

# c-Myb is required for progenitor cell homeostasis in colonic crypts

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The colonic crypt is the functional unit of the colon mucosa with a central role in ion and water reabsorption. Under steady-state conditions, the distal colonic crypt harbors a single stem cell at its base that gives rise to highly proliferative progenitor cells that differentiate into columnar, goblet, and endocrine cells. The role of c-Myb in crypt homeostasis has not been elucidated. Here we have studied three genetically distinct hypomorphic *c-myb* mutant mouse strains, all of which show reduced colonic crypt size. The mutations target the key domains of the transcription factor: the DNA binding, transactivation, and negative regulatory domains. *In vivo* proliferation and cell cycle marker studies suggest that these mice have a progenitor cell proliferation defect mediated in part by reduced *Cyclin E1* expression. To independently assess the extent to which *c-myb* is required for colonic crypt homeostasis we also generated a novel tissue-specific mouse model to allow the deletion of *c-myb* in adult colon, and using these mice we show that c-Myb is required for crypt integrity, normal differentiation, and steady-state proliferation.

colon | hypomorphs | A33 | stem cells | p27

Colonic crypts are exquisite structures that generate a vast and continuous epithelial surface allowing efficient water and ion reabsorption. Crypt cells are rapidly renewed, consisting of comparable numbers of columnar cells and mucin-producing goblet cells and fewer enteroendocrine cells. Mouse colonic crypts consist of  $\approx 500$  cells, of which stem cells located at the base of the crypt give rise to a dividing population that differentiate into the three lineages (1). This process takes 4–7 days, during which progenitor cells proliferate and progressively differentiate while migrating toward the top of the crypts where many of the cells undergo apoptosis (2). During development, differentiation of the murine intestine occurs in a proximal to distal wave between embryonic day 15 (E15) and E19. Crypts continue to lengthen and divide by fission until 28 days postnatal, after which time homeostasis is attained (3).

The rapid expansion of multiple lineages building up to homeostasis in the adult parallels processes in the hematopoietic system where stem cells give rise to progenitor cells and finally terminally differentiated cells. Colonic crypts offer an attractive model in which to study stem and progenitor cells because these cells occupy discrete positions within crypts. Specifically, distal colonic crypt stem cells reside at the crypt base from where progenitor cells arise. In the proximal colon, stem cells are found several cells above the crypt base, in parallel to that observed in small intestinal crypt-villi structures. Thus, understanding these apparently simple structural features affords the opportunity to identify defects in progenitor cell replacement and turnover and has been used in numerous examples of mutant and gene-knockout mouse lines.

*c-myb* is expressed early in the development of colonic mucosa and persists in the adult colonic epithelia in both mouse and human

(4). We used *c-myb*<sup>-/-</sup> embryos (5) to indirectly investigate the role of *c-myb* in colon development (6). The effect of the *c-myb* deletion on intestinal development could not be assessed *in situ* because mice with targeted disruptions of both *c-myb* alleles die at E15 because of a severe defect in fetal liver hematopoiesis (5). However, dissecting colon and small intestine from *c-myb*<sup>-/-</sup> embryos at E14 and transplanting the tissue under the kidney capsule of recipient adult mice allowed the development of these tissues to proceed. We found that colon tissue from the *c-myb*<sup>-/-</sup> embryos developed with profound epithelial disorganization such that normal crypts failed to form (6).

In contrast to *c-myb*<sup>-/-</sup> embryos, *c-myb*<sup>+/-</sup> mice develop normally, but under cytotoxic stress produced by treatment with sublethal to lethal doses of cytotoxic drugs or radiation, profound differences from the wild-type response are evident (7). As such, this model has been a powerful means of revealing a biological role for c-Myb in the colon. Thus, we have found that both alleles of *c-myb* are required for crypt survival and recovery. In this study we have investigated a unique series of mouse mutants that affect the three key domains of the c-Myb protein as well as a novel tissue-specific inducible *c-myb* knockout model to show that *c-myb* is essential to normal colonic crypt proliferation and architectural integrity in adult mice.

