Published in final edited form as:

Cancer Res. 2009 June 15; 69(12): 4999–5006. doi:10.1158/0008-5472.CAN-09-0262.

PUMA Suppresses Intestinal Tumorigenesis in Mice

Wei Qiu¹, Eleanor B. Carson-Walter³, Shih Fan Kuan¹, Lin Zhang², and Jian Yu¹

¹ Department of Pathology, University of Pittsburgh and Cancer Institute, Pittsburgh, Pennsylvania

² Department of Pharmacology and Chemical Biology, University of Pittsburgh and Cancer Institute, Pittsburgh, Pennsylvania

³ Department of Neurosurgery, University of Rochester, Rochester, New York

Abstract

Defective apoptosis contributes to tumorigenesis, although the critical molecular targets remain to be fully characterized. PUMA, a BH3-only protein essential for p53-dependent apoptosis, has been shown to suppress lymphomagenesis. In this study, we investigated the role of PUMA in intestinal tumorigenesis using two animal models. In the azoxymethane (AOM)/dextran sulfate sodium salt model, *PUMA* deficiency increased the multiplicity and size of colon tumors but reduced the frequency of β -catenin hotspot mutations. The absence of *PUMA* led to a significantly elevated incidence of precursor lesions induced by AOM. AOM was found to induce p53-dependent PUMA expression and PUMA-dependent apoptosis in the colonic crypts and stem cell compartment. Furthermore, *PUMA* deficiency significantly enhanced the formation of spontaneous macroadenomas and microadenomas in the distal small intestine and colon of *APC*^{Min/+} mice. These results show an essential role of PUMA-mediated apoptosis in suppressing intestinal tumorigenesis in mice.

Introduction

Colon carcinogenesis is a multistep process involving the accumulation of a series of genetic and epigenetic alterations in normal colonic epithelial cells (1). Some of the most frequent changes found in colon cancer include mutations in the tumor suppressors *APC* (adenomatous polyposis coli) and *p53*, and those in the oncogenes β -catenin, *K-ras*, *BRAF*, and *PI3K*, many of which are capable of suppressing apoptosis (1). Overexpression of the antiapoptotic proteins such as Bcl-2, Bcl-xL, and survivin are also common in cancer (2). Apoptosis is a major mechanism regulating the turnover of intestinal epithelial cells, and is progressively inhibited in colon carcinogenesis (3,4). Decreased apoptosis is a hallmark of cancer, leading to increased risk of clonal expansion of cells containing critical alterations (4), and subsequently driving other tumorigenic events, including extension of life span, accumulation of further genetic mutations, growth under stress conditions, and tumor angiogenesis (5). *In vivo* studies directly examining the role of apoptosis in colon tumorigenesis are still largely lacking.

We and others have shown that the Bcl-2 family protein PUMA (p53 up-regulated modulator of apoptosis) is a major mediator of p53-dependent apoptosis through the mitochondrial pathway in multiple cell and tissue types, including intestinal epithelial cells (6–10). *PUMA*

Requests for reprints: Jian Yu, Hillman Cancer Center Research Pavilion, Suite 2.26h, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-7786; Fax: 412-623-7778; yuj2@upmc.edu.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

expression is transcriptionally induced by apoptotic stimuli through both p53-dependent and p53-independent pathways (7,11,12). Several lines of evidence suggest the potential role of PUMA in tumor suppression. *p53* deficiency abrogated the induction of PUMA by radiation and various chemotherapeutic drugs (7,13,14). *PUMA* shRNAs suppressed p53-dependent apoptosis, promoted oncogenic transformation of primary murine fibroblasts by *E1A/ras*, and dramatically accelerated Eμ-*myc*-induced lymphomagenesis (15,16). Reduced expression of PUMA has been reported in subcutaneous melanomas and Burkitt lymphomas, and was correlated with worsened prognosis (16,17). However, its role in solid tumors has not been firmly established.

In the current study, we investigated the role of PUMA in suppressing intestinal carcinogenesis using two mouse models, the carcinogen-induced tumor model [azoxymethane (AOM) and dextran sulfate sodium salt (DSS), (AOM/DSS)] and the *APC*^{Min/+} spontaneous tumor model. We found that *PUMA* deficiency enhances tumor formation in both models. p53-dependent *PUMA* induction is largely responsible for an acute apoptotic response induced by AOM at the bottom of colonic crypts where the colonic stem cells reside. *PUMA* deficiency increases the incidence of precursor lesion aberrant crypt foci (ACF) induced by AOM in the colon, or that of microadenomas in the small intestine of *APC*^{Min/+} mice. These data show that PUMA-mediated apoptosis suppresses tumor initiation in the gut.

