

Pathway-specific tumor suppression: Reduction of p27 accelerates gastrointestinal tumorigenesis in *Apc* mutant mice, but not in *Smad3* mutant mice

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Summary

Expression of the cyclin-dependent kinase inhibitor p27^{Kip1} (p27) is frequently reduced in human colorectal cancer, and this correlates with poor patient prognosis. To clarify the role of p27 in gastrointestinal (GI) cancer, we measured p27 expression, as well as the effect of germline deletion of p27, in 3 different mouse models of GI neoplasia. p27 expression was frequently reduced in GI tumors arising in 1,2-dimethylhydrazine (DMH) treated mice, and in *Apc* mutant *Min*⁺ mice, but not in GI tumors arising in *Smad3* mutant mice. Germline deletion of p27 resulted in accelerated tumor development and increased tumor cell proliferation in both DMH treated and *Min*⁺ mice, but not in *Smad3* mutant mice. p27 deficiency also led to increased adenoma to adenocarcinoma progression. These results indicate that reduction of p27 cooperates with mutations in *Apc* but not in *Smad3* during GI tumorigenesis. Thus, tumor suppression by p27 is contingent on the specific oncogenic pathway that drives tumor development.

Introduction

p27^{Kip1} belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors that includes p21^{Cip1} and p57^{Kip2} (Sherr and Roberts, 1999). The Cdk inhibitors bind to cyclin/Cdk complexes, block the activation of Cdks, and inhibit cell cycle progression. p27 inhibits most cyclin/Cdk complexes, although it is most prominently linked to inhibition of cyclinE/Cdk2 (Polyak et al., 1994b). Endogenous expression of p27 in cell lines causes cell cycle arrest in G1 (Polyak et al., 1994b; Toyoshima and Hunter, 1994). Mitogen withdrawal, treatment of cells with TGF- β , or cadherin-mediated cell-cell contact lead to increased p27 binding to cyclinE/Cdk2 and cyclinA/Cdk2 complexes, and inhibition of G1/S progression (Polyak et al., 1994a; St. Croix et al., 1998; Levenberg et al., 1999).

The role of p27 in cancer is poorly defined. Reduced p27 expression is an unfavorable prognostic marker in many human cancers, including tumors of the colon, stomach, breast, lung, prostate, and ovary (Lloyd et al., 1999; Philipp-Staheli et al., 2001). Loss of p27 in tumors is also correlated with tumor aggressiveness, depth of tumor cell invasion, and poor state of

differentiation (Mori et al., 1997; Yasui et al., 1997; Kim et al., 2000; Singh et al., 1998). With regard to colon cancer, the median five-year survival rates for patients with colorectal cancer are dramatically reduced if tumors have low versus high p27 expression (Loda et al., 1997). Other studies have linked low p27 expression to more advanced colon cancer stage and to more poorly differentiated tumors (Ciaparrone et al., 1998; Sgambato et al., 1999). In matched pairs of primary and metastatic colorectal tumors, metastatic cells showed reduced p27 expression relative to the primary tumor (Thomas et al., 1998).

Point mutations in the coding region of the *p27* gene (*CDKN1B*) are rare in human tumors (Ponce-Castaneda et al., 1995; Kawamata et al., 1995; Pietenpol et al., 1995), although loss of heterozygosity (LOH) is observed in some tumor types (Stegmaier et al., 1995; Pietenpol et al., 1995; Hatta et al., 1997). The lack of tumor-associated mutations in *CDKN1B* has hindered efforts to establish a causal role of p27 in tumor progression. However, experiments in p27 deficient mice have established p27 as a haploinsufficient tumor suppressor. *p27* null ($-/-$) mice are predisposed to spontaneous pituitary adenomas (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996),

SIGNIFICANCE

The role of p27 in GI cancer is poorly understood, particularly the association of reduced p27 expression with poor clinical outcome. Our findings demonstrate a causal connection between reduction of p27 in GI tumors and increased proliferation, tumor growth, and tumor-associated mortality. Colon tumors from p27 deficient mice also showed more aggressive phenotypes, including complete invasion into the serosal space and lymphatic vessel penetration. This suggests a role for p27 in the early stages of metastasis, a finding that could have clinical implications. Furthermore, tumor suppression by p27 is not universal, but depends on the primary genetic lesion fueling tumor growth. This suggests that further stratification of human tumors according to defined molecular alterations, in combination with p27 staining, may improve prognostic sensitivity.

as well as radiation- and ENU-induced tumors in multiple epithelial tissues, with colon adenomas and adenocarcinomas being a prominent tumor type (Fero et al., 1998). *p27* heterozygous mice (+/-) show an intermediate susceptibility to the same tumor types.

Our first aim in the present study was to address the cellular mechanism by which *p27* suppresses colon tumorigenesis. We treated wild-type and *p27* knockout mice with the carcinogen 1,2-dimethylhydrazine (DMH), an alkylating agent that induces adenomas and adenocarcinomas specifically in the colon. These tumors closely parallel human colonic neoplasia in clinical and pathological features (Ahnén, 1985; LaMont and O'Gorman, 1978). In wild-type mice, *p27* protein expression was reduced in a subset of DMH-induced colon tumors. Both *p27*+/- and -/- mice showed accelerated colon tumor development, increased tumor cell proliferation, and enhanced adenoma to adenocarcinoma progression, indicating a prominent tumor suppressing function of *p27* in colonic neoplasia.

Our second aim was to determine if *p27* deficiency cooperates with mutations in two central oncogenic pathways during GI neoplasia: the Wnt/Apc and TGF- β /Smad3 pathways. The APC protein is part of the Wnt signaling pathway, and mutation of the APC gene plays a key role early in the development of both human and murine intestinal neoplasia. Individuals with familial adenomatous polyposis bear a germline mutation in APC and are highly predisposed to colorectal cancer (Grodén et al., 1991; Nishisho et al., 1991), and somatic mutations in APC are found in the majority of sporadic human colorectal tumors (Fearon and Vogelstein, 1990; Polakis, 1997). Likewise, mice bearing a germline mutation in *Apc*, e.g., *Min*+ mice, are highly prone to intestinal neoplasia (Su et al., 1992).

The TGF- β pathway is also frequently altered in colorectal cancer (Akhurst and Derynck, 2001). Many colorectal cancer cells are resistant to growth inhibition by TGF- β , due to mutations in members of the TGF β /SMAD signaling pathway (Markowitz et al., 1995; Miyaki et al., 1999). SMAD3 is one of several intracellular signaling proteins that mediate the inhibition of epithelial cell proliferation by TGF- β (Zhou et al., 1999). Mice with a targeted disruption in *Smad3* or *TGF- β 1* develop colorectal adenocarcinomas (Zhu et al., 1998; Engle et al., 1999), indicating an important role for TGF- β signaling in both human and murine GI neoplasia.

To clarify the role of *p27* in both the Wnt/Apc and TGF- β /Smad signaling pathways during GI tumor development, we generated *p27/Apc* and *p27/Smad3* compound mutant mice. Germline deletion of *p27* markedly accelerated the rate of development of intestinal tumors bearing mutations in *Apc*, but had no measurable effect in tumors bearing mutations in *Smad3*.

Results

p27 deficiency reduces latency and increases malignancy of chemically induced colorectal tumors

To specifically address the role of *p27* in colorectal cancer, cohorts of *p27*-/-, *p27*+/-, and wild-type littermates were treated with DMH and sacrificed when moribund. Overall tumor-free survival was significantly reduced in *p27*-/- mice, and to an intermediate extent in *p27*+/- mice, relative to wild-type

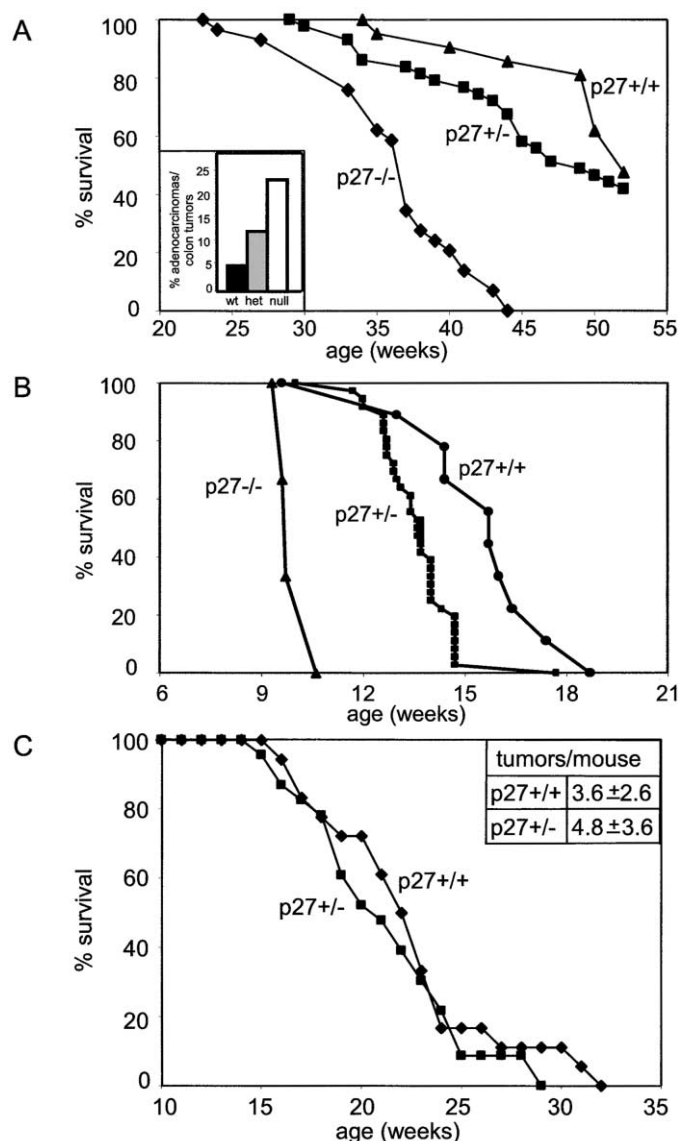


Figure 1. *p27* reduction decreases tumor-free survival of DMH-treated and *Min*+ mice but not *Smad3*-/- mice

