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# Targeted Inactivation of *p27$^{kip1}$* Is Sufficient for Large and Small Intestinal Tumorigenesis in the Mouse, Which Can Be Augmented by a Western-Style High-Risk Diet[1]

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## ABSTRACT

**Mice with a targeted inactivation of both alleles of the cyclin-dependent kinase inhibitor *p27$^{kip1}$* developed both small and large intestinal adenomas when fed a control AIN-76A diet. A Western-style diet that is high in fat and phosphate and low in calcium and vitamin D was also able to initiate adenoma formation in wild-type mice. The combination of *p27$^{kip1}$* inactivation and the Western-style diet was additive in terms of tumor incidence, frequency and size, and in reducing the life span of the mice. The genetic and dietary combination also resulted in development of adenocarcinoma. Tumor formation was linked to a disruption in homeostasis of the intestinal mucosa, involving increased cell proliferation and decreased apoptosis. There was also decreased goblet cell differentiation as assessed by alcian blue staining and expression of the *Muc2* gene, especially in mice fed the Western-style diet, although this differentiation lineage was still present as indicated by expression and staining for intestinal trefoil factor. The inactivation of *p27$^{kip1}$* and the consequent disruption of normal colonic cell maturation in the mucosa were associated with modestly elevated *c-myc*, *cdk4*, and *cyclin D1* expression. These data establish a fundamental role for *p27$^{kip1}$* in maintenance of intestinal cell homeostasis and in suppressing tumor formation. The data also emphasize the critical role that dietary factors can have in both tumor initiation and progression through interaction with pathways that normally maintain intestinal homeostasis.**

## INTRODUCTION

Intestinal tumorigenesis arises from perturbations in homeostasis of the intestinal mucosa. This is manifest most clearly as increased cell proliferation in the mucosa, as well as an expanded proliferative compartment, which is normally restricted to the lower two-thirds of the crypt architecture (1, 2). Observations regarding the molecular mechanisms of intestinal tumorigenesis are consistent with this cell biology. *Cyclin D1* and *c-myc*, two genes that encode products drive cells through the cell proliferation cycle in the intestinal mucosa, are direct targets of transcriptional regulation by β-catenin-Tcf4 signaling (3–5). This signaling pathway is nearly uniformly up-regulated in colon tumors because of loss of function of the *APC* tumor suppressor gene, whose encoded protein, in a complex with axin and glycogen synthase kinase 3β, normally targets β-catenin for degradation (6–9). In addition, up-regulation of the pathway in colon tumors because of mutations in β-catenin itself have also been observed (6).

This fundamental role of enhanced cell cycling in intestinal tumor formation implies that negative regulators of the cell cycle, such as *p21$^{WAF1/cip1}$* and *p27$^{kip1}$* would be effective modulators of tumor formation, and indeed, this has been observed. We (10) demonstrated that the targeted inactivation of *p21* increased intestinal tumor formation initiated by an inherited *Apc* mutation in *Apc1638$^{+/-}$* mice, and Philipp-Staheli *et al.* (11) demonstrated that inactivation of *p27* increased intestinal tumors in the *ApcMin$^{+/-}$* mouse. In each case, the respective cdki[3] was haplo-insufficient for suppression of *Apc*-initiated tumors.

We also reported that the inactivation of *p21* in the mouse was additive with a Western-style stress diet, a diet that mimics major dietary risk factors for colon cancer in Western societies, in augmenting *Apc*-initiated tumor formation (10). In the present study, we extended this investigation to the cdki *p27* for several reasons: first, although p21 and p27 are both cdkis, they do not serve identical functions in regulating multifactor complexes that regulate cell cycle progression (12, 13); second, p27 has been demonstrated to have significant functions in colonic cell differentiation outside of its role in regulating cell proliferation, with forced increases and decreases in expression linked to stimulation and inhibition, respectively, of differentiation along the absorptive cell lineage (14, 15); third, decreased expression of p27 is associated with human colon tumor progression and poorer prognosis (16–19), which has not been reported for p21; and fourth, it has been found recently that although *p21* is uniformly up-regulated in the differentiation of the goblet cell lineage as well as in the differentiation of intestinal cells that exhibit transepithelial cell transport, *p27* elevation is much more pronounced in cells of the latter phenotype (A. Velcich, personal communication).

Surprisingly, we found that unlike the case for *p21*, inactivation of *p27* in the absence of an inherited *Apc* mutation was sufficient to cause development of mouse tumors in both the duodenum and large intestine. This intestinal tumorigenesis by inactivation of *p27* was also augmented substantially by feeding the mice the Western-style diet, and was associated with increased proliferation and decreased apoptosis, as well as a decrease in recognizable goblet cells, in the intestinal mucosa. Moreover, both the inactivation of *p27* and the Western-style diet modestly increased *cyclin D1* and *c-myc* expression, as well as a down-stream target of *c-myc*, *cdk4*.

## MATERIALS AND METHODS

*p27$^{+/-}$* mice, reported previously (20), were mated with each other to generate *p27$^{+/+}$* or *p27$^{-/-}$* mice. Genotyping was done using a PCR-based assay of mouse tail DNA, as reported previously. At weaning (~3–4 weeks), the littermates of different genotype were randomized to dietary groups and fed *ad libitum* either AIN-76A control diet or a Western-style diet that is formulated on the basis of nutrient density to mimic major risk factors for colon cancer in the Western-style diet: high in fat and phosphate and low in calcium and vitamin D (21, 22). Diets were from Harlan-Teklad (Madison, WI).