## Results

### Fully Functional c-Myb Is Required for Normal Colonic Crypt Length.

Three genetically distinct mouse lines with mutations in the *c-myb* locus have been generated in two separate studies after saturation mutagenesis with ENU (8, 9). These mice have mutations in the three well characterized functional domains of the c-Myb protein, the DNA binding domain (Plt3), transactivation domain (M303V), and negative regulatory domain/leucine-rich motif (Plt4) noted in Fig. 1A. The c-Myb proteins encoded by these mutant loci have suboptimal transactivation capacity (8, 9).

When longitudinal sections of colonic crypts were examined from each of the three hypomorphic mutants their reduced length was immediately obvious. Fig. 1B shows that, compared with wild-type distal colonic crypts, *c-myb*<sup>Plt3/plt3</sup>, *c-myb*<sup>Plt4/plt4</sup>,

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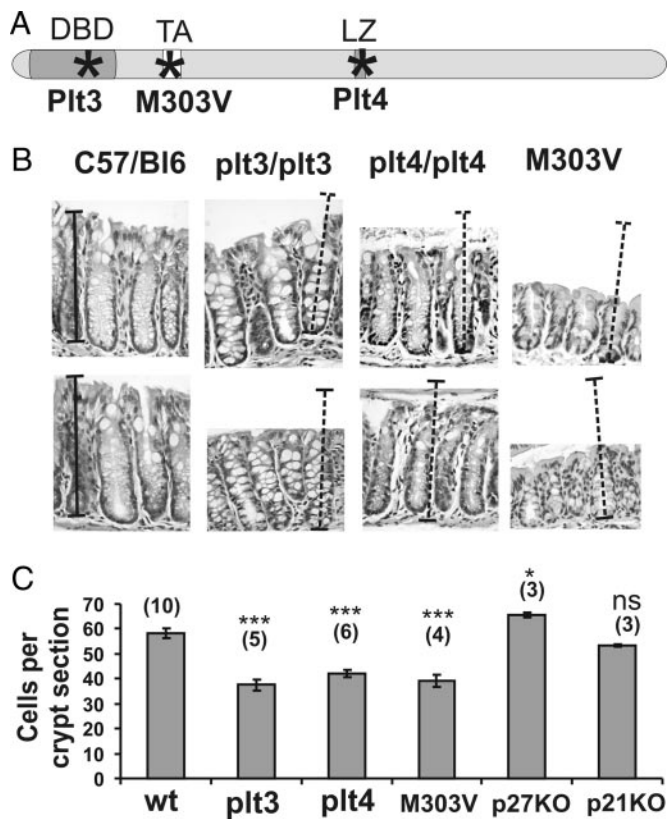
The authors declare no conflict of interest.

Abbreviations: PCNA, proliferating cell nuclear antigen; PAS, periodic acid/Schiff reagent; MEF, mouse embryonic fibroblast; En, embryonic day n.

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**Fig. 1.** Hypomorphic *c-myb* mutant mice have shorter crypts than wild-type mice. (A) Murine *c-Myb* diagram marking (\*) the location of amino acid substitutions within the DNA binding domain (Plt3), the leucine zipper region (Plt4) (8), and the transactivation domain (M303V) (9). (B) H&E-stained sections of wild-type, *plt3/plt3*, *plt4/plt4*, and *M303V/M303V* distal colons show that the mutant crypts are shorter than wild type. Relative length size bars are shown layered over normal distal colonic crypts, and these have been transferred to panels representing the three hypomorphs. (C) Morphometric analysis of the sections indicates that the hypomorphic mutant crypts are significantly shorter than wild-type C57BL/6 crypts whereas *p27*<sup>-/-</sup> crypts are significantly longer. *p21*<sup>-/-</sup> crypts are indistinguishable from wild type. Bars represent mean ± SEM (number of mice analyzed). \*\*\*,  $P > 0.0001$  (ANOVA).

and *c-myb*<sup>M303/M303V</sup> crypts retain overall crypt architecture but are of reduced length and display an apparent excess of goblet cells compared with that observed in wild-type crypts as evidenced by the enlarged vacuoles after H&E staining.