A: Survival of DMH-treated mice is significantly decreased with reduction of *p27* gene dosage (log rank test: wt versus *p27*-/- $p < 0.0001$, *p27*+/- versus *p27*-/- $p < 0.0001$, wt versus *p27*+/- $p = 0.2759$). The insert shows that *p27* reduction leads to a higher percentage of adenocarcinomas. Due to different age at sacrifice, this likely underestimates the frequency for *p27* deficient mice. **B:** Survival rate of *Min*+ mice is decreased with *p27* reduction (log rank test: wt versus *p27*-/- $p < 0.005$, wt versus *p27*+/- $p < 0.001$). **C:** Survival curves for *Smad3* null mice show no difference between *Smad3*-/-*p27*+/- and *Smad3*-/-*p27*+/- mice (log rank test: $p = 0.4$). The insert shows similar tumor multiplicities between the two *p27* genotypes (2-sample t test: $p = 0.10$).

littermate controls (Figure 1A). The primary cause of morbidity was intestinal obstruction and rectal prolapse as a result of tumors of the colon and rectal region. Other tumor types also reduced survival in *p27*-deficient mice, including uterine tumors of several histologic subtypes and pituitary adenomas (data not shown). Survival plots of the subset of animals that were

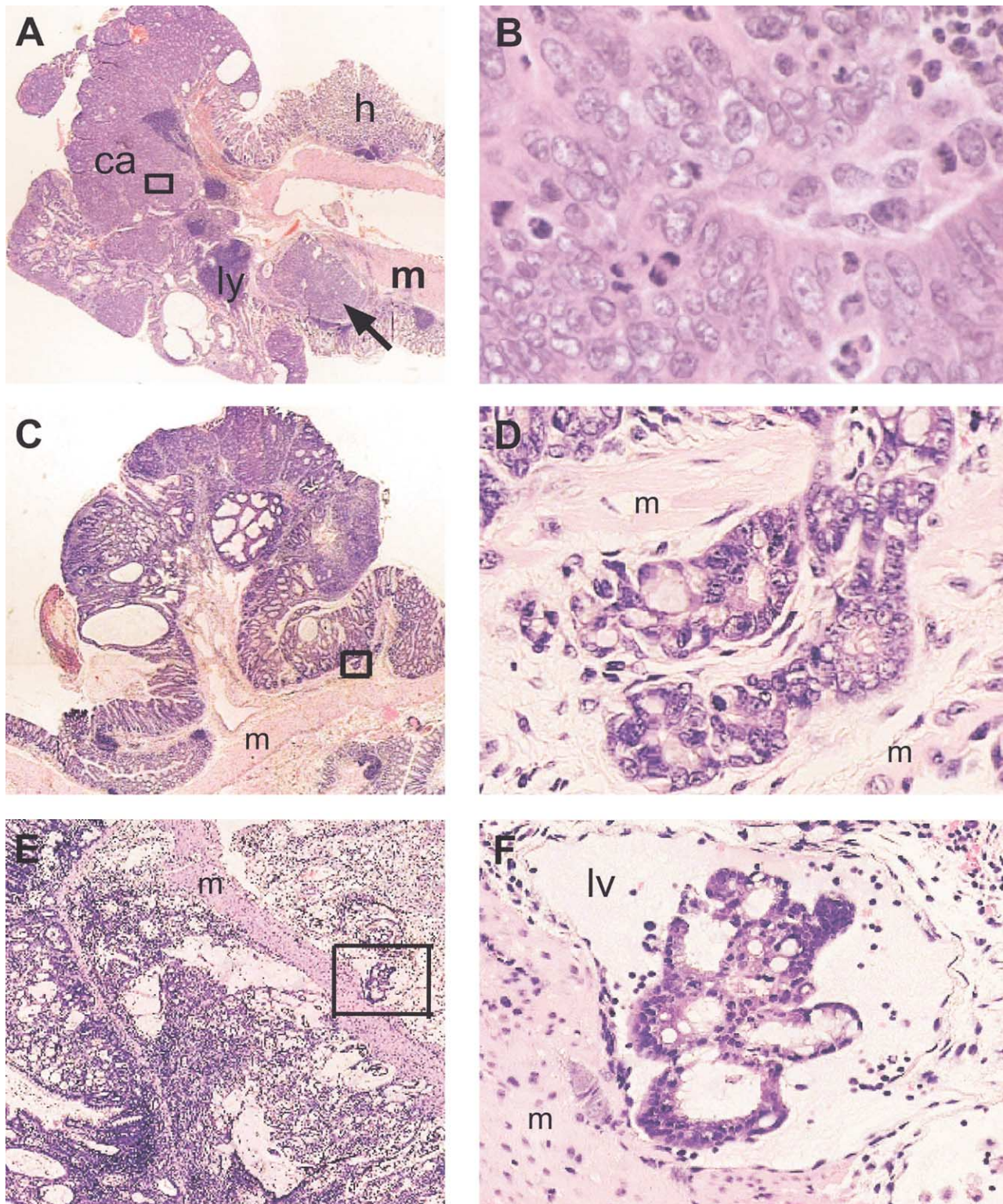


Figure 2. Colon tumors from p27 deficient mice demonstrate more aggressive features

Shown are H&E stained sections of colon tumors from DMH treated p27^{-/-} mice on the left with the corresponding high-power views (indicated by box) on the right. **A:** A morphologically complex neoplasm demonstrating an adenocarcinomatous change with invasion into the submucosa and muscularis (m). These changes exist adjacent to areas of focal hyperplasia (h). Lymphoid cell aggregates (ly) are also present in scattered locations. **B:** Dysplastic cells, abundant mitotic figures, and infiltrating immune cells within the tumor are shown. **C:** A large colonic neoplasm with focal invasion of the submucosa (m), shown in **D**. **E:** A highly malignant diffusely distributed adenocarcinoma with tubular and scirrhous properties extends throughout the submucosa and serosa. A raft of neoplastic cells is present within a serosal lymphatic vessel (lv) (**F**).

sacrificed only due to complications from colorectal tumors were similar to Figure 1A, and showed that p27 deficiency specifically accelerated colon tumor development and associated mortality, and did so in a p27 gene dosage-dependent manner.

Colorectal tumors from p27^{-/-} (n = 15), p27^{+/-} (n = 24), and p27^{+/+} (n = 10) littermates were evaluated for histopathologic features. The majority of tumors from wild-type mice were classified as adenomas, whereas the incidence of colorectal adenocarcinomas, as well as the ratio of adenocarcinomas to adenomas, were both significantly increased in p27 deficient mice (Figure 1A, insert). In addition to increased carcinoma in situ, a number of adenocarcinomas from p27^{-/-} and p27^{+/-} mice showed clear evidence of invasion through the submucosa and muscularis propria into the serosal space (Figures 2A–2D). In more severe cases, lymphatic vessel invasion by tumor cells was observed (Figures 2E and 2F). Thus, germline reduction of p27 decreased the latency for DMH-induced colorectal tumor development and resulted in more aggressive tumor behavior.

Individual DMH-induced tumors are likely to harbor different combinations of mutations in *k-ras* (Jacoby et al., 1991), *β-catenin* (Blum et al., 2001), *Apc*, or other oncogenes or tumor suppressor genes. This genetic diversity between tumors is likely to closely mirror human colorectal neoplasia, but it also prohibits the analysis of the tumor-suppressing role of p27 in a defined genetic setting or molecular pathway. In addition, the use of chemical or physical agents in tumor induction could have other nongenotoxic effects that influence p27 levels or other pathways. To determine the effect of p27 levels on GI tumor development in the absence of exogenous agents, as well as to determine if tumor suppression by p27 depends on the genetic makeup of the tumor, we used defined genetic models of GI neoplasia.

