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# Short Communication

## Early Alteration of Cell-Cycle-Regulated Gene Expression in Colorectal Neoplasia

Kornelia Polyak,*† Stanley R. Hamilton,†‡ Bert Vogelstein,*† and Kenneth W. Kinzler†

*From the Howard Hughes Medical Institute,* Oncology Center,† and Department of Pathology,‡ The Johns Hopkins University School of Medicine, Baltimore, Maryland*

***Aberrant crypt foci with dysplasia are thought to be the first detectable lesions of colorectal neoplasia. Because cell cycle disruption appears crucial for tumorigenesis, we analyzed the immunohistochemical expression patterns of the prototype cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and the proliferation marker Ki67 in the early stages of colorectal tumorigenesis. In colorectal epithelium, $p21^{WAF1/CIP1}$ expression was undetectable in the lower third of the crypts, where Ki67 was expressed, but then sharply increased as cells passed out of the proliferating zone and migrated toward the lumen. Hyperplastic polyps retained this normal compartmentalized pattern. In contrast, markedly decreased $p21^{WAF1/CIP1}$ immunostaining was observed in dysplastic aberrant crypt foci as well as in small adenomas. Moreover, the compartmentalization of Ki67 and $p21^{WAF1/CIP1}$ was lost, as Ki67 expression extended into the small p21-expressing zone at the top of the crypts. These data suggest that the dysregulated expression of cell-cycle-controlling genes and the consequent release from normal cell cycle controls may represent an essential early step in colorectal neoplasia. (Am J Pathol 1996, 149:381–387)***

Colorectal tumors progress through a series of well defined clinical stages.[1] At the histopathological level, the smallest recognizable abnormal entity is the aberrant crypt focus (ACF). These lesions were originally described in rodent tumor model systems as a morphological forerunner of cancers induced by colorectal carcinogens.[2,3] In humans, two types of ACF have been observed after methylene blue staining of whole mount mucosa preparations.[4–6] The more common type of ACF is hypercellular, but at the light microscopic level, the cells themselves appear to be normal. For this reason, such ACFs are referred to as hyperplastic or nondysplastic. Such lesions uniformly contain K-*ras* gene mutations but rarely if ever contain *APC* mutations and appear unlikely to progress to clinically important lesions.[6–8] Less common, but perhaps more important, are the dysplastic ACFs, which share the intra- and intercellular hallmarks of dysplasia with adenomas and carcinomas. It is therefore believed that dysplastic ACFs are the precursors of adenomas, which in turn are the precursors of carcinomas. This idea is consistent with the following observations. First, familial adenomatous polyposis (FAP) patients, who develop numerous adenomas, also develop numerous dysplastic ACFs, which have been termed microadenomas.[9,10] Second, the incidence of dysplastic ACFs after carcinogen treatment of rodents parallels that of carcinomas,[2,3] and the ACFs precede carcinoma development.[11] Third, one human dysplastic ACF that was examined in detail contained an *APC* mutation but not a K-*ras* gene mutation.[6] This genetic profile is identical to that observed in small adenomas.[6,12–16]

Polyps, although much larger than ACFs, are categorized as hyperplastic (metaplastic) and dysplastic, with only the dysplastic lesions (adenomas) dis-

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Address reprint requests to Dr. Kenneth Kinzler, Oncology Center, Research Lab, Room 109, The Johns Hopkins University School of Medicine, 424 North Bond Street, Baltimore, MD 21231.

playing *APC* mutations and a propensity for progression.[6,15,16] FAP patients, who have germline *APC* mutations,[17,18] develop large numbers of adenomas but are not at high risk for hyperplastic polyps. It is possible that hyperplastic ACFs are the precursors of hyperplastic polyps, but the fact that hyperplastic polyps do not generally contain K-*ras* gene mutations argues against this conjecture.[6]

Over the past several years it has become clear that escape from normal cell cycle control is critical for tumorigenesis (reviewed in Refs. 19–21). The timing of this escape, however, remains largely unexplored. The cell cycle in all eukaryotic cells is regulated by protein complexes containing cyclin-dependent kinases and their cyclin partners (reviewed in Ref. 22). A recently identified group of small proteins, the cyclin-dependent kinase inhibitors, has been shown to negatively regulate cyclin-dependent kinase activity and thereby play a crucial role in the regulation of cell proliferation and differentiation (reviewed in Ref. 23). The prototype of these inhibitors is $p21^{WAF1/CIP1}$, which binds to and inhibits most known cyclin-dependent-kinase-cyclin complexes and the transcription of which is regulated in part by p53.[24–27] Previous analyses of $p21^{WAF1/CIP1}$ have demonstrated that large adenomas and carcinomas express $p21^{WAF1/CIP1}$ in an aberrant pattern with respect to proliferation.[28] To determine the stage of tumor progression at which this abnormality occurs, we have characterized p21 expression patterns in the earliest recognizable stages of colorectal neoplasia.