Materials and Methods

Mice and Treatment

The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Detailed information on the generation of *PUMA*^{+/+}, *PUMA*^{-/-} (F6 on C57BL/6 background), *APC*^{Min/+}/*PUMA*^{-/-}, and *p53*^{-/-} mice is described in Supplementary Materials and Methods.

AOM and DSS treatment—Six- to 10-wk-old littermates were injected i.p. with a single dose of AOM (Sigma; 12.5 mg/kg). After 7 d, 2.5% DSS (MP Biomedicals) was given in the drinking water, followed by 14 d of regular water. The cycle was repeated twice. Following the last cycle, mice were given regular water for 2 mo and sacrificed (Fig. S1). Some mice were injected i.p. with 100 mg/kg of bromodeoxyuridine (BrdUrd) 2 h prior to sacrifice to analyze crypt proliferation.

AOM treatment—Six- to 10-wk-old mice were injected i.p. with AOM (10 mg/kg) or saline (control) once a week for 6 wk. Animals were sacrificed 4 wk after the last AOM injection (18). To determine the levels of apoptosis induced by AOM, mice were injected i.p. with 15 mg/kg of AOM and sacrificed 8 h later. BrdUrd (100 mg/kg) was i.p. injected into the mice 2 h prior to euthanasia, or 2 h before AOM treatment in some experiments (72-h tracing experiments) to analyze crypt proliferation.

Total RNA Extraction and Real-time Reverse Transcriptase-PCR

Total RNA and cDNA were prepared from the freshly scraped intestinal mucosa as described (9). Real-time PCR was performed on a Mini Opticon Real-time PCR System (Bio-Rad) with SYBR Green (Invitrogen). The primers used for *PUMA* and *β-actin* have been described (9). Three mice were included in each group. Melting curve and agarose gel electrophoresis of the PCR products were used to verify the specificity of PCR amplification.

Western Blotting

Total protein extracts were purified and analyzed by NuPage gel (Invitrogen) electrophoresis as previously described (10). Primary antibodies included those for PUMA (Abcam) and β -actin (Sigma). Three mice were used in each group.

RNA *In situ* Hybridization

The protocol has been previously described (10). Digoxigenin-labeled *PUMA* and *Lgr5* antisense RNA probes were generated by PCR amplification with incorporation of a T7 promoter (5'-CTAATACGACTCACTATAGGGAGA) into the antisense primer as previously reported (ref. ¹⁰; Table S1).

Tumor Histology

Macroadenomas (polyps; >0.5 mm) induced by AOM/DSS were scored under a stereomicroscope using fixed colons. Histology grading was done by a board-certified pathologist according to established criteria using 5 μ m H&E sections (19). Macroadenomas in *APC*^{Min/+} mice on the AIN93G diet (Dyets, Inc.) were scored at 22 wk as described above. The microadenomas in *APC*^{Min/+} mice were scored at 5 wk using H&E sections of "Swiss-rolled" tissue under a light microscope (20). ACF were identified using methylene blue-stained colons (18). Details are described in the Supplementary Materials and Methods.

Immunostaining

Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) staining, BrdUrd, and Ki67 immunohistochemistry were performed on 5 μ m sections as previously described (10). The TUNEL or BrdUrd-positive cells were scored in 100 crypt sections and reported as mean $\pm$ SE. Three mice were used in each group.

Statistical Analysis

Data were analyzed by ANOVA in which multiple comparisons were performed using the method of least significant difference. Data on the frequency of β -catenin mutations were analyzed by χ^2 test. Differences were considered significant if the probability of the difference occurring by chance was less than 5 in 100 ($P < 0.05$).

Results

PUMA deficiency increased colon tumor formation in the AOM/DSS model

AOM and DSS are two commonly used colonic carcinogens in rodents. Both can induce tumors alone in the distal colon of rodents, although the incidence and multiplicity in some commonly used inbred mouse strains such as C57BL/6 are rather low even after an extended time (8–12 months; refs. 21,22). Recently, DSS has been shown to promote AOM-induced colon cancer in C57BL/6 mice within 4 months (23,24). To determine whether *PUMA* plays a role in colon carcinogenesis, we compared the tumor incidence, size, and grade in wild-type (WT) and *PUMA*-knockout (*PUMA* KO) littermates using a modified AOM/DSS model (Fig. S1A; ref. 24). All the mice in this protocol developed tumors in 16 weeks following the initial exposure to AOM (Fig. 1A; Fig. S1A). The tumor incidence in *PUMA* KO mice increased by 1.5-fold compared with WT mice (5.7 ± 1.6 versus 2.1 ± 1.1) with the majority of tumors located in the middle and distal colon (Fig. 1A and B). The size of tumors also significantly increased in *PUMA* KO mice compared with that in WT mice (Fig. 1C). Histopathologic evaluation indicated that the tumors developed in *PUMA* KO mice had higher grades (Table 1). For example, 91% of tumors in WT mice were adenomas with low-grade dysplasia, and none (0%) were adenocarcinomas (Table 1; Fig. S1B). In contrast, 86% of tumors in *PUMA* KO mice

were adenomas with high-grade dysplasia, and nearly 10% were adenocarcinomas (Table 1; Fig. S1B).