Reduction of p27 accelerates tumor growth and decreases survival of *Min*⁺ mice

Multiple intestinal neoplasia (*Min*⁺) mice bear a heterozygous mutation in the *Apc* gene, and these mice spontaneously develop numerous adenomas in both the small intestine and colon (Su et al., 1992; Shoemaker et al., 1997). *Min*⁺ mice were crossed to p27-deficient mice, and spontaneous tumor development was compared between the single and compound mutant animals. *Min*⁺ mice developed symptoms of tumor burden, including anemia and lethargy, starting at 16 weeks of age, whereas *Min*⁺ p27^{+/-} mice became moribund earlier, between 12–14 weeks of age, and *Min*⁺ p27^{-/-} mice had an even shorter latency, succumbing by around 9 weeks of age (Figure 1B). Morbidity in all genotypes was due to extensive tumor development throughout the small intestine and colon. Both *Min*⁺ p27^{-/-} and *Min*⁺ p27^{+/-} mice carried a greater tumor burden throughout the GI tract relative to *Min*⁺ mice (Figures 3A and 3B). For example, 9-week-old *Min*⁺ p27^{-/-}, *Min*⁺ p27^{+/-}, and *Min*⁺ mice had an average (± standard deviation) of 101 (±49), 35 (±13), and 21 (±9.6) tumors per mouse, respectively. Reduction of p27 also resulted in increased tumor size (Figures 4A–4C). Of colon tumors from *Min*⁺ p27^{-/-} mice, 42% (5/12) were ≥ 3 mm in size by 9 weeks of age compared to 0/6 in *Min*⁺ mice. Of small intestinal tumors from *Min*⁺ p27^{-/-} mice, 29% (115/393) were ≥ 2 mm in size by 9 weeks of age, compared to 0/100 in *Min*⁺ mice. Tumor multiplicity and size in *Min*⁺ p27^{+/-} mice was also significantly increased compared to *Min*⁺ mice (Figures 4B–4C).

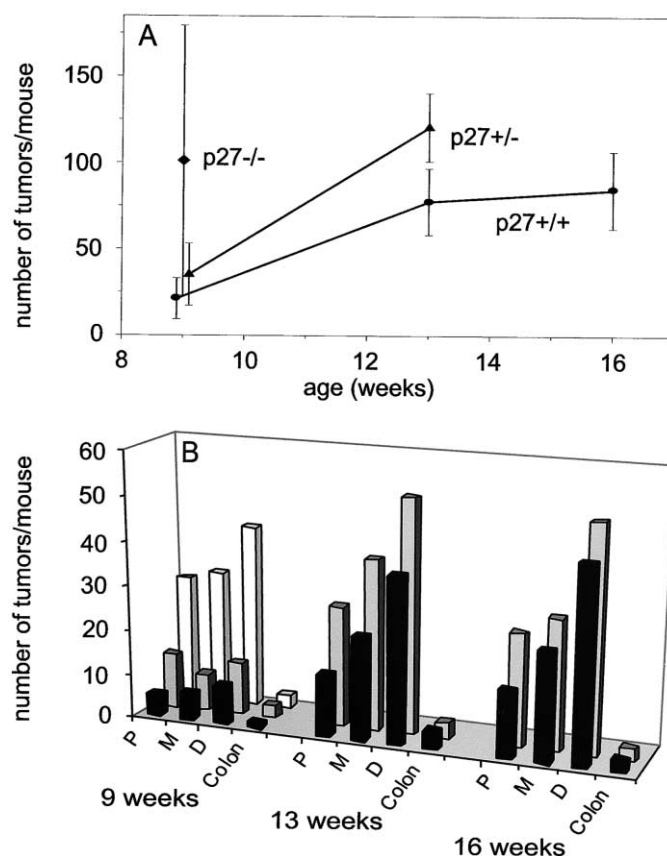


Figure 3. Reduction of p27 increases tumor multiplicity in *Min*⁺ mice

A: Tumor multiplicity throughout the GI tract is increased in *Min*⁺ p27^{-/-} mice and *Min*⁺ p27^{+/-} as compared to *Min*⁺ p27^{+/+} mice. Vertical bars represent t-based 95% confidence intervals. p27^{-/-} versus p27^{+/+} at 9 weeks $p = 0.002$, p27^{+/-} versus p27^{+/+} at 13 weeks $p = 0.07$ using the two sample t-test. **B:** Reduction of p27 has the largest relative effect on tumor number in the proximal region of the small intestine (white p27^{-/-}, gray p27^{+/-}, black p27^{+/+}). D, distal; M, middle; P, proximal.

Importantly, p27^{-/-} mice with two wild-type copies of *Apc* did not develop GI tumors up to one year of age. These results demonstrate a potent synergistic interaction between reduction of p27 and mutation in *Apc* during GI neoplasia.

Intestinal neoplasms from *Min*⁺ mice had morphologic features ranging from adenomatous hyperplasia to (most commonly) adenoma, with no difference in tumor histology noted between p27 genotypes (Figure 4A). However, the very early lethality of p27 deficient mice in this study (9–10 weeks of age) might well preclude detection of an effect of p27 on malignant progression.

Reduction of p27 does not enhance tumor development in *Smad3* mutant mice

To address whether intestinal tumor suppression by p27 is a general phenomenon or if it is specific to the oncogenic pathway driving tumor development, we next examined *Smad3*^{-/-} knockout mice. *Smad3*^{-/-} mice are defective in the TGF-β signaling pathway, and spontaneously develop intestinal adenocarcinomas (Zhu et al., 1998). p27 deficient mice were crossed to *Smad3* deficient mice to generate compound mutant animals.