Animals were maintained on diet for 36 weeks or until they exhibited significant weight loss or other signs of extensive tumor formation. Mice were killed by $CO_2$ overdose and cervical dislocation. The gastrointestinal tract (from stomach to rectum) was opened longitudinally, except for the stomach, which was cut along the greater curvature. The dissected organs were inspected under a dissecting microscope, and the number and location of tumors and macroscopic features of mucosal growth were recorded as described previously (10). Tissues were routinely fixed in formalin and embedded in paraffin

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[3] The abbreviations used are: cdki, cyclin-dependent kinase inhibitor; RT-PCR, reverse transcription-PCR; Itf, intestinal trefoil factor.



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for histopathological analysis and snap-frozen in liquid $N_2$ for isolation of RNA and protein used in quantitative real time RT-PCR and Western blot analysis, respectively. Proliferation, apoptosis, and goblet cell differentiation in the mucosa were assayed by scoring the percent mitotic cells, the percent terminal deoxynucleotidyl transferase-mediated nick end labeled-positive cells, and the percent alcian blue-stained cells, respectively, as previously described in detail (10).

Total RNA was isolated from the frozen tissues using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA). The quantity of RNA was determined spectrophotometrically. cDNA was synthesized from Dnase-treated total RNA using TaqMan Multiscrible Reverse Transcriptase (Applied Biosystems, Inc., Foster City, CA). Purified RNA (2 $\mu$g) was brought to 10 $\mu$l with RNase-free $H_2O$. Reverse transcription mixture (40 $\mu$l) was added, containing 1× TaqMan reverse transcriptase buffer, 5.5 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphate mixture, 2.5 $\mu$M random hexamers, 0.4 units/$\mu$l RNase inhibitor and 1.25 units/$\mu$l reverse transcriptase. The thermal cycler was programmed with the following conditions: incubation for 10 min at 25°C; reverse transcription at 48°C for 30 min; and reverse transcriptase inactivation for 5 min at 95°C.

Quantitative PCR analysis was done using the ABI Prism 7900-HT Sequence Detection System (96-well) and monitored in real time. Sixty ng of cDNA were used as template, to which was added SYBR green dye, 3.0 mM $MgCl_2$, 0.5 $\mu$M deoxynucleotide triphosphate mixture, 0.025 units/$\mu$l AmpliTaq Gold DNA polymerase, 0.01 units/$\mu$l AmpErase UNG, 0.1 $\mu$M primers, and $dH_2O$ to a final volume of 30 $\mu$l. The following primers were used for relative quantification of target gene expression: *β-actin*, forward 5′-acccacactgtgcccatctac-3′ and reverse 5′-gccatctcctgctcgaagtc-3′; $p27^{kip1}$, forward 5′-tcaaacgtgagagtgtctaacgg-3′ and reverse 5′-aggggcttatgattctgaaagtcg-3′; $p21^{WAF1/cip1}$, forward 5′-gacagtgagcagttgcg-3′ and reverse 5′-ctcagacaccagagtgc-3′; *cdk4*, forward 5′-accgtcaagctggctgacttt-3 and reverse 5′-tacagccaacgctccacatg-3′; and *c-myc*, forward 5′-gacaagaggcggacacacaa-3′ and reverse 5′-ggatgtaggcggtggctttt-3′. The primers for assay of *Muc2* and *Itf* were as described previously (23). Experiments were performed in duplicate for each sample. Each PCR run consisted of a five-point calibration curve and a control containing no template. *β-Actin* was used as an internal control. The thermal cycling conditions were composed of an AmpliTaq Gold activation step at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 15 s and annealing/extension at 60°C for 1 min. The relative quantification of target gene was acquired by analyzing the generated data using SDS2.0 software (Applied Biosystems, Inc.). The identity of the expected PCR products was confirmed by agarose gel electrophoresis.

Western blot analysis of steady-state levels of specific proteins was done by standard methods, as we have previously described (24), using the following primary antibodies for detection: anti-c-myc (N-262) and anti-cdk4 (H-22; both from Santa Cruz Biotechnology, Santa Cruz, CA); anti-cyclin D1 (AM29; Zymed Labs, South San Francisco, CA); and anti-$\beta$-actin (Sigma, St. Louis, MO). Signal was detected by the enhanced chemiluminescence technique (Amersham Life Science, Piscataway, NJ). Immunohistochemical staining for Itf was previously described in detail (23). Briefly, 4-$\mu$g formalin-fixed and paraffin-embedded sections were deparaffinized and rehydrated, quenched with 1.5% $H_2O_2$, blocked with 10% normal goat serum, and probed with rabbit anti-Itf polyclonal antibody (kindly provided by Catherine Tomasetto, Strasbourg, France). Detection was with biotinylated antirabbit IgG (Santa Cruz Biotechnology), followed by incubation with avidin-biotin complex method (Vector Labs, Burlingame, CA) and the substrate 3′,5′-diaminobenzidine combined with hematoxylin counterstaining.