## Materials and Methods

### Colorectal Tissues

Specimens were obtained from colonoscopic biopsies, and surgical resections were performed at The Johns Hopkins Hospital and were frozen in OCT embedding medium (Miles, Elkhart, IN). Specimen usage was approved by the Johns Hopkins Joint Committee on Clinical Investigation Institutional Reviewer Board. To obtain ACFs, sheets of mucosa were dissected from the muscularis propria, stained with methylene blue, and observed under a microscope. ACFs were identified and marked by tattoo powder.[29] Dysplastic ACFs were collected from five FAP patients (ages 19 to 38 years). Twelve adenomas (2 to 5 mm in size) were obtained from six sporadic (ages 63 to 75 years) and four FAP patients (ages 19 to 37 years), and six hyperplastic polyps and six hyperplastic ACFs were collected from eight sporadic patients (ages 46 to 78 years).

### Monoclonal Antibodies

Generation and characterization of the anti-p21 monoclonal antibody EA10[28] and the anti-hMSH2 antibody FE11[30–32] have been described previously. The Ki67-specific monoclonal antibody MIB against a proliferation-associated antigen not expressed in quiescent cells was obtained from Oncogene Science (Cambridge, MA).

### Immunohistochemistry

Immunohistochemistry was performed with standard techniques.[28,30] Briefly, frozen sections at 6 $\mu$m were fixed in Histochoice (Amresco, Solon, OH) for 5 minutes at room temperature and stored in phosphate-buffered saline at 4°C until use. Endogenous peroxidase activity was blocked in some experiments by Immunopure peroxidase suppressor (Pierce, Rockford, IL) following the manufacturer's protocol. Nonspecific primary antibody binding was blocked by preincubating the sections in filtered goat serum for 1 hour. Primary antibody incubations were performed at room temperature for 12 to 16 hours and were followed by incubation with a biotin-conjugated goat anti-mouse secondary antibody (Pierce). Staining was accomplished with the ABC horseradish peroxidase method (Vector Laboratories, Burlingame, CA). Sections were counterstained with 0.5% methyl green, dehydrated, and then mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ).

## Results

### Colorectal Epithelium

In normal appearing epithelium from FAP and non-FAP patients, p21 expression was absent in the rapidly proliferating cells and the stem cells located within the lower third of the crypts. There was a relatively sharp demarcation at which the replicating compartment ended and p21 expression began. This expression of p21 continued and in some glands appeared to increase slightly as cells migrated toward the top of the crypt toward the surface epithelium (Figure 1C). Ki67 is a commonly used marker of cellular proliferation that is expressed in actively growing cells at all phases of the cell cycle but not in quiescent cells.[33,34] Intense Ki67 staining was observed in the nuclei of the proliferating cells in the lower third of the crypt, whereas the p21-positive cells above this point uniformly lacked expression of this antigen (Figure 1B). Another proliferation marker was provided by the protein encoded by the hMSH2