Morphometric analysis showed that all three hypomorphic mutant mice had significantly reduced cell numbers per crypt compared with wild-type C57BL/6 mice ( $P < 0.0001$ , ANOVA; for each hypomorph) (Fig. 1C). When crypts were examined for spontaneous apoptosis there was no indication that increased cell death could account for the shortened crypt length as assessed by the presence of apoptotic bodies or activated caspase-3-positive cells (data not shown). Therefore, these observations suggested that there may be a role for *c-myb* in driving proliferation of colonic crypts.

To highlight the role of proliferation regulators in maintaining crypt length, we also examined the impact on crypt length when two negative regulators of growth were deleted. We first examined *p27* because its expression appears to be a reciprocal to the high *c-myb* expression observed at the crypt base (4, 10) where *p27* expression is low at the base and increases toward the colon lumen (11). Second, we examined *p21* as there is an inverse relationship between *c-myb* expression and *p21* expression during colon cell differentiation (12). To investigate whether the loss of expression of these genes had an effect on crypt length, cells

per longitudinal section were quantified. Histological examination of *p27*<sup>-/-</sup> crypts showed they were significantly longer than wild type when sections were observed and this was confirmed by counting crypt cells in longitudinal sections ( $P = 0.05$ , ANOVA). In contrast, *p21*<sup>-/-</sup> crypts were indistinguishable from normal controls but markedly different to *p27*<sup>-/-</sup> crypts ( $P = 0.0004$ , ANOVA).

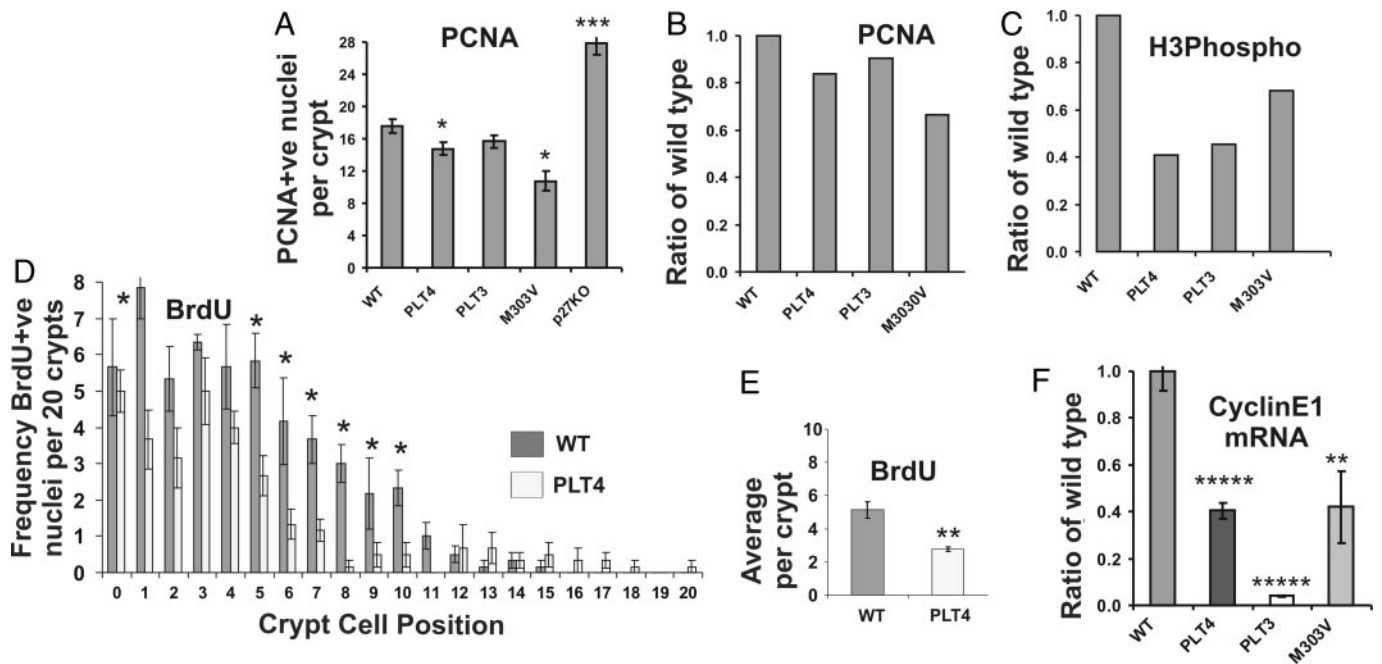
**Disrupted Differentiation and Retarded Proliferation in Hypomorphic Crypts.** The observed defects in colonic crypt morphology raised the prospect that the crypts in hypomorphic *c-myb* mutant mice had a defect in cytodifferentiation and/or cell proliferation. To test this, sections were stained with periodic acid/Schiff reagent (PAS) that detects mucins thereby identifying goblet cells, one of the two predominant cell types within the colonic crypt. PAS-positive cells were readily observed in wild-type, *plt3/plt3* and *plt4/plt4* crypts indicating that the goblet cell lineage was generated in the presence of *c-myb* hypomorphic mutations. However, there was a consistent trend toward over-representation of this cell type in crypts whereby PAS-positive cells were predominant in mutant versus wild-type colonic crypts. In contrast, *p27*<sup>-/-</sup> crypts appeared to have reduced PAS staining [supporting information (SI) Fig. 6A]. Reduced cell density and spatial disorder of cells at the crypt base was also apparent. Determining the actual frequency of goblet cells for each mutant was problematic because the boundaries of the goblet cell cytoplasm and released mucin are ill-defined. Nevertheless, when mucin content was indirectly assessed by using MetaMorph software that allows relative levels of PAS staining compared with hematoxylin staining of nuclei, it was determined that mucin content was significantly increased in two of the hypomorphic crypts, and thus goblet cells were implicitly over-represented (SI Fig. 6B). The third lineage generated within the colonic crypt is the enteroendocrine cell type that is readily detected by immunohistochemistry after staining sections with anti-ChromograninA antibody. This cell type, like columnar cells, was in deficit in the hypomorphic mutant colonic crypts (SI Fig. 7).