## RESULTS

Heterozygote mice that harbor a single inactivated *p27* allele were bred to generate mice that were either $p27^{+/+}$ or $p27^{-/-}$, and these mice were then maintained, after weaning, on AIN-76A control diet or a Western-style diet that is formulated on the principal of nutrient density to mimic the intake of major dietary risk factors for colorectal cancer (high fat and phosphate, low calcium, and vitamin D; Refs. 21, 22). Fig. 1 illustrates that the homozygous inactivation of *p27* was sufficient to initiate tumor formation in both the duodenum and the large intestine of mice on the control diet. Fig. 1*a* shows two tumors, both of which are adenomas, in the duodenum of a $p27^{-/-}$ mouse fed

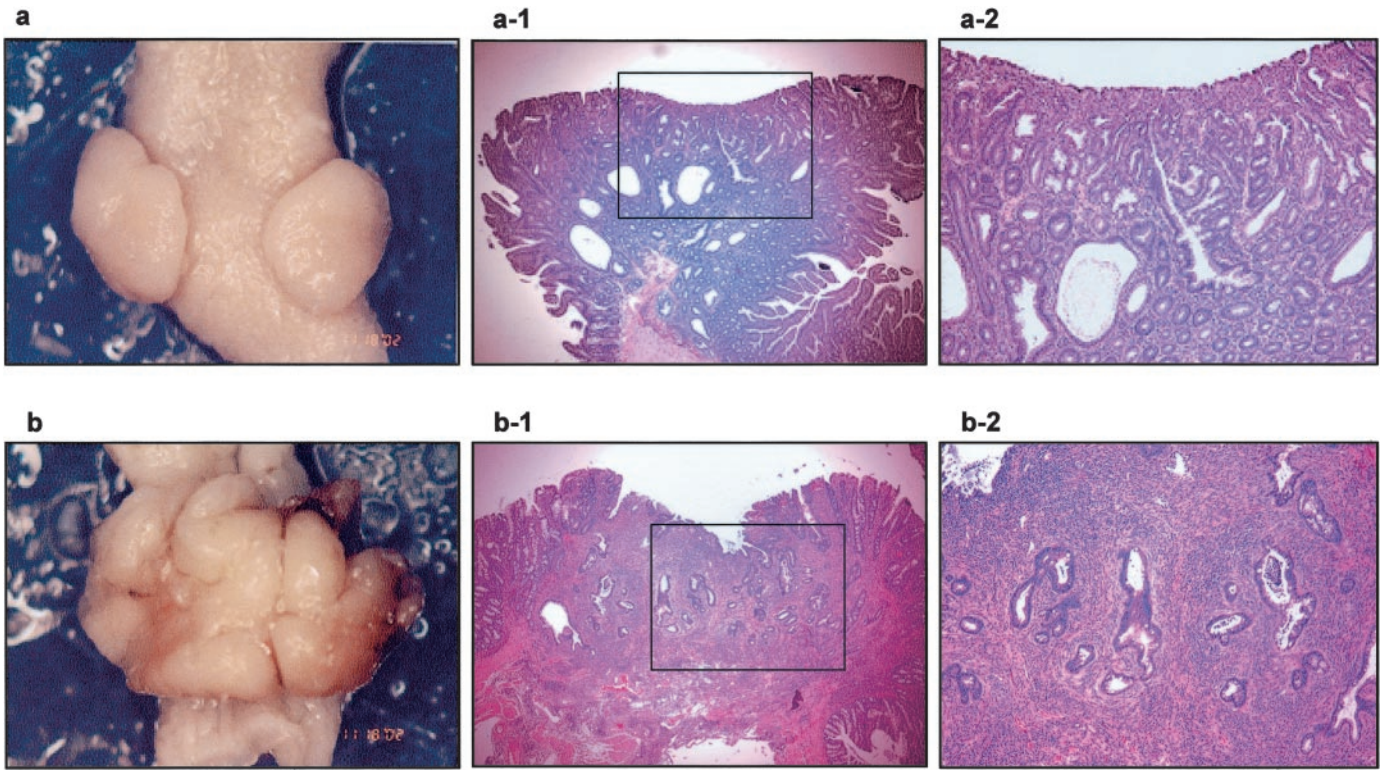


Fig. 1. Histopathology of tumors in $p27^{-/-}$ mice. *a*, gross of pathology of two tumors in the duodenum. Histopathological features of one of the adenomas shown in *a*: *a-1* (×40) and *a-2* (×100). *b*, a large adenocarcinoma of the colon. Histopathological features of the tumor in b are shown in *b-1* (×40) and *b-2* (×100). Note that the layers of the intestinal wall were destroyed with infiltration of the carcinoma.



