The Related Retinoblastoma (pRb) and p130 Proteins Cooperate to Regulate Homeostasis in the Intestinal Epithelium

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pRb, p107, and p130 are related proteins that play a central role in the regulation of cell cycle progression and terminal differentiation in mammalian cells. Nevertheless, it is still largely unclear how these proteins achieve this regulation in vivo. The intestinal epithelium is an ideal in vivo system in which to study the molecular pathways that regulate proliferation and differentiation because it exists in a constant state of development throughout an animal’s lifetime. We studied the phenotypic effects on the intestinal epithelium of mutating Rb and p107 or p130. Although mutating these genes singly had little or no effect, loss of pRb and p107 or p130 together produced chronic hyperplasia and dysplasia of the small intestinal and colonic epithelium. In Rb/p130 double mutants this hyperplasia was associated with defects in terminal differentiation of specific cell types and was dependent on the increased proliferation seen in the epithelium of mutant animals. At the molecular level, dysregulation of the Rb pathway led to an increase in the expression of the epithelium throughout an organism’s lifetime. The absence of Cdx1 function in Rb/p130 double mutant mice partially reverted the histologic phenotype by suppressing ectopic mitosis in the epithelium. These studies implicate the Rb pathway as a regulator of epithelial homeostasis in the intestine.

Normal adult tissues maintain a constant cell number by regulating the relative amounts of proliferation and apoptosis. The intestinal epithelium is a highly proliferative tissue; the human small intestinal epithelium undergoes 10^11 mitoses per day (1). In this tissue homeostasis is preserved through strict regulation of proliferation, differentiation, migration, and exfoliation. The position of a cell within the crypt/villus axis directly reflects its cell cycle and differentiation states. Cell proliferation normally occurs in the undifferentiated cells near the base of the crypts of Lieberkühn. The progeny of these undifferentiated cells migrate out of the crypt and into the villus and in the process exit the cell cycle and become terminally differentiated. Differentiation of the intestinal epithelium is controlled by several transcription factors including hairy and enhancer of split 1 (Hes1), atonal 1 (Atoh1/Math1), and E74-like factor 3 (Eif3/Ese1) (2). The spatial orientation of cells within the crypt/villus axis and the high rate of cell turnover within the tissue provide a unique opportunity to study the development of the intestinal epithelium throughout an organism’s lifetime.

pRb, p107, and p130 comprise a family of cell cycle regulators known as the “pocket proteins.” The predominant function of the pocket proteins is to control the G1/S transition through negative regulation of the E2F family of transcription factors (3). In addition, this protein family plays an important role in regulating other cellular processes, such as terminal differentiation and senescence (4). The regulation of differentiation by pRb appears to occur independently of E2F’s (5); instead, pRb promotes differentiation through interaction with tissue-specific transcription factors. For example, pRb interacts with CCAAT enhancer-binding protein α to promote adipocyte differentiation and core binding factor α1 to promote osteoblast differentiation (6, 7). The pRb-related proteins p107 and p130 also appear to play a role in differentiation. Mice mutant for p107 and p130 have cartilage and bone differentiation defects and fail to develop terminally differentiated keratinocytes (8, 9). In addition, the pocket proteins directly interact with histone deacetylases and regulate their ability to alter chromatin structure around specific promoters (10, 11). In adipocytes a complex consisting of pRb and histone deacetylase 3 prevents differentiation by inhibiting the expression of peroxisome proliferator-activated receptor γ (12).

Given the importance of the pocket proteins in regulating cell cycle progression and differentiation, it is not surprising that mutations in the RB gene are frequent in many human tumor types (3). RB mutations are common in tumors such as retinoblastoma, osteosarcoma, and small cell lung cancer. In contrast to RB, it is unclear whether p107 or p130 act as tumor suppressor genes. Although P130 expression is lost in a subset of ovarian carcinomas and non-small cell lung cancers, a causal relationship between P130 mutations and these types of cancer has not been established (13, 14).

Studies of mice with targeted alleles of the genes encoding Rb, p107, and p130 have uncovered functional relationships between the pocket proteins. Although p107+/− and p130−/− mice are viable and fertile, mutation of p107 and p130 in combination is embryonic lethal (8). In addition to displaying functional redundancy, the pocket proteins exhibit striking functional compensation. For example, in the absence of p130, p107 expression is up-regulated in peripheral T lymphocytes, and this up-regulation results in increased association of p107 with E2F4 (15). Clearly, the pocket proteins function in a complex, multi-tiered molecular pathway to protect cells from inappropriate cell cycle progression.