We analyzed size-matched tumors from WT and *PUMA* KO mice for apoptosis and proliferation. Apoptosis was found to be reduced in the tumors from *PUMA* KO mice (Fig. S2A; data not shown). Reduction of apoptosis was even more pronounced in the center of larger tumors found in *PUMA* KO mice, suggesting resistance to nutrient deprivation and hypoxia (7,25). Proliferation only increased slightly in the tumors from *PUMA* KO mice (Fig. S2B). These results suggest that PUMA suppresses AOM/DSS-induced colon tumorigenesis through apoptosis induction.

***PUMA* deficiency increased ACF formation induced by AOM**

The elevated tumor incidence in *PUMA* KO mice suggests a role for PUMA in suppressing tumor initiation. Several studies suggested that ACF are likely to be precursor lesions and a risk factor for colon cancer in both human and rodents (26). We therefore analyzed the incidence of ACF induced by AOM in WT and *PUMA* KO mice. ACF incidence increased by ~4-fold in *PUMA* KO mice (33 ± 4.5 versus 7.3 ± 1.5 ; Fig. 1D). These results indicate that PUMA potently suppresses tumor initiation induced by genotoxin.

***PUMA* deficiency reduced the frequency of β -catenin mutations in AOM/DSS-induced colon tumors**

Mutations in β -catenin or APC lead to the stabilization of β -catenin and oncogenic transformation by interfering with the phosphorylation and degradation of β -catenin mediated by glycogen synthase kinase-3 β (27). Correspondingly, frequent mutations at codons 33, 34, 37, and 41 of β -catenin, within the glycogen synthase kinase-3 β phosphorylation motif, have been reported in AOM-induced colon tumors in rats and mice (21). We therefore sequenced exon 3 of β -catenin in the tumors from WT and *PUMA* KO mice using laser capture microdissected tumor tissues. We found that 44% (7 of 16) of tumors in WT mice had β -catenin mutations distributed at all four codons, whereas 12% (2 of 17) of those in *PUMA* KO mice had mutations in only codon 33 (Fig. S3; $P < 0.05$). No hotspot (codons 12 and 13) mutations in *K-ras* or p53 overexpression were found in the tumors from either group (Fig. S3; data not shown; ref. 28). We also analyzed β -catenin localization in the tumors that were negative for hotspot mutations by immunohistochemistry. Intense nuclear β -catenin staining was found in 2 out of 9 (~22%) tumors in WT mice, and 5 out of 15 (~33%) tumors in *PUMA* KO mice. These data suggest that preexisting apoptotic deficiency affects mutation frequency or spectrum in AOM/DSS-induced colon tumors.

AOM rapidly induced colonic *PUMA* expression

AOM is known to induce DNA damage (29). We therefore analyzed the expression of PUMA in the colonic mucosa of WT mice after AOM injection. Using quantitative reverse transcriptase-PCR, we found that *PUMA* mRNA was induced by 4-fold at 8 hours compared with untreated mice (Fig. 2A). PUMA protein levels were also substantially elevated in the mice after AOM treatment (Fig. 2B). RNA *in situ* hybridization was then used to identify the specific cell types in which *PUMA* expression was induced. *PUMA* mRNA was highly induced in the lower one-third of the crypts as early as 8 hours after AOM treatment, with the highest concentration at the bottom of the crypts (Fig. 2C). The basal levels of *PUMA* were undetectable (Fig. 2C). Recently, the orphan receptor *Lgr5* was reported as a specific stem cell marker for the small intestine and colon (30). Using RNA *in situ* hybridization, we detected *Lgr5* expression in the bottom of the crypts as reported (Fig. 2D; ref. 30), where robust *PUMA* induction was observed (Fig. 2C). These results indicate that AOM treatment rapidly induces *PUMA* in the colonic crypts and the stem cell compartment in particular.