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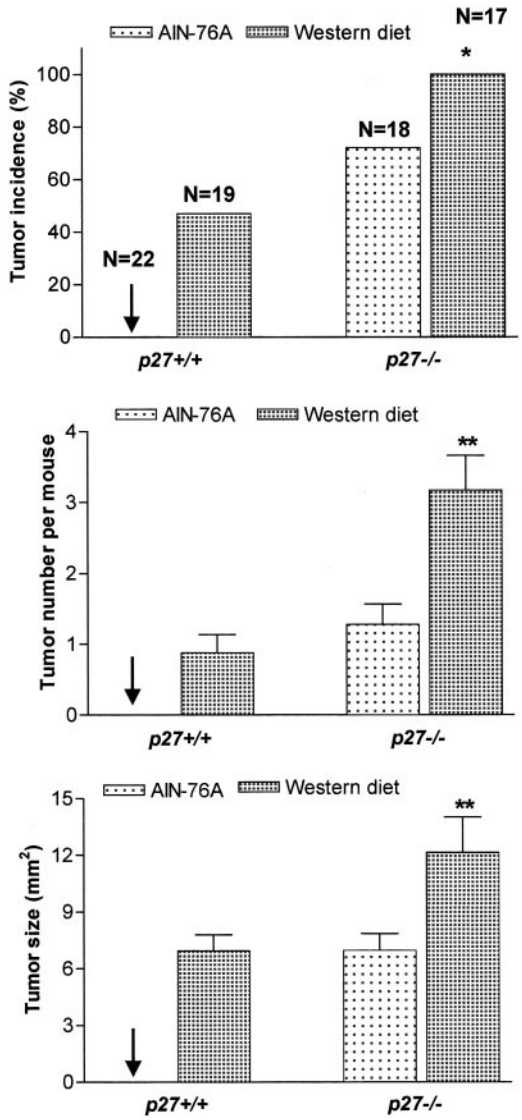


Fig. 2. Tumor formation in the intestine. The incidence, frequency, and size of intestinal tumors in $p27^{+/+ \text{ or } -/-}$ mice fed AIN-76A or a Western-style diet for 36 weeks. (*, $P < 0.01$, **, $P < 0.05$ comparison to $p27^{+/+}$ mice fed the Western-style diet or $p27^{-/-}$ mice fed the AIN-76A diet, respectively. N, number of mice studied in each group. *Arrow* indicates 0 tumors for $p27^{+/+}$ mice fed the control diet).

the control diet. The initiation by inactivation of *p27* was substantial, with 72% of the $p27^{-/-}$ mice developing tumors, at an average frequency of 1.28 tumors/mouse with an average size by 36 weeks of age of ~7 $mm^2$ (Fig. 2). As with initiation of intestinal tumor formation in the mouse by targeted inactivation of the *Apc* gene (25), the Western-style diet was highly effective in augmenting this tumorigenesis. This was especially clear in terms of tumor frequency and size, each of which approximately doubled in the $p27^{-/-}$ mice maintained on the Western-style diet compared with the AIN-76A diet (Fig. 2). Interestingly, although wild-type mice, as expected, never developed tumors when maintained on the control diet, ~50% of the wild-type mice developed tumors when fed the Western-style diet for 36 weeks, with a frequency approaching one tumor/mouse and average size of 6.9 $mm^2$. This was one-third the average number and one-half the average size of tumors that developed when the $p27^{-/-}$ mice were maintained on the same Western-style diet (Fig. 2). Although it has been previously reported that the Western-style diet has a major effect on tumor progression, not initiation, the observation that mice fed the Western-style diet develop a low incidence of tumors suggests that the diet can also initiate genetic or epigenetic changes that initiate tumorigenesis. Tumor initiation has been reported recently for a variation of the Western-style diet that also includes a decrease in methyl group donors (26).

The data of Table 1 illustrate that tumor formation in the $p27^{-/-}$ mice was approximately equivalent in the large and the small intestine. The majority of the tumors that develop in both the duodenum and the large intestine were adenomas, with most being tubular adenomas and some progressing to tubulovillous adenomas (Table 1, Fig. 1*a*). We also detected adenocarcinomas in both the duodenum and the large intestine of the $p27^{-/-}$ mice maintained on the Western-style diet (Table 1, Fig. 1*b*). Fig. 1*b* is a large adenocarcinoma in the colon of a $p27^{-/-}$ mouse fed the Western-style stress diet. In this case, the layers of the intestinal wall have been destroyed by infiltration of the carcinoma. Polyps were found in the stomach of the $p27^{-/-}$ mice fed the Western diet (Table 1), but no neoplastic growths were found in the jejunum or ileum of any of the mice (data not shown).

Fig. 3 shows the consequence of this tumor formation on the life span of the mice. The experiment was carried out for 36 weeks after weaning, and in this time, either the inactivation of *p27* or the Western-style diet were effective in reducing mouse life span ($P = 0.007$ and $P = 0.039$ for the $p27^{-/-}$ mice or the Western-style diet/wild-type mice, respectively, compared with the wild-type mice fed the control diet). The effects of the Western diet combined with *p27* inactivation on decreasing life span were additive, consistent with our prior report on inactivation of *p21* combined with the Western-style diet (10).

To gain insight into the mechanisms by which tumors form in these

Table 1 *Distribution and histological feature of tumors in the gastrointestinal tract*

| | $p27^{+/+}$ mice | | $p27^{-/-}$ mice | |
|---|---|---|---|---|
| Organ/diet | AIN-76A | Western diet | AIN-76A | Western diet |
| Large Intestine | 0[a] | 14 (13TA, 1TVA)[b] | 10 (10TA) | 24 (22TA, 2AC) |
| Duodenum | 0 | 2 (1TA, 1TVA) | 13 (11TA, 2TVA) | 20 (18TA, 2AC) |
| Stomach | 0 | 0 | 0 | 6 (6 P) |

[a] Total number of tumors in each group.
[b] TVA, tubulovillous adenoma; TA, tubular adenoma; AC, adenocarcinoma; P, squamous cell papilloma.