When sections were stained for proliferating cell nuclear antigen (PCNA) to assess cell proliferation it was evident that the three *c-myb* hypomorphs had fewer PCNA-positive cells when quantified by crypt position. Because the presumed stem cell is located at the very crypt apex of the distal colon this cell position was often PCNA-negative. For counting purposes such cells were allocated the zero cell position within the crypt (Fig. 2A). At least 20 crypts were assessed for PCNA stained nuclei enumerating positive cells on both sides of each crypt. When this was done *plt4/plt4* and *plt3/plt3* crypts showed significantly fewer PCNA-positive cells at positions 4–7 and 12–18, respectively (Figs. 2C and 3D), compared with wild type (Fig. 2B). In contrast *M303V/M303V* crypts showed significantly less PCNA closer to the base of the crypt (positions 2–7) (Fig. 2E). In accord with the significantly longer crypts in *p27*<sup>-/-</sup> mice the extent of PCNA staining was greater at positions 9–21 suggesting that in the absence of *p27* proliferation is sustained for longer, as cells migrate to the luminal surface of the colon (Fig. 2F). Collectively these data imply that each hypomorphic *c-myb* mutant has a proliferative defect within the progenitor cell zone and perhaps these defects are in different positions within this zone.

#### ***c-myb* Hypomorphic Crypts Show Different Defects in Proliferation.**

From the proliferation profiles shown in Fig. 2 it would appear that the *plt4* and *plt3* mutations affect the progenitor cell region of crypts while the *M303V* mutation influences proliferation closer to the crypt base where stem cells are located. Both *plt4* and *M303V* mutants showed significantly fewer, and *p27*<sup>-/-</sup> mice had significantly more, PCNA-positive cells within the crypts when the total frequency was assessed (Fig. 3A). The relative





