Crypt-restricted proliferation and commitment to the Paneth cell lineage following *Apc* loss in the mouse intestine

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Accepted 14 January 2005

Development 132, 1443-1451
Published by The Company of Biologists 2005
doi:10.1242/dev.01700

Summary

Loss of Apc appears to be one of the major events initiating colorectal cancer. However, the first events responsible for this initiation process are not well defined and the ways in which different epithelial cell types respond to Apc loss are unknown. We used a conditional gene-ablation approach in transgenic mice expressing tamoxifen-dependent Cre recombinase all along the crypt-villus axis to analyze the immediate effects of Apc loss in the small intestinal epithelium, both in the stem-cell compartment and in postmitotic epithelial cells. Within 4 days, Apc loss induced a dramatic enlargement of the crypt compartment associated with intense cell proliferation, apoptosis and impairment of cell migration. This result confirms the gatekeeper role of Apc in the intestinal epithelium in vivo. Although Apc deletion activated β-catenin signaling in the villi, we observed neither proliferation nor morphological change in this compartment. This highlights the dramatic difference in the responses of immature and differentiated epithelial cells to aberrant β-catenin signaling. These distinct biological responses were confirmed by molecular analyses, revealing that Myc and cyclin D1, two canonical β-catenin target genes, were induced in distinct compartments. We also showed that Apc is a crucial determinant of cell fate in the murine intestinal epithelium. Apc loss perturbs differentiation along the enterocyte, goblet and enteroendocrine lineages, and promotes commitment to the Paneth cell lineage through β-catenin/Tcf4-mediated transcriptional control of specific markers of Paneth cells, the cryptdin/defensin genes.

Key words: APC, β-Catenin, Intestine, Paneth cells, Differentiation

Introduction

The adult intestine is maintained by proliferation of stem cells that reside in the crypt compartment. Stem cell progeny undergo terminal differentiation to form the four differentiated cell types of the intestine (Stappenbeck et al., 1998). Differentiation occurs during cell migration along the crypt-villus axis. Bi-directional migration occurs in the epithelium of the small intestine. Enterocytes, goblet cells and enteroendocrine cells differentiate upon reaching the top of the villus, whereas Paneth cells migrate towards the bottom of the crypt. Thus, the proliferation and differentiation processes are compartmentalized and coupled to migration.

Numerous factors that regulate the stem cell compartment and stem cell differentiation have been identified (Sancho et al., 2003; van de Wetering et al., 2002). Wnt/β-catenin signaling appears to control intestinal homeostasis. Activation of the β-catenin signaling pathway results in the formation of the β-catenin-Tcf complex, which controls the transcription of several target genes (Bienz and Clevers, 2000; Giles et al., 2003). This signaling pathway is tightly regulated by several inhibitors, including the well-known tumor suppressor gene *APC* (Polakis, 1997). Inactivation of Wnt signaling, either by ablation of the *Tcf4* gene or by ectopic expression of Dkk1, a secreted Wnt inhibitor, results in depletion of stem cell compartments in the small intestine, indicating that β-catenin signaling plays a role in stem cell maintenance (Korinek et al., 1998; Kuhnert et al., 2004; Pinto et al., 2003). Familial adenomatous polyposis (FAP), an autosomal dominant condition characterized by the development of hundreds or thousands of polyps in the colon and rectum, is caused by constitutive activation of Wnt signaling by mutations in the *APC* gene. Mutations in *APC* are also responsible for most sporadic colorectal cancers (Fodde et al., 2001). APC has been defined as the gatekeeper of the intestine (Kinzler and Vogelstein, 1996). The distribution of APC is in agreement with the role of β-catenin signaling in maintaining stem cell properties and controlling differentiation in the intestine. A gradient of APC is observed along the crypt-villus axis, where it counteracts β-catenin signaling and allows differentiation.

Enhanced β-catenin signaling is common to normal intestinal stem cell compartments and colorectal cancer (Batlle et al., 2002; Sancho et al., 2003). This suggests that colorectal cancers associated with APC mutations result from excessive proliferation and/or lack of differentiation. It is still unclear

whether tumor formation occurs in the crypt or in well-differentiated cells of the villus (Preston et al., 2003; Shih et al., 2001). Sequential analyses of the cellular and molecular consequences of *Apc* inactivation may elucidate the nature of the pre-malignant disease. Therefore, we analyzed the immediate effects of *Apc* loss in the intestinal epithelium all along the crypt-villus axis. We used a conditional-knockout approach based on the Cre/loxP system and an inducible Cre recombinase ($CreER^{T2}$) driven by the villin promoter to disrupt the *Apc* gene in adult Apc-floxed mice (Colnot et al., 2004a). The villin promoter directs transgene expression specifically in the intestinal epithelium (el Marjou et al., 2004).

Materials and methods

Animal treatments

The generation of $Apc^{lox/lox}$ mice and Vil-$CreER^{T2}$ transgenic mice has been described previously (Colnot et al., 2004a; el Marjou et al., 2004). All experiments involving animals were carried out in accordance with French government regulations. Cre recombinase was activated by a single injection of tamoxifen (TAM) solution (1 mg) (Sigma). At different time points after TAM injection, the mice were killed and the intestine collected.

Collection of patient samples

Ten primary colorectal cancers and the adjacent tissues were collected at the Laennec Hospital (Paris, France).

Immunohistochemistry

Immunostaining was performed on a paraffin wax-embedded Swiss roll, as previously described (Colnot et al., 2004b). We used polyclonal primary antibodies directed against APC C20 (Santa Cruz Biotechnologies, 1/250), caspase3 (Cell Signalling, 1/200), Ki67 (Novocastra, 1/300), c-Myc N-262 (Santa Cruz, 1/50), chromogranin A+B (Progen, 1/50) and Villin (S. Robine, Institut Cochin, Paris), and monoclonal primary antibodies directed against β-catenin (BD Biosciences, 1/100), E-cadherin (Zymed, 1/400) and cyclin D1 (Dako, 1/50). For APC immunostaining, a catalyzed signal amplification system based on the Dako CSA kit was used. Frozen sections were stained with the Cre antibody (Covance, 1/400) and UEA1-FITC (Sigma, 1/100).

Western blotting

Western blots were performed as previously described, and probed with anti-β-catenin (1/500), polyclonal anti-c-Myc N-262 (1/200) and monoclonal anti-cylin D1 (1/250) antibodies (Ovejero et al., 2004).

In situ hybridization

Partial cryptdin 5 and lysozyme cDNAs were obtained by RT-PCR and then subcloned. In situ hybridization was performed using a digoxigenin-labeled probe (Roche) and detected using an anti-digoxigenin antibody (Roche, 1/4000).

RNA extraction and analyses

Extraction of total RNA and reverse transcription were performed as previously described (Ovejero et al., 2004). Cre recombinase activity was detected by allele-specific RT-PCR (Colnot et al., 2004b). Real-time quantitative RT-PCR was carried out with a LightCycler instrument using the LightCycler-fastStart DNA Master SYBR Green I Kit (Roche Diagnostics). Quantification was performed in duplicate and expressed relative to 18s rRNA. For mouse samples, gene expression levels are expressed relative to in wild-type small intestines. For human samples, data are expressed as the ratio of gene expression levels in the tumor to gene expression levels in the non-tumor counterpart.

Cell lines and transfection

Human embryonic kidney cells 293T were grown in DMEM with 10% penicillin-streptomycin (Invitrogen). After 24 hours in culture (80% confluence), 293T cells were cotransfected with 0.2 μg of the indicated reporter plasmid plus 0.5 μg of Δ89β-catenin expression vector, and/or 0.25 μg of Tcf4 expression vector and 20 ng of TK-Renilla reporter vector, using Lipofectamine 2000 (Invitrogen). Total amounts of plasmids were kept constant by adding the empty DNA vector when necessary. Reporter activity was determined by using the dual luciferase reporter assay system (Promega). Experiments were performed in duplicate and repeated at least three times.

Plasmid constructs

Cryptdin/defensin promoter constructs were synthesized from mouse and human genomic DNA by PCR and subcloned into the pGL3-basic luciferase reporter vector (Promega). The proximal mutated Tcf-binding sites at nucleotides –159 (C-binding site), –141 (B-binding site) and –130 (A-binding site) from AT to GC (HD6C and HD6ABC) were constructed from the HD6-200 plasmid using the QuikChange Site-directed mutagenesis kit (Stratagene).

Oligonucleotides

The sequences of the primers used in this study are available upon request.

Results

Epithelium-specific inactivation of *Apc* all along the crypt-villus axis

To study the initial stage of adenoma formation and to characterize the early molecular and cellular effects of Apc loss, we conditionally inactivated *Apc* all along the crypt-villus axis. We used a conditional *Apc* knockout model, $Apc^{lox/lox}$, in which exon 14 of *Apc* was flanked by *LoxP* sites (Fig. 1A), and transgenic mice that expressed the Cre recombinase conditionally and specifically in the intestinal epithelium all along the crypt-villus axis (Vil-$CreER^{T2}$) (Colnot et al., 2004a; Colnot et al., 2004b; el Marjou et al., 2004). The Vil-$CreER^{T2}$ transgene encodes a Cre recombinase that is active only after TAM injection. Multiple crossings generated $Apc^{lox/lox}$;Vil-$CreER^{T2}$ (mutant) and $Apc^{lox/+}$;Vil-$CreER^{T2}$ (control) mice. In the absence of TAM treatment, both mutant and control mice developed normally, showing no basal Cre recombinase activity (data not shown).

Two days after a single injection of TAM, we observed intense translocation of the Cre recombinase from the cytoplasm to the nucleus of epithelial cells all along the crypt-villus axis in both mutant and control mice (Fig. 1B). However, as previously described (el Marjou et al., 2004), the labeling was strongest in the villi, consistent with the gradient of the endogenous villin gene expression (Fig. 1B). RT-PCR detected the Cre-mediated *Apc* deletion in mutant mice two days after TAM treatment; this mutation was preponderant on day 4 (Fig. 1B). As expected, it was associated with loss of Apc protein all along the crypt-villus axis in the mutant mice, whereas Apc staining was normal in control mice (Fig. 1B). We checked that Apc deletion was restricted to the small intestine and colon of mutant mice (data not shown).

Loss of Apc along the crypt-villus axis of the small intestine disturbs only the crypt architecture

Four days after one injection of TAM, the mutant mice appeared unwell. Given the moribund phenotype of the mutant

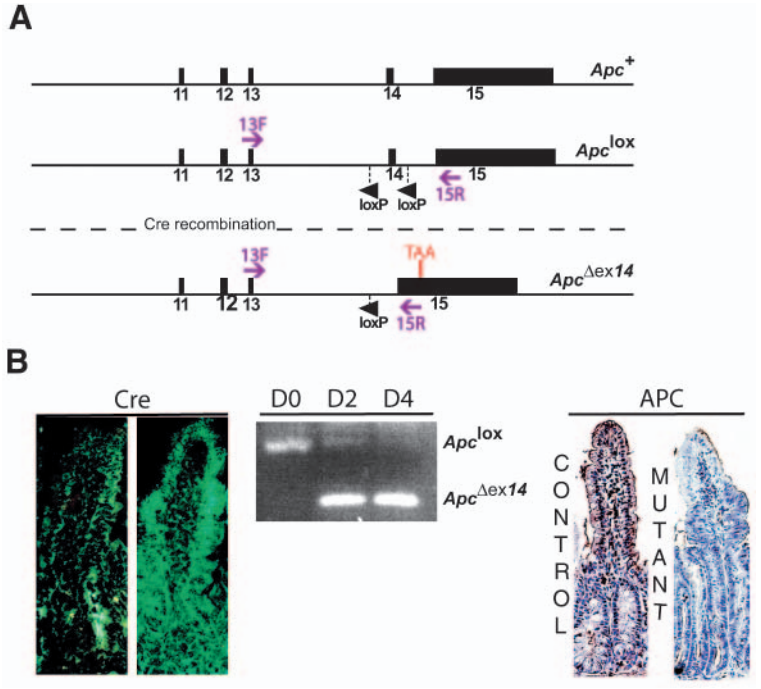

Fig. 1. TAM injection induces epithelium-specific inactivation of Apc all along the crypt-villus axis. (A) Structure of the conditionally targeted allele of Apc. Exons 11 to 15 are indicated as black bars. The positions of the primers used for the detection of each allele are indicated: 13F, 15R. In floxed mice, induction of Cre recombination by TAM injection induced exon 14 deletion, generating a frameshift mutation at codon 580 (appearance of the stop codon TAA). (B, left) Cre expression in the small intestine before (D0) and 2 days after (D2) TAM injection in mutant mice. (Middle) RT-PCR analyses on days 0 (D0), 2 (D2) and 4 (D4) in mutant mice reveal the floxed (Apc^{lox}) and deleted ($Apc^{\Delta ex14}$) *Apc* alleles. (Right) Immunostaining of APC on day 4 in control and mutant mice, showing loss of APC protein in the mutant mice.

mice, we killed them. The proximal distal extension of the small intestine of mutant mice appeared normal in length but was consistently wider than that of control mice. No apparent macroscopic phenotype was observed in the colon.