The functions of pRb, p107, and p130 in the intestinal epithelium remain largely uncharacterized. However, studies of the laboratory mouse have provided some insight into the function of the pocket proteins in this tissue. Enterocytes expressing SV40 large T antigen, which...
bonds to and inactivates all of the pocket proteins, reenter the cell cycle and display an increase in p53-independent apoptosis (16, 17). Interestingly, the expression of large T antigen did not appear to induce the de-differentiation of enterocytes (16). Nevertheless, these studies were limited to assessing the function of the pocket proteins in differentiated enterocytes and not any of the other cell types in the epithelium, thereby preventing a broad study of cell cycle exit and differentiation. In addition, large T antigen has multiple cellular targets and may not fully inactivate the three pocket proteins. Additional studies are required to appreciate the function of the pocket proteins in the intestinal epithelium.

Homeostasis in the intestinal epithelium is achieved in part through regulation of proliferation and differentiation, making it an ideal system for studying the molecular pathways that control these processes in vivo. By using mice with conditional and germ-line null mutations in the pocket protein genes, we have evaluated their role in regulating homeostasis in this tissue. We found that mutation of the pocket protein genes alters the development of the small intestinal and colonic epithelium. This study implicates the pocket proteins as regulators of both proliferation and differentiation, making it an ideal system to appreciate the function of the pocket proteins in the intestinal epithelium.

MATERIALS AND METHODS

Mouse Strains—Cdx1 knock-out mice (18) were the generous gift of Dr. Jacqueline Deschamps (Hubrecht Laboratory, The Netherlands). The Fabp4fl/fl-Cre transgenic mouse strain (19) was obtained from the Mouse Models of Human Cancer Consortium Repository. Rbfl/fl and p130 mutant mice have been described previously (8, 20). For each animal, the entire intestinal tract was removed, flushed with 1× PBS, and fixed overnight in 10% neutral-buffered formalin. Tissue for histologic analysis was removed from the identical location in all animals. For colonic sections, tissue was taken from the medial colon, ~4 cm proximal to the rectum. For small intestinal sections, tissue was taken within 1 cm of the ileo-cecal junction. In our experience with Fabp4-Cre mice, nearly 100% of the crypts in this region of the small intestine are Cre-positive. For cell cycle analysis, animals were injected with 30 mg/kg 5-bromo-2-deoxyuridine (BrduRd) 1 h before sacrifice.

Immunohistochemistry—Fluorescence immunohistochemistry was performed using a modified citric acid unmasking protocol. Briefly, slides were deparaffinized in xylene and rehydrated through ethanol. Slides were microwaved for 25 min in citrate buffer, pH 6.0, and then cooled in running tap water. After incubation in 1× PBS plus 0.1% Tween 20 for 10 min, slides were blocked in 10% native donkey serum for 1 h at room temperature. Primary antibody (diluted 1:100 in 1× PBS plus 0.1% Tween 20) was added, and slides were incubated overnight at 4 °C. The following day, slides were washed three times in 1× PBS plus 0.1% Tween 20. Secondary antibody (diluted 1:200 in 1× PBS plus 0.1% Tween 20) was added, and slides were incubated for 1 h at 37 °C. Slides were again washed, counterstained with propidium iodide or 4′,6-diamidino-2-phenylindole, and mounted in 1× PBS.

For BrduRd immunohistochemistry, samples were deparaffinized and microwaved in citrate followed by standard detection with 3,3′-diaminobenzidine using a kit from Vector Laboratories (Burlingame, CA). Samples stained for BrduRd were counterstained with hematoxylin.

Primary antibodies used were as follows: rabbit α-Rb C15 (Santa Cruz Biotechnology), rabbit α-p107 C18 (Santa Cruz Biotechnology), rabbit α-p130 C20 (Santa Cruz Biotechnology), goat α-1-FABP N20 (Santa Cruz Biotechnology), rabbit α-lysozyme (ICN), rabbit α-chromogranin A (Abcam), rabbit α-Ki67 (Novocastra), goat α-Mcm6 C20 (Santa Cruz Biotechnology), rabbit α-phospho-histone H3 (Cell Signaling Technology), rabbit α-cleaved caspase 3 (Cell Signaling Technology), and mouse α-BrduRd (BD Biosciences).

Specificity of Rb, p107, p130, and Cdx1 antibodies was confirmed by hybridizing to tissue sections from intestine lacking the relevant protein (data not shown). In all cases each antibody detected specific nuclear staining. In some instances, nonspecific background staining was detected in the cytoplasm. Secondary antibodies for immunofluorescence were raised in donkey and labeled either with Alexa Fluor 488 or Alexa Fluor 594.

Western Blotting—Western blots were performed on purified intestinal epithelium. The purification was performed with a protocol modified from Whitehead et al. (21). Briefly, intestines were removed, flushed with ice-cold 1× PBS, and opened lengthwise. Tissues were then incubated in 3 mm EDTA, 50 μm dithiothreitol for 1 h on ice at 4 °C. Tissues were gently washed in ice-cold 1× PBS then transferred to new 1× PBS. Intestinal epithelium was dislodged by vigorous shaking. Epithelial cells were isolated by centrifugation at 1500 rpm for 5 min. After decanting of supernatant, cells were resuspended in Nonidet P-40 buffer (100 mm NaCl, 100 mm Tris-HCl, pH 8.0, 1% Nonidet P-40) and incubated on ice for 10 min. Samples were then centrifuged to remove insoluble matter and quickly frozen in liquid nitrogen.