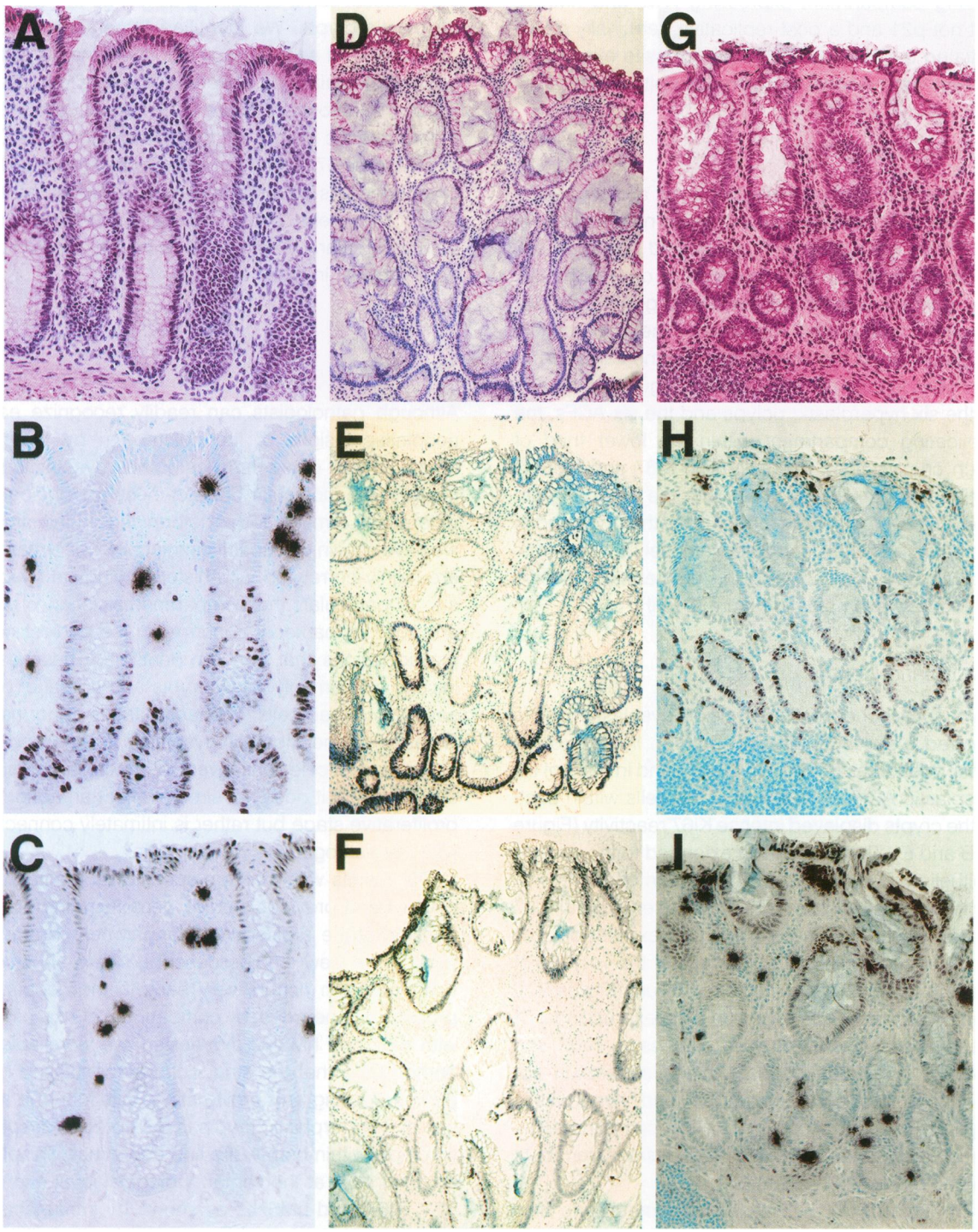


Figure 1. *Expression of Ki67 and p21 in normal and hyperplastic crypts. Colonic mucosa from familial adenomatous polyposis patient* (A *to* C), *sporadic hyperplastic polyps* (D *to* F) *and nondysplastic ACFs* (G *to* I) *from non-FAP patients stained with H&E* (A, D, *and* G), *anti-Ki67 antibody* (B, E, *and* H), *and anti-p21 antibody* (C, F, *and* I). *Proliferation is confined to the lower third of the crypts whereas p21 expression is observed in the upper third of the crypts and in the surface epithelium. Peroxidase-positive cells in the interstitium represent inflammatory cells.*

mismatch repair gene.[30–32] Repair mediated by the mismatch repair system appears most active during DNA replication, and accordingly, expression of hMSH2 is largely confined to the proliferative compartment of the crypt.[30] The Ki67 and hMSH2 expression patterns overlapped considerably, although the hMSH2 reactivity extended further toward the top of the crypt (data not shown). Thus, a prolif-

erating compartment expressing Ki67 and hMSH2 but not p21 and a post-replicative, terminally differentiated compartment with the opposite expression pattern were evident in colorectal epithelium.

### *Hyperplastic Crypts*

The crypts in six hyperplastic polyps and six hyperplastic ACFs contained an increased number of cells and were elongated with a serrated sawtooth appearance and with protuberance of crowded cells. The epithelium included distended goblet and absorptive cells without dysplasia. The hyperplastic crypts retained the normal compartmentalization of p21 seen in colorectal epithelium (Figure 1). In each of the six hyperplastic polyps and the six ACFs, the replicating compartment within the lower third of each crypt stained intensely with Ki67 and hMSH2 antibodies but did not react with the anti-p21 antibody. Conversely, cells further up in the crypt and in the surface epithelium stained intensely with the anti-p21 antibody but not with the proliferation-specific antibodies (examples in Figure 1, D–I).