PUMA deficiency abrogated apoptosis induced by AOM in the colonic crypts

To determine whether PUMA mediates AOM-induced apoptosis (31), we compared apoptosis in the colonic crypts of WT and *PUMA* KO mice (Fig. 3A). Apoptosis in the crypts markedly increased at 8 hours after AOM treatment in WT mice, but this increase was blocked by >90% in *PUMA* KO mice (Fig. 3B). In contrast, apoptosis in the submucosa was present at similarly low levels in both groups after AOM treatment (Fig. 3B). The basal level of apoptosis in these two groups of mice was negligible (<0.02 cells/crypt) and was not significantly different. Interestingly, AOM-induced apoptosis occurred primarily at the bottom of crypts where *Lgr5*-positive cells are located, resembling the patterns of *PUMA* induction (Figs. 2C, D and 3B). Active caspase-3 staining also confirmed the location of apoptotic cells (Fig. 3C). *PUMA* deficiency did not affect crypt proliferation measured by BrdUrd (Fig. S4) or Ki67 staining (data not shown) either at 8 or 72 hours after AOM treatment. These results show that PUMA is a major mediator of AOM-induced apoptosis in the colonic crypts and stem cell compartment.

PUMA mediated p53-dependent apoptosis induced by AOM in the colonic crypts

Induction of apoptosis is an essential function of p53 as a tumor suppressor in response to DNA damage. Although a large number of proapoptotic genes can be regulated by p53, only a few have been shown to be required for p53-dependent apoptosis (32). To determine whether p53 is required for PUMA induction by AOM, we compared PUMA expression in WT and *p53*-knockout (*p53* KO) mice at 8 hours after AOM treatment. The induction of *PUMA* mRNA and protein was completely abrogated in *p53* KO mice, which was most evident at the bottom of the crypts (Fig. 4A and B). Apoptosis in the crypts was reduced by >90% in *p53* KO mice, a level similar to that observed in *PUMA* KO mice (Figs. 3 and 4C, D). These results suggest that p53-dependent PUMA induction is primarily responsible for AOM-induced apoptosis in the colonic crypts and stem cell compartment.

PUMA deficiency increased tumor formation in *APC*^{Min/+} mice

APC^{Min/+} mice develop a large number of intestinal adenomas on a C57BL/6 background, mostly in the distal small intestine (33). To examine the potential role of PUMA in spontaneous intestinal tumorigenesis, we generated *APC*^{Min/+}/*PUMA* KO mice. The sex- and age-matched cohorts of *APC*^{Min/+} and *APC*^{Min/+}/*PUMA* KO mice were analyzed for tumor incidence at the age of 5 months on a high-fat diet (AIN93G). The number of macroadenomas (polyps ≥ 0.5 mm in diameter) in the colon of *APC*^{Min/+}/*PUMA* KO mice (4.2 ± 1.0) was significantly elevated compared with that in *APC*^{Min/+} mice (2.0 ± 0.7 ; Fig. 5A and Fig. S5). A smaller, yet statistically significant increase (64.4 ± 4.0 versus 47.6 ± 6.3) in the overall number of macroadenomas in the small intestine of *APC*^{Min/+}/*PUMA* KO mice was also found (Fig. 5B). Further anatomic stratification revealed that macroadenomas in the distal small intestine significantly increased in *APC*^{Min/+}/*PUMA* KO mice (43.3 ± 5.0) compared with *APC*^{Min/+} mice (24.6 ± 4.3), whereas those in the proximal and middle small intestine did not (Fig. 5C). No significant difference in the size of polyps between these two groups was found either in the small intestine (Fig. 5C) or colon (data not shown). Consistent with the role of PUMA-mediated apoptosis in suppressing tumor initiation, microadenomas (20) in the small intestine of *APC*^{Min/+}/*PUMA* KO mice at an age of 4 to 5 weeks increased compared with *APC*^{Min/+} mice (47 ± 2.5 versus 33 ± 3.6 ; Fig. 5D). These results indicate that loss of PUMA leads to increased susceptibility of developing early neoplastic lesions in the absence of acute DNA damage in the intestinal tract.

Discussion

Our studies indicate that the BH3-only protein PUMA suppresses intestinal tumorigenesis induced by either carcinogens or germ line mutation in the *APC* tumor suppressor gene. PUMA

is induced by AOM and mediates apoptosis in a p53-dependent manner in the colonic crypts and stem cell compartment. Several earlier studies showed that an intact p53 pathway suppresses AOM or DSS-induced colon carcinogenesis (34–36). Our study suggests that this suppression is likely mediated by PUMA. On the other hand, p53 was reported to have little effect on the incidence or the progression of adenomas in *APC*^{Min/+} mice, or on their spontaneous apoptosis (37,38). However, the role of p53 in this model cannot be ruled out due to genetic background or modifiers (39). Enhanced tumorigenesis found in *APC*^{Min/+}/*PUMA* KO mice suggests a p53-independent function of PUMA in tumor suppression. Therefore, PUMA-mediated apoptosis through either p53-dependent (AOM/DSS model) or p53-independent mechanism (*APC*^{Min/+} mice) serves as a critical roadblock to tumorigenesis. Furthermore, our data suggest that PUMA-mediated apoptosis plays a more significant role in suppressing colonic carcinogenesis induced by DNA damage and tissue injury associated with inflammation. This is consistent with the prominent role of PUMA in mediating apoptosis in this tissue, as opposed to other members in the extended BH3-only protein family (6–10). These findings provide direct evidence supporting apoptosis induction as an important mechanism for tumor suppression in the intestinal tract (40).