Polyacrylamide gels (8 or 12%) were loaded with 25 or 40 μg of purified epithelial protein. Western blots were detected with ECL Plus (Amersham Biosciences). Primary antibodies were as follows: mouse α-Rb (BD Pharmingen) at 1:400 dilution, rabbit α-p107 C18 (Santa Cruz Biotechnology) at 1:1000 dilution, rabbit α-p130 C20 (Santa Cruz Biotechnology) at 1:1000 dilution, mouse α-actin C4 (Abcam) at 1:3000 dilution, rabbit α-Cdx1 CPSP (22) at 1:4000 dilution, and mouse α-Cdx2 (BioGenex) at 1:1000 dilution.

RNA Isolation and Taqman Analysis—Before RNA isolation, intestinal epithelium was purified using the modified EDTA protocol (see “Western blotting” above). RNA was isolated using Trizol reagent according to the manufacturer’s instructions (Invitrogen). CDNA was generated from 1 μg of total epithelial RNA using Superscript III reverse transcriptase (Invitrogen). Quantitation of p107 and Cdx1 expression was performed using a Taqman gene expression assay (Applied Biosystems). Quantitative PCR reactions were run and scored on an ABI Prism 7000 Sequence detection system.

For each genotypic class, RNA from at least 2 animals was analyzed. All reactions were done in quadruplicate. To normalize p107 and Cdx1 expression levels between samples, its expression relative to TATA binding protein (Tbp) was assessed. For the control group, Rbfl/flC2C6, the average expression level relative to Tbp was determined. Then, for each of the experimental groups, each single reaction was compared with the average of the control to generate a mean relative expression level for that group. Statistical analysis was performed using the Mstast computer program (mcardle.oncology.wisc.edu/mstast).

Quantitative Analysis of Tissue Kinetics—The heights of the Ki67- and BrduRd-positive zones were determined by counting the number of cell positions from the bottom of the crypt that the highest-staining cell occupied. For phospho-histone H3 staining, entopic cell positions were defined (based on staining in wild-type colon) as the bottom 10 cell positions of the crypt. Ectopic cell positions were defined as any positive staining above the tenth position. For each assay, at least 50 crypts, originating from tissue sections of 2–3 different mice, were scored for each genotype. Only crypts for which a good longitudinal section was
RESULTS

Little is known about the role of the pocket proteins in regulating homeostasis in the intestinal epithelium. To understand the function of the pocket proteins in this context, we first analyzed the pattern of expression within the crypts and villi of pRb, p107, and p130. In the colon, nuclear pRb was found in all epithelial cells, with higher expression in cells toward the bottom of the crypt (Fig. 1A). In contrast to the ubiquitous expression of pRb, p107 was expressed predominantly in the lower half of the crypt, whereas p130 is expressed in the upper portion of the crypt and the epithelium lining the lumen (Fig. 1, B and C). The expression patterns of pRb, p107, and p130 were similar in the small intestinal epithelium. The undifferentiated cells in the small intestinal crypt expressed pRb and p107 (Fig. 1, D and E), whereas the differentiated cells in the villi expressed Rb and p130 (Fig. 1, D and F).

Although immunohistochemistry allowed us to assess the localization of pocket proteins within the epithelium, it did not give us an indication of the relative expression levels of these proteins. We analyzed by Western blot the expression of pRb, p107, and p130 in purified intestinal epithelium to determine whether/how functional compensation occurs in this tissue. Because loss of Rb function is lethal during embryogenesis (23–25), we utilized a conditional allele of Rb (Rb2lox) (20) and a transgenic mouse strain that expresses Cre recombinase in the intestinal epithelium (Fabpl-Cre) (19). In this strain of mice, Cre is expressed mosaically within the intestinal epithelium, with little or no expression in the proximal small intestine and relatively high expression in the distal small intestine and colon. It is this mosaic Cre expression that may account for the ability to detect pRb, albeit at reduced levels, in “Rb-mutant” intestinal epithelium (Fig. 1G).

We found that pRb and p130 levels remained constant, even when one or another pocket protein was mutated (Fig. 1G). By contrast, the levels of p107 increased dramatically upon mutation of Rb or p130 (Fig. 1G). In Rb/p130 double mutant intestine, the levels of p107 were even higher (Fig. 1G). This increase p107 expression was evident not only at the protein level but also in RNA isolated from mutant intestinal epithelium (Fig. 1H). These data indicate that in the intestinal epithelium, loss of Rb and/or p130 function is associated with compensatory up-regulation of p107 and that this compensation most likely occurs at the level of p107 transcription.