**Fig. 3.** Proliferation markers indicate that hypomorphic *c-myb* mice have a defect in cell cycle progression. (A) Collectively, the total number of PCNA-positive cells per crypt for each strain of mice was significantly different for all mutants with the exception of PLT3 mutants. (B) To allow a further assessment of a potential defect in cell cycle progression each data set was analyzed as a ratio to wild type to show the reduced PCNA staining in mutants. (C) This type of analysis was extended to include phospho-histone-3 expression whereby it would appear that the ratios of this marker of G<sub>2</sub>/M phase cells are less than those observed for cycling PCNA-stained cells. (D and E) PLT4 mutant and wild-type mice were further assessed for the extent of BrdU staining on the basis of crypt position (D) and total per crypt (E). Both analyses suggested significantly less S-phase progression in PLT4 mutant mice compared with wild-type littermates. Bars represent mean  $\pm$  SEM. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  (ANOVA). (F) Real-time RT-PCR studies on colonic crypt RNA isolated from wild-type ( $n = 6$ ), PLT3 ( $n = 3$ ), PLT4 ( $n = 3$ ), and M303V ( $n = 3$ ) mice show that *Cyclin E1* expression is lower in these hypomorphic mutant mice, perhaps explaining the defect in cell cycle progression particularly at the crypt base.

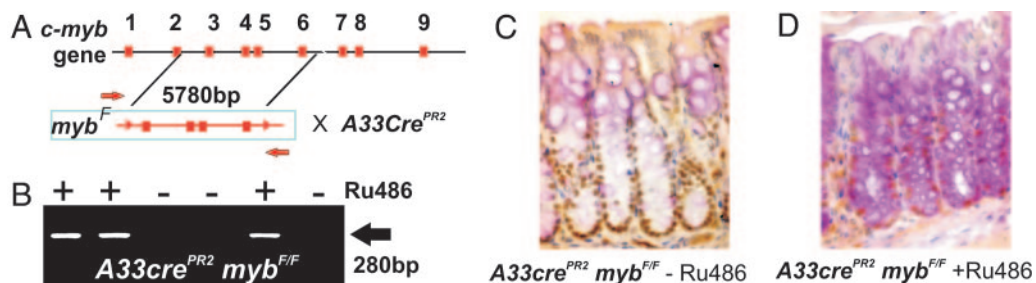
***c-myb* Deletion Leads to Reduced Proliferation and Disrupted Differentiation.** It was suggested above that crypts in the hypomorphic mutant mice had an over-representation of goblet cells. This distortion of differentiation was far more evident in the conditional deletion mutants. Furthermore, the expanded vacuoles associated with goblet cells appear to be a feature of the knockout crypts and as judged by PAS content there was significantly more mucin present in the *c-Myb*-deleted crypts (SI Fig. 8C). Typical examples of abnormal crypts present after recombination and disruption of the *c-myb* locus are shown Fig. 4 C and D.

In view of the proliferation defects observed with the hypomorphic mutant mice colon sections *A33cre*  $\times$  *myb*<sup>F/F</sup> mice plus RU486 were also stained for PCNA. Micrographs of colon sections stained for PCNA highlight the reduced proliferation after Cre-mediated recombination when compared with *A33*

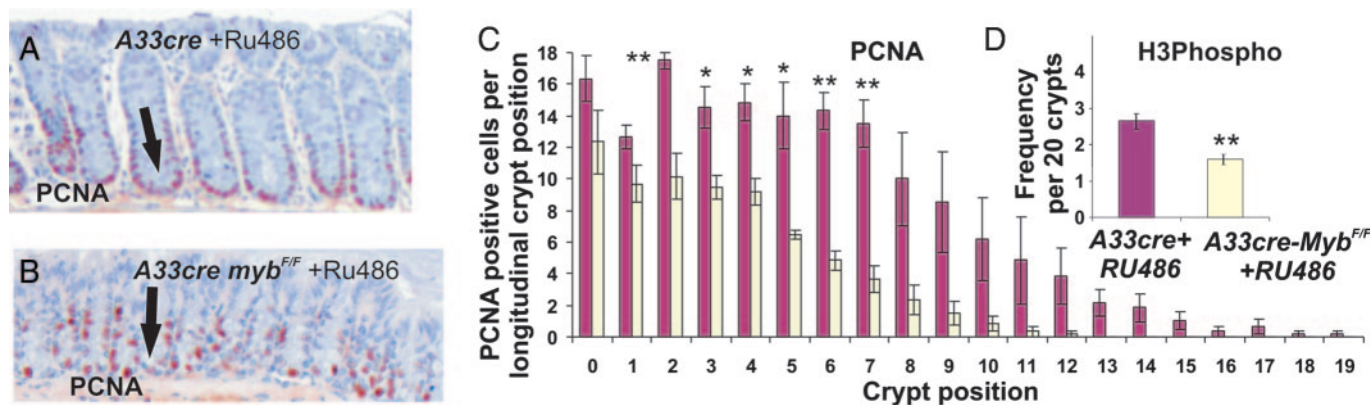
transgenic mice alone (Fig. 5 A and B). Quantitation of PCNA staining in discernable crypts in mutant and control mice is shown for each crypt cell position, indicating that after Cre induction with RU486, the proportion of cells per crypt is significantly reduced (Fig. 5C). Crypt staining for phospho-histone-3 expression to assess cell cycle progression indicated that the crypts with deleted *c-myb* had fewer cells in G<sub>2</sub>/M phase of the cell cycle compared with control crypts (Fig. 5D).