Previous studies have established that p53-dependent induction of PUMA is required for apoptotic response to a wide range of stresses (6–10). However, the evidence for PUMA in tumor suppression, particularly in solid tumors, is still very limited. Unlike *p53* KO mice, *PUMA* KO mice are not overtly tumor-prone (41,42). Our data suggest that p53-mediated PUMA induction serves as a safeguard to inhibit tumorigenesis in response to DNA damage, which is mutagenic if left unrepaired, or may present as oncogenic stress following error-prone repair of either tumor suppressors or oncogenes (43). Interestingly, *p21*-deficient mice developed significantly higher numbers of ACF and showed less apoptosis in the colon after AOM treatment compared with WT mice (18). It is not clear how *p21* deficiency inhibits apoptosis in this setting, as *p21* deficiency in human colon cancer cells triggered a PUMA-dependent killing in response to DNA damage (7). Loss of PUMA does not affect crypt proliferation after AOM treatment, nor does loss of p53 or p21 (18,35). These findings support a hypothesis that p53 suppresses colon carcinogenesis primarily through the induction of apoptosis in response to perhaps chronic DNA damage due to exposure to the mutagens in the colonic contents, and oncogenic mutations of *APC*, *β-catenin*, or *K-ras* as suggested (40). Therefore, loss of apoptotic response to DNA damage (35) or abnormal oncogenic stimulation might be the most important mechanism leading to tumorigenesis in this organ system. This might also explain why the small intestine is much less prone to carcinogenesis, perhaps due to the more limited life span of differentiated cells, and reduced carcinogen exposure in terms of both - concentration and duration.

The function or regulation of PUMA in response to DNA damage is likely to be severely impaired in most colon cancers given the prevalence of *p53* mutations and the overexpression of anti-apoptotic protein Bcl-xL (1,44,45). This might explain why reduced expression or mutation of *PUMA* is not found in colon cancer. Unlike in human colon cancer (46), the mutations in *K-ras* and *p53* are rare in rodent colon tumors induced by AOM (28). Most of the tumors found in *APC*^{Min/+} mice are in the small intestine. This might be explained by differences in development, diet, life span, environment, and carcinogen exposure between the mouse models and human. It is well established that the Wnt/β-catenin pathway is antiapoptotic and can stimulate the expression of Bcl-2 like proteins in colon cancer cells (27,47). Therefore, *PUMA* deficiency might bypass the need of activating β-catenin mutations to inhibit apoptosis and facilitate tumor initiation, which might explain the reduced frequency of β-catenin mutations found in AOM/DSS-induced *PUMA*-deficient tumors. However, activation of the β-catenin pathway in AOM/DSS-induced tumors clearly involves alternative mechanisms that remain to be defined. It would be interesting to identify other genetic or epigenetic alterations that cooperate with *PUMA* deficiency through global genetic or genomic analysis. PUMA

antagonizes all five known members of the antiapoptotic Bcl-2 family of proteins, several of which are overexpressed in colon (48). We would predict that tumors from the *PUMA*-deficient mice are less likely to overexpress these proteins. Such studies might reveal important pathways and interactions in tumor biology and therapy, and provide a better molecular understanding of tumor heterogeneity.

Our data provide a model to test the provocative cancer stem cell hypothesis. Colonic epithelium is constantly renewed, with the loss of differentiated cells at the top of the crypts through apoptosis to maintain homeostasis (3). It is unclear whether colon cancer is derived from stem cells that have endured critical mutations in a certain sequence (1,46), or from more differentiated cells that have acquired mutations conferring stem cell-like properties, and the relative contribution of both mechanisms. A recent study showed that selective targeting of *APC* in the intestinal stem cells, but not in rapidly proliferating progenitors, results in rapid intestinal tumorigenesis in mice (49). Our data suggest that *PUMA*-mediated apoptosis protects colonic stem cells against carcinogenesis. Using recently published stem cell marking mice (30), we will be able to trace the cellular origin of colon tumors. It is possible that a pre-existing deficiency in apoptosis can affect the types of cells that give rise to tumors as a result of a shifted mutation spectrum. Moreover, mutation and phenotype connection in the precursor lesions in humans is supported by a recent report, in which *BRAF* mutations are associated with serrated-ACF and polyps, but not *K-ras* mutations (50). Genetically engineered mice that are resistant to apoptosis make a great model system to further explore this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: NIH grants CA106348 and CA121105, American Cancer Society grant RSG-07-156-01-CNE (L. Zhang), and NIH grants CA129829, U19-A1068021 (Pilot Project), and from ACGT, FAMRI, and the Hillman Foundation (J. Yu).