We went on to examine the intestinal phenotype of mice with muta-
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In mice singly mutant for p107 or p130 was not markedly different from wild type at the histologic level. In the small intestinal epithelium there was no apparent phenotype due to mutation of p107 or p130 (Fig. 2, F and I). The colonic epithelium from p107- and p130-mutant mice displayed focal hyperplasias that manifested as a minor increase in the depth of otherwise normal crypts (Fig. 2, E and I). Nevertheless, these hyperplasias were extremely mild and did not progress to neoplasia, even in animals greater than 18 months of age.

Mice double mutant for Rb and p107 or p130 developed significant hyperplasia in the small intestine and colon. This hyperplasia was present throughout the regions of the intestinal epithelium in which Cre was expressed and did not develop as focal lesions. In the small intestine the dysplasia was associated with severe dysmorphogenesis of the crypt/villus axis (Fig. 2, H and L). In the colon the crypts were elongated, and the surface epithelium often displayed a serrated border. In both the small intestine and colon, the orientation of the crypts relative to the muscularis was skewed such that it was often difficult to obtain perfectly longitudinal sections (see Fig. 2L).

Age of onset was extremely variable in both classes of double mutant animals. This is probably due to the mixed genetic background of the animals. In general, the hyperplasia was detectable by 6 months of age. Interestingly, the hyperplasia did not appear to significantly affect the health of the animals, which routinely lived 18–24 months. In no cases did the hyperplasia progress to neoplasia.

The presence of chronic hyperplasia in Rb/p130 double mutant mice indicates that these pocket proteins play an important role in regulating development and homeostasis of the intestinal epithelium. This observation led us to analyze the cellular and molecular bases for the phenotype of the mutant animals. We chose to focus our efforts on characterizing the phenotype of Rb/p130 double mutant animals because their phenotype was significantly more robust than that of Rb/p107 mutant animals.

Mutations of the pocket proteins have been found to affect the proliferative and apoptotic indices of many different tissues, for example in the retina (26). We used immunohistochemistry to analyze cell cycle kinetics in the intestinal epithelium of our mutant mice. Ki67 is expressed by cells that remain active in the cell cycle. In wild-type mice, Ki67-positive cells were restricted to the bottom 7 cell positions in the colonic crypt (Fig. 3A). The Ki67-positive zone was expanded to 11 cell positions in Rb and p130 mutant colon and 18 cell positions in double mutant colon (Fig. 3A). Thus, as reported for a variety of other cell types, mutation of the Rb pathway caused a delay in the ability of colonic epithelial cells to exit the cell cycle and enter Go.

Loss of Rb function enhances apoptosis in a cell autonomous manner in the peripheral nervous system, lens, and retina (26, 27). Furthermore, inactivation of the entire Rb family by expression of large T antigen in the intestinal epithelium leads to an increase in apoptosis (17). Surprisingly, we found no difference in the apoptotic indices between wild-type, Rb mutant, p130 mutant, or double-mutant colons by staining for activated caspase 3 (CC3) (data not shown). It appears, therefore, that the induction of apoptosis in response to pRb loss is a tissue-specific phenomenon.

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![Diagram of intestinal epithelium](image)

**FIGURE 2. Histology of wild-type and mutant epithelia.** Genotypes are as follows: wild-type (A and B), Fabpl-Cre;Rb<sup>2lox/2lox</sup> (C and D), Rb<sup>2lox/2lox</sup>p107<sup>−/−</sup> (E and F), Fabpl-Cre;Rb<sup>2lox/2lox</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (G and H), Rb<sup>2lox/2lox</sup>p130<sup>−/−</sup> (I and J), Fabpl-Cre; Rb<sup>2lox/2lox</sup>;p130<sup>−/−</sup> (K and L). The histology of Rb-mutant colon (denoted Rb<sup>−/−</sup>) and small intestine (C and D) is indistinguishable from wild type (A and B). This result was confirmed in Rb<sup>−/−</sup>; Rosa26/+ ↔ wild-type chimeric mice (Panel C, inset). p107- and p130-mutant small intestines (F and J) are also identical to wild type, whereas the colons shows regions of focal hyperplasia (E and H), both the colons (G and K) and small intestines (H and L) of Rb/p107 and Rb/p130 double-mutant animals are hyperplastic. The small intestine also shows a significant dysmorphogenesis of the crypt/villus axis. H and L, the phenotype of Rb/p130 mutant animals is somewhat more severe than Rb/p107 mutant animals. Scale bar, 50 μm.

In mice in which Rb was mutated or ablated in the intestinal epithelium, we found that the histologically normal crypts were indeed deficient for pRb (data not shown). We also confirmed that loss of Rb function produces histologically normal crypts by injecting Rb<sup>−/−</sup>; Rosa26/+ embryonic stem cells into wild-type blastocysts to generate chimeric mice (Fig. 2C, inset). In these chimeric animals the lacZ+, Rb-mutant crypts are histologically normal. The failure of Rb-mutant intestinal epithelium to exhibit even a subtle histological phenotype may be due to functional overlap with p107 and/or p130.