### *Dysplastic Crypts*

Immunohistochemical analysis of dysplastic crypts showed a strikingly different pattern. In each of 12 small adenomas (2 to 5 mm in size) and in each of six dysplastic ACFs, virtually all of the cells within each of the crypts displayed intense Ki67 reactivity (Figure 2, B and E). This expression extended to the surface epithelium in most cases. The pattern of expression of hMSH2 was very similar to that observed with Ki67 (data not shown). In contrast, p21 expression was either absent or limited to the cells at the very top of the crypts and to the surface epithelium (Figure 2, C and F). Moreover, the region of cells expressing p21 clearly overlapped with that expressing Ki67 and hMSH2, in marked contrast to the separation observed in colorectal epithelium and hyperplastic crypts. The disruption of p21/Ki67 compartmentalization was observed in dysplastic lesions from both sporadic and FAP patients.

The smallest ACF obtained after methylene blue staining contained approximately 20 to 100 crypts each. To extend the analysis further, we identified microscopic ACFs by hematoxylin and eosin (H&E) staining of mucosal specimens from FAP patients. The vast majority of colonic crypts from these patients were normal, both with respect to histopathological characteristics and staining with Ki67, hMSH2, and p21 antibodies. Occasionally, however, a small ACF was identified histopathologically within the mucosa, some containing as few as two or three dysplastic crypts. We identified three such lesions and found that each already displayed the disrupted compartmentalization observed in larger dysplastic lesions. As shown in Figure 2, G–I, most cells within the dysplastic crypts of these microscopic ACFs expressed Ki67 regardless of their position within the crypt, whereas p21 staining was limited to the top layer of epithelial cells. The p21-expressing compartment clearly overlapped with the proliferative compartment in these lesions.

## *Discussion*

Although pathologists can readily recognize early neoplastic lesions of the colorectum by the histopathological characteristics of their dysplastic epithelium, the molecular basis of these morphological differences remains to be determined. In this study, we have shown that specific molecular abnormalities are highly correlated with histopathological dysplasia. In particular, the compartmentalization of p21 and Ki67 is markedly abnormal in the earliest neoplastic lesions that can be identified. These early lesions contain only a few crypts and a total of only several hundred cells. The fact that this abnormality is observed specifically in dysplastic lesions, and not in hyperplastic lesions even of much larger size, suggests that it does not simply reflect an abnormal proliferative stage but rather is intimately connected with the pathogenesis of neoplasia.

Our results with markers specific for proliferating cells extend previous studies demonstrating an expansion of the proliferative compartment in adenomas.[35] The new studies described here show that this expansion occurs very early in the neoplastic process. Furthermore, by comparing p21 expression with Ki67 and hMSH2 expression, we were able to demonstrate that the strict compartmentalization of p21-expressing and proliferating cells found in normal and hyperplastic crypts is disrupted in dysplastic crypts within both ACFs and adenomas. Dysplastic crypt cells at the same topological level express p21, Ki67, and hMSH2, as shown by immunohistochemistry of serial sections, suggesting that all three proteins are expressed within the same cell. Thus, the control system that activates p21 during colonic cell migration is defective, and conversely, the control system that inactivates Ki67 and hMSH2 is similarly abnormal in dysplastic colonic lesions.

Previous studies have demonstrated that the expression of cell-cycle-controlling genes, including cyclins, cyclin-dependent kinases, and cyclin-de-