**Discussion**

We have taken advantage of the unprecedented opportunity to employ mutant mice with specific *c-myb* mutations in the three key functionally defined domains to examine the effect of *c-Myb* functional loss in adult colonic crypts. The complete ablation of *c-Myb* function by germ-line deletion has until now precluded analysis of the role of *c-myb* in tissues other than the hemato-



**Fig. 4.** Tissue-specific conditional deletion of *c-myb* in distal colon leads to a reduction of proliferation in colonic crypts. (A) Schematic of the strategy for the conditional deletion of the *c-myb* locus (14) whereby exons 3–6 are excised after the activation of *A33Cre*<sup>PR2</sup> activity by RU486. (B) An image of an agarose gel with ethidium bromide-stained PCR products generated by using primers (marked by red arrows in A) indicated successful deletion of exons 3–6 in the presence of RU486 (+). Costaining of colonic crypts for *c-Myb* (C) and PAS (D) shows that the intensity of PAS staining (as an indicator of goblet cell differentiation) increases with the loss of *c-Myb*.



**Fig. 5.** *c-myb* is required for proliferation in colonic crypts. (A and B) Colon sections were stained for PCNA in nonrecombined colonic crypts (A) and in recombined crypts where reduced proliferation is evident (B). (C) Quantitation of PCNA staining in discernable crypts in nonrecombined and RU486-treated mice is shown for each crypt cell position, indicating that after Cre activation with RU486 the proportion of cells per crypt is significantly reduced. (D) Crypts stained for phospho-histone-3 to examine cell cycle progression into the G<sub>2</sub>/M phases of cell cycle indicate that the crypts with deleted *c-myb* had fewer cells in the G<sub>2</sub>/M phase of the cell cycle compared with unrecombined crypts. Bars represent mean  $\pm$  SEM ( $n = 3$  per treatment). \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  (ANOVA).

poietic system (5). Indeed, the recent use of tissue-specific knockout and hypomorphic mutants has expanded the repertoire of approaches available for the analysis of *c-Myb* in adult mouse hematopoiesis (8, 9, 14–16). We further report here the tissue-specific ablation of *c-myb* in the colon showing a more severe phenotype than observed with the *c-myb* hypomorphs.

It would appear that *c-myb* mutations that affect the hematopoietic system in terms of progenitor cell production and lineage commitment also affect colonic crypt morphogenesis and homeostasis. The three hypomorphic mutants studied allow sufficient *c-Myb* functional activity to permit development beyond critical checkpoints like the ability to make sufficient definitive red blood cells and subsequent survival to adulthood. These biological defects do not appear to relate to mutations in any one functional domain. Perhaps the unifying basis for these mutations generating similar biological outcomes is due to disruptions of interactions that *c-Myb* has with other regulators of crypt morphology.