The intestinal epithelium in mice singly mutant for p107 or p130 was not markedly different from wild type at the histologic level. In the small intestinal epithelium there was no apparent phenotype due to mutation of p107 or p130 (Fig. 2, F and I). The colonic epithelium from p107- and p130-mutant mice displayed focal hyperplasias that manifested as a minor increase in the depth of otherwise normal crypts (Fig. 2, E and I). Nevertheless, these hyperplasias were extremely mild and did not progress to neoplasia, even in animals greater than 18 months of age.

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A common consequence of Rb inactivation is ectopic entry into S-phase (24). Inactivation of all pocket proteins through expression of large T antigen in differentiated enterocytes leads to ectopic S-phase entry in small intestinal villi (16). To test for ectopic S-phase entry in
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...colons, BrdUrd-positive cells were found only within the bottom nine cell positions (Fig. 3, B). Cells that enter S-phase at ectopic positions within the crypt might also progress to mitosis. To determine whether the ectopic BrdUrd staining in mutant colons leads to ectopic cell cycling, we measured the mitotic index by immunostaining for phosphorylated histone H3. As staining in mutant colons leads to ectopic cell cycling, we measured the mitotic index for phosphorylated histone H3 staining for mitotic cells both at entopic positions (C and D) and at ectopic positions (Fig. 3, C). Quantitative differences between genotypic classes were determined by Wilcoxon Rank Sum analysis: the asterisk (*) denotes p < 0.05 versus wild-type, and the double asterisk (**) denotes p < 0.01 versus wild-type and single mutants.

Our analysis of the tissue kinetics in mutant epithelium revealed that all mutant animals exhibited ectopic S-phase entry and mitoses without increasing the frequency of apoptosis. Given that only Rb/p130 double mutant mice present with hyperplasia, these observations indicate that changes in the growth kinetics of the epithelium are not sufficient to markedly alter the development of the intestinal epithelium.

One feature of the hyperplasia that develops in Rb/p130 double mutant animals is an apparent defect in goblet cell maturation. In the hyperplastic tissue the goblet cells commonly have smaller mucin droplets, slight nuclear enlargement, and greater variations in the polarity of the nucleus and mucin droplet compared with "normal" goblet cells. To expand on this observation we examined the development of other differentiated cell types within the intestinal epithelium. We used antibodies to the following proteins to detect specific cell types by immunohistochemistry: liver fatty acid binding protein (Fabpl) for enterocytes, mucin 2 (Muc2) for goblet cells, lysozyme (Lyzs) for Paneth cells, and chromogranin A (Chga) for enteroendocrine cells. Of particular interest were enteroendocrine cells, since loss of Rb function is associated with defective neuroendocrine differentiation in other tissues (28, 29). To our surprise, all of the differentiated cells could be detected in the epithelium of Rb/p130 double mutant mice (Fig. 4, E–H).

Although most of the cell types in the epithelium appeared to begin to differentiate, the morphology of the goblet cells led us to believe that the epithelial cells were not completely, or terminally, differentiated. To address this issue, we analyzed wild-type and mutant intestinal epithelium for expression of a marker for terminal differentiation. We found that Mcm6 was expressed in the cells in the small intestinal and colonic crypts of wild-type mice (Fig. 4, I and K). At the cellular level, Mcm6 was found in the nuclei of undifferentiated cells but not in the nuclei of terminally differentiated Paneth cells at the base of the crypt (Fig. 4M) nor in the nuclei of terminally differentiated goblet cells migrating out of the crypt (Fig. 4O). It was from these observations that we have defined Mcm6 as a marker of non-terminally differentiated cells. In Rb and p130 single mutant animals we could not detect any abnormalities in Mcm6 staining. However, Mcm6 was expressed throughout the crypt/villus axis in Rb/p130 double mutant mice, indicating that the epithelium in these mice has largely failed to terminally differentiate (Fig. 4, J and L). In Paneth cells there was no nuclear Mcm6 (Fig. 4N), indicating that this cell type does indeed terminally differentiate in Rb/p130 double mutants. In goblet cells, however, Mcm6 was still highly expressed (Fig. 4P). We conclude that goblet cells as well as enteroctyes (data not shown) fail to properly complete the terminal differentiation process in Rb/p130 double mutant animals.