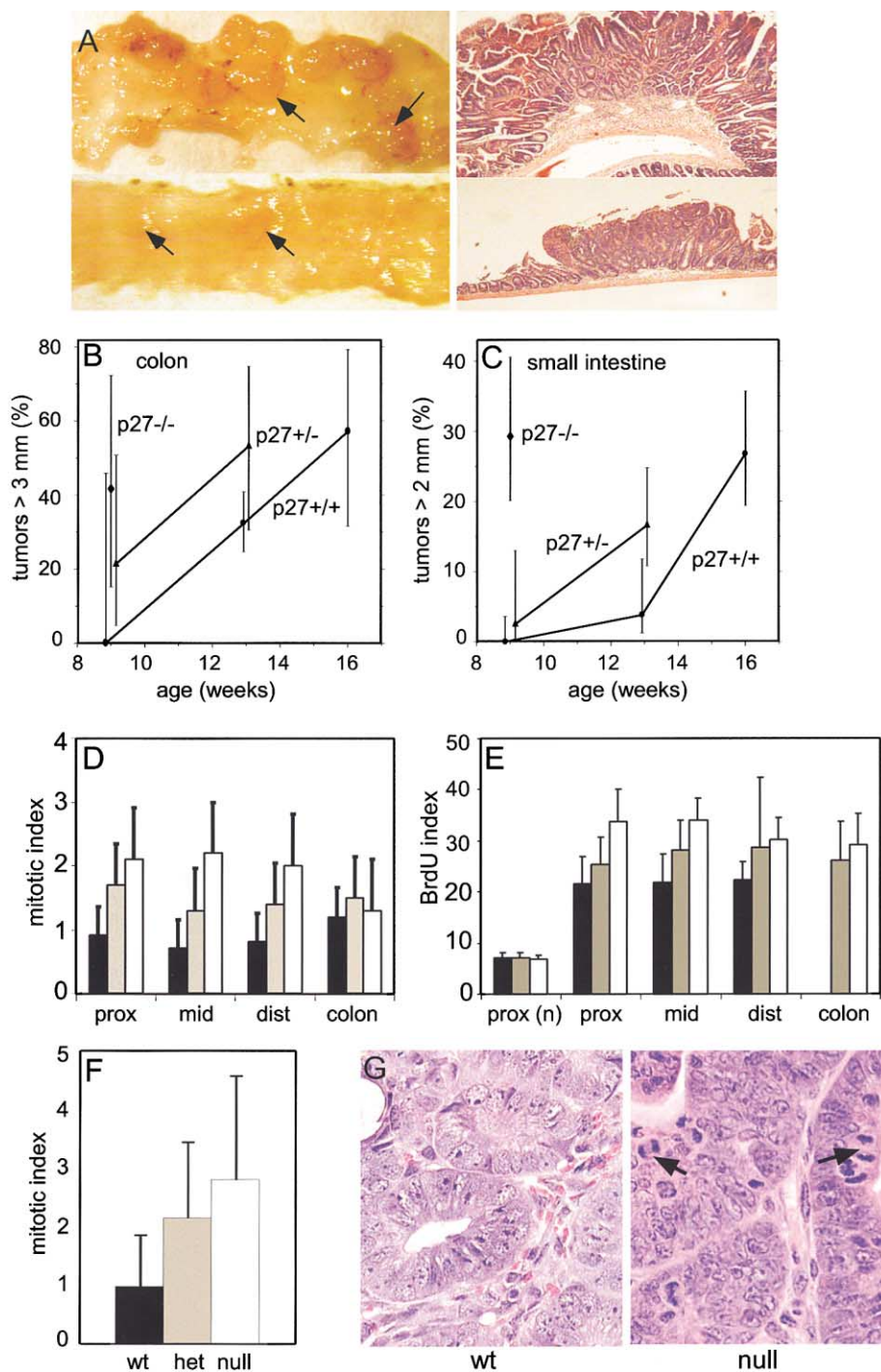
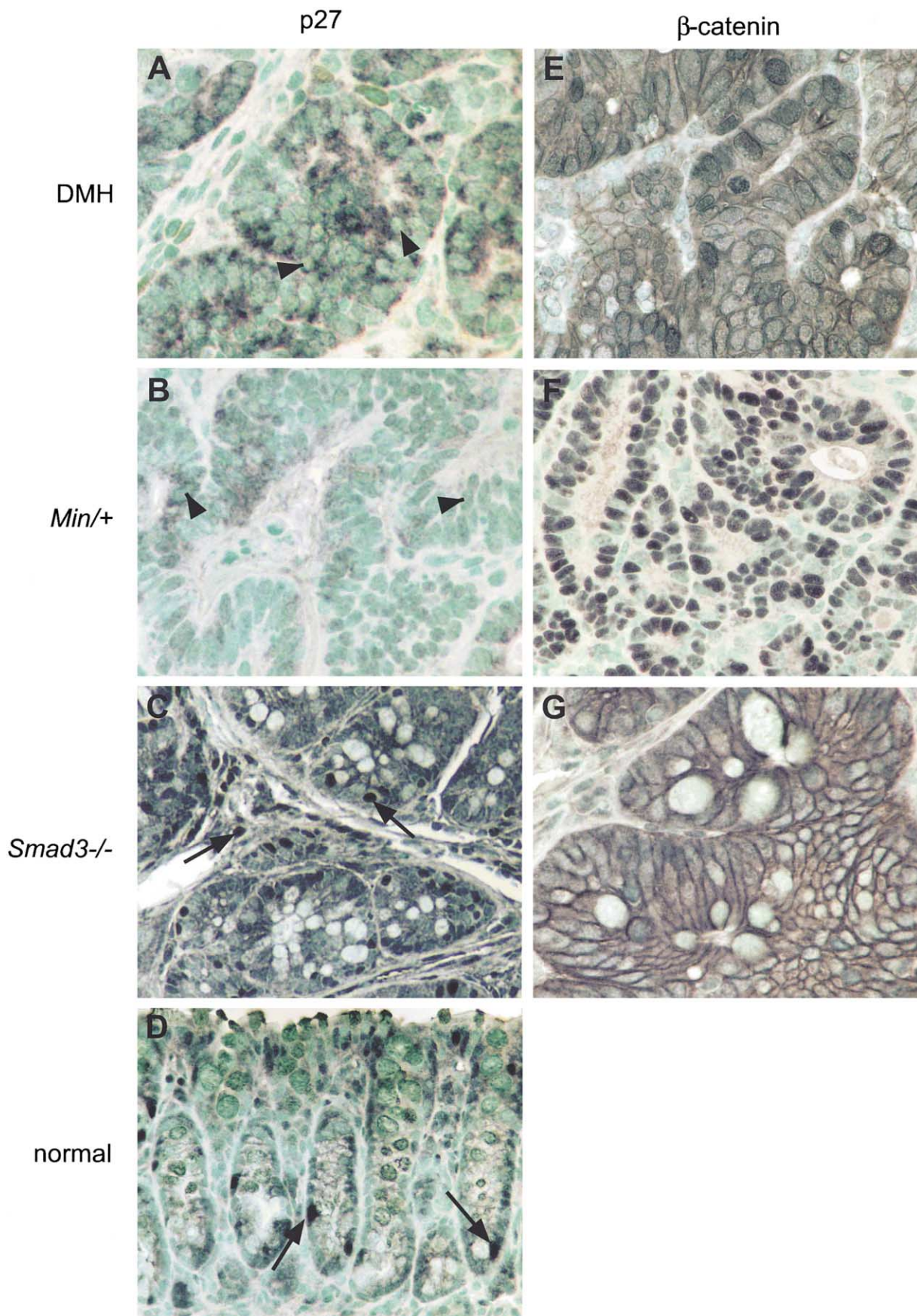


Figure 4. Reduction of p27 leads to larger tumor size and increased proliferative index in *Min/+* mice and DMH-treated mice

A: Adenomas in the small intestine of *Min/+p27^{-/-}* mice (top) are larger and more numerous than in *Min/+p27^{+/+}* mice (bottom) at nine weeks of age. Arrows point to tumors. H&E stained sections of typical adenomas from *Min/+p27^{-/-}* (upper right) and *Min/+* mice (lower right) are shown. The percentage of large size class tumors per mouse in colon (**B**) and small intestine (**C**) is significantly greater in p27 deficient mice. Vertical bars represent t-based 95% confidence intervals. p-values are: colon: p = 0.11 for p27^{-/-} versus wt at 9 weeks, p = 0.09 for p27^{+/-} versus wt at 13 weeks; small intestine: p < 0.001 for p27^{-/-} versus wt at 9 weeks, p = 0.02 for p27^{+/-} versus wt at 13 weeks. Mitotic (**D**) and BrdU (**E**) labeling indices are higher in adenomas from *Min/+p27^{-/-}* and *Min/+p27^{+/-}* mice relative to *Min/+* mice (white p27^{-/-}, gray p27^{+/-}, black p27^{+/+}). Values are the mean \pm standard deviation. The difference between the mitotic indices of adenomas from *Min/+* mice and *Min/+p27^{-/-}* mice was highly significant in all sections of the small intestine but not in the colon. For *Min/+p27^{+/+}* versus *Min/+p27^{-/-}* mice, p = 0.07 (distal), 0.18 (middle), 0.01 (proximal); for *Min/+p27^{+/+}* versus *Min/+p27^{-/-}* mice, p = 0.002 (distal), 0.001 (middle), 0.001 (proximal) (Wilcoxon rank sum test). The following total number of powerfields was counted per p27 genotype: for mitotic index, 83 (wt), 110 (p27^{+/-}), 55 (p27^{-/-}); for BrdU index, 90 (wt), 84 (p27^{+/-}), 40 (p27^{-/-}) in tumors; the following number of crypts was counted for normal tissue: 365 (wt), 478 (p27^{+/-}), 452 (p27^{-/-}). **F:** The mitotic index within colon adenomas from DMH treated mice is also significantly higher in mice with reduced p27 (Wilcoxon rank sum test: wt versus p27^{-/-} p < 0.0327, p27^{+/-} versus p27^{-/-} p < 0.3951, wt versus p27^{+/-} p = 0.0316). Mitotic figures were counted in H&E stained sections of colon adenomas (**G**).

Smad3^{-/-} p27^{-/-} offspring were generated in fewer than expected numbers and died at around 4 weeks of age prior to colorectal tumor development, indicating a nontumorigenic interaction between *Smad3* and p27. These results will be described elsewhere. *Smad3^{-/-}* and *Smad3^{-/-} p27^{+/-}* littermates were sacrificed when moribund, between 15–30 weeks of age. The primary cause of morbidity was anemia due to bleeding of large tumors within the cecum or colon, and rectal prolapse. However, in contrast to the two previous studies, there was no measurable difference in tumor-free survival between

Smad3^{-/-} and *Smad3^{-/-} p27^{+/-}* mice (Figure 1C). There was also no significant difference in tumor multiplicity (Figure 1C, insert) or in tumor size (data not shown) between p27 genotypes. Cecal and colon tumors arising in *Smad3^{-/-}* and *Smad3^{-/-} p27^{+/-}* mice were classified as both adenomas and adenocarcinomas, with no difference in histological subtypes noted between groups. Enlarged lymph nodes were frequently observed in tumor bearing mice, but we did not observe metastatic cells in any of the lymph nodes (n = 27), or other major organ sites examined.