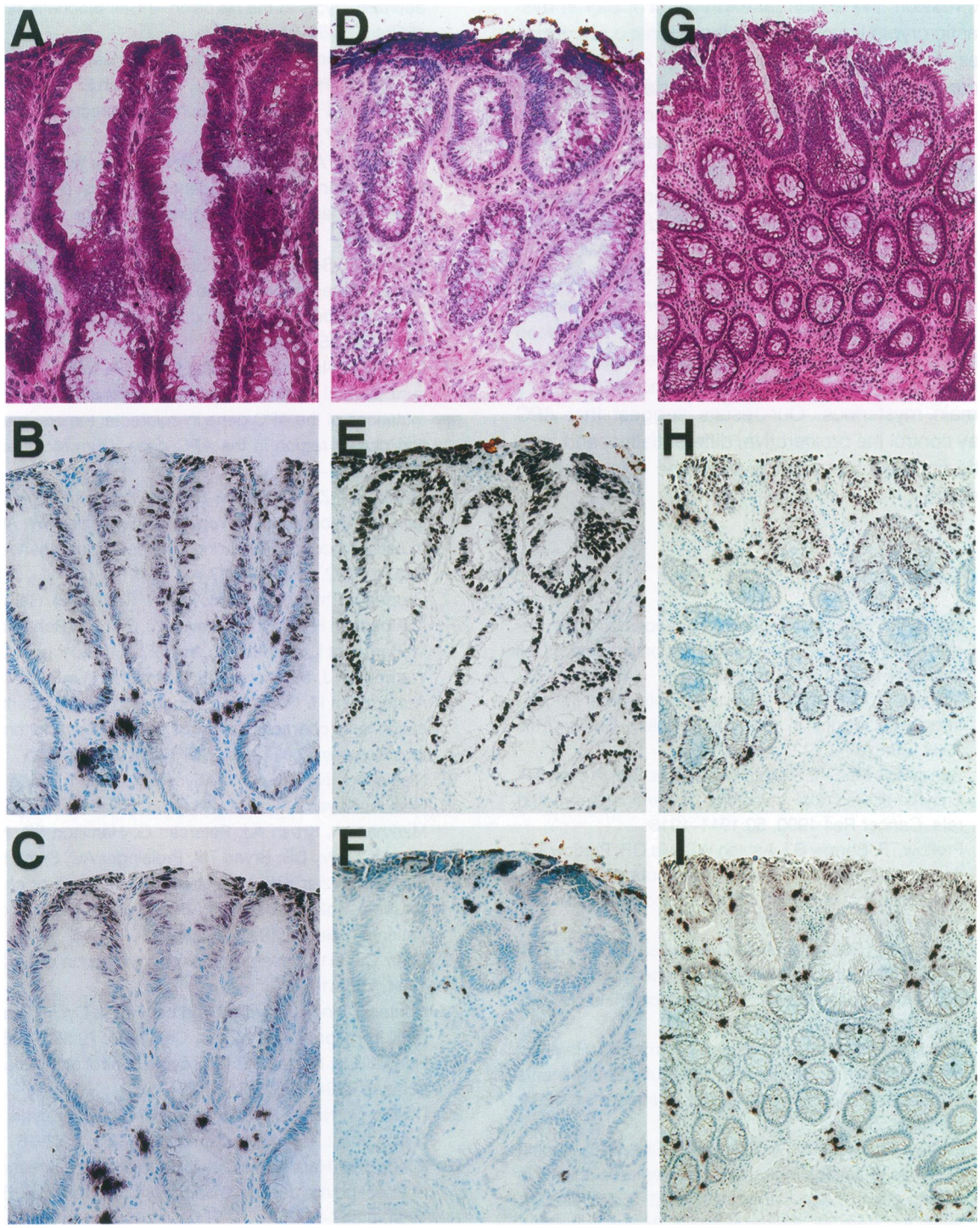


Figure 2. *Expression of Ki67 and p21 in dysplastic crypts. A small adenoma* (A *to* C), *a dysplastic ACF* (D *to* F), *and a microscopic ACF* (G *to* I) *from FAP patients stained with H&E* (A, D, *and* G), *anti-Ki67 antibody* (B, E, *and* H), *and anti-p21 antibody* (C, F, *and* I). *In contrast to colorectal epithelium and hyperplastic crypts, proliferating cells and cells expressing p21 are topologically unrestricted in dysplastic crypts. Peroxidase-positive cells in the interstitium represent inflammatory cells.*

pendent kinase inhibitors is often altered in cancers (reviewed in Refs. 19–21). Most of these alterations, however, appear to occur at a relatively late stage of tumorigenesis. Our results indicate that an abnormality of p21 expression occurs very early in this process in colorectal neoplasia, perhaps at the initiating stage. The biochemical basis of this disruption is uncertain. Although p53 is a major regulator of p21

expression, the abnormal expression of p21 in dysplastic crypts is unlikely to reflect genetic abnormalities of p53 for at least two reasons. First, the basal expression of p21 in normal colorectal epithelium is independent of p53, at least in the mouse.[28,36] Second, alterations of p53 do not usually occur until much later in tumorigenesis, often at the adenoma-carcinoma transition.[37,38] The only specific genetic abnormality known to occur in early dysplastic colorectal lesions is *APC* inactivation.[6,8,15,16] *APC* alterations are inherited in FAP patients[17,18,39,40] and are thought to be the initiating somatic event in sporadic colorectal neoplasms. Although much has been learned about mutations of *APC*, the effects of these mutations on colorectal physiology and biochemistry remain mysterious. Our results suggest that *APC* may control the proliferative, differentiative, and apototic compartmentalization within the colorectal epithelium.

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