The ability of pRb to promote differentiation is dependent at least in part on its ability to regulate the expression of tissue-specific transcription factors (30). pRb can also bind to and regulate the function of transcription factors, for example Elf1 during T cell development (31). We sought to determine whether intestinal transcription factors act as mediators of the hyperplastic phenotype in Rb/p130 double mutant mice. To determine whether the pocket proteins regulate intestinal epithelial homeostasis through specific transcription factors, we examined the relationship between pRb and p130 and several transcription factors thought to regulate differentiation in the intestinal epithelium, namely Math1, Elf3, Cdx1, and Cdx2. We found that the levels of Elf3 were unchanged in single and double mutant intestinal epithelium (data not shown). By contrast, we found that Math1 was overexpressed in intestinal epithelium that was mutant for both Rb and p130 (Fig. 5A). Finally, Cdx1 and Cdx2 were overexpressed in tissues that were mutant for Rb both alone and in combination with p130 mutation (Fig. 5A). In the case of Cdx1, we found that up-regulation occurred at the level of transcription, indicating that pRb might negatively regulate Cdx1 gene expression (Fig. 5B). These results demonstrate that, as in other tissues, the pocket proteins regulate the expression of transcription factors that function in differentiation.

Given that the levels of Math1, Cdx1, Cdx2 are increased in single and/or double mutant intestine, we sought to determine whether this overexpression was required for the homeostatic defect in double mutant mice. We chose to focus our further efforts on Cdx1 for three reasons. First, the upstream regulators of Cdx1 function are poorly characterized, and we reasoned that the up-regulation of Cdx1 by loss of Rb function warranted further investigation in this regard. Second, among the few characterized downstream targets of Cdx1 is CyclinD1, which regulates pocket protein function (32). Finally, mutations in Cdx1, unlike Cdx2 and Math1, are homozygous-viable, allowing us to test genetically whether overexpression of Cdx1 was required for the phenotype of double mutant animals. To characterize the role of Cdx1...
in the hyperplasia induced by loss of pRb and p130, we generated *Fabpl-Cre;Rb<sup>2lox/2lox</sup>;p130<sup>-/-;Cdxi<sup>-/-</sup></sup> mice and analyzed the histology of the intestinal epithelium. The intestinal phenotype of *Cdxi* knock-out mice is poorly characterized. We found that the small intestinal epithelium of *Cdxi<sup>-/-</sup></sup> mice displays a slight lengthening of the crypt-villus axis that is characterized by very prominent goblet cells (Fig. 6, A–D). Heterozygosity for *Cdxi* did not affect the phenotype of *Rb/p130* double-mutant intestine, but complete loss of *Cdxi* significantly diminished the mutant phenotype in all triple mutant animals (Fig. 6, C–D). Specifically, the overall morphology of the crypts was similar to *Cdxi* knock-out mice.

We characterized two homeostatic defects in Rb-mutant intestinal epithelium. First, we found that epithelium that was mutant for *Rb* and/or *p130* exhibited ectopic mitoses. Second, we found that epithelium mutant for *Rb* and *p130* exhibited a defect in terminal differentiation. We sought to determine whether loss of *Cdxi* function reverts either of these cellular phenotypes to wild type. Staining for Mcm6 revealed that loss of *Cdxi* function does not revert the terminal differentiation defect in the colon or small intestinal of *Rb/p130* double mutant mice (Fig. 6, E and F). Nevertheless, we found that whereas loss of *Cdxi* had no effect on the frequency of entopic mitoses in the colon of *Rb/p130* double mutant mice, the frequency of ectopic mitosis was reduced in triple mutant epithelium (Fig. 6, G and H). Interestingly, loss of *Cdxi* on an otherwise wild-type background does not significantly affect the occurrence of entopic or ectopic mitosis (Fig. 6, G and H). Thus, loss of *Cdxi* function reverts the histologic phenotype of *Rb/p130* mutant mice by lowering the frequency of ectopic mitosis.
Although these genes are not frequently mutated in neoplastic lesions of animals. Our observations provide the first direct evidence for the regression of epithelium and appears to persist throughout the lifespan of the mutant mice. Cdx1 and Cdx2 are overexpressed in the small intestinal epithelium in response to mutation of Rb but are unaffected when p130 is mutated singly. Rb tagman analysis of Cdx1 expression. Tissues that are mutant for Rb (Rb<sup>+/−</sup>) express high levels of Cdx1 RNA when compared with wild type. Red, Fabpl-Cre;Rb<sup>+/2lox</sup>;p130<sup>−/−</sup>; green, Rb<sup>+/2lox</sup>;p130<sup>−/−</sup>; blue, Fabpl-Cre; Rb<sup>−/2lox</sup>;p130<sup>−/−</sup>. Quantitative differences between genotypic classes were determined by Wilcoxon Rank Sum analysis; the asterisk (*) denotes p < 0.05 versus wild-type.

### DISCUSSION

Using transgenic mice with conditional and germ-line mutations in Rb and/or p107 and p130, we have assessed the role that these genes play in regulating homeostasis in the intestinal epithelium. Although the pocket protein family has been extensively studied in other contexts, its function in the mammalian intestinal epithelium had not been comprehensively characterized. Our studies indicate that, whereas functional compensation by family members may occur, this family of proteins plays an important role in maintaining intestinal epithelial homeostasis through its ability to regulate proliferation and terminal differentiation.