Histological studies revealed signs of dysplasia in the crypt compartment all along the small intestine of mutant mice (Fig. 2A). These signs included nuclear pseudostratification, enlargement of the nuclei, prominent nucleoli, and basophil accumulation. We observed numerous apoptotic bodies (Fig. 3A), and an impairment of differentiation associated with loss of mucosecretion (Fig. 2A). The crypt compartment of mutant mice was greatly expanded. Histological analysis revealed no effect of TAM injection in the control mice (Fig. 2A). The phenotype appeared less drastic in the colon (Fig. 2B). We observed some rare lesions in the proximal colon, which presented similar histological characteristics to the small intestinal dysplasia (Fig. 2B). The reason of the distinct severity of phenotype in mutant mice between the small intestine and the colon is unclear. It could be due to a longer cell cycle in the large intestine than in the small intestine (Potten and Hendry, 1995). However it is important to note that all mouse models with activated β-catenin signaling present less adenomatous polyps in the colon than in the small intestine (Harada et al., 1999; Yamada et al., 2002). As the colonic lesions appeared infrequent, we choose to focus our studies on the effect of Apc loss on the small intestine.

The dysplasia observed in mutant mice affected the entire small intestine (visible at low magnification, Fig. 2A). As Cre recombinase expression is restricted to the intestine (el Marjou et al., 2004), it is highly probable that the small intestinal phenotype explains the moribund status of the mutant mice.

We then analyzed the small intestine of control and mutant mice at various time points after a single TAM injection (from day 2 to day 4) (Fig. 2C). No histological changes were observed in the control intestinal epithelium at any stage. Although the mutant epithelium appeared grossly normal on day 2, an enlarged crypt compartment was visible on day 3 and was more pronounced on day 4. We examined the proliferative status of the small intestine epithelium by immunohistochemistry for the S-phase marker Ki-67 (Fig. 2C). In the control epithelium, proliferative cells were restricted to the crypt compartment. By contrast, in the mutant mucosa, the Ki-67-positive zone was greatly expanded between days 2 and 4, with Ki-67 staining present in all the cells of the abnormal crypts (at day 4, 28.6±1.7 Ki-67-positive cells per crypt section in the control; 94.2±7.6 Ki-67-positive cells per crypt section in the mutant; P=0.04 Mann Whitney). Ki-67 staining was restricted to the expanded proliferative compartment and was negative in the villi of mutant mice (Fig. 2C).

Only occasional apoptotic cells were found in control crypts. Hematoxylin and Eosin staining revealed a much higher number of apoptotic cells in the elongated crypts of the mutant mice. Caspase 3 immunostaining confirmed that apoptosis was enhanced in the mutant (3.3±0.8 caspase 3-positive cells per crypt section in the control, 11.2±0.8 caspase 3-positive cells per crypt section in the mutant; P=0.03 Mann Whitney) (Fig. 3A).

BrdU experiments were carried out to study the kinetics of epithelial cell migration within the gastrointestinal tracts of mutant and control mice. Mice were given a single BrdU injection 2 days after TAM injection and were killed 2 or 24 hours later. We evaluated the rate of cell migration by comparing the labeling of BrdU at 2 and 24 hours. At 24 hours, the epithelial cells from the mutant mice had clearly not migrated as far as those from the control mice (Fig. 3B). Thus, BrdU labeling showed that *Apc* loss slowed down the migration of epithelial cells along the crypt-villus axis in the mutant epithelium (Fig. 3B).

These findings show that inactivation of Apc is sufficient to alter the intestinal epithelium architecture dramatically within three days, especially in the crypt compartment, resulting in a large dysplasia-like zone. These morphological changes were associated with an increase in cell proliferation and cell death, and with impaired cell migration along the crypt-villus axis. By contrast, no proliferative phenotype was induced in the villi compartment, which did not reveal any morphological changes.