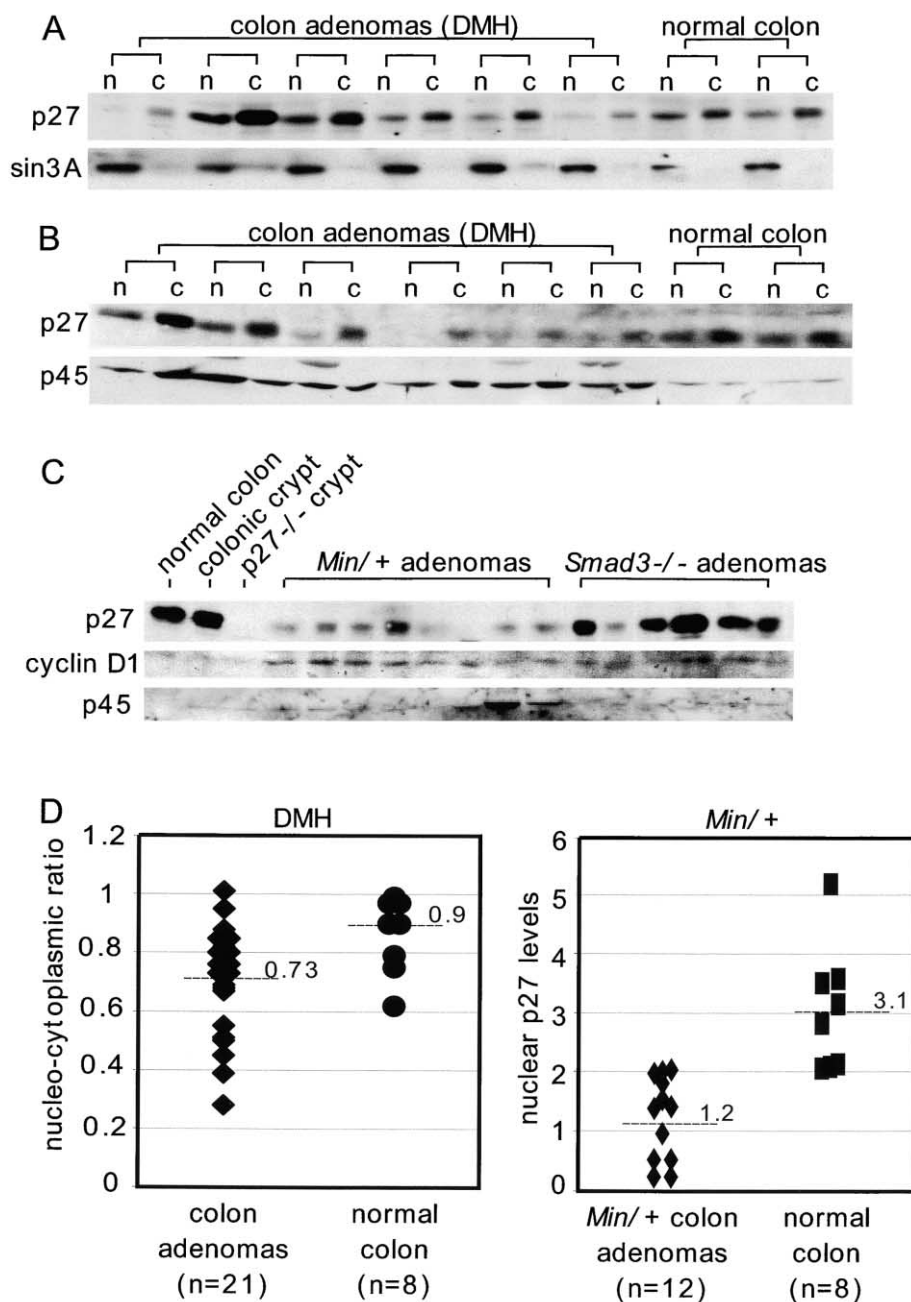


Figure 6. Reduced p27 expression in DMH-induced and Min/+ colon adenomas but not in Smad3^{-/-} adenomas

A: Western blot analysis of nuclear (n) and cytoplasmic (c) extracts from colon adenomas of DMH-treated mice was performed using an anti-p27 polyclonal antibody. Normal colon tissues were from age-matched wild-type C57BL/6 mice. The Sin3A blot confirms complete cellular fractionation as well as equal loading for nuclear extracts. Ponceau S staining was used in all Western blots to confirm equal loading. **B:** Skp2 (p45) protein levels are increased in DMH-induced colon tumors, but do not correlate with p27 levels. Antibodies against nuclear proteins Sin3A and Max were used to confirm complete cellular fractionation. No contamination of cytoplasmic fractions by nuclear proteins was detected (see Figure 6A and data not shown). **C:** Western blot analysis of nuclear extracts shows reduced expression of p27 protein in Min/+ adenomas but abundant expression in Smad3^{-/-} adenomas compared to normal colon or normal colonic crypt epithelial cell extracts. Cyclin D1 levels are increased in colon tumors but are not differentially regulated in Min/+ versus Smad3^{-/-} mice. p45 is more abundant in normal colonic crypt cells than in total colon tissue, and is strongly increased in a subset of Min/+ adenoma samples. **D:** Densitometric measurement of Western blots with 21 DMH-induced colon tumors and 8 normal colon samples from Min/+ mice were used to derive densitometric measurements for nuclear p27 levels. Note reduced levels of nuclear p27 in a subset of DMH induced tumors and reduced nuclear levels of p27 in Min/+ adenomas.

Nuclear p27 is reduced in adenomas and adenocarcinomas from DMH-treated and Min/+ mice, but not Smad3^{-/-} mice

Given the significant effect of p27-deficiency on GI tumor development in DMH-treated and Min/+ mice, but not Smad3^{-/-}

mice, we next determined whether p27 protein expression was altered in tumors arising in p27 wild-type mice from these same three models. Normal intestinal epithelial cells showed diffuse nuclear and cytoplasmic staining for p27. Very intense nuclear p27 staining was seen in the occasional cell within the crypt,

Figure 5. p27 and β -catenin expression patterns in colon tumors

A and E: Section of a colon adenoma from DMH treated mouse probed with p27 or β -catenin antibody, respectively. Note decreased nuclear staining for p27 with some remaining cytoplasmic staining, and nuclear and cytoplasmic staining for β -catenin within tumor cells. **B and F:** Colon adenoma from a Min/+ mouse. Note overall reduction of p27 staining along with prominent nuclear β -catenin staining. **C and G:** Colon adenoma from a Smad3^{-/-} mouse. Note strong nuclear staining for p27 throughout section along with weaker cytoplasmic staining. β -catenin staining is exclusively cytoplasmic. **D:** Normal colonic mucosa stained for p27. Note strong nuclear staining scattered throughout bottom third of crypts and weaker overall cytoplasmic staining. Arrows: p27 positive nuclei; arrow heads: p27 negative nuclei.

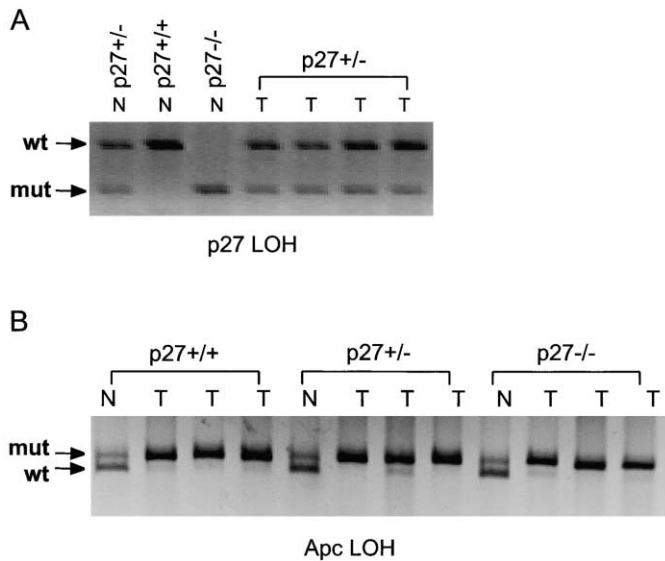


Figure 7. LOH analysis of *p27* (*Cdkn1b*) and *Apc* in GI tumors

A: Semiquantitative PCR amplification using *p27* specific primers reveals no LOH of wild-type *p27* allele in colon tumors from *p27*^{+/-} mice. **B:** The wild-type *Apc* allele is lost in intestinal tumors from *Min*^{+/+} mice independent of *p27* genotype. Representative results of a PCR analysis using *Apc* specific primers are shown. (T = tumor, N = normal tissue).

generally 1–2 per crypt cross-section (Figure 5D). This pattern of *p27* staining differs from Ki-67 staining of proliferating cells, which is localized at the bottom third of the crypt, and from *p21*/*Cip1* staining, which is localized to cells in the upper region of the crypt (El-Deiry et al., 1995). The staining pattern for *p27* was similar in tumors from DMH-treated and *Min*^{+/+} mice. Large areas of most tumors showed noticeably reduced or undetectable nuclear *p27* staining (Figures 5A and 5B). Highly differentiated, glandular structures of the tumors tended to show more prominent nuclear *p27* staining, whereas pseudoglandular structures or more poorly differentiated areas lacked nuclear *p27* staining. *p27* staining was present in mesenchymal cells within the tumor, and prominent nuclear *p27* staining was observed in infiltrating lymphocytes. Overall, the distribution of *p27* staining in normal colon tissue and tumor tissue from both DMH-treated and *Min*^{+/+} mice bears close resemblance to staining seen in human tissue (Ciaparrone et al., 1998). In marked contrast, most adenomas and adenocarcinomas from *Smad3*^{-/-} mice displayed strong nuclear *p27* staining within most cells of the tumor (Figure 5C).