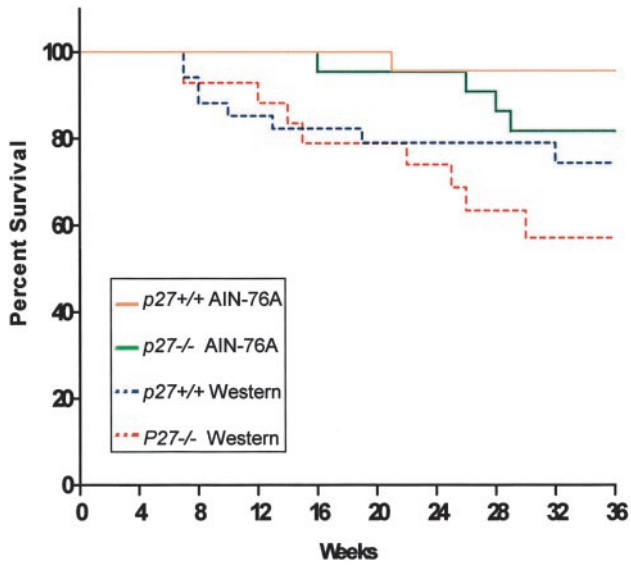


Fig. 3. Survival of $p27^{+/-}$ or $^{-/-}$ mice fed the AIN-76A or Western-style diet. Mice were fed the control or Western-style diet and survival analyzed using Kaplan-Maier plots of the data.



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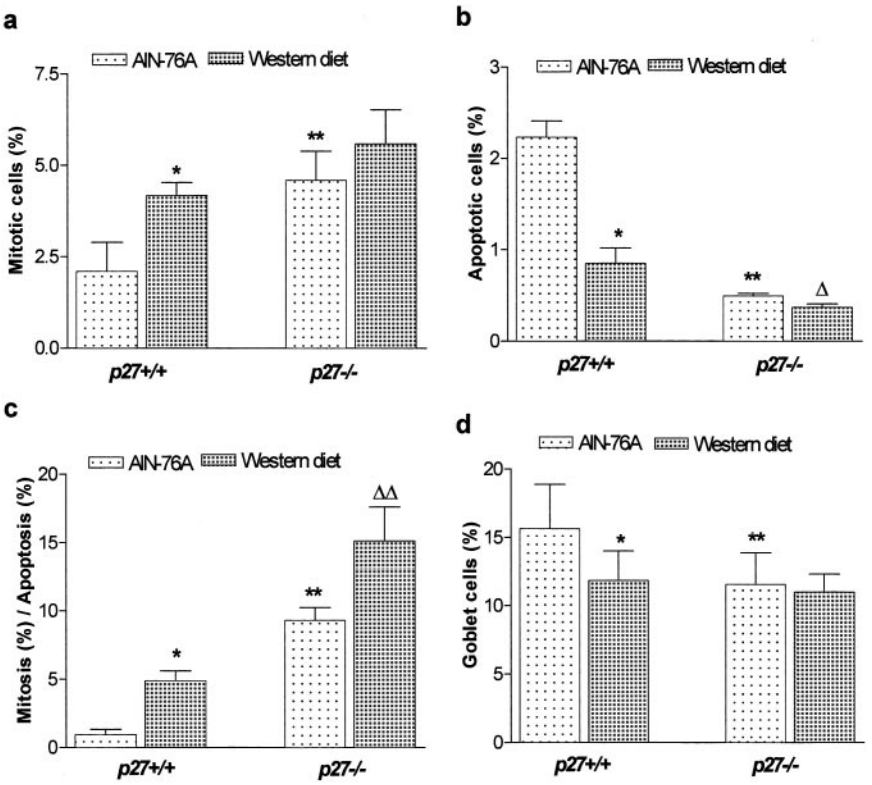


Fig. 4. Disruption of homeostasis in the intestinal mucosa. The percent cells in the intestinal mucosa were scored as mitotic after H&E staining, apoptotic, after terminal deoxynucleotidyl transferase-mediated nick end labeling staining, or goblet cell, after staining for mucin with alcian blue. Proliferation (*a*), apoptosis (*b*), the ratio of proliferating to apoptotic cells (*c*), and number of goblet cells (*d*) in the duodenal mucosa of $p27^{+/+}$ or $^{-/-}$ mice fed AIN-76A or Western-style diet for 36 weeks. (*, $P < 0.01$; **, $P < 0.05$ comparison to $p27^{+/+}$ mice fed AIN-76A diet; △, $P < 0.01$, △△, $P < 0.05$ comparison to $p27^{+/+}$ mice fed Western-style diet by Mann-Whitney test.)

mice, we assayed the levels of cell proliferation and apoptosis in both the duodenum and the large intestine. Only the data for the duodenum are shown, but similar results were found for the large intestine. Consistent with a wealth of data that intestinal tumorigenesis is initiated by a disruption of homeostasis in the mucosa, there were alterations in both these parameters and in the resulting ratio between proliferating and apoptotic cells (Fig. 4, *a–c*). The increase in proliferation was approximately equal for the $p27^{-/-}$ mice or the Western-style diet/wild-type mice when each was compared with the wild-type mice fed control diet (Fig. 4*a*). Similarly, the decrease in apoptosis was equivalent for the genetic defect and the dietary perturbation individually (Fig. 4*b*). This is consistent with the genetic defect and the dietary perturbation each initiating approximately the same number of tumors (Fig. 2). Only modest further changes in proliferation or apoptosis were seen when the $p27^{-/-}$ mice were fed the Western-style diet, but a more substantial further increase was seen when the ratio of the two markers was calculated (Fig. 4*c*). Interestingly, the Western-style diet alone, or the inactivation of *p27*, whether in combination with the control or Western diet, decreased the number of detectable goblet cells in the mucosa by ~30% (Fig. 4*d*), an observation also made for inactivation of *p21* (10). However, although there was a decrease in the number of alcian blue-stained goblet cells, decreased mRNA for the major colonic mucin peptide, Muc2, was seen only in the intestinal mucosa from mice that were fed the Western-style diet (Fig. 5*a*). Moreover, this decreased *Muc2* mRNA content was not paralleled by decreased expression of the expression of *Itf*, another marker of goblet cell differentiation (Fig. 5*b*). Immunohistochemical evaluation of Itf staining confirmed this observation: there was no decrease in the intensity of staining for Itf or position of cells that express Itf in either the $p27^{+/+}$ or $^{-/-}$ mice fed either diet (colon data shown in Fig. 5*c*; similar results not shown for the duodenum).

The increase in proliferation in the intestinal mucosa of mice in which *p27* was inactivated or mice fed the Western-style diet or both was reflected in alteration of the machinery that drives cells through the cell cycle. First, as expected, targeted inactivation of the p27 gene resulted in no detectable *p27* mRNA in the mucosa of the large intestine of $p27^{-/-}$ mice (Fig. 6*a*; similar data were obtained for the mucosa of the duodenum, data not shown). Second, there were modest increases in *c-myc* mRNA expression that are likely linked to the increases in cell proliferation in the mucosa (Fig. 6*b*). This reached statistical significance for the $p27^{-/-}$ mice fed the Western-style diet compared with wild-type mice fed control diet. These increases were also reflected at the protein level, especially in mice of either wild type or −/− genotype maintained on the Western diet (Fig. 7). There were also modest increases in *cdk4* mRNA levels that were statistically significant (Fig. 6*c*), consistent with the report that this gene is a downstream target of *c-myc* (27). Again, these increases in cdk4 were also seen by Western blot analysis. Finally, at the protein level, cyclin D1 showed paralleled increases, especially in the $p27^{-/-}$ mice fed the western diet (Fig. 7), which have the greatest tumor development and the poorest survival. Overall, these changes in the cell cycle machinery were modest, although the cyclin D1 changes exceeded 5-fold in the $p27^{-/-}$ mice fed the Western-style diet, reflecting the alterations in cell maturation that are linked in this model to the development of only one to a few tumors over 36 weeks.

## DISCUSSION

The principal finding we report is that the targeted inactivation of *p27* was sufficient to initiate tumor formation in the duodenum and the large intestine of the mouse. This role of *p27* inactivation as an initiator of intestinal tumors has not been previously reported, although it has been reported that *p27* inactivation is sufficient for development of pituitary tumors (28). We also observed that, similar to other genetic initiators of tumor formation, maintaining the $p27^{-/-}$ mice on a Western-style diet high in fat and phosphate and low in calcium and vitamin D could profoundly increase the number and size



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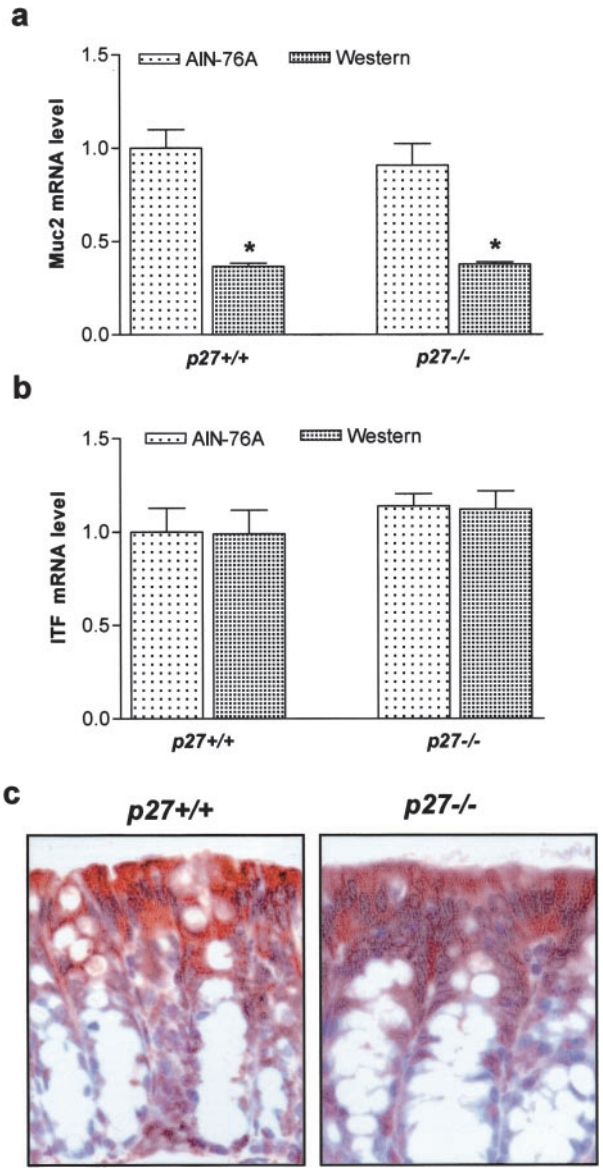


