Defective Myb Function Ablates Cyclin E1 Expression and Perturbs Intestinal Carcinogenesis

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Abstract

Cyclin E1 is essential for the reentry of quiescent cells into the cell cycle. When hypomorphic mutant Myb mice (Myb<sup>Hmo</sup>) were examined, it was noted that Cyclin E1 (Ccne1) expression was reduced. Furthermore, the induction of Ccne1 in recovering intestinal epithelia following radiation-induced damage was ablated in Myb-mutant mice. These data prompted us to investigate whether Myb directly regulated Ccne1 and to examine whether elevated Myb in colorectal cancer is responsible for Cyclin E1–driven tumor growth. Here, it was found that Myb/MYB and Ccne1/CCNE1 expressions were coupled in both mouse and human adenomas. In addition, the low molecular weight Cyclin E1 was the predominant form in intestinal crypts and adenomatous polyposis coli (Apc)–mutant adenomas. Chromatin immunoprecipitation (ChIP) analysis confirmed that Myb bound directly to the Ccne1 promoter and regulated its endogenous expression. In contrast, Myb<sup>Hmo</sup> served as a dominant-negative factor that inhibited wild-type Myb and this was not apparently compensated for by the transcription factor E2F in intestinal epithelial cells. Myb<sup>Hmo</sup>/Plt<sup>þ</sup> mice died prematurely on an Apc<sup>Min</sup>/ background associated with hematopoietic defects, including a myelodysplasia; nevertheless, Apc<sup>Min</sup>/ mice were protected from intestinal tumorigenesis when crossed to Myb<sup>Hmo</sup> mice. Knockdown of CCNE1 transcript in murine colorectal cancer cells stabilized chromosome ploidy and decreased tumor formation. These data suggest that Cyclin E1 expression is Myb dependent in normal and transformed intestinal epithelial cells, consistent with a cell-cycle progression and chromosome instability role in cancer.

Implications: This study demonstrates that Myb regulates Cyclin E1 expression in normal gastrointestinal tract epithelial cells and is required during intestinal tumorigenesis. Mