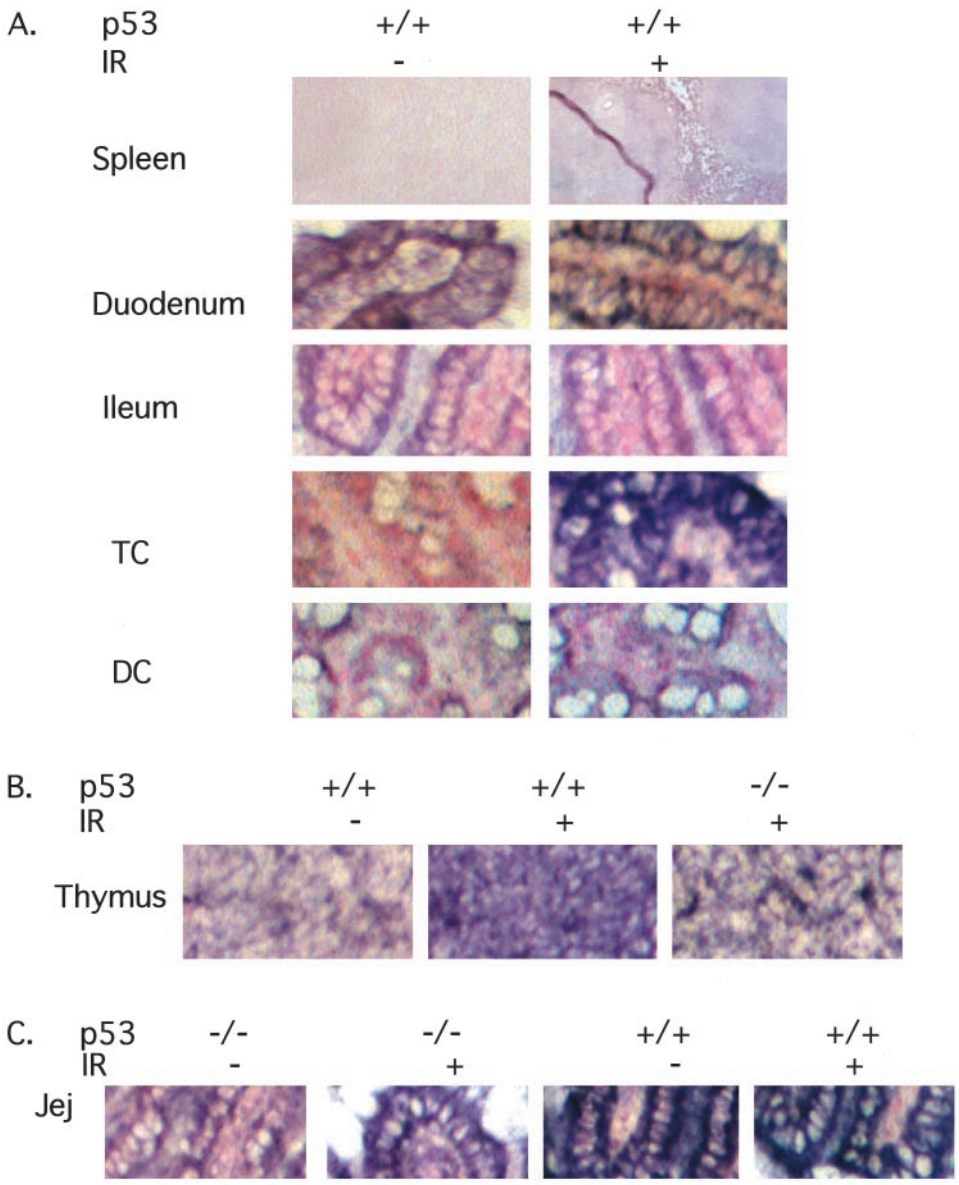

Fig. 6. Basal and p53-dependent γ-irradiation-induced Bid expression *in vivo*. *A*, *Bid* mRNA expression in mouse spleen, duodenum, ileum, transverse colon, and descending colon. Increased *blue color* is noted in irradiated $p53^{+/+}$ red pulp of spleen and in the transverse colon. The images of nonirradiated and irradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. TC = transverse colon, DC = descending colon. *B*, *Bid* mRNA expression in mouse thymus. Increased *blue color* is noted in irradiated $p53^{+/+}$ thymus with a slight increase in irradiated $p53^{-/-}$ thymus. The image of nonirradiated $p53^{-/-}$ thymus (data not shown) is similar to the nonirradiated $p53^{+/+}$ thymus. *C*, *Bid* mRNA expression in mouse jejunum. Increased *blue color* is noted in irradiated $p53^{-/-}$ jejunum as compared with the nonirradiated $p53^{-/-}$ jejunum. There is high basal expression of *Bid* mRNA in the $p53^{+/+}$ jejunum with no additional increase after irradiation. Jej = jejunum.

liver from irradiated $p53^{+/+}$ mice as compared with the corresponding tissues from irradiated $p53^{-/-}$ mice or nonirradiated control $p53^{+/+}$ or $p53^{-/-}$ mice. In the case of liver, we believe the apparently exclusive preference for $p21^{WAF1}$ activation, and not other p53 targets that could induce apoptosis, may, in part, explain the absence of detectable apoptotic death after radiation exposure. The p53 response profile and apparent lack of apoptosis in the liver does not exclude other types of toxicity such as necrotic death or inflammatory responses. The *in situ* hybridization results reveal remarkable variation in the selectivity of p53 for *in vivo* transactivation and the resulting gene expression patterns may correlate with the response to genotoxic stress.

γ-Irradiation induces a large variety of DNA lesions, including single- and double-strand breaks, base, and sugar damage (44, 45). The apoptotic pathway activated by γ-irradiation in proliferating cells is known to involve transcription of a variety of genes, among which the tumor suppressor gene p53 is one of the most relevant (46–48). Upon activation, p53 transactivates its target genes to induce cell growth arrest and/or apoptosis, responses that must be finely balanced in order to protect cells with intact genomes and at the same eliminate excess or damaged cells. The requirement of subtle regulation is reflected here by the complex tissue- and cell-specific responses. Our studies further expand previous work (31, 33, 34, 49, 50) examining p53-dependent apoptosis in tissues of spleen, thymus, and gut. We previously showed (34) that *KILLER/DR5* and $p21^{WAF1}$ induction by γ-irradiation was p53 dependent in spleen, thymus, and small intestine and that there was some variation in the magnitude of gene induction using real-time reverse transcription-PCR-based measurements on bulk tissue mRNA. We have not only confirmed these observations but also provided extensive *in situ* studies on three new targets of p53 (*Noxa*, *Puma*, and *Bid*) along with *KILLER/DR5* and $p21^{WAF1}$ in spleen, thymus, and liver, and we further investigated various portions of small and large intestines. Interestingly, the response to γ-irradiation in the spleen revealed evidence for p53-dependent cell death throughout the organ but various proapoptotic (BH3-domain containing) p53 targets were up-regulated in separate compartments; *Bid* and *Noxa* were expressed in the red pulp of the spleen, whereas *Puma* was induced in the white pulp. The molecular basis for compartmentalized regulation of specific p53 targets within a given tissue is not understood at present. In itself the compartmentalization suggests candidate

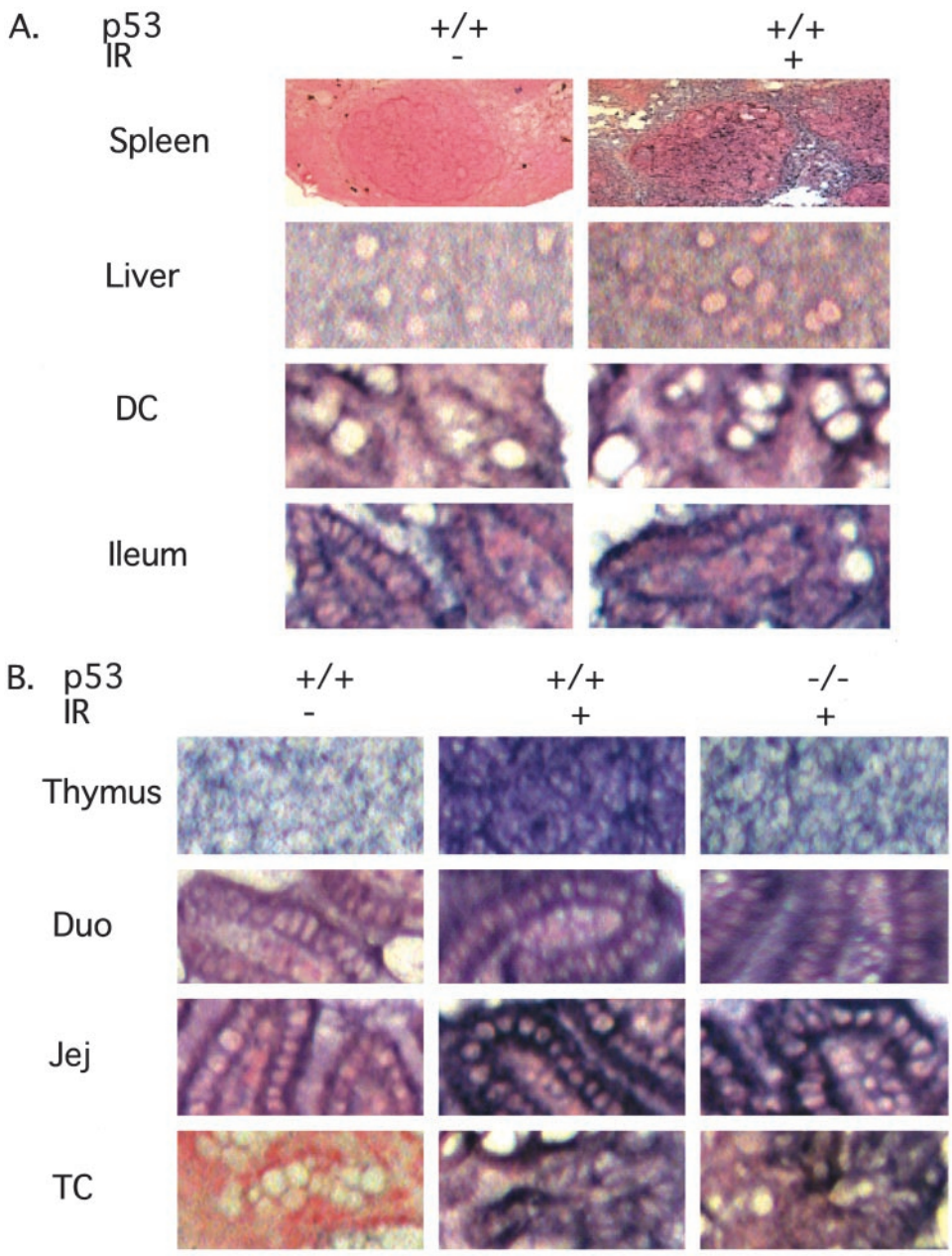

Fig. 7. Tissue specificity and p53 dependence of *Noxa* gene induction after γ-irradiation *in vivo*. *A*, *Noxa* mRNA expression in mouse spleen, liver, descending colon, and ileum. *Noxa* mRNA expression is increased in irradiated $p53^{+/+}$ red pulp of spleen but not in the irradiated $p53^{-/-}$ spleen. *Noxa* mRNA is expressed in the descending colon at higher levels in the ileum and is not additionally increased by irradiation in either of the $p53^{+/+}$ or $p53^{-/-}$ corresponding tissues. The images of nonirradiated and irradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. DC = descending colon. *B*, *Noxa* mRNA expression in mouse thymus, duodenum, jejunum, and transverse colon. *Noxa* mRNA expression is expressed in unirradiated thymus, increased additionally in irradiated $p53^{+/+}$ thymus, and slightly increased in irradiated $p53^{-/-}$ thymus. *Noxa* mRNA expression is detectable in unirradiated duodenum and jejunum and is up-regulated to a similar extent in $p53^{+/+}$ and $p53^{-/-}$ duodenum and jejunum, although the level of *Noxa* mRNA expression appears higher in irradiated jejunum *versus* duodenum. *Noxa* expression was not detectable in unirradiated transverse colon and was induced by irradiation regardless of p53 status. The images of nonirradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. Duo = duodenum, Jej = jejunum, TC = transverse colon.

mediators of death in certain locations (because they are induced there) and also suggests that there may be unique functions of certain p53 targets in certain situations *in vivo*. Possible mechanisms for additional investigation for the selectivity of p53 for different targets in different tissue compartments within the spleen include (*a*) potential differences in p53 modification in the radiation response in different tissue compartments, and (*b*) potential tissue compartment-specific proteins that may positively or negatively regulate the selectivity of p53 for either particular DNA binding sequences or the transactivation of particular target genes.

In response to apoptotic signals, p53 protein is stabilized and activated, leading to transcriptional activation of multiple target genes that cause apoptosis of cells. These include death receptors, including *Fas/Apo* or *KILLER/DR5* or proteins that are involved in mitochondria-mediated apoptosis, including *Bax*, *Noxa*, *Puma*, and *p53Aip1*. Activation of mitochondria-mediated apoptosis represents a major antitumor response of p53 (51). Among the identified target genes of p53, *Bax* encodes a proapoptotic *Bcl-2* family member that can activate mitochondria-mediated apoptosis (25). However, in *Bax*-deficient mice, DNA damage-induced apoptosis occurs normally in thymocytes (52). Our data that *Noxa* was strongly induced by γ-irradiation in $p53^{+/+}$ thymus may, in part, explain why apoptosis still occurs in *Bax*-deficient thymus.

The small intestine represents one of the most rapidly proliferating tissues of the body, with cell division occurring approximately every 5 min in each crypt (53). Despite its high proliferation rate, cancers rarely develop in the small intestine, suggesting that this tissue contains an efficient mechanism for regulating cell growth (54). Our data shows that all the proapoptotic p53 target genes studied here were to, at some extent, expressed in jejunum and ileum, and we suspect they may act synergistically to contribute to the strong wtp53-dependent caspase-3 activation in jejunum and ileum after γ-irradiation. In contrast to the jejunum and ileum, there are less proapoptotic p53 targets expressed in the transverse and descending colon, which may correlate with the relatively low caspase-3 activity detected after irradiation. Liver is the only tissue studied here that did not display

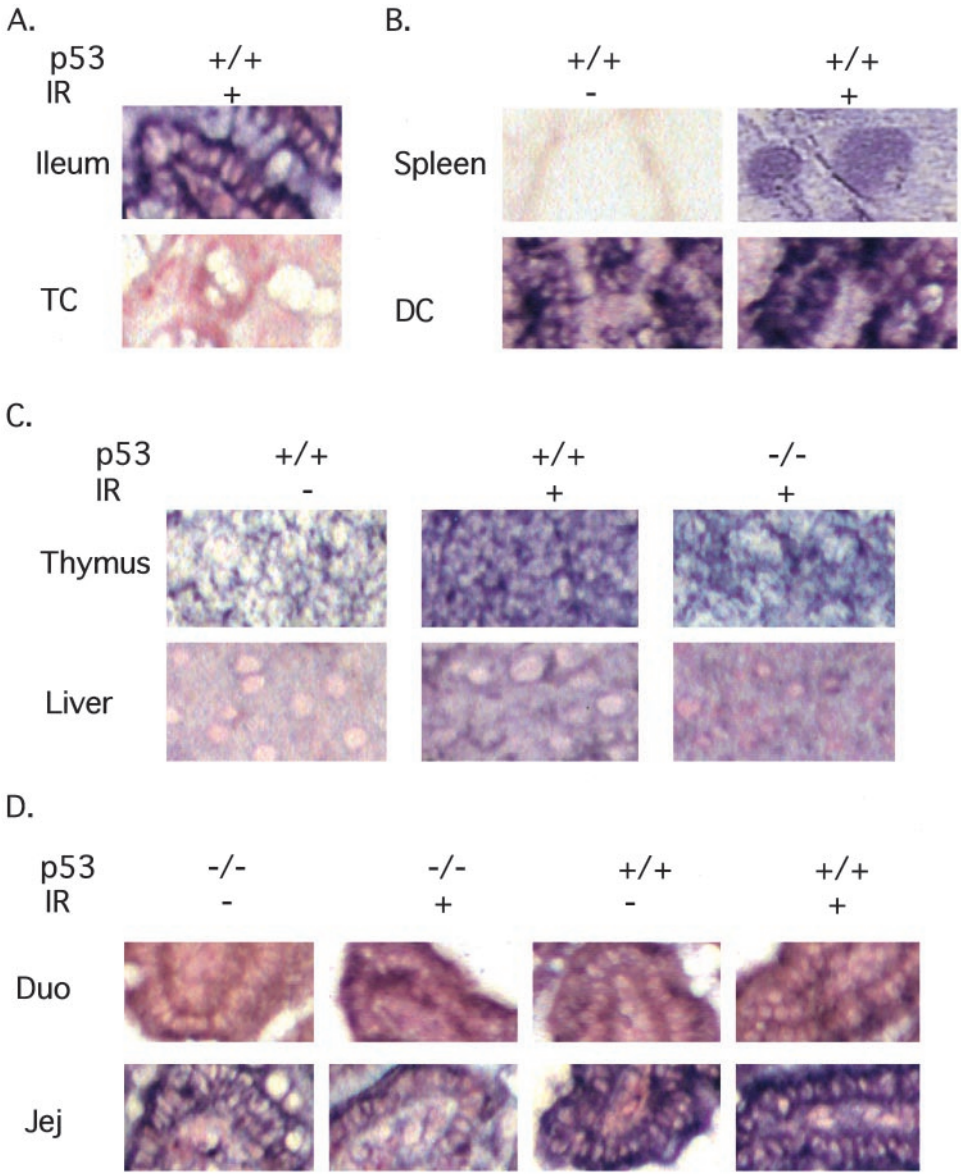

Fig. 8. Tissue specificity and p53 dependence of *Puma* gene induction after γ-irradiation *in vivo*. *A*, *Puma* mRNA expression in mouse ileum and transverse colon. Expression is noted in ileum but not in transverse colon with no detectable difference either between nonirradiated and irradiated tissues or between $p53^{+/+}$ and $p53^{-/-}$ ileum. The images of nonirradiated and irradiated $p53^{-/-}$ tissues (data not shown) and nonirradiated $p53^{+/+}$ tissues (data not shown) are similar to the irradiated $p53^{+/+}$ tissues (shown here). TC = transverse colon. *B*, *Puma* mRNA expression in mouse spleen and descending colon. Expression is highly increased in irradiated $p53^{+/+}$ splenic white pulp and is only very slightly increased in irradiated $p53^{+/+}$ descending colon. The images of nonirradiated and irradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues (shown here). DC = descending colon. *C*, *Puma* mRNA expression in mouse thymus and liver. Expression is elevated in irradiated thymus with high elevation in $p53^{+/+}$ thymus and a slight increase in irradiated $p53^{+/+}$ liver. The images of nonirradiated $p53^{-/-}$ tissues (data not shown) are similar to the nonirradiated $p53^{+/+}$ tissues. *D*, *Puma* mRNA expression in mouse duodenum and jejunum. The *purple color* is observed in all sections of duodenum and jejunum but much higher in $p53^{+/+}$ sections with a little irradiation response in $p53^{-/-}$ duodenum only. Duo = duodenum, Jej = jejunum.

caspase-3 activation in the presence of wtp53 after γ-irradiation. This is in agreement with previous studies showing that liver does not undergo apoptosis after γ-irradiation (33, 55). However, significant $p21^{WAF1}$ expression was induced in $p53^{+/+}$ liver (Fig. 4), whereas none of the other proapoptotic targets studied here was significantly up-regulated. $p21^{WAF1}$ seems to play an important role in determining whether a cell should undergo apoptosis or survive with $p21^{WAF1}$ expression appearing to favor growth or growth arrest over cell death (56–59). Therefore, the pattern of p53 target gene expression in liver may explain, at least in part, the inability of γ-irradiation to induce caspase-3 activation, and this correlates with the resistance of liver to radiation-induced apoptosis.

