

Colonic tumorigenesis in *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice is linked to premature separation of sister chromatids and enhanced genomic instability

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Edited by Bert Vogelstein, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, and approved January 14, 2005 (received for review October 20, 2004)

Faithful chromosome segregation is essential for the maintenance of genetic stability during cell division and it is at least partly monitored by the spindle checkpoint, a surveillance mechanism preventing the cell from prematurely entering anaphase. The adenomatous polyposis coli (*Apc*) gene also plays an important role in regulating genomic stability, as mutations of *Apc* cause aneuploidy. Here we show that whereas *Apc*^{Min/+} mice developed many adenomatous polyps, mostly in the small intestine, by 3 mo of age; *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice developed 10 times more colonic tumors than *Apc*^{Min/+} mice. The colonic tumors in *BubR1*^{+/-}*Apc*^{Min/+} mice were in higher grades than those observed in *Apc*^{Min/+} mice. Consistently, *BubR1*^{+/-}*Apc*^{Min/+} murine embryonic fibroblasts (MEFs) contained more β -catenin and proliferated at a faster rate than WT or *BubR1*^{+/-} MEFs. Moreover, *BubR1*^{+/-}*Apc*^{Min/+} MEFs slipped through mitosis in the presence of nocodazole and exhibited a higher rate of genomic instability than that of WT or *BubR1*^{+/-} or *Apc*^{Min/+} MEFs, accompanied by premature separation of sister chromatids. Together, our studies suggest that *BubR1* and *Apc* functionally interact in regulating metaphase–anaphase transition, deregulation of which may play a key role in genomic instability and development and progression of colorectal cancer.

polyposis | genetic instability | colon cancer | spindle checkpoint | mitosis

Genetic instability is an integral component of human neoplasia. High-fidelity DNA replication and faithful chromosome segregation are fundamental processes that allow cells to transmit their genetic information to progeny. Failures in maintaining genetic stability inevitably cause either cell death or abnormal phenotypes such as malignancy. Missegregation of chromosomes may result from various causes, including defects of spindle checkpoint components and abnormal centrosomal duplication. In fact, a significant fraction of cancer cells that exhibit aneuploidy either harbor defects in cell cycle checkpoint pathways (1, 2) or contain an abnormal centrosome number (3).

Aneuploidy occurs frequently in colorectal cancer. Colorectal tumors exhibit a defect in chromosome segregation, leading to frequent gains or losses of chromosomes ($>10^2$ per chromosome per generation) (4). Chromosome instability has been detected in the smallest adenoma, suggesting that chromosome instability may occur at very early stages of colorectal carcinogenesis (5). Extensive research during the past has led to the identification of genes that play a major role in the development of colorectal cancer. For example, mutations or deletions of the adenomatous polyposis coli (*Apc*) gene, encoding a 310-kDa cytoplasmic protein (6, 7), are commonly found in inherited familial adenomatous polyposis patients and in sporadic colorectal cancers (8, 9). Such mutations appear to be an early event during colorectal tumorigenesis (10). Mouse models have been developed to study the role of *Apc* mutations (truncated *Apc*) in affecting cell proliferation or loss of *Apc* as a contributing factor in formation

of polyps (11, 12). The importance of the *Apc* gene in intestinal cancer development by regulating the stability of β -catenin has been well established by efforts of many laboratories (13, 14). However, *Apc* mutations are detected in preneoplastic lesions, the earliest stages of polyp formation, 7–15 years ahead of the malignant tumor formation (15). It is therefore likely that loss of *Apc* at the early stage promotes tumor formation at least in part by mechanism(s) independent of the proliferation pathway mediated by β -catenin. Recent studies suggest that a loss of WT *Apc* correlates with chromosome instability (16, 17), a hallmark of colon polyposis. C-terminal *Apc* truncations similar to those found in colon tumors correlate with chromosome instability in mouse embryonic cells (17). Although the molecular mechanism by which *Apc* regulates chromosomal segregation remains to be established, the existing evidence that *Apc* associates with spindle checkpoint components and localizes to kinetochores and centrosomes (16, 18) strongly suggests a role of this protein in controlling metaphase–anaphase transition.