Western blot analysis confirmed these histologic findings. In normal colon tissue, abundant *p27* was detected in both nuclear and cytoplasmic fractions (Figure 6A). Approximately one-third of DMH-induced colon adenomas and adenocarcinomas showed markedly reduced levels of nuclear *p27*, but retained abundant cytoplasmic *p27*. Representative tumor samples are shown in Figure 6A. Densitometric analysis confirmed that, on average, these tumors had reduced nuclear/cytoplasmic ratios of *p27* relative to normal colon tissue (Figure 6D). The majority of tumors in both the small intestine and colon from *Min*^{+/+} mice also showed reduced levels of *p27* in both the nuclear and cytoplasmic fractions compared to normal intestine

and colon (Figures 6C and 6D). In marked contrast, *p27* levels remained high in tumor lysates from *Smad3*^{-/-} mice, similar to that seen in normal intestinal tissue (Figure 6C), confirming the immunostaining results (Figures 5A–5C). Thus, reduction of nuclear *p27* protein is seen in a subset of intestinal and colonic adenomas and adenocarcinomas from DMH treated and *Min*^{+/+} mice, but not in *Smad3* mutant mice.

These findings, together with the genetic results described above, indicate that reduced *p27* expression that is observed in intestinal tumor cells is not coincidental but rather is causally related to increased tumor growth. In tumors that show reduced *p27* expression, e.g., in the DMH-treated and *Min*^{+/+} mice, germline reduction of *p27* leads to increased tumor growth, whereas tumors from *Smad3*^{-/-} mice do not show reduction of *p27* expression and deletion of *p27* has no measurable effect on tumor development. Thus, the selective pressure for reducing *p27* expression varies depending on the predominant genetic lesion driving tumor development.

***p27*-deficiency enhances proliferation in GI tumors from DMH-treated and *Min*^{+/+} mice**

The earlier appearance of GI tumors in *p27*-deficient mice in the DMH and *Min* study, and the known function of *p27* as an inhibitor of cell cycle progression, suggested that one mechanism of tumor suppression by *p27* may be to regulate tumor cell proliferation. Indeed, the mitotic index in adenomas from *p27*^{+/-} and *p27*^{-/-} mice was 2- to 3-fold greater than the mitotic index in adenomas from wild-type mice (Figures 4D–4G). This effect of *p27* on tumor cell proliferation was seen in both DMH-induced colon tumors and in small intestinal tumors from *Min*^{+/+} mice. BrdU labeling index of S-phase cells confirmed these findings (Figure 4E). The apoptotic index in adenomas did not differ between *p27* genotypes (data not shown). In contrast, crypt cells of normal intestinal tissue displayed similar mitotic and BrdU labeling indices between *p27* genotypes (Figure 4E). These data indicate that *p27* functions to retard intestinal and colonic adenoma growth by reducing tumor cell proliferation, and it does so in a *p27* gene dosage dependent manner.

***Apc*, but not *p27*, shows loss of heterozygosity in tumors**

p27 heterozygous mice showed an intermediate effect with respect to the phenotypes described above, including the timing of tumor-related mortality (Figures 1A and 1B), tumor cell proliferation (Figure 4), and tumor malignancy (Figure 1A). In contrast to *Apc* and other tumor suppressor genes that show LOH in tumors, loss of the wild-type *p27* allele has not been observed in tumors from *p27* heterozygous mice (Fero et al., 1998). To confirm this in the current study, we tested for LOH of *p27* by semiquantitative PCR. This analysis showed that 13 out of 13 DMH-induced tumors from *p27*^{+/-} mice and ten out of ten tumors from *Min*^{+/+}*p27*^{+/-} retained the wild-type *p27* allele (Figure 7A and data not shown). In both studies, Western blot analysis of tumor extracts of *p27*^{+/-} mice showed that *p27* protein expression was retained, but at reduced levels compared to wild-type, in all tumors examined (data not shown). Thus, *p27* suppresses intestinal adenoma and colon adenocarcinoma development in a quantitative manner.

Close to 100% of adenomas from *Min*^{+/+} mice show complete loss of the wild-type *Apc* allele, usually via chromosomal nondisjunction (Luongo et al., 1994). A possible mechanism for

synergy between p27 and Apc is that reduction of p27 might obviate the need for homozygous inactivation of *Apc*, thereby accelerating tumor development. However, all adenomas analyzed from *Min/+* ($n = 7$), *Min/+ p27+/-* ($n = 10$), and *Min/+ p27-/-* ($n = 6$) mice showed loss of the wild-type *Apc* allele (Figure 7B). Thus, reduction of p27 does not functionally substitute for loss of *Apc*; homozygous mutation of *Apc* is still required for tumor development, independent of p27 status.

Nuclear accumulation of β -catenin in tumors from *Min/+*, but not *Smad3* mutant mice

To further define the molecular differences in the *Min/+* and *Smad3* tumors, we examined β -catenin and cyclin D1 expression patterns. The Apc protein functions as part of a complex that binds to β -catenin, leading to its phosphorylation and subsequent ubiquitin-mediated degradation (Polakis, 2000). Mutations in Apc, such as those that occur in sporadic colorectal cancers, disrupt the Apc: β -catenin interaction, leading to β -catenin stabilization and its nuclear accumulation. Nuclear β -catenin, in turn, leads to activation of the Tcf/Lef-1 transcription factors and induction of target genes such as cyclin D1 and *myc*. This pathway is clearly important in oncogenesis, as mutations in β -catenin are detected in both human and murine tumors, resulting in its stabilization and nuclear accumulation (Polakis, 2000). Tissue sections from DMH, *Min/+*, and *Smad3-/-* induced tumors were stained for β -catenin and examined for β -catenin localization. In normal GI, diffuse cytoplasmic β -catenin staining was seen in intestinal epithelial cells. A similar pattern of β -catenin staining was seen in all tumors from *Smad3* mutant mice ($n = 11$) (Figure 5G). In contrast, all tumors examined from *Min/+* mice ($n = 5$) displayed prominent nuclear β -catenin staining (Figure 5F), consistent with mutation of *Apc* in these tumors. DMH-induced tumors displayed both cytoplasmic and nuclear β -catenin staining (Figure 5E). Thus, in DMH-induced and *Min/+* GI tumors, nuclear accumulation of β -catenin is a prominent feature, while in *Smad3* tumors, nuclear β -catenin is not observed. This result confirms that different molecular pathways drive tumorigenesis in the *Min/+* compared to the *Smad3* mutant mice. Cyclin D1 has been identified as one of many β -catenin/Tcf/Lef1-target genes (Tetsu and McCormick, 1999), and its expression is increased in some colon tumors. Relative to normal colonic tissue, cyclin D1 expression was increased in adenomas from both *Min/+* and *Smad3-/-* mice (Figure 6C). This indicates that cyclin D1 can be upregulated in tumors, independent of β -catenin activation.

Expression of Skp2/p45 is increased in GI tumors

p27 is regulated at several levels, including transcription, translation, and protein stability (Philipp-Staheli et al., 2001). However, the basis for the variation in p27 expression seen between tumors is not understood, although where examined, posttranslational control is cited (Loda et al., 1997; Chiarle et al., 2000). Skp2 is an F box adapter protein that targets p27 for ubiquitin-mediated degradation (Carrano et al., 1999). To determine if p27 abundance correlated with Skp2 expression, we measured Skp2 levels in tumors from the three models. Relative to normal colon tissue, Skp2 was markedly increased in all GI tumors examined from DMH treated mice (Figure 6B). Immunoblots with nuclear proteins Sin3A and Max confirmed that cytoplasmic fractions were not contaminated by nuclear proteins (Figure 6A and data not shown). However, there was no clear association

between levels of p27 and Skp2, as tumors with low or high p27 both showed similar high levels of Skp2. Skp2 expression was slightly increased in adenomas from both *Min/+* and *Smad3-/-* mice, with several *Min/+* tumors showing abundant Skp2 (Figure 6C). Here again, there was no clear correlation between levels of Skp2 and p27. Thus, the strongest association with p27 levels in GI tumors was not with Skp2, but rather with the primary genetic lesion driving tumor development. p27 levels were much lower in tumors with *Apc* mutations relative to tumors with *Smad3* mutations.

Discussion

We used several carcinogen protocols and two tumor prone mouse models to address the role of p27 in the natural history of intestinal neoplasia. While p27 deficient mice do not spontaneously develop intestinal tumors, they show markedly increased predisposition to adenoma and adenocarcinoma development in the small intestine and colon in response to four diverse carcinogens: ENU, γ -radiation (Fero et al., 1998), DMBA (Philipp et al., 1999), and DMH (this study). These agents produce a variety of genetic lesions that likely result in cancer-initiating mutations in a variety of oncogenes or tumor suppressor genes. The apparent requirement for carcinogen treatment suggests that tumor suppression by p27 may be contingent on mutational activation in one or more oncogenic pathways. To determine if there was specificity to the oncogenic pathway that p27 might interact with, we used two genetic models of GI neoplasia. Reduction of p27 greatly accelerated the rate of development of intestinal tumors with preexisting mutations in *Apc*, but showed little or no effect in tumors with *Smad3* mutations. Thus, tumor suppression by p27 is not universal, but is contingent on the specific genetic pathway altered in the tumor cells.