We found that in all three cases crypt lengths in distal colonic mucosa of *c-myb* hypomorphic mice were significantly shorter than wild type. Small intestine crypt and villus formation appear normal consistent with our previous studies (6) (data not shown). Moreover the proliferative activity of the colonic crypts was significantly reduced as measured by PCNA and phospho-histone-3 staining on the basis of overall proliferation and crypt cell position. Essentially *plt3/plt3* and *plt4/plt4* mutant mice showed premature termination of proliferation within the crypt by position 10 while the M303V/M303V mutant appears to have a defect in proliferation closer to the crypt base. These are indicative of either accelerated differentiation and/or retarded cell cycling. Defining the precise demarcation between stem and transit amplifying cells within the distal colonic crypt underpins an ongoing debate, however from the data presented here it is suggested that defective *c-Myb* function directly affects transit amplifying cell proliferation. Previous studies using irradiated and 5-Fluorouracil exposed *c-myb* heterozygous mice indicate that normal *c-Myb* function is also required for the replacement of transit amplifying cells and this need has to be met by stem cells (7). In contrast to shorter crypts in *c-myb* hypomorphs the deletion of the cell cycle regulator *p27*, but not *p21*, led to crypt lengthening. This 26% increase in length over normal controls may simply reflect the overall larger size (25%) manifested by the *p27*<sup>-/-</sup> mice reported elsewhere (17). Nevertheless, these data suggest that *p27* and *c-Myb* are part of the network of cell cycle regulators that govern crypt homeostasis.

The role of *c-myb* in controlling differentiation in colon has to be considered based on the observations reported here and previous studies in mouse and human colon cancer cell lines where exogenous *c-myb* expression blocks cytodifferentiation (18). Consistent with this role is the observation that *c-myb* expression is found in immature columnar cells (4, 19) and reduced *c-Myb* function might be expected to allow an over-representation of goblet cells as well as mucin production. We also found that endocrine cell numbers as assessed by positive staining for ChromograninA were in deficit in the hypomorphic colonic crypts. The goblet defect in particular was most evident in the tissue-specific knockout crypts. As all three cell lineages arise from a single stem cell these differentiation data suggest that *c-Myb* may regulate the balanced differentiation in the colonic crypt.

Separate expression profiling studies in MEFs was consistent with *Cyclin E1* being a *c-Myb* target and analysis of the mouse and human *Cyclin E1* promoters identifies a number of high affinity *c-Myb* binding sites (data not shown). From the analysis of isolated colonic crypts from the hypomorphic mutant mouse crypts we propose that in colonic crypts *Cyclin E1* is a direct *c-Myb* target gene required for cell cycle entry of stem and/or progenitor cells. Because the principle defect in the viable *Cyclin E1* knockout is a failure to enter the cell cycle from quiescence (20), we suggest that a similar defect in cell cycle entry would account for some of the defects observed in the *c-myb* hypomorphic mutants described here.

Recently, another intestine-specific cre-mediated recombination mouse model has been used to delete *c-myc* expression, most particularly in the small intestine (21). This study unexpectedly showed that *c-myc* is required for small intestine crypt development but not homeostasis. In contrast, we show here that *c-myb* is required for homeostasis and, as previously suggested, *c-myb* is required for colonic crypt development but not small intestinal morphogenesis during embryogenesis (6). These contrasting observations are puzzling because *c-myc* is subject to *c-myb* regulation in reporter studies (22), but clearly the *in vivo* studies suggest that the regulation and role of both of these protooncogenes is distinct. It was also interesting to find that the deletion of *c-myb* expression as well as reactivation of the *Rosa* locus by A33-Cre recombination (data not shown) was incomplete within the colon. *Rosa* reactivation was not observed in bone marrow, spleen, lung, or liver (M.E., R.G.R., and J.K.H., unpublished data). Thus, the progressive and apparently stochastic *c-Myb* deletion in the crypts leads to reduced proliferation and distorted differentiation that is intrinsic to the colonic crypts.

Finally, the observations reported here might also be considered in the context that *c-Myb* is overexpressed in colon cancers (23), which may be due in rare cases to gene amplification (24) or mutations within the transcriptional attenuator region (25). Thus, the antithesis to the low *c-myb* expression and reduced proliferation in the mutant mice might be unrestricted proliferation in colon cancers in part driven by *c-Myb*.

## Materials and Methods

**Mice.**  $p27^{-/-}$  (26) mice were maintained on a C57BL/6J background and housed in a specific pathogen-free Thoren racking system (Thoren Caging Systems, Hazelton, PA).  $p21^{-/-}$  mice (27) were maintained on a mixed 129/C57BL/6J background and were a gift from David Vaux (The Walter and Eliza Hall Institute). C57BL/6J *c-myb*<sup>blt3/plt3</sup> and *c-myb*<sup>blt4/plt4</sup> mice (8) were also maintained at The Walter and Eliza Hall Institute. *c-myb*<sup>M303V/M303V</sup> mice (9) were housed at the Novartis Genomics Institute (San Diego, CA).