We thank other members of our labs for helpful discussion and advice, Hongtao Liu for breeding mice, and Gerard P. Zambetti at St. Jude Children's Research Hospital for providing *PUMA* KO mice.

References

1. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004;10:789–99. [PubMed: 15286780]
2. Yu J, Zhang L. Apoptosis in human cancer cells. *Curr Opin Oncol* 2004;16:19–24. [PubMed: 14685088]
3. Hall PA, Coates PJ, Ansari B, Hopwood D. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci* 1994;107:3569–77. [PubMed: 7706406]
4. Bedi A, Pasricha PJ, Akhtar AJ, et al. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 1995;55:1811–6. [PubMed: 7728743]
5. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70. [PubMed: 10647931]
6. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. *PUMA* induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 2001;7:673–82. [PubMed: 11463391]
7. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. *PUMA* mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci U S A* 2003;100:1931–6. [PubMed: 12574499]
8. Yu J, Wang P, Ming L, Wood MA, Zhang L. *SMAC/Diablo* mediates the proapoptotic function of *PUMA* by regulating *PUMA*-induced mitochondrial events. *Oncogene* 2007;26:4189–98. [PubMed: 17237824]

9. Wu B, Qiu W, Wang P, et al. p53 independent induction of PUMA mediates intestinal apoptosis in response to ischaemia-reperfusion. *Gut* 2007;56:645–54. [PubMed: 17127703]
10. Qiu W, Carson-Walter EB, Liu H, et al. PUMA regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. *Cell Stem Cell* 2008;2:576–83. [PubMed: 18522850]
11. Wang P, Yu J, Zhang L. The nuclear function of p53 is required for PUMA-mediated apoptosis induced by DNA damage. *Proc Natl Acad Sci U S A* 2007;104:4054–9. [PubMed: 17360476]
12. Ming L, Sakaida T, Yue W, Jha A, Zhang L, Yu J. Sp1 and p73 activate PUMA following serum starvation. *Carcinogenesis* 2008;29:1878–84. [PubMed: 18579560]
13. Yu J, Yue W, Wu B, Zhang L. PUMA sensitizes lung cancer cells to chemotherapeutic agents and irradiation. *Clin Cancer Res* 2006;12:2928–36. [PubMed: 16675590]
14. Sun Q, Sakaida T, Yue W, Gollin SM, Yu J. Chemosensitization of head and neck cancer cells by PUMA. *Mol Cancer Ther* 2007;6:3180–8. [PubMed: 18089712]
15. Hemann MT, Zilfou JT, Zhao Z, Burgess DJ, Hannon GJ, Lowe SW. Suppression of tumorigenesis by the p53 target PUMA. *Proc Natl Acad Sci U S A* 2004;101:9333–8. [PubMed: 15192153]
16. Garrison SP, Jeffers JR, Yang C, et al. Selection against PUMA gene expression in Myc-driven B cell lymphomagenesis. *Mol Cell Biol* 2008;28:5391–403. [PubMed: 18573879]
17. Karst AM, Dai DL, Martinka M, Li G. PUMA expression is significantly reduced in human cutaneous melanomas. *Oncogene* 2005;24:1111–6. [PubMed: 15690057]
18. Poole AJ, Heap D, Carroll RE, Tyner AL. Tumor suppressor functions for the Cdk inhibitor p21 in the mouse colon. *Oncogene* 2004;23:8128–34. [PubMed: 15377995]
19. Boivin GP, Washington K, Yang K, et al. Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology* 2003;124:762–77. [PubMed: 12612914]
20. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990;247:322–4. [PubMed: 2296722]
21. Takahashi M, Nakatsugi S, Sugimura T, Wakabayashi K. Frequent mutations of the β -catenin gene in mouse colon tumors induced by azoxymethane. *Carcinogenesis* 2000;21:1117–20. [PubMed: 10836998]
22. Hibi T, Ogata H, Sakuraba A. Animal models of inflammatory bowel disease. *J Gastroenterol* 2002;37:409–17. [PubMed: 12108674]
23. Suzuki R, Kohno H, Sugie S, Tanaka T. Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate. *Cancer Sci* 2004;95:721–7. [PubMed: 15471557]
24. Greten FR, Eckmann L, Greten TF, et al. IKK β links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004;118:285–96. [PubMed: 15294155]
25. Ming L, Sakaida T, Yue W, Jha A, Zhang L, Yu J. Sp1 and p73 activate PUMA following serum starvation. *Carcinogenesis* 2008;29:1878–84. [PubMed: 18579560]
26. Stevens RG, Swede H, Rosenberg DW. Epidemiology of colonic aberrant crypt foci: review and analysis of existing studies. *Cancer Lett* 2007;252:171–83. [PubMed: 17182176]
27. Clevers H. Wnt/ β -catenin signaling in development and disease. *Cell* 2006;127:469–80. [PubMed: 17081971]
28. Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Sci* 2004;95:475–80. [PubMed: 15182426]
29. Feinberg A, Zedeck MS. Production of a highly reactive alkylating agent from the organospecific carcinogen methylazoxymethanol by alcohol dehydrogenase. *Cancer Res* 1980;40:4446–50. [PubMed: 7002292]
30. Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007;449:1003–7. [PubMed: 17934449]
31. Hirose Y, Yoshimi N, Makita H, Hara A, Tanaka T, Mori H. Early alterations of apoptosis and cell proliferation in azoxymethane-initiated rat colonic epithelium. *Jpn J Cancer Res* 1996;87:575–82. [PubMed: 8766520]
32. Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun* 2005;331:851–8. [PubMed: 15865941]

33. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 1992;256:668–70. [PubMed: 1350108]
34. Nambiar PR, Giardina C, Guda K, Aizu W, Raja R, Rosenberg DW. Role of the alternating reading frame (P19)-p53 pathway in an *in vivo* murine colon tumor model. *Cancer Res* 2002;62:3667–74. [PubMed: 12097273]
35. Hu Y, Le Leu RK, Young GP. Absence of acute apoptotic response to genotoxic carcinogens in p53-deficient mice is associated with increased susceptibility to azoxymethane-induced colon tumours. *Int J Cancer* 2005;115:561–7. [PubMed: 15700305]
36. Fujii S, Fujimori T, Kawamata H, et al. Development of colonic neoplasia in p53 deficient mice with experimental colitis induced by dextran sulphate sodium. *Gut* 2004;53:710–6. [PubMed: 15082590]
37. Fazeli A, Steen RG, Dickinson SL, et al. Effects of p53 mutations on apoptosis in mouse intestinal and human colonic adenomas. *Proc Natl Acad Sci U S A* 1997;94:10199–204. [PubMed: 9294187]
38. Clarke AR, Cummings MC, Harrison DJ. Interaction between murine germline mutations in p53 and APC predisposes to pancreatic neoplasia but not to increased intestinal malignancy. *Oncogene* 1995;11:1913–20. [PubMed: 7478622]
39. Halberg RB, Katzung DS, Hoff PD, et al. Tumorigenesis in the multiple intestinal neoplasia mouse: redundancy of negative regulators and specificity of modifiers. *Proc Natl Acad Sci U S A* 2000;97:3461–6. [PubMed: 10716720]
40. Rupnarain C, Dlamini Z, Naicker S, Bhoola K. Colon cancer: genomics and apoptotic events. *Biol Chem* 2004;385:449–64. [PubMed: 15255176]
41. Jeffers JR, Parganas E, Lee Y, et al. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* 2003;4:321–8. [PubMed: 14585359]
42. Villunger A, Michalak EM, Coultas L, et al. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 2003;302:1036–8. [PubMed: 14500851]
43. Wood RD, Mitchell M, Lindahl T. Human DNA repair genes, 2005. *Mutat Res* 2005;577:275–83. [PubMed: 15922366]
44. Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. *Science* 2000;290:989–92. [PubMed: 11062132]
45. Ming L, Wang P, Bank A, Yu J, Zhang L. PUMA dissociates Bax and BCL-XL to induce apoptosis in colon cancer cells. *J Biol Chem* 2006;281:16034–42. [PubMed: 16608847]
46. Vogelstein B, Kinzler KW. The multistep nature of cancer. *Trends Genet* 1993;9:138–41. [PubMed: 8516849]
47. Li Q, Dashwood WM, Zhong X, Nakagama H, Dashwood RH. Bcl-2 overexpression in PhIP-induced colon tumors: cloning of the rat Bcl-2 promoter and characterization of a pathway involving β -catenin, c-Myc and E2F1. *Oncogene* 2007;26:6194–202. [PubMed: 17404573]
48. Labi V, Erlacher M, Kiessling S, Villunger A. BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. *Cell Death Differ* 2006;13:1325–38. [PubMed: 16645634]
49. Barker N, Ridgway RA, van Es JH, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 2008;457:608–11. [PubMed: 19092804]
50. Rosenberg DW, Yang S, Pleau DC, et al. Mutations in BRAF and KRAS differentially distinguish serrated versus non-serrated hyperplastic aberrant crypt foci in humans. *Cancer Res* 2007;67:3551–4. [PubMed: 17440063]

Figure 1.