Fig. 5. Analysis of goblet cell markers. In *a* and *b*, quantitative real-time RT-PCR was used to assay level of Muc2 and Itf mRNA, respectively, in the flat mucosa of the colon of $p27^{+/+}$ or $^{-/-}$ mice fed AIN76A or a Western-style diet. *c*, immunohistochemical analysis of Itf protein expression (*brown stain*, ×100) in the flat mucosa of the colon.

of intestinal tumors. This observation, along with the fact that the Western diet by itself in these wild-type mice can initiate tumor formation, emphasizes the critical role that diet plays in intestinal tumor formation in developed countries.

As detailed in the "Introduction," the functions of p21 and p27, especially as regards intestinal cell differentiation, are distinct, and there is a clearer link of decreased expression of p27 to human colon tumor progression and poorer outcome (16–19). It is therefore important that inactivation of *p27* was able to initiate tumor formation in the mouse intestine in the absence of an *Apc* mutation but that inactivation of *p21* was not sufficient for tumor formation, although it was effective in enhancing Apc-initiated tumors (10, 29). This function of initiation by inactivation of *p27* is consistent with the fact that $p27^{-/-}$ mice also develop pituitary hyperplasia and tumors but that p21 mice do not develop tumors in any tissue (30).

A previous study (11) has demonstrated that inactivation of *p27* can augment intestinal tumorigenesis initiated by inactivation of *Apc* or by dimethylhydrazine but did not report intestinal tumor formation by *p27* inactivation alone. However, there were several differences between that study and the experiments presented here. First, data were reported only for $p27^{-/-}$ mice that were initiated by either carcinogen treatment or Apc inactivation, and the age of the $p27^{-/-}$ mice when sacrificed in the absence of any initiating event was not clear. The experiments reported here were in mice maintained on diet for 36 weeks after weaning (~38–40 weeks of age). Second, there is a background strain difference between the $p27^{-/-}$ mice used in the prior study and those used here (129S1/sv × C57Bl/6). Third, because of our interest in the interaction of nutritional and genetic factors in tumor development, we feed our control mice a completely defined diet (AIN76A) that is formulated for maximum growth of the animals, whereas the diet used in the prior study was not specified. Therefore, although these and other potential modulators of tumor formation need to be addressed in experiments with a combinatorial design, the important fact is that inactivation of *p27* is capable of initiating intestinal tumor formation.

It is tempting to attribute all of the effects of the inactivation of

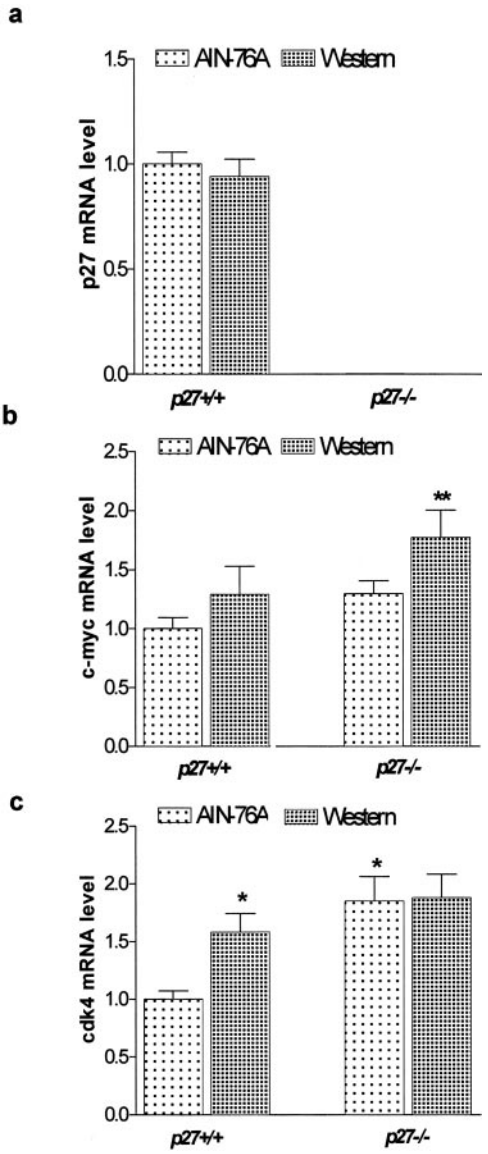


Fig. 6. Gene expression in the intestinal mucosa. Quantitative real-time RT-PCR was used to assay the relative level of *p27*, *c-myc*, and *cdk4* mRNA in the flat mucosa of $p27^{+/+}$ or $^{-/-}$ mice fed AIN-76A or Western-style diet. (*, $P < 0.05$ comparison to $p27^{+/+}$ mice fed AIN-76A diet; **, $P < 0.05$ comparison to $p27^{+/+}$ mice fed Western-style diet by Mann-Whitney test.)