We found that the pocket proteins are expressed asymmetrically within the crypts of the mouse small intestine and colon (Fig. 1, A–F). For example, pRb is expressed more in the bottom of the crypt than at the top of the colonic crypt, a pattern that is consistent with its expression in human colon (33). The patterns of expression for all of these proteins are consistent with their expression patterns in other cell/tissue types; pRb is expressed in both cycling and quiescent cells, whereas p107 is expressed in cycling cells and p130 is mostly expressed in cells that have exited the cell cycle (3).

Compensatory up-regulation of p107 expression is common in cells that have lost pRb (20) or p130 (15, 34). Loss of Rb or p130 is thought to release activating E2Fs that in turn activate transcription of the p107 gene (20). This model is consistent with our finding that in the intestinal epithelium loss of pRb and/or p130 leads to up-regulation of p107 gene expression (Fig. 1, G and H). It is not clear whether this compensatory up-regulation of p107 is responsible for the lack of phenotype in Rb- and p130-mutant epithelium. However, the increase in p107 expression seen in Rb/p130 double mutant epithelium is clearly insufficient to suppress hyperplastic growth.

Mice that fail to express pRb and p107 or p130 in the intestinal epithelium exhibit chronic hyperplasia (Fig. 2). This is a developmental phenotype that is present throughout the Cre-positive regions of the epithelium and appears to persist throughout the lifespan of the mutant animals. Our observations provide the first direct evidence for the regulation of intestinal epithelial homeostasis by the pocket proteins. Although these genes are not frequently mutated in neoplastic lesions of the human colon (i.e., adenomas or carcinomas), the retinoblastoma pathway may be a good candidate for causative mutations in sporadic hyperplastic lesions of human colon. Colorectal hyperplastic polyps are extremely common, with a prevalence of 10–35% in the Western world (35). Historically, hyperplastic polyps have been considered to have very low malignant potential. More recently, however, these benign polyps have been proposed to represent the first stage in an alternative route of colorectal cancer, with progression to a serrated adenoma occurring before the development of a frank adenocarcinoma (35). Approximately 50% of hyperplastic polyps have mutations in the Ras/mitogen-activated protein kinase pathway, either in BRAF or in KRAS (36). The remaining 50% of hyperplastic polyps have an unknown molecular etiology. Based on our observations, it will be of interest to screen human hyperplastic polyps for alterations in the retinoblastoma pathway.

Colons in which Rb and/or p130 were inactivated appeared to exhibit an equivalent effect on cell kinetics. In all cases when Rb and/or p130 were mutated, the intestinal epithelium displayed an increase in ectopic S-phase entry and mitosis (Fig. 3). This result is perplexing given that both Rb and p130 must be inactive to significantly affect homeostasis in this tissue. It is clear from this analysis that simply altering the growth kinetics of the tissue is not sufficient to alter homeostasis in the intestinal epithelium.

If the phenotype of Rb/p130 double mutant animals is not simply a function of increased proliferation, what else underlies the developmental defect in the intestinal epithelium? The answer may lie in the poorly characterized relationship between terminal differentiation and exfoliation in the intestinal epithelium. Exfoliation is a critical regulator of homeostasis in this tissue; in the mouse small intestine 1400 cells are exfoliated from each crypt every 24 h (37). It is conceivable that the exfoliation of cells from the intestinal epithelium is dependent upon their differentiation state, with cells that have not terminally differentiated being retained. When cells in the epithelium experience a delay in the terminal differentiation process, they would also experience a delay in exfoliation. At the tissue level a decrease in the rate of exfoliation would manifest as hyperplasia. Indeed, hyperplastic polyps from human colon are proposed to exhibit a defect in exfoliation (38). Similarly, the hyperplasia in our mutant mice is consistent with a link between terminal differentiation and exfoliation. Nevertheless, further experiments are required to test formally whether exfoliation in the intestinal epithelium is linked to differentiation state.

The ability of a cell to terminally differentiate requires it to leave the cell cycle and enter quiescence. Mice with a germ line mutation of Rb exhibit defects in cell cycle exit during terminal differentiation (39, 40). Recent work also has demonstrated a role for pRb in the establishment and maintenance of the G<sub>0</sub> state (20, 41). We have found that pRb and p130 do not appear to be required for the initial development of goblet cells, enterocytes, Paneth cells, or enteroendocrine cells. Nevertheless, continued expression of Mcm6 in goblet cells and enterocytes is a clear
indication that these cell types fail to terminally differentiate in the absence of pRb and p130. It is known from other systems that Rb function is not required for the initial specification of differentiated fate but is instead required for complete cell cycle withdrawal associated with terminal differentiation (30). Indeed, our observations parallel the situation in the skin of *p107/H11002; p130/H11002* mice. In these mice keratinocytes begin to differentiate but never complete terminal differentiation (9).