PUMA deficiency enhanced tumorigenesis induced by AOM/DSS in the mouse colon. **A**, quantification of colon tumor burden in WT and *PUMA* KO mice at 4 mo in the AOM/DSS model. Columns, mean; bars, SD ($n = 12$ in each group; *, $P < 0.001$). **B**, representative pictures of AOM and DSS-induced colon tumors in WT and *PUMA* KO mice. **C**, quantification of tumor size in WT and *PUMA* KO mice. Columns, mean; bars, SD ($n = 12$ in each group; *, $P < 0.05$). **D**, quantification of ACF in WT and *PUMA* KO mice at 10 wk after the initial AOM injection. Columns, mean; bars, SD ($n = 4$ in each group; *, $P < 0.001$). Arrowheads, examples of ACF visualized by methylene blue staining (left, magnification, $\times 40$) and a typical ACF involving multiple crypts surrounded by normal crypts on an H&E section (right, magnification, $\times 400$).

Figure 2.

AOM treatment induced PUMA expression in the colonic crypts and stem cells. *A*, *PUMA* mRNA expression in the colonic mucosa of WT mice at 8 h following injection with either saline (*Un*) or 15 mg/kg of AOM was analyzed by quantitative real-time reverse transcriptase-PCR. *Columns*, mean; *bars*, SD ($n = 3$ in each group; *, $P < 0.001$). *B*, PUMA protein expression in the colonic mucosa of WT mice was determined by Western blotting 8 h after AOM injection. β -Actin was used as the control for loading ($n = 3$ mice in each group). *C*, *PUMA* mRNA (red) *in situ* hybridization in the colon of WT mice following indicated treatment (magnification, $\times 600$). *Right*, an enlarged view of the selected area. *D*, *Lgr5* mRNA (red) *in situ* hybridization in the colon of WT mice without any treatment (magnification, $\times 600$).

Figure 3.

PUMA mediated AOM-induced apoptosis in the colonic crypts and stem cells. The colon tissue was harvested 8 h after 15 mg/kg of AOM injection. *A*, apoptotic index in the colonic crypts was measured by counting 100 crypts/mouse following TUNEL staining. *Columns*, mean; *bars*, SD ($n = 3$ in each group; *, $P < 0.0001$). *B*, examples of TUNEL staining (*brown*) in the colon of WT and *PUMA* KO mice (magnification, $\times 600$). *C*, active caspase 3 immunohistochemistry (*brown*) in the colon of WT and *PUMA* KO mice (magnification, $\times 400$).

Figure 4.

PUMA mediated p53-dependent apoptosis induced by AOM in the colonic crypts. *A*, *PUMA* mRNA expression in the colonic mucosa was evaluated by quantitative real-time reverse transcriptase-PCR in WT and *p53* KO mice at the indicated time points after AOM treatment. *Columns*, mean; *bars*, SD ($n = 3$ in each group; *, $P < 0.01$). *Right*, *PUMA* protein expression in the colonic mucosa was determined by Western blotting at the indicated time points after AOM treatment. β -Actin was used as the control for loading. *B*, *PUMA* mRNA (red) *in situ* hybridization in the colon of WT and *p53* KO mice 8 h after AOM treatment (magnification, $\times 600$). *C*, apoptotic index was calculated by counting 100 crypts following TUNEL staining. *Columns*, mean; *bars*, SD ($n = 3$ in each group; *, $P < 0.001$). *D*, representative pictures of TUNEL staining in WT and *p53* KO mice 8 h after AOM treatment (magnification, $\times 400$).

Figure 5. *PUMA* deficiency enhanced tumorigenesis in *APC^{Min/+}* mice. *A*, polyps in the colon (≥ 0.5 mm in diameter) were scored under a stereoscope in age-matched (22 wk) and sex-matched *APC^{Min/+}* and *APC^{Min/+}/PUMA KO* mice. *B*, polyps in the small intestine were scored as in *A*. *C*, *left*, polyps in the small intestine stratified by anatomic region. *Right*, the size distribution of polyps in the small intestine. *Columns*, mean; *bars*, SD ($n = 14$ in each group in *A–C*). *D*, the number of microadenomas scored on H&E sections in the small intestine and colon of mice at 5 wk of age ($n = 5$ in each group).

Table 1

Histologic grades of AOM/DSS-induced colon tumors

WT mice	<i>PUMA</i> KO mice
3 LGD	1 LGD, 1 HGD
2 LGD	3 HGD
1 LGD	3 HGD, 1 AC
2 LGD	4 HGD, 1 AC
1 LGD	1 LGD, 3 HGD
1 LGD, 1 HGD	3 HGD

Abbreviations: LGD, adenoma with low-grade dysplasia; HGD, adenoma with high-grade dysplasia; AC, adenocarcinoma ($n = 6$ in each group).