Pathways

p27 protein levels were reduced in a significant fraction of adenomas from *Min/+* mice and germline reduction of p27 greatly accelerated the rate of development of tumors from *Min/+* mice. This indicates that p27 is inhibitory, and that there is strong selective pressure to reduce p27 expression in mutant *Apc*-driven tumorigenesis. What is the basis of the synergism between p27 and Apc? p27 binds to cyclin E/cdk2 complexes and inhibits cdk2 activity which can block cell cycle progression. The increased proliferation seen in p27 deficient tumors from *Min/+* mice indicates that tumor development driven by mutation in *Apc* is likely limited by cdk2 activity, and this is relieved by reduction of p27. Mutation in *Apc* contributes to tumor development in at least two ways. The Apc protein is a component of the Wnt signaling pathway and normally functions to target β -catenin for degradation. Mutational inactivation of *Apc* results in accumulation of β -catenin in the nucleus, leading to activation of Tcf/Lef-1 transcription factors and expression of target genes, including cyclin D1 and *c-myc* (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Both β -catenin and cyclin D1 levels were increased in *Min/+* tumors. However, the fact that cyclin D1 was also increased in *Smad3* mutant tumors, yet reduction of p27 did not cooperate with this model, suggests that the synergy between p27 and the Apc/ β -catenin pathway is independent of cyclin D1 levels.

Apc also appears to contribute to tumorigenesis via an effect

on chromosomal instability. Loss of *Apc* was recently shown to disrupt the interactions between the kinetochores and spindle microtubules leading to chromosomal instability (Kaplan et al., 2001; Fodde et al., 2001). Although we did not observe LOH of *p27* in tumors from *Min/+ p27+/-* mice, we did observe LOH of *Apc* in tumors from *Min/+ p27-/-*, *p27+/-*, and *p27* wild-type mice. This indicates that complete loss of *Apc* was still required for tumorigenesis regardless of *p27* levels, and that loss of *p27* did not functionally substitute for loss of *Apc*. It is possible that *p27* deficiency accelerates genome instability in *Apc* mutant tumors, although the effect of *p27* on proliferation seems the most likely explanation for the observed synergistic interaction between *Apc* and *p27*.

The contrast in frequency of LOH between *p27* (0/23 tumors) and *Apc* (23/23 tumors) within the same tumors highlights the difference in gene dosage sensitivity between these two tumor suppressor genes. Loss of one allele of *p27* is sufficient to confer a strong tumor growth phenotype, whereas loss of both *Apc* alleles appears to be required for tumor development. These two genes, at least in this context, appear to represent two extremes of what is likely a continuum of dosage sensitivity of tumor suppressor genes, ranging from haploinsufficient to recessive.

Smad3 is an intracellular mediator of TGF- β function and acts as a nuclear transcriptional activator (Massague, 1998). Cells lacking Smad3 are deficient in TGF- β -mediated migration and growth arrest responses (Ashcroft et al., 1999; Datto et al., 1999). *p27* was initially discovered in complexes with cyclin E/cdk2 and cyclin D2/cdk4 in TGF- β arrested Mv1Lu mink epithelial cells, implicating *p27* in proliferation arrest signaling from TGF- β (Polyak et al., 1994a). However, subsequent studies showed that cells lacking *p27* still respond to growth inhibition by TGF- β (Nakayama et al., 1996). Other cdk inhibitors such as p15 and p21 are induced by TGF- β and contribute to the proliferation arrest of TGF- β treated cells (Reynisdottir et al., 1995; Hannon and Beach, 1994). TGF- β signaling is inhibitory to GI tumor development, as both TGF β and Smad3 deficient mice spontaneously develop GI neoplasms. In contrast to what was observed in the *Min/+* model, *p27* expression was not reduced and germline reduction of *p27* had no apparent effect on tumor growth in *Smad3* mutant mice. This suggests that *p27* is not inhibitory, and there is little selective pressure to reduce *p27* expression in tumors with mutations in *Smad3*. This also indicates that *p27* expression in GI tumors does not require active TGF- β signaling. The synthetic lethality observed in *Smad3/p27* compound null mice formally demonstrates that *p27* is not solely regulated by Smad3 and that these proteins lie on distinct pathways.

Mechanisms

The cellular mechanism for tumor suppression by *p27* has not been established. Early reports on human solid tumors did not detect consistent associations between *p27* levels and proliferation, as measured by proliferation markers such as Ki-67 staining (Porter et al., 1997; Catzavelos et al., 1997; Loda et al., 1997; Esposito et al., 1997). However, some tumor types, such as lymphomas, show an inverse correlation between *p27* staining and proliferative fraction (Sanchez-Beato et al., 1997; Quintanilla-Martinez et al., 1998). Here, we show in two separate mouse models, using both mitotic counts and BrdU labeling, that *p27* deficiency resulted in increased tumor cell proliferation.

Moreover, this effect was *p27* gene dosage dependent. This is consistent with the known function of *p27* as a Cdk inhibitor and indicates that one mechanism of tumor suppression by *p27* is to control tumor cell proliferation. Difficulty in detecting this link in human solid tumors could be due to imprecise measurement of *p27* expression through the use of immunohistochemistry, the broad cell cycle distribution of Ki-67 expression as a proliferation marker, or considerable genetic and phenotypic heterogeneity between tumors. In addition, *p27* appears to play additional roles in tumor suppression, beyond control of proliferation.

In addition to increasing tumor proliferation and growth, reduction of *p27* also resulted in an increase in malignant progression of tumors. A greater percentage of colon tumors from DMH-treated *p27* deficient mice were adenocarcinomas, with features that included complete invasion through the muscularis into the serosal space, and lymphatic vessel penetration. These more aggressive phenotypes were not observed in tumors from *p27* wild-type littermates. This suggests that an additional tumor suppressing function(s) of *p27* may be to control tumor cell differentiation, migration, and/or invasion. *p27* has been shown to induce differentiation of several cell types, including intestinal epithelial cells and colon carcinoma cells (Quaroni et al., 2000; Baldassarre et al., 1999; Yamamoto et al., 1999; Hauser et al., 1997), and cells lacking *p27* show impaired differentiation (Casaccia-Bonnel et al., 1997; de Koning et al., 2000; Zhang et al., 1998). *p27* is also implicated in cell adhesion, in that *p27* is upregulated when cells are grown in suspension or in response to E-cadherin or N-cadherin-mediated growth suppression (St. Croix et al., 1996, 1998; Fang et al., 1996; Levenberg et al., 1999). These attributes of *p27* may have contributed to the loss of differentiation and enhanced progression seen in *p27* deficient tumors. *p27* deficiency may confer the ability to proliferate in the absence of proper extracellular matrix signaling that would occur as tumor cells invade through the basement membrane or within lymphatic vessels, and hence facilitate cell survival and clonal expansion during metastatic spread.

Enhanced malignant progression was also observed in chemically induced skin tumors from *p27* deficient mice (Philipp et al., 1999) and is of considerable interest, given the correlation between reduced *p27* expression and increased tumor grade and metastasis seen in human colorectal, gastric, and other cancers. Mortality from colorectal cancer is typically due to extensive metastatic spread of the primary tumor to distal sites. The association of reduced *p27* expression in tumors with poor patient survival may be due to a role of *p27* in regulating metastatic spread of the tumor. The *p27* deficient mouse model should prove useful to further test this idea.

The observation that *p27* shows pathway-dependent tumor suppression may also have clinical implications. Although it is well established that *p27* expression levels in human tumors correlate with patient survival, this correlation is imperfect. There may be a subset of tumors with defined genetic alterations that progress independent of *p27* expression levels. Stratification of tumors based on defined genetic alterations, in combination with *p27* staining, may further improve prognostic sensitivity.

Experimental procedures

Mice

Inbred 129/Sv *p27* deficient mice were obtained from J. Roberts and genotyped as described (Fero et al., 1996). The *p27* knockout allele was back-

crossed to the NIH strain for seven generations. 129/Sv p27^{+/-} mice were crossed to NIH p27^{+/-} mice to generate the F1 littermates used for carcinogen treatment. Mice were injected with 1,2-dimethylhydrazine (DMH) (15 mg per kg body weight, s.c.) once weekly for 12 weeks starting at 8 weeks of age. DMH was dissolved in 0.001 M EDTA and adjusted to pH 6.5 using 8 N NaOH. Following the last treatment, mice were observed as described below.