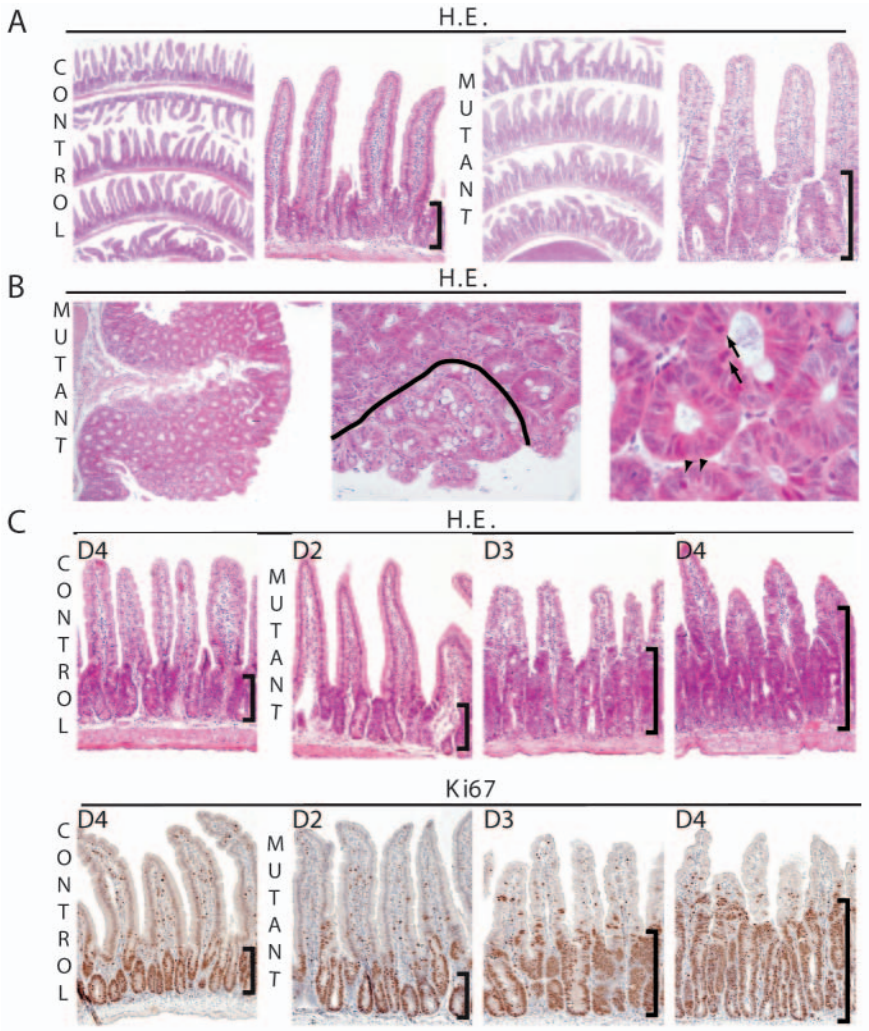

Fig. 2. Loss of Apc along the crypt-villus axis induces progressive crypt extension associated with intense proliferation. (A) Representative Hematoxylin and Eosin (H&E)-stained sections of jejunum from control (left) and mutant mice (right) at day 4, shown at low and high magnification. (B) Representative H&E-stained sections of proximal colon from mutant mice (day 4), shown at low, medium and high magnification. Line indicates the junction between dysplastic (top section) and normal areas (bottom section). Arrows and arrowheads indicate mitotic and apoptotic cells, respectively. (C) Representative H&E- (top) and Ki-67- (bottom) stained sections of jejunum from control (day 4, D4) and mutant (D2, D3, D4) mice. Abnormal crypt extension and similar Ki67 staining were observed all along the small intestine from the duodenum to the ileum in the mutant mice. Brackets indicate the dysplastic zone.

Loss of Apc induces distinct patterns of β-catenin signaling along the crypt-villus axis

All the cellular defects described above were restricted to the proliferative compartment; they did not appear to affect the differentiated villi. We thus analyzed the subcellular distribution of β-catenin as an indicator of activation of the β-catenin signaling pathway. As expected, cytoplasmic and nuclear β-catenin staining was observed in the aberrant highly proliferative area (Fig. 4A). However, β-catenin cell membrane staining was stronger in the upper part of the mutant villi than in the control, and precipitates accumulated in the cytosol at the basolateral membrane (Fig. 4A). To analyze whether aberrant activation of β-catenin signaling was readily induced all along the crypt-villus axis, we analyzed the expression of two well-established target genes of β-catenin/Tcf4 signaling: Myc (previously known as c-myc) and cyclin D1 (He et al., 1998; Tetsu and McCormick, 1999). As expected, the accumulation of β-catenin in the small intestine of the mutant mice was associated with the induction of Myc and cyclin D1, both at the RNA and protein levels (Fig. 4B). Interestingly, four days after TAM injection, Myc and cyclin D1 were not found in the same compartments along the crypt-villus axis in the small intestine of the mutant mice. Myc was localized mainly in the aberrant crypt compartment and in the bottom part of the villi all along the small intestine (Fig. 4C, parts a-c), whereas cyclin D1 was mainly present in the upper part of the villi, where β-catenin accumulated at the membrane (Fig. 4C, parts b-d; Fig. 4A). It is possible that β-catenin nuclear staining was not observed because this gene is transiently expressed or expressed at a level too low to be readily detected.

Thus, loss of Apc was associated with the aberrant activation of the Wnt pathway both in the proliferative compartment and in the differentiated villus compartment in the mutant mice. However the response to Wnt deregulation differed dramatically between the two compartments: Myc expression was induced in the proliferative compartment whereas cyclin D1 expression was induced in the villus compartment.

Effects of Apc loss on intestinal epithelial differentiation: commitment to Paneth cell lineage

We then investigated the consequences of Apc loss on the differentiation of all four epithelial lineages found in the small intestine: enterocytes, goblet cells, enteroendocrine cells and Paneth cells. We used the villin marker for the enterocyte lineage. In controls, villin was uniformly localized at the brush border of enterocytes all along the villus (Fig. 5A). In TAM-treated mutants, only the epithelial cells of the upper part of villi, corresponding to the non-proliferative area, expressed villin. Staining with UEA-I, a marker for goblet cells, showed that goblet cells were dramatically less numerous in the intestinal epithelium of the mutant mice than in that of the control mice. Similarly, chromogranin labeling, indicative of differentiation along the enteroendocrine lineage, was slightly weaker in the mutant mice (Fig. 5A). These results indicate that loss of Apc rapidly induces excessive proliferation, associated with a lack of differentiation to the three epithelial intestinal lineages, enterocytes, goblet and enteroendocrine. This result is in agreement with the role of the Wnt pathway in maintaining stem cell properties in the intestine (van de Wetering et al., 2002).

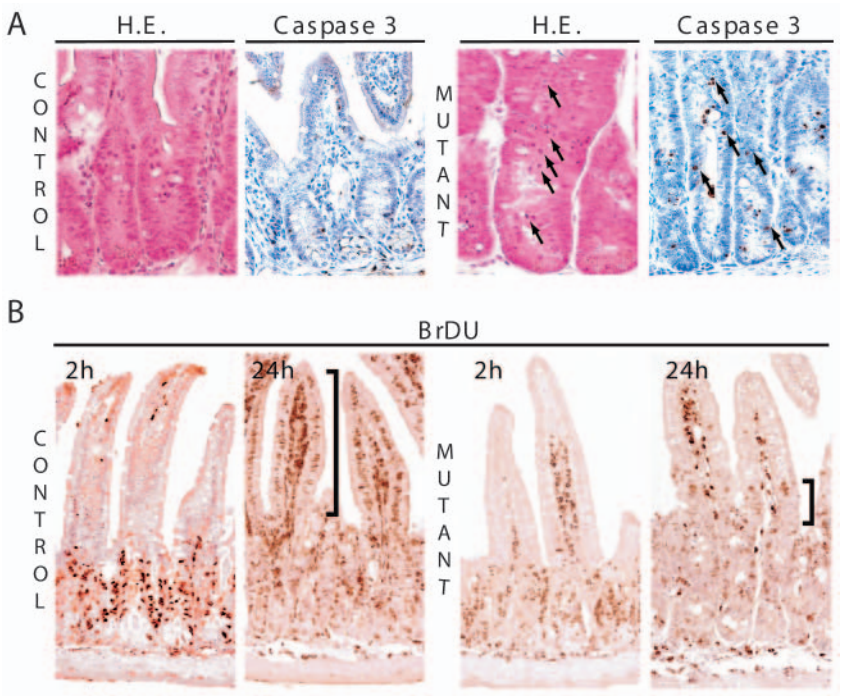

Fig. 3. Apc deficiency activates apoptosis in the crypts and slows down cell migration. (A) High magnification images of H&E-stained sections and sections immunostained for active caspase 3 from control and mutant mice (D4). Arrows indicate apoptotic cells. (B) BrdU staining at 2 and 24 hours after injection in control and mutant mice. Brackets indicate the position of the BrdU-stained cells that have migrated in the villi in 24 hours.