The role of p53 in radiosensitivity is complex. In some cases, expression of wtp53 is associated with an increase in the sensitivity to anticancer treatment (60–63). Although loss of p53 function can also result in increased sensitivity to anticancer treatment in other situations (55, 64). In the current study, all patterns of gene expression demonstrate distinct tissue/cell type specificity as well as some overlap in gene expression/induction of p53 targets, which seems to correlate well with the level of caspase-3 activation in particular tissues. Therefore, our study supports the concept that wtp53 contributes to radiosensitivity. It appears that induction of more proapoptotic target genes resulted in a stronger apoptotic response, *e.g.*, in jejunum and ileum. In the future, additional p53 targets need to be analyzed, and in fact, it would be extremely useful to perform microarray analyses investigating global gene expression patterns in the radiation response of different tissues. In addition to the striking patterns of tissue specificity, the present studies provide a framework within which future studies can analyze p53 or proapoptotic targets of interest. The patterns of gene expression may suggest markers of tissue responsiveness to therapeutic manipulations and may provide essential clues to modulate tissue toxicity. In this regard, it will be of interest to determine whether tumors that arise from a given tissue maintain the genetically programmed p53 activation response profile as compared with the normal tissue of origin. If they do, one could envision scenarios where (transient) blockade of a proapoptotic p53 target not involved in the therapeutic response of a metastatic tumor may protect normal tissues exposed to chemotherapy or radiotherapy where the proapoptotic target gene induction contributes to toxicity. Another important direction that emerges from our studies involves additional work to understand the relationship, if any, between the observed strong DNA damage p53-mediated apoptotic response in the

small bowel of mice and the observed low incidence of intestinal tumors in humans. We hypothesize that (prolonged) blockade of the p53-dependent apoptotic response to genotoxic stress in the small intestine may influence tumor susceptibility, especially in backgrounds where small intestinal tumors occur such as in *min* mice or individuals with familial polyposis and especially if the p53 response is activated by various endogenous or exogenous exposures. Experimentally, this may be approached through either small intestine-specific deletion of p53 or through small intestine-specific expression of potent antiapoptotic genes. In summary, the present studies provide a foundation for future studies to analyze the genetic basis, therapeutic implications, and tissue specificity of the *in vivo* p53-mediated stress response.

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