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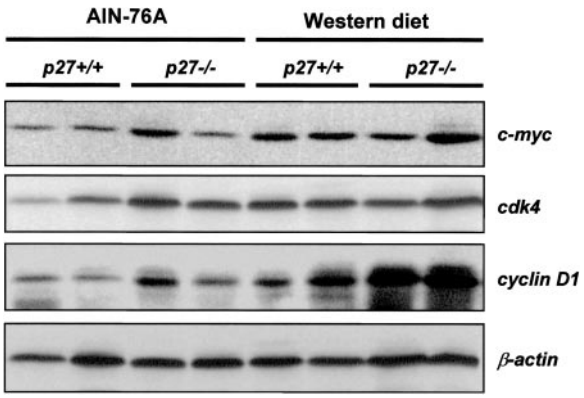


Fig. 7. Western blot analysis of protein. Western blots were used to analyze expression of c-myc, cdk4, and cyclin D1 protein in the colonic mucosa of $p27^{+/+}$ or $^{-/-}$ mice fed AIN76A or a Western-style diet. Analysis is shown for two different mice from each genetic/dietary group.

these cdkis to their activity in inhibiting cell proliferation in the normal mucosa, and perhaps in modulating apoptosis and, indeed, expected alterations in these markers of cell maturation are seen in the flat mucosa of both the $p21^{-/-}$ (10) and $p27^{-/-}$ mice (Fig. 4, *a* and *b*). However, the role of loss of these inhibitors of the cell cycle may be more complex in intestinal tumor formation. As described in the introduction, the role of the *Apc* gene product in regulating β-catenin-Tcf4 signaling is paramount in the initiation of intestinal tumor formation (9). Therefore, the phenotype of mice that have a targeted inactivation of the Tcf4 gene is highly instructive. $Tcf4^{-/-}$ mice die soon after birth, essentially because of differentiation of small intestinal stem cells (31). The loss of the stem cell population abrogates repopulation of the epithelial mucosa upon cell loss and hence the animals bleed to death. This therefore points to a fundamental role of the β-catenin-Tcf4 signaling pathway in intestinal cell differentiation, as well as in proliferation. In confirmation of this hypothesis, down-regulation of this signaling pathway accompanied Caco-2 colon carcinoma cell differentiation (32). Furthermore, forced down-regulation of this signaling pathway in Caco-2 cells by introduction of a wild-type *Apc* allele, overexpression of E-cadherin, or expression of a dominant negative for Tcf4 was effective in elevating the promoter activity of some genes that are markers for differentiation of intestinal epithelial cells (32). More recently, van de Wetering *et al.* (33) have clearly demonstrated that β-catenin-Tcf4 signaling directly regulates differentiation of intestinal epithelial cells as well as intestinal cell proliferation. Moreover, these investigators showed that both *c-myc* and *p21* are key players in that high levels of c-myc repress p21 expression, favoring proliferation, whereas low levels of c-myc permit levels of p21 to rise, promoting differentiation (33). Interestingly, in the $Apc1638^{+/-}$, $p21^{-/-}$ mice in which inactivation of *p21* leads to increased *Apc*-initiated tumor formation, there was a significant decrease in alcian blue-stained intestinal goblet cells (10). It is important that we demonstrated in this work that inactivation of *p27* also decreased alcian blue-stained cells. However, this was not paralleled by loss of cells that stain for Itf, another goblet cell specific marker. Therefore, similar to work we have reported on tumor formation in mice with a targeted inactivation of the *Muc2* gene in which there was also loss of mucin staining but not Itf expression (23), it may be the loss of mucin itself, and not complete ablation of the goblet cell lineage, that is linked to tumorigenesis. However, whether loss of *p27* plays a direct role in regulating cell differentiation and mucin synthesis or an indirect role through failure of cells to arrest in the cell cycle as they migrate up the crypt is not yet clear.

The alterations in mRNA expression in the mucosa of components that drive cells through the cell cycle was significant and was also reflected in altered protein levels, although the changes were modest. This likely reflects the significant, although relatively small, shifts in cell maturation in the mucosa (proliferation, apoptosis, and differentiation) that characterize elevation of risk for tumor formation. However, it is important to note that there may be a highly significant link between such changes and the probability of the animals developing a single to a few tumors over 36 weeks of age. This is also precisely the situation in human populations in which >90% of the individuals who develop colon cancer also develop only a single tumor in six to seven decades of life, during which time there are $\sim 10^{12}$ cell divisions in the colon, with no major abnormalities in the molecular or cell biology or functioning of the intestinal mucosa over this time span. Thus, the level of perturbation of homeostasis in the intestinal mucosa and the interactions detected between the genetic pathways and dietary factors in tumor development in this system are likely to be highly relevant to the processes that determine risk for colonic cancer in western society.

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# Targeted Inactivation of *p27$^{kip1}$* Is Sufficient for Large and Small Intestinal Tumorigenesis in the Mouse, Which Can Be Augmented by a Western-Style High-Risk Diet

WanCai Yang, Laura Bancroft, Courtney Nicholas, et al.

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