We suspected that the results for the fourth lineage, the Paneth cell lineage, would be different for several reasons. First, in controls, a large amount of β-catenin accumulates in the nuclei of Paneth cells and these cells are positive for Tcf4 (Fig. 5B, part f) (Sancho et al., 2003). Second, we recently constructed subtractive cDNA libraries of normal mucosa and colonic adenomas of $Apc^{+/-}$ mice (Colnot et al., 2004b). Although there are no Paneth cells in the mouse colon, we detected two Paneth cell markers in the colonic polyps: cryptdins and lysozyme. We identified several clones encoding Paneth-specific cryptdin and cryptdin-related genes: cryptdin 1, 5 and 6 (Defcr1, 5 and 6 – Mouse Genome Informatics), crs4c-2 (Defcr-rs7 – Mouse Genome Informatics) and crs1c (P.A. and B.R., unpublished). Cryptdins and lysozyme overexpression has been confirmed by in situ hybridization (Fig. 5B, parts c,d,h,i). These arguments prompted us to analyze the profiles of lysozyme and crytpdins in control and mutant mice following TAM injection. In control mice, Paneth cells were located exclusively in the small intestine at the base of the crypts, as shown by Hematoxylin and Eosin staining (Fig. 5B, part a). In situ hybridization revealed that both of these markers were expressed after TAM treatment in the mutant, essentially in the dysplasia-like zone (Fig. 5B, parts b,e,g,j). However, most of these cells did not present any typical granules according to Hematoxylin and Eosin staining (data not shown) and were negative for UEA-I (Fig. 5A), showing that they have not undergone full differentiation to the Paneth cell lineage.

Cryptdin/defensin family genes are direct targets of β-catenin

To analyze further this commitment to the Paneth cell lineage, we investigated whether the strong and rapid induction of

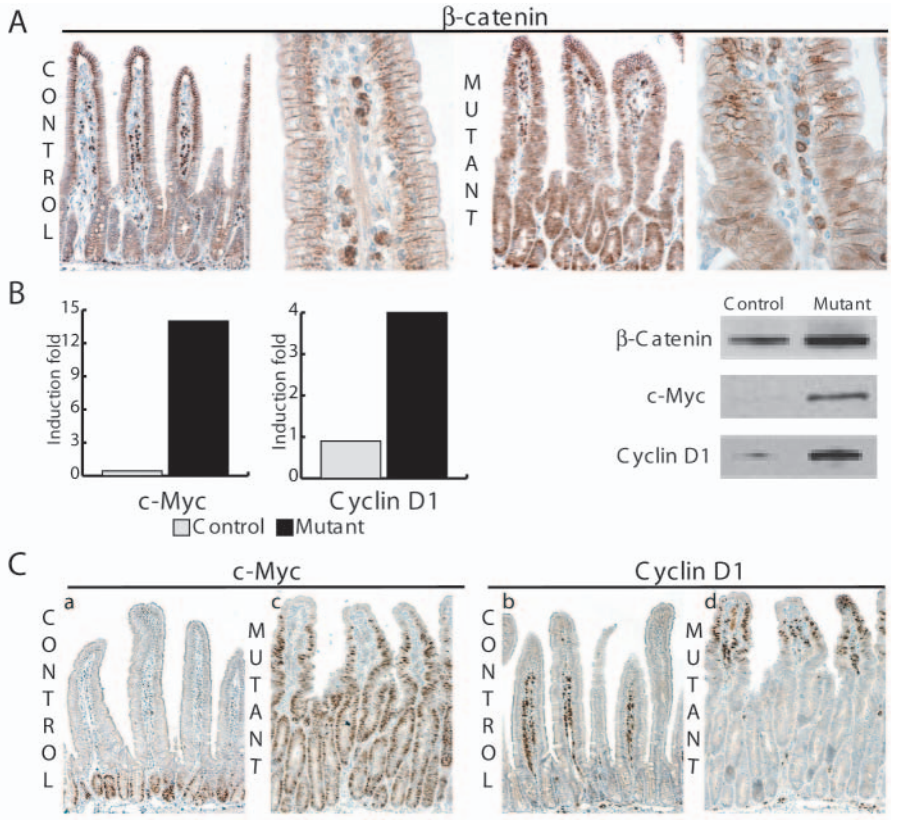

Fig. 4. Distinct profiles of β-catenin localization associated with distinct profiles of Myc and cyclin D1. (A) β-Catenin immunostaining of jejunum sections from control and mutant mice (D4), shown at low and high magnification. (B, left) Levels of Myc and cyclin D1 transcripts in the jejunums of control and mutant mice on day 4, as measured by RT-PCR. The level of each transcript is expressed relative to that in wild-type mice. (B, right) Western blot analysis of β-catenin, Myc and Cyclin D1 in the jejunums of control and mutant mice on day 4. (C) Myc (a,c) and Cyclin D1 (b,d) staining in sections of jejunum from control (a,b) and mutant mice (c,d) on day 4. Similar staining patterns were observed all along the small intestine.