C57BL/6J *Apc*^{Min/+} mice were obtained from Jackson Laboratory and genotyped as described (Dietrich et al., 1993). The p27 knockout allele, which was originally on a mixed 129/Sv x C57BL/6 genetic background, was backcrossed 14 times onto the C57BL/6 strain. As the *Mom1* locus (Modifier of *Min-1*) on Chromosome 4 has a strong influence on the severity of the *Min* phenotype, we verified that the C57BL/6 p27 deficient mice indeed carried the C57BL/6 *Mom1* allele (e.g., *Mom1*^{S/S}) by PCR (Gould et al., 1996). C57BL/6 *Min*^{+/+} mice were crossed to C57BL/6 p27^{+/-} mice to generate *Min*^{+/+} p27^{+/-} which were intercrossed to generate *Min*^{+/+} littermates of all three p27 genotypes and observed as described below.

129/Sv *Smad3*^{+/-} mice were obtained from J. Graff and genotyped as described (Zhu et al., 1998). The p27 knockout allele, which was originally on a mixed 129/Sv x C57BL/6 genetic background, was backcrossed 14 times onto the 129/Sv strain and genotyped as described (Fero et al., 1998). 129/Sv *Smad3*^{+/-} mice were crossed to 129/Sv p27^{+/-} mice to generate *Smad3*^{+/-} p27^{+/-} mice. These were intercrossed to generate *Smad3*^{-/-} p27^{+/-}, *Smad3*^{-/-} p27^{+/+}, and *Smad3*^{-/-} p27^{-/-} mice. However, only 3 out of 219 total offspring were of *Smad3*^{-/-} p27^{-/-} genotype, and these did not live past 30 days of age.

All mice were observed daily and sacrificed when moribund, which included the following criteria: excessive loss of weight, abdominal swelling, rectal prolapse or bleeding, or anemia. Morbidity was scored as tumor-related if tumors resulted in intestinal obstruction or hemorrhaging, or were >1 cm³. The entire small intestine, cecum, and colon was cut longitudinally and fixed on a bibulous paper. Intestinal tumors were enumerated using a dissecting microscope.

Histopathology and immunohistochemistry

Sections of tumors were removed and flash-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for 4–6 hr and embedded in paraffin. After high temperature antigen retrieval in 10 mM citrate buffer (pH 6.0), 5 mm sections were stained for p27 (mouse monoclonal antibody, Neomarkers, Fremont, CA) for 1 hr, for β -catenin (rabbit polyclonal antibody, Neomarkers, Fremont, CA) for 1 hr, or for Ki67 (mouse monoclonal antibody, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) for 1 hr. Standard avidin-biotin peroxidase complex (ABC) techniques were used for primary antibody detection (biotinylated goat anti-rabbit antibody, Vector Labs Inc., Burlingame, CA; streptavidin ABC, DAKO Corp., Carpinteria, CA). The slides were developed in DAB/NiCl₂, then counterstained with methyl green. Controls included: no primary antibody and/or normal rabbit serum, and tissues from p27 null mice. p27 and Ki67 staining were performed on serial sections.

Kinetic analysis of tumor cells

Mice were injected with BrdU (1 mg/10 g body wt, i.p.) (Sigma) and sacrificed 1 hr later. For BrdU staining, 4 μ m paraffin sections were deparaffinized and rehydrated. The sections were denatured with 2N HCl and treated with 0.1% trypsin (Sigma). Sections were then stained with a mouse monoclonal anti-BrdU (DAKO) antibody followed by an anti-mouse IgG1 horseradish peroxidase-linked antibody (Southern Biotechnologies). Sections were developed with DAB/NiCl₂ (Sigma) and counterstained with methyl green. BrdU-labeled cells were counted at 400 \times magnification and expressed as positive cells per crypt for normal GI or per 400 \times powerfield for tumors. Alternatively, mitotic figures were counted in H&E stained sections of intestinal adenomas. Proliferation in DMH treated colon adenomas was established by counting 50–100 400 \times powerfields per genotype. One to two adenomas per mouse and 6–12 mice per genotype were evaluated. For *Min*^{+/+} mice, a total of 10 powerfields were counted at 400 \times magnification in the colon and in three distinct sections of the small intestine. At least 10 adenomas per mouse and three mice per genotype were evaluated.

Western blot analysis

Nuclear and cytoplasmic protein extracts were prepared as described (Schreiber et al., 1989) with the following modifications. Pieces of tumors

and normal tissues were minced with a razor blade and dissolved in buffer A (Schreiber et al., 1989) and further homogenized for 1 min on ice (PowerGen 125, Fisher Scientific). Buffers A and C both contained 1 mM DTT, 0.4 mg/ml Pefablock, 25 mg/ml Aprotinin, 10 mg/ml Pepstatin, and 10 mg/ml Leupeptin (Boehringer Mannheim) to inhibit proteases, and in addition, buffer C contained 25% glycerol. Protein concentrations were standardized using the Bradford assay (BioRad) and equal loading was confirmed by Ponceau S staining of PVDF membranes after electroblotting (BioRad). The antibodies used for Western blotting were: rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) against p27 (sc-528), cyclin D1 (sc-753), p45^{Skp2} (sc-7164), and Sin3A (sc-994) for cellular fractionation and loading control. Anti- α -tubulin (clone B-5-1-2, Sigma) and anti-max antibodies (generous gift of Dr. R. Eisenmann) served as additional controls for the completeness of cellular fractionation. Blots were developed using chemiluminescence protein detection kits for alkaline phosphatase (Tropix, Bedford, MA) or horseradish peroxidase (Pierce, Rockford, IL). Densitometric analysis of Western blots was performed using ImageQuant software for Macintosh, version 1.2.

Tumor DNA isolation and LOH analysis

DNA was extracted from frozen tumors using BioRad InstaGene Matrix. 10–15 mg of tumor tissue was digested in 100 μ l volume with 1 mg/ml Proteinase K over night at 37°C followed by deactivation of Proteinase K at 95°C. The supernatant was used as a DNA template for the following two PCR protocols. Retention of the remaining p27 allele in tumor tissue from p27^{+/-} mice was analyzed using primers F (5' GAGCAGACGCCCAAGA AGC 3'), R (5' TGGAAACCCTGTGCCATCTCTAT 3'), and N (5' CCTTCTATGG CCTTCTTGACG 3'). Tumor samples were amplified in a 25 μ l reaction containing 500 ng of DNA, 2.5 μ l of 10 \times PCR buffer, 2.7 mM MgCl₂, 0.5 mM dNTP, 0.1 mM of each of the three primers, F, R, and N, and 1 unit of Taq polymerase at the following conditions: first cycle –94°C for 3 min; 40 cycles –93°C for 45 s, 55°C for 60 s, 65°C for 90 s; last cycle –65°C for 10 min. Samples were run on a 1% agarose gel stained with ethidium bromide.

Loss of the wild-type *Apc* allele was analyzed using primers F (5' TCTCGTCTTGAGAAAGA-CAGAAGCT3') and R (5' GATACCTCTTCCAAA GCCTTGGCTAT 3') designed to introduce a HindIII restriction site without altering the *Apc* point mutation at nucleotide position 2,549. Tumor DNA was amplified in a 25 μ l reaction containing 100 ng of DNA, 2.5 μ l of 10 \times PCR buffer (500 mM KCl, 100 mM Tris, and 1% TritonX-100), 2.5 mM MgCl₂, 0.5 mM dNTP, 0.1 mM primers, and 1 unit of Taq polymerase. Samples were amplified using the following conditions: first cycle –94°C for 3 min; 40 cycles –93°C for 30 s, 55°C for 45 s, 65°C for 1 min; last cycle –65°C for 10 min. 10 μ l of postamplification samples were digested with HindIII (10 units) overnight and run on a agarose gel (2% agarose/3% NuSieve) stained with ethidium bromide.

Statistical methods

Kaplan-Meier survival curves were used to display the time to tumor morbidity or mortality. Corresponding two-sample logrank statistics were used to test for rate differences between genotype groups. Brookmeyer-Crowley confidence limits (Brookmeyer and Crowley, 1982) accompany estimates of median latency time to tumor morbidity or mortality. Two sample t tests for samples with unequal variance were used for comparison of mean tumor numbers in *Min*^{+/+} mice. Logistic regression-based estimates, confidence intervals, and hypothesis tests were used to display and compare the proportion of GI tumors with minimum specified diameter at selected follow-up times. The robust sandwich variance estimator (Huber, 1967) was used in these models to account for the nonindependence of tumor size observations from the same mouse. Exact binomial confidence limits were alternatively used where the observed group proportion was zero, and for lower GI tumor observations at 9 weeks of age due to small numbers. Fisher's exact test was used for comparison involving these groups. 95% confidence limits and p values are 2-sided.

Acknowledgments

We thank J. Graff and J. Roberts for providing *Smad3* and p27 knockout mice, respectively, and M. Fero, J. Roberts, and P. Porter for constructive comments on the manuscript. Funding from the American Cancer Society,

the Life Possibilities Fund, and the NIH to C.J.K. is gratefully acknowledged. S.R.P. was supported by the NIH Molecular Training Program in Cancer Research through the University of Washington. This work is dedicated to the memory of Lois Kemp.

Received: December 18, 2001

Revised: March 18, 2002

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