To begin to understand the molecular nature of the hyperplasia in double mutant epithelium, we examined the expression of transcription factors that regulate differentiation in this tissue. We found that Math1 was overexpressed in the intestinal epithelium of *Rb/p130* double mutant animals (Fig. 5A). Math1 is regulator of secretory cell differentiation; Math1-null mice fail to develop any of the differentiated secretory cells (goblet, Paneth, or enteroendocrine) in the intestinal epithelium (42). Based on the phenotype of the knock-out mouse, one would expect intestinal epithelium overexpressing Math1 to be “hyper-differentiated” rather than “hypo-differentiated,” as are the *Rb/p130* double mutant intestines. Overexpression of Math1, however, has been shown to inhibit differentiation during cerebellar development (43). Thus, the finding that overexpression of Math1 correlates with the hyperplasia in mutant animals suggests that this protein may play an important role in the mutant phenotype. Further experiments are required to formally test this hypothesis.

We also found that loss of pRb leads to up-regulation of Cdx1 and Cdx2 (Fig. 5), homeobox-containing proteins whose expression in adult animals is restricted to the intestinal epithelium; Cdx1 is expressed predominantly in the crypt, and Cdx2 is expressed in the upper crypt and villus (22, 44). Null mutations in Cdx2 are embryonic lethal in mice (45, 46). Nevertheless, loss of Cdx2 function in the intestinal epithelium of adult mice leads to gastric metaplasia, whereas overexpression of Cdx2 in the gastric epithelium leads to intestinal metaplasia (45, 47). In addition, overexpression of CDX2 in cultured colorectal cancer cells induces
markers of differentiation (48). The molecules that mediate Cdx2 function are not known. However, Cdx2 is known to induce the expression of Kruppel-like factor 4 (Klf4), a transcription factor that regulates goblet cell differentiation (49). Testing the role of Cdx2 downstream of Rb will require the generation of animals with compound conditional mutations.

We found that the up-regulation of Cdx1 in Rb-mutant intestine occurs at the level of transcription. Nevertheless, the Cdx1 promoter does not appear to have any E2F binding sites. This observation indicates that pRb indirectly regulates Cdx1 expression or else directly regulates Cdx1 expression through interaction with transcription factors other than the E2Fs. Additional studies are required to distinguish between these alternative possibilities.

Regardless of the mechanism by which pRb negatively regulates Cdx1, our data indicated that overexpression of Cdx1 was required for the hyperproliferation seen in Rb-mutant animals. What is the relationship between Cdx1 and epithelial proliferation? In vivo analyses of Cdx1 function have not provided much insight into its role in regulating cellular proliferation. Cdx1 knock-out mice exhibit a defect in vertebral development because of its expression in early embryos but no characterized intestinal phenotype (18). In human colon cancer cell lines, Cdx1 has been reported to inhibit proliferation by directly inhibiting the expression of CYCLIN-D1 (32) and by inhibiting transcriptional activity of β-catenin/Tcf (50). These reports are consistent with the proposed function of Cdx1 as a tumor suppressor gene in human colon (51). Other data indicate, however, that Cdx1 is an oncogene. In rat IEC-6 cells, expression of Cdx1 promotes cell growth through up-regulation of pancreatitis-associated protein 1 (52) and by activating Ras (52, 53).

We found that loss of Cdx1 function suppressed hyperplastic growth in Rb/p130 double mutant mice (Fig. 6D). To our surprise loss of Cdx1 suppressed the ectopic mitoses in Rb/p130 double mutant animals rather than the terminal differentiation defect. Our results indicate that expression of Cdx1 is necessary for the severe hyperplasia in double-mutant mice. By combining our data with previously published studies on Cdx1 function, we propose a model in which Cdx1 and pRb function in a negative feedback loop to control proliferation in the intestinal epithelium (Fig. 7). This model predicts that in the presence of a functional Rb pathway, overexpression of Cdx1 would induce cell cycle arrest by inhibiting CyclinD1 (32). However, loss of the negative regulatory function of pRb would release Cdx1 to promote proliferation, perhaps through pancreatitis-associated protein 1 and/or Ras (52, 53).

Our studies demonstrate for the first time that pRb and p130 regulate homeostasis in the intestinal epithelium. These results also indicate that intestinal epithelial hyperplasia does not simply result from altered growth kinetics (i.e., an increase in proliferation) or a defect in terminal differentiation but, rather, a combination of the two. Furthermore, we have found that expression of Cdx1 is required for the hyperplastic phenotype of Rb/p130 double mutant mice. The role that other transcription factors play in regulating homeostasis in the absence of pRb and p130 remains to be determined. The phenotype of intestinal epithelium with mutations in Rb and p130 is very similar to colorectal hyperplastic polyps and serrated adenomas, indicating that mutations in these genes may be responsible for a subset of these non-neoplastic lesions in human colon.

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