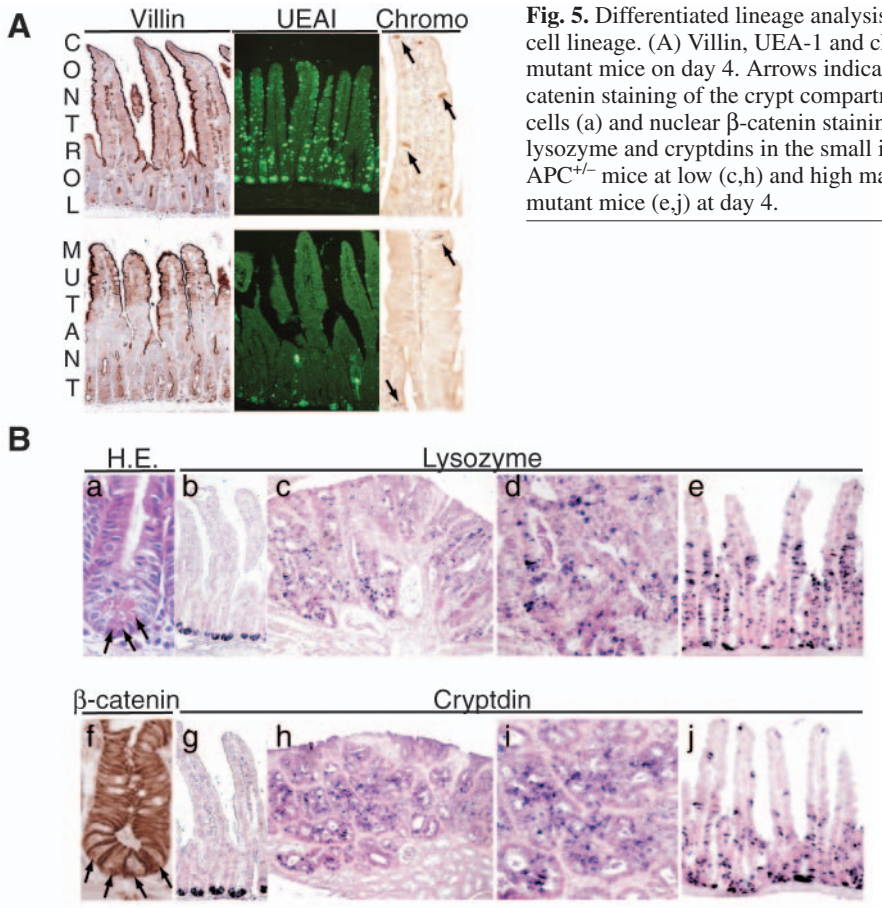

Fig. 5. Differentiated lineage analysis in intestinal mucosa: commitment to the Paneth cell lineage. (A) Villin, UEA-1 and chromogranin staining in sections from control and mutant mice on day 4. Arrows indicate chromogranin-positive cells. (B) H&E and β-catenin staining of the crypt compartment of control mice (a,f). Arrows indicate Paneth cells (a) and nuclear β-catenin staining in the Paneth cells (f). In situ hybridization of lysozyme and cryptdins in the small intestine of control mice (b,g), in colon polyps from $APC^{+/-}$ mice at low (c,h) and high magnification (d,i), and in the small intestine of mutant mice (e,j) at day 4.

lysozyme and crytpdins could be controlled by β-catenin signaling. In contrast with lysozyme, a GenBank search for regulatory elements revealed multiple consensus β-catenin/Tcf-binding sites in the promoters of several cryptdin genes, all of which are located close to the TATA box (Fig. 6A). The probability of uncovering a Tcf-binding consensus sequence (T/A T/A CAA T/A G) by chance is one per 2048 nucleotides. We found at least one Tcf motif in the first 300 bp of the promoter regions of several cryptdin genes. In addition, both human cryptdin genes, α defensin 5 and 6 (HD5, HD6), contain at least three Tcf-binding sites in the first 300 nucleotides that includes the promoter. This situation is reminiscent of the transcriptional control of a group of hair-specific keratin genes. Indeed, all these genes have a Lef/Tcf1 consensus motif positioned close to the promoter (Zhou et al., 1995). This was the first clue suggesting the involvement of the β-catenin signaling in hair follicle lineage differentiation.

We then performed luciferase reporter assays in 293T cells with different promoters of the cryptdin/defensin family. We transiently transfected 293T cells with different constructs containing different lengths of regulatory sequences of the cryptdin or defensin genes: cryptdin 5-2500, HD5-1000 and HD6-2500. Co-transfection with activated β-catenin and Tcf4 plasmids increased the basal activity of these promoters (Fig. 6B). We choose to focus on HD6. To localize the promoter region responsive to β-catenin/Tcf4, we tested various lengths of HD6 regulatory sequence: HD6-1000 and HD6-200. In these cells, co-expression of activated β-catenin and Tcf4 resulted in a 2.8-fold increase in luciferase activity with the HD6-1000 construct, and in a 6.7-fold increase with the HD6-200 construct (Fig. 6C). We next tested the functional significance of the potential Tcf4-binding sites in the smallest HD6 construct (HD6-200) by suppressing one or all three Tcf4-binding sites (Fig. 6C). The luciferase activity of the wild-type sequence was affected by altering one binding site and was strongly suppressed by mutating all three binding sites. Hence, cryptdin/defensin genes are regulated by a β-catenin-Tcf4-dependent pathway.

Our data indicate that Apc is a crucial determinant of intestinal epithelium cell fate. Activation of the β-catenin signaling pathway following Apc loss alters the terminal differentiation of the enterocyte, goblet and enteroendocrine lineages, and promotes differentiation along the Paneth cell lineage. This de novo differentiation is partly mediated by the transcriptional control of specific markers of Paneth cells: the cryptdin/defensin genes by β-catenin/Tcf signaling.

HD6 is induced in human colon cancers

The demonstration that the cryptdin/defensin genes are the direct targets of β-catenin and are overexpressed in colon tumors of $Apc^{+/-}$ mice led us to investigate whether these genes are also induced in human colon cancers. We thus analyzed the levels of defensins HD5 and HD6 (Fig. 7). First, we compared Myc and cyclin D1 levels in 10 tumor samples and in patient-matched normal tissues (Fig. 7). Real-time RT-PCR revealed that Myc and cyclin D1 mRNA levels were elevated in nine out of ten tumors. This is probably due to enhanced β-catenin-mediated transcription. We found elevated HD6 mRNA levels in all nine of the tumors in which Myc and cyclin D1 had accumulated. However, virtually no HD5 mRNA was detectable in any of the samples. Given that the regulatory sequences of these two genes do not share a high degree of homology (except Tcf-binding sites), we hypothesise that transcriptional regulations of HD5 and HD6 are different. HD5 expression may be activated by multiple pathways. These results suggest that HD6 expression and β-catenin activation are related events in human colon cancer, and that HD6 could be used as a new molecular marker of human colon tumors.