**Conditional Deletion Construct and RU486.** To generate the intestine-specific *c-myb* mutants, we used mice in which *c-myb* exons 3–6 were flanked with loxP sites (14). Mice harboring two *c-myb*<sup>F</sup> alleles were crossed with transgenic mice possessing the Cre recombinase gene under the control of the endogenous A33 promoter (M.E., R.G.R., and J.K.H., unpublished data). The cre-cDNA was fused to a mutant progesterone ligand-binding domain to allow activation by RU486 (Mifepristone; Sigma, St. Louis, MO). RU486 was prepared in mouse chow (1.6 g/kg). Confirmation that the Cre was activated by RU486 was established by using *Rosa* reporter mouse and by deletion-specific *c-myb* PCR (14).

**BrdU Labeling and Morphometric Analysis.** BrdU (Sigma) was delivered by i.p. injection at 100 mg/kg to mice, which were killed 2 h later. Colons were fixed in 4% buffered formalin, processed for sectioning, and stained with mouse monoclonal anti-BrdU (2  $\mu$ g/ml; Roche) detected by biotinylated goat anti-mouse IgG (1:200; Vector Laboratories), and staining was visualized with diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. BrdU-positive cells were counted under high power on a Nikon E800 microscope with Magnifire digital camera, and the image was displayed on a computer monitor. Results were expressed as the average number of BrdU-positive crypt cells (20) per animal and by crypt position.

For *c-Myb* and H&E staining colon sections were fixed in methacarn for 2 h and transferred to 70% ethanol, embedded, sectioned, and stained with H&E. Full crypts exposing a lumen and identifiable base were scored. Crypt cells per 40–50 longi-

tudinal sections were scored as previously described (7). Sample groups were subjected to one-way ANOVA using Origin software. The anti-PCNA antibody PC-10 (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:100 dilution to identify PCNA followed by goat anti-mouse-HRP at 1:250 (Bio-Rad) and developed with Pierce metal enhanced detection reagent. *c-Myb* was visualized by using Mab1.1 and processed as described previously (4) using antigen retrieval by boiling slides in 1 mM EDTA in a pressure cooker for 3 min. Rabbit anti-ChromograninA was used at 1:100 (SC-13090; Santa Cruz Biotechnology) after citrate buffer antigen retrieval. Donkey anti-goat HRP at 1:250 (Santa Cruz Biotechnology) was used as a secondary antibody. Phospho-histone-3 (Upstate Biotechnology) staining was performed by immunofluorescence at a final titer of 1:200.

Mucin content was estimated by using integrated morphometric analysis based on pixel density of differential color staining of pink-PAS-positive cytoplasm corrected for blue-nuclear staining over five microscopic fields at  $\times 40$  magnification on at least three mice per genotype. This was done by using the MetaMorph computer program (Universal Imaging, Downingtown, PA) in a comparable manner to a previous report (28).

**Real-Time RT-PCR.** Colonic crypts were isolated from mouse colons by using the method described elsewhere (29). cDNA was prepared in 20- $\mu$ l volumes by the addition of 1  $\mu$ l of random hexamers (1  $\mu$ g/ $\mu$ l) and 6  $\mu$ l of diethyl pyrocarbonate-treated H<sub>2</sub>O to 4  $\mu$ l of RNA. cDNA was prepared by using SuperScript III (Promega) according to the manufacturer's instructions. Eight microliters of cDNA (1:10 dilution) was combined with 10  $\mu$ l of SyBr Green PCR Master Mix (Applied Biosystems) and 200 nM each sense and antisense oligonucleotides (Geneworks, Adelaide, Australia) and amplified by using temperatures of 50°C for 2 min and 95°C for 10 min. These initial steps were followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Expression of all genes was compared with  $\beta_2$ -microglobulin to determine relative levels of mRNA transcripts. *Cyclin E1* primers: sense, 5'-TTT CTG CAG CGC CAT CCT; antisense, 5'-GCA CAC CTC CAT CAG CCA A-3'.

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