The spindle checkpoint plays an essential role in the maintenance of chromosomal segregation (19). Haploinsufficiency of many spindle checkpoint components results in enhanced genomic instability and tumor formation (20–22). A couple of recent studies show that a compromised *BubR1* activity due to specific germ-line mutations causes aneuploidy, infertility, and/or early onset of malignancies (23) and strongly suggest that spindle checkpoint failure is an underlying cause for the development of certain diseases. In this paper, we demonstrate that *BubR1*^{+/-}*Apc*^{Min/+} (*BubR1* is also termed *Bub1B*) compound mutant mice developed colonic tumors at an accelerated rate and that this enhanced tumorigenicity was associated with premature separation of sister chromatids and greatly elevated chromosomal instability.

Materials and Methods

Generation of *Apc*^{Min/+}*BubR1*^{+/-} Mice. We used our previously developed *BubR1*^{+/-} mice (21) and *Apc*^{Min/+} mice purchased from The Jackson Laboratory to generate *Apc*^{Min/+}*BubR1*^{+/-} genotype mice. All of the mice were housed in a pathogen-free barrier environment for the duration of the study. We intercrossed female *BubR1*^{+/-} with male *Apc*^{Min/+} to obtain mouse progenies having genotypes of *BubR1*^{+/+}*Apc*^{+/+}, *BubR1*^{+/+}*Apc*^{Min/+}, *BubR1*^{+/-}*Apc*^{+/+}, and *BubR1*^{+/-}*Apc*^{Min/+}. *BubR1* genotyping of each animal was carried by PCR according to previously published procedures (25). *Apc* genotyping was

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: *Apc*, adenomatous polyposis coli; MEF, murine embryonic fibroblast.

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carried out by using primers obtained from The Jackson Laboratory.

Mouse Intestinal Tumor Analysis. Age-matched mice with genotypes of *BubR1*^{+/+}*Apc*^{+/+}, *BubR1*^{+/+}*Apc*^{Min/+}, *BubR1*^{+/-}*Apc*^{+/+}, or *BubR1*^{+/-}*Apc*^{Min/+} were selected to assess the small and large intestinal tumorigenesis patterns. In brief, at 5 wk of age, WT mice or mice deficient in either *Apc* or *BubR1* or both were fed semipurified AIN-76A diet. At 12 wk of age, all of the mice were killed by CO₂, and their intestines were removed and flushed with Krebs–Ringer solution. Intestines were opened and analyzed for number, location, and size of tumors with the help of a dissecting microscope. Small intestinal and colonic tumors were further histologically analyzed by the hematoxylin/eosin staining procedure.

Maintenance and Treatment of Murine Embryonic Fibroblasts (MEFs). MEFs were derived from embryonic day 14.5 fetuses produced from intercrosses of *BubR1*^{+/-} and *Apc*^{Min/+} mice as described (25). Four different genotypes of MEFs (namely, WT, *BubR1*^{+/-}, *Apc*^{Min/+}, and *BubR1*^{+/-}*Apc*^{Min/+}) were obtained. MEFs were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS, 100 μg/ml penicillin, 50 μg/ml streptomycin sulfate, and 0.2 mM 2-mercaptoethanol. MEFs were also treated with nocodazole (0.4 μg/ml) for various times.

Western Blot Analysis. Total proteins were prepared from MEFs treated with vehicle or with various agents as above. Equal amounts of total proteins were analyzed by SDS/PAGE. Proteins fractionated on the denaturing gels were transferred to nitrocellulose membranes, which were blotted with antibodies to

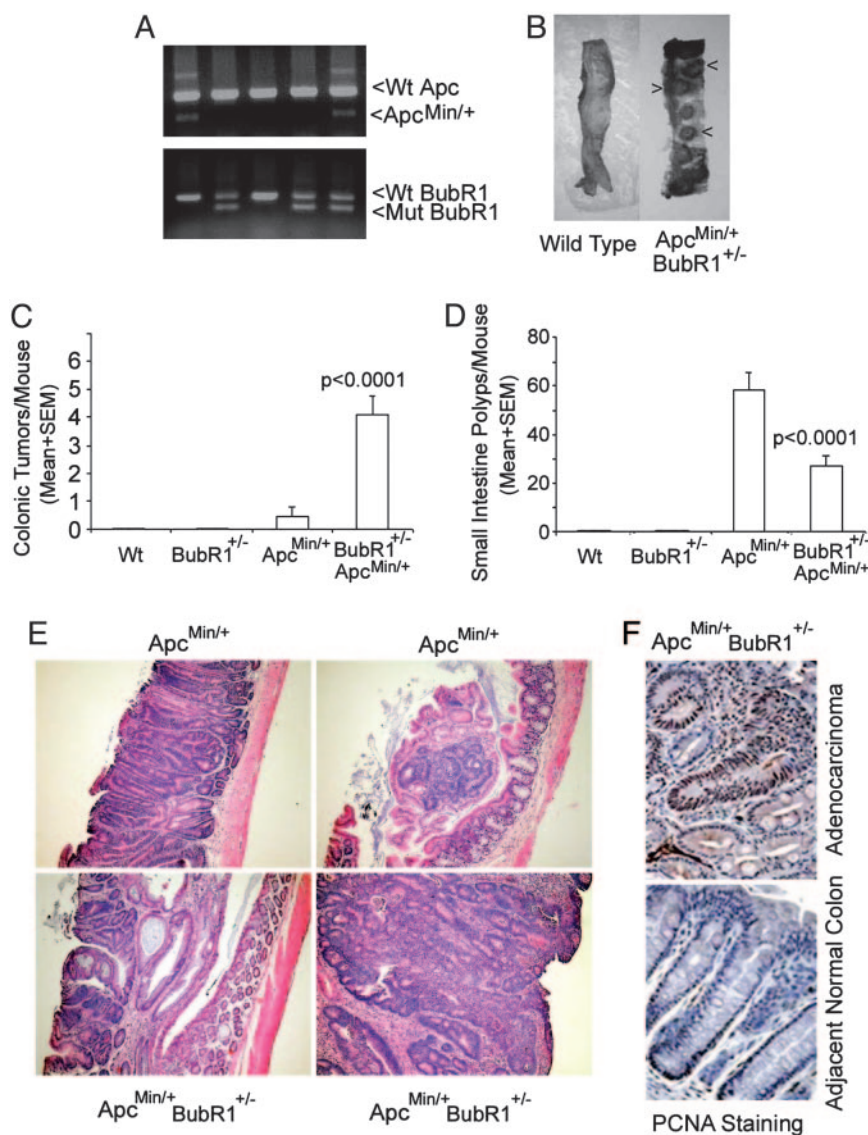


Fig. 1. Haploinsufficiency of *BubR1* and *Apc* results in an increased formation of colonic tumor polyps. (A) Genomic DNA samples isolated from mouse tails were subjected to genotyping by PCR using primers that detect both WT (Wt) and mutant alleles of *Apc* or *BubR1*. (B) Dissection micrographs of representative colons from WT and *BubR1*^{+/-}*Apc*^{Min/+} mice at 3 mo of age. Arrows denote the colonic tumor masses. (C and D) Mice of various genotypes at 3 mo of age were killed. Intestines from each mouse were examined under a dissection microscope for tumor polyps. Average numbers of tumor masses in colon (C) and the small intestine (D) from WT, *BubR1*^{+/-}, *Apc*^{Min/+}, and *BubR1*^{+/-}*Apc*^{Min/+} mice are shown. No visible intestinal tumor masses were detected in WT or *BubR1*^{+/-} mice. (E) Hematoxylin/eosin-stained sections of tumor masses from *Apc*^{Min/+} (Upper) and *BubR1*^{+/-}*Apc*^{Min/+} (Lower) mice. (×400.) (F) Sections of colonic tumors from *BubR1*^{+/-}*Apc*^{Min/+} mice were subjected to immunohistochemical studies after staining with IgGs to proliferating cell nuclear antigen. (×400.) Typical malignant tissues (Upper) and normal colon tissues adjacent to the adenocarcinomas (Lower) are presented.

Table 1. Colonic tumor numbers in individual mice of various genotypes

Genotype	No. of tumors in individual mice																Mean \pm SEM		
WT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>BubR1</i> ^{+/-}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Apc</i> ^{Min/+}	2	0	1	1	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0.4 \pm 0.6
<i>BubR1</i> ^{+/-} <i>Apc</i> ^{Min/+} *	7	4	6	3	3	4	2	2	5	5									4.1 \pm 1.7

*There were fewer mice in the *BubR1*^{+/-}*Apc*^{Min/+} compound mutant group because of a limited availability of age-matched mice as a result of embryonic lethality.

Apc (Abcam, Cambridge, MA), BubR1, β -catenin (Cell Signaling Technology, Beverly, MA), and β -actin (Sigma). Immunoreactive bands were detected with an appropriate second antibody and visualized with a chemiluminescence kit (Pierce).

Fluorescence Microscopy and Immunohistochemistry. Immunostaining of MEFs was carried out as described (21). In brief, cells fixed in 4% paraformaldehyde were treated with 0.1% Triton X-100 on ice and then washed three times with ice-cold PBS. After blocking with 2.0% BSA in PBS for 15 min on ice, cells were stained with DAPI (1 μ g/ml, Fluka). Fluorescence microscopy was performed on a Nikon microscope, and images were captured by using a digital camera (Optronics International, Chelmsford, MA). For immunohistochemistry, sections from formalin-fixed, paraffin-embedded mouse colon tissues were prepared and incubated with 0.3% hydrogen peroxide for 10 min and then stained with the primary antibody to proliferating cell nuclear antigen, cyclin D1, Apc, or BubR1 for 1 h. After incubation with an appropriate second antibody, the sections were washed and incubated with a preformed avidin-biotinylated enzyme complex for 30 min by using an ABC kit (Vector Laboratories) followed by color development with diaminobenzidine tetrahydrochloride. The stained colon sections were counterstained with hematoxylin. For TUNEL assays, formalin-fixed, paraffin-embedded sections from the intestines of various genotypes were prepared. *In situ* end labeling (or TUNEL) was performed by using an apoptosis assay kit (Oncogene Science) according to the manufacturer's instructions. For TUNEL assays, sections from lymph nodes of WT mice were used as positive controls, whereas sections incubated with non-immune sera were used as negative controls. Cells undergoing apoptosis manifested as *in situ* end labeling were examined under a microscope. Three slides per group were reviewed.

Cytogenetics. MEFs seeded for 24 h were treated with Colcemid (0.04 μ g/ml) for 3 h to arrest cells in metaphase. Cells detached from the culture plates with trypsin/EDTA were incubated in 75 mM KCl for 20 min at 37°C. These cells were then fixed in three changes of methanol/acetic acid (3:1), and the fixed cell pellets were used for slide spread. Slides were air dried for at least 2 d at 37°C before examination. For MEFs of each genotype, at least 50 metaphase spreads were examined.

Cell Viability Assay. Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. MEFs (2×10^4 cells per well) were seeded on 96-well plate in triplicates. After a 24-h culture at 37°C, the culture medium was aspirated and a fresh medium was added to a final volume of 200 μ l. MTT (20 μ l, Sigma, 2 mg/ml in PBS) was then added to each well. After the cells were incubated at 37°C for 4 h, the medium was removed. MTT formazan precipitates were dissolved in 100 μ l of DMSO with mechanical shaking for 10 min. The absorbance of dissolved samples was measured at 570 nm by using a plate reader.

Results

To determine whether *BubR1* is directly involved in colon carcinogenesis, we introduced the *BubR1* mutation into *Apc*^{Min/+} mice through cross-breeding *BubR1*^{+/-} mice with *Apc*^{Min/+} mice. The genotypes of offspring were determined through PCR as described (25), and typical results of genotyping are shown in Fig. 1A. As expected, no mice with a homozygous mutation of either *BubR1* or *Apc* were obtained. The detected frequency of newborn mice heterozygous for both *BubR1* and *Apc* was 13%, that is approximately one-half of the percentage predicted by the Mendelian segregation rule, indicating a certain extent of embryonic lethality. Because *Apc*^{Min/+} mice typically develop numerous adenomas in the small intestine by 3 mo of age, we asked whether *BubR1*^{+/-}*Apc*^{Min/+} mice would be more susceptible to tumor development, especially in the large intestine. An examination of intestines obtained from mice of various genetic backgrounds revealed that WT or *BubR1*^{+/-} mice developed no visible polyps, whereas some *Apc*^{Min/+} mice sporadically developed one or two visible polyps (Table 1). On the other hand, age-matched *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice developed many tumor masses in the large intestine (Fig. 1B). In fact, the average number (4.1 \pm 1.7) of colonic tumor masses in *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice was \approx 10 times of that (0.4 \pm 0.6) in *Apc*^{Min/+} mice (Table 1 and Fig. 1C). Interestingly, there were significantly fewer polyps in small intestines of *BubR1*^{+/-}*Apc*^{Min/+} mice than in *Apc*^{Min/+} mice (Fig. 1D). Histological analysis revealed that tumors from *Apc*^{Min/+} mice were either well defined tubular adenomas or tubular adenomas within the lamina propria (Fig. 1E Upper). On the other hand, tumor masses from *BubR1*^{+/-}*Apc*^{Min/+} colons were adenocarcinomas that were either poorly or moderately differentiated with cribriform tumor glands, stratified epithelial cells, and necrotic tissues (Fig. 1E Lower), indicating that colonic tumors from *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice had progressed to much higher grades than those from *Apc*^{Min/+} mice. Immunohistochemical analysis revealed that tumor cells from *BubR1*^{+/-}*Apc*^{Min/+} colons uniformly exhibited strong nuclear staining for proliferating cell nuclear antigen, whereas normal colonic epithelia showed proliferating cell nuclear antigen staining primarily in stem cells of basal crypts (Fig. 1F Upper), suggesting that mutations in both *BubR1* and *Apc* genes confer the growth advantage for colonic tumors.

To examine levels of BubR1 and Apc expression in mutant mice, we derived murine fibroblasts from embryos with various genetic backgrounds. As expected, both *Apc*^{Min/+} and *BubR1*^{+/-}*Apc*^{Min/+} MEFs expressed \approx 50% less Apc protein than WT or *BubR1*^{+/-} MEFs; similarly, *BubR1*^{+/-} and *BubR1*^{+/-}*Apc*^{Min/+} cells contained at least 50% less BubR1 protein (Fig. 2A). Because the stability of β -catenin, a key component in the Wnt pathway, is regulated by the Apc complex, we examined β -catenin levels in various MEFs. Immunoblot analysis revealed that MEFs with an *Apc* mutation contained more β -catenin (Fig. 2B), thus in agreement with the role of *Apc* in negative regulation of this molecule. Moreover, because cyclin D1 is a target of β -catenin that was elevated in *Apc*-deficient cells, we analyzed cyclin D1 levels in colonic tissues of various genetic backgrounds by immunocytochemistry. We observed that

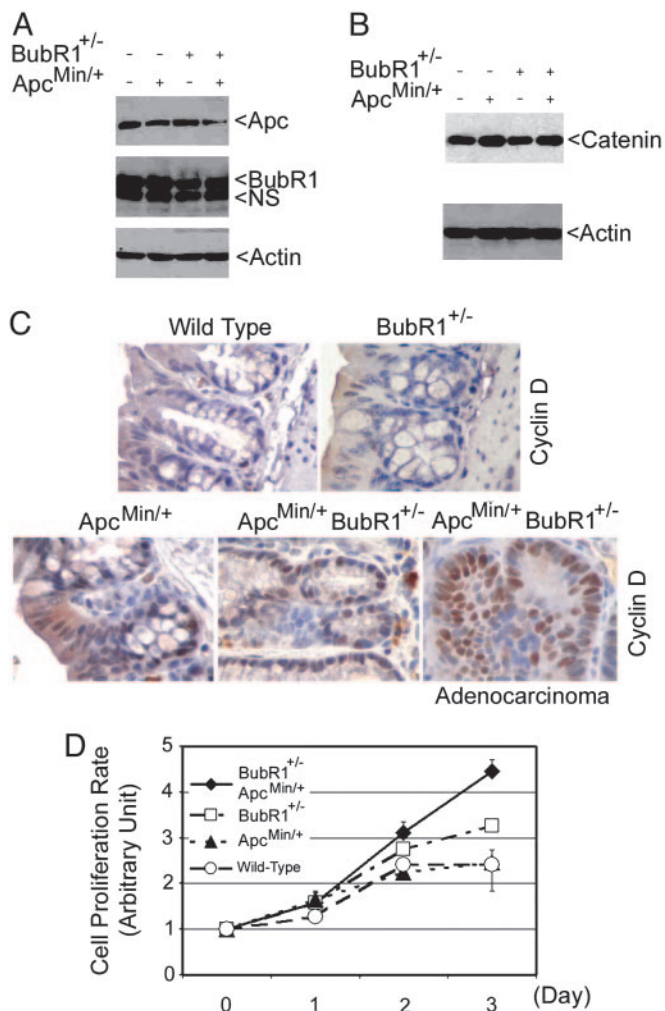


Fig. 2. *BubR1*^{+/-}*Apc*^{Min/+} MEFs proliferate at an accelerated rate. (A) Paired MEFs were lysed and an equal amount of cell lysates were blotted for Apc, BubR1, or β -actin. Arrow NS denotes a nonspecific cross-reactive band. (B) Equal amount of cell lysates from MEFs of various genotypes were blotted for β -catenin and β -actin. (C) Sections of normal colons from mice of various genotypes were subjected to immunohistochemical studies after staining with IgGs to cyclin D1. A typical adenocarcinoma section from *BubR1*^{+/-}*Apc*^{Min/+} mice that was stained with cyclin D1 is also presented. (D) MEFs of various genotypes were subjected to cell proliferation assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. The data are summarized from three independent experiments.

whereas no significant cyclin D1 staining was seen in normal colonic epithelia from WT or *BubR1*^{+/-} mice, strong cyclin D1 staining was detected in basal crypts of colonic epithelia from *BubR1*^{+/-}*Apc*^{Min/+} colons as well as *Apc*^{Min/+} colons (Fig. 2C). Furthermore, a high level of cyclin D1 was present in colonic adenocarcinomas from *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice (Fig. 2C). Consistently, cell proliferation assays showed that *BubR1*^{+/-}*Apc*^{Min/+} cells grew at an accelerated rate compared with WT MEFs or MEFs with a single gene mutation (Fig. 2D).

We were rather puzzled about the reduced number of small intestinal tumors in *BubR1*^{+/-}*Apc*^{Min/+} compound mutant mice. One possible reason is a difference of *BubR1* expression between small intestine and colon. Immunohistochemistry revealed that although epithelia from both small intestine and colon were stained with BubR1, both the cytoplasm and the nucleus of epithelial cells from the small intestine but not from the colon were strongly stained with BubR1 (Fig. 3A), suggesting that the

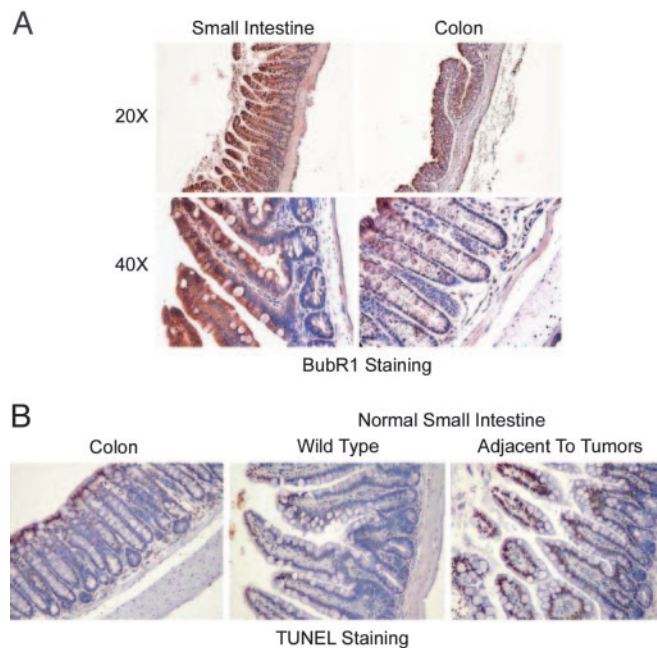


Fig. 3. Analysis of *BubR1* expression and apoptosis in small intestines by immunohistochemistry. (A) Sections of paraffin-embedded small intestine and colon from WT mice were stained with antibody to BubR1. Representative images at various magnifications are presented. Samples from at least three mice were examined. (B) Sections of paraffin-embedded small intestine and colon samples from *BubR1*^{+/-}*Apc*^{Min/+} mice were examined for apoptosis by using a TUNEL kit. Representative images from three independent samples are presented.

small intestine may tolerate *BubR1* deficiency better than the colon because of a higher level of *BubR1* expression. A second possibility is that chromosomal instability that occurred because of *BubR1* haploinsufficiency would induce apoptosis at an accelerated rate in the small intestine. Our studies showed that some apoptosis, as revealed by TUNEL staining, occurred in the apical portion of epithelia of both colon and small intestine and that there was no significantly enhanced apoptosis in normal small intestinal tissues compared with those of colon (Fig. 3B). However, we observed that small intestinal tissues adjacent to the tumors exhibited significantly elevated apoptotic signals, suggesting that the enhanced programmed cell death may partly mediate the reduction in the tumor number in mice deficient in *BubR1* and *Apc*.

Because the spindle checkpoint failure causes mitotic slippage (21), we next determined the mitotic index in various MEFs treated with nocodazole for various times. We observed that whereas a significant fraction of WT MEFs were arrested at mitosis 14 h after nocodazole treatment, MEFs with either an *Apc* or a *BubR1* mutation significantly compromised the mitotic arrest induced by the spindle poison; consistent with the enhanced proliferation rate as shown in Fig. 2 C and D, MEFs with both *Apc* and *BubR1* mutations exhibited little mitotic arrest in the presence of nocodazole (Fig. 4A). Enhanced mitotic slippage often results in genomic instability (21). Micronuclei analysis revealed that there was an increased rate of micronuclei formation in *BubR1*^{+/-}*Apc*^{Min/+} MEFs (Fig. 4B and C) compared with WT MEFs. The enhanced genomic instability was also confirmed by chromosomal counts on metaphase spreads (Fig. 4D). *BubR1*^{+/-}*Apc*^{Min/+} MEFs showed a significant increase in the frequency of aneuploid metaphase compared with WT MEFs, which have a very stable karyotype of 40 (diploid); although many *BubR1*^{+/-} and *Apc*^{Min/+} MEFs also exhibited an unstable karyotype, there were fewer *BubR1*^{+/-}

K-Ras, *Apc*, and *p53* that are critical for regulating cell proliferation or cell cycle checkpoint control. In humans, the development from early adenomas to metastatic carcinomas takes somewhere from 20 to 40 years; it is believed that genetic instability plays a key role in accelerating the rate of mutation in cancerous cells (4). Our studies show that haploinsufficiency of *BubR1* not only significantly increases the frequency of formation of colonic polyps but also enhances their progression toward more malignant phenotypes in *Apc^{Min/+}* mice (Fig. 1). Given that *BubR1* primarily functions in the spindle checkpoint that monitors chromosomal segregation, it is likely that chromosomal instability due to *BubR1* deficiency is the driving force for both expansion of proliferative cell population and accelerated progression of colonic tumors in *Apc*-deficient mice.

It is unexpected that enhanced colonic tumor development in *BubR1^{+/-}Apc^{Min/+}* mice is correlated with a significant decrease in the number of adenomas in the small intestine (Fig. 1D). Although the exact mechanism for the suppression of tumor formation by *BubR1* deficiency in the small intestine of *Apc^{Min/+}* mice remains unclear it is most likely due to enhanced cell death as a consequence of accelerated chromosomal instability. Theoretically, cells with chromosomal instability have two fates. (i) Most cells with chromosomal instability will undergo apoptosis because of severe imbalance of genetic content within the cell. Many *in vivo* and *in vitro* studies support this notion. For example, enhanced apoptosis occurs in normal small intestinal tissues adjacent to tumors from *BubR1^{+/-}Apc^{Min/+}* mice (Fig.

3B). Ectopic expression of *Bub1* dominant-negative mutant induces apoptosis *in vitro* (29). In addition, homozygous deletions of spindle checkpoint genes such as *BubR1* and *Mad2* result in embryonic lethality, which is accompanied by enhanced apoptosis (25, 30). (ii) A small fraction of cells with missegregated chromosomes will survive and proliferate at an accelerated rate because of a right combination of genetic contents; these cells eventually give rise to tumors *in vivo*. Somehow, the microenvironment in *Apc^{Min/+}* colon either tolerates chromosomal instability better or confers more resistance to apoptosis when the spindle checkpoint is compromised (i.e., *BubR1* deficiency), resulting in dramatic increase in colonic tumor formation.

In conclusion, our studies demonstrate that there is a functional interaction between *BubR1* and *Apc* genes *in vivo* and that *BubR1* deficiency confers the susceptibility of *Apc^{Min/+}* mice to develop colonic tumors, supporting the notion that enhanced chromosomal instability due to spindle checkpoint failure may play a key role in the development and progression of colorectal cancer.

We thank Drs. Rulong Sheng and Gita Ramaswamy for advice on our histological studies and Dr. Christoph Lengauer for critical reading of the manuscript. This work was supported in part by grants from the National Institutes of Health to W.D. (CA90658) and to C.V.R. (CA80003). Y.-M.Y. was supported in part by an R03 grant (CA110057) from the National Institutes Health.

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