Discussion

Loss of Apc has been described as being one of the major events that initiates colorectal cancer, and it appears to be associated with a multitude of changes in cell signaling

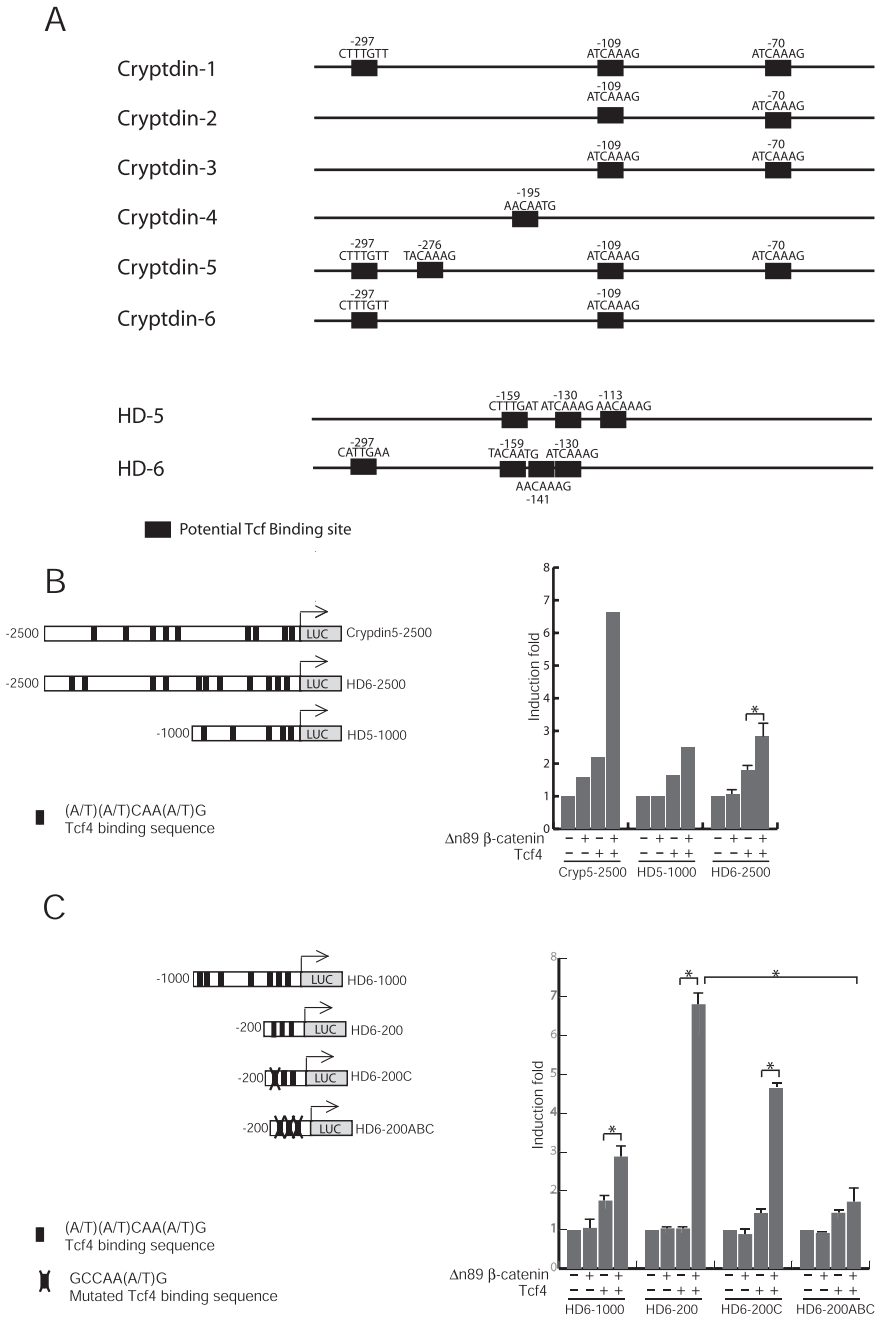

Fig. 6. Cryptdin/defensin family genes are direct targets of β-catenin signaling. (A) Consensus Tcf-binding sites (black boxes) are present in 300 bp regions of promoter sequences of distinct cryptdin and defensin genes. (B, left) Schematic representation of cryptdin/defensin promoter constructs showing the putative Tcf-binding sites (black boxes). (B, right) Responses of various promoter fragments (cryptdin5-2500, HD5-1000 and HD6-2500) to activated β-catenin and Tcf4 in 293T cells (**P*<0.05). (C) HD6 promoter constructs of various lengths showing the putative Tcf-binding sites (left), mutated or not, and their response to β-catenin/Tcf4 (right, **P*<0.05).

molecules that are responsible for tumor progression (Fodde et al., 2001). It is important to determine the biological relevance of the changes observed during tumor progression; for example, are they causal or just associated events? To identify the first events responsible for the initiation process, we created a mouse model in which the *Apc* gene was conditionally inactivated. We found that loss of Apc in intestinal epithelial cells immediately led to hyperproliferation associated with induction of apoptosis and slowed cellular migration. The rapid appearance of the phenotype and the complete penetrance observed in all the crypt units suggest that loss of Apc is sufficient to lead to these drastic cellular alterations that are the properties of premalignant cells. These results confirmed that *Apc* is a crucial gatekeeper gene of the intestinal epithelium in vivo (Kinzler and Vogelstein, 1996).

Similar results were recently obtained by Sansom et al., using a conditional deletion of Apc by expression of an inducible Cre recombinase dependent on β-naphthoflavone treatments (Sansom et al., 2004). However in Sansom's study, the Apc deletion was restricted to the proliferative compartment of the small intestine, which meant it was not possible to analyze the consequence of Wnt deregulation in differentiated villus cells. Our experiments show that the crypt and the villus react differently to β-catenin signaling. In the crypt compartment, which contains the progenitor stem cells and the cellular amplification pools, the immediate consequences of Apc loss were severe. Within three days, we detected an expanded compartment with dysplastic-like cells. Myc expression was strongly induced in this compartment, confirming the crucial in vivo role of Myc in the maintenance of the crypt progenitor phenotype (van de Wetering et al., 2002). By contrast, the villus cells appeared to be resistant to morphological alterations. At least in the first four days after Apc loss, the villus cells presented no proliferation or morphological changes, although activation of the β-catenin signaling pathway was supported by the induction of cyclin D1. However, this was not sufficient to allow the differentiated epithelial cells of the villus to re-enter the cell cycle, as Ki67 stained very few cells in this compartment. Accordingly, in human colorectal cancer, cyclin D1 is predominantly expressed in non-proliferative cells at the invasion front (Jung et al., 2001). Thus, our results support the bottom-up proposal based on adenomatous crypts from FAP patients: tumorigenesis takes place among the stem cell population in the crypt base and the transformed stem cells subsequently expand (Preston et al., 2003). The local microenvironment or niche may be critical for inducing proliferation in response to activation of β-catenin signaling.

The distinct biological responses of the proliferative and the

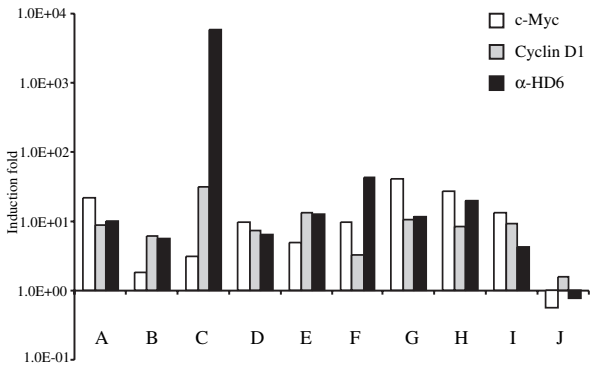

Fig. 7. Induction of HD6 in human cancers. Levels of Myc, cyclin D1 and HD6 transcripts in 10 human colon cancers (A-J) and matched normal mucosa samples, as measured by quantitative RT-PCR. Results are expressed as a ratio of the levels in tumors to in their normal counterparts.

differentiated compartments imply that β-catenin signaling induces different target genes. Indeed, we observed different expression patterns for two canonical target genes of β-catenin. Several hypotheses, which possibly overlap, can be proposed to explain these molecular differences: the level of β-catenin signaling and the cell context (i.e. the cell type and the cell environment). The heterogeneity of target gene transcription may be the result of distinct critical thresholds of β-catenin signaling. Several studies have indicated that a minimum amount of β-catenin is necessary for the transcriptional activation of target genes (Kielman et al., 2002; Muller et al., 2002). Using distinct ES-cell lines harboring different APC mutations, Kielman et al. showed that the differentiation capacity of ES cells depends on the amount of active nuclear β-catenin (Kielman et al., 2002). Alternatively, complex regulation could be due to crosstalk with other signaling pathways resulting from the dialogue between epithelial cells and their environment (Israsena et al., 2004; Muller et al., 2002). Our results demonstrate that the effects of β-catenin signaling on gene expression have context-specific outcomes in terms of cell behavior.

Another important point highlighted by our results concerns the role of Apc in cell fate in the intestinal epithelium. We were not surprised that enhanced β-catenin/Tcf4 activity, known to impose a crypt progenitor phenotype, prevented the terminal differentiation for three epithelial intestinal lineages: enterocytes, goblet cells and enteroendocrine cells. However, our results provide strong evidence that Apc promotes, rather than prevents, the differentiation of the Paneth cell lineage. This was shown by the strong induction of Paneth cell markers both in the aberrant crypt areas and in the colonic tumors of $Apc^{+/-}$ mice. In addition, we demonstrated that the cryptdin/defensin genes are targets of β-catenin signaling. Previous studies demonstrated that activation of β-catenin-Tcf signaling is implicated in the proper allocation of Paneth cells via the regulation of expression of Eph ligand and receptors (Batlle et al., 2002). Our studies show that it might also play a role in commitment to the Paneth cell lineage. Thus, β-catenin signaling might control this lineage at two levels: cell fate determination and the spatial localization in the intestine. To date, three specific Paneth cell markers have been described as target genes of β-catenin signaling: MMP7, Eph and cryptdin genes. Interestingly, Wnt signaling has been shown to regulate positively the defensin/cryptdin genes in two other systems: ES cell lines with mutations in the *Apc* gene express different Paneth cell markers, such as cryptdin (Kielman et al., 2002), and activation of the β-catenin signaling pathway in lung progenitors can induce a switch in lineage commitment and, in particular, is responsible for the expression of Paneth cell markers, MMP7 and cryptdins (Okubo and Hogan, 2004). Our results and these studies suggest that Wnt signaling can induce differentiation towards the Paneth lineage.

Although β-catenin signaling induces different Paneth cell markers, it does not appear to be sufficient for the complete differentiation of Paneth cells, as shown by the presence of typical secretory granules (Batlle et al., 2002; Crawford et al., 1999). We thus propose that β-catenin activation leads to commitment to the Paneth cell lineage. This is consistent with the results of a previous study showing that, during mouse development, the differentiation of the Paneth cell lineage involves the sequential expression of specific markers (Bry et al., 1994). Cryptdin-positive cells first appear in the intervillus epithelium of the small intestine at E15. Cells with the morphological appearance of Paneth cells only appear on P7. Our model makes it possible to mimic the first step of Paneth cell lineage differentiation.

It is possible that immature Paneth cells are a molecular target that may allow the early detection of colon cancer. Accordingly, our experiments with human cancer samples showed that HD6 might be such a molecular marker. Despite extensive examinations, the biological role of Paneth cells has not been clearly defined. Many studies have suggested that Paneth cells play a role in intestinal host defense through their production of antimicrobial factors such as cryptdin/defensin (Porter et al., 2002). There is also weaker evidence suggesting that Paneth cells participate in stem cell protection and crypt formation. The expression patterns of Paneth cell products support the notion that the emergence of this cell lineage in the small intestine coincides with crypt morphogenesis (Bry et al., 1994). In our study, we observed an association between increased proliferation in the crypts and the accumulation of 'Paneth-like cells' following *Apc* gene ablation, which reinforces this hypothesis.

Our study confirms that Apc is a crucial gatekeeper in intestinal homeostasis in vivo. Loss of function of the gene encoding this tumor suppressor rapidly disrupts the balance between cell proliferation, death and differentiation, and impairs cell migration. These events are believed to be the hallmarks of the initiation of intestinal tumorigenesis. Activation of the β-catenin signaling pathway also promotes commitment to the Paneth cell lineage.

We thank B. Terris for pathological studies, E. Bloch-Gallego and M. Hidalgo-Sanchez for advice with in situ hybridization, and P. Polakis for generously providing the N89β-catenin-pCAN and Tcf4 expression vectors. We thank T. Decaens and P. Billuart for helpful discussions. This work was supported by INSERM, Aventis, the Ligue Nationale contre le Cancer and the Association pour la Recherche contre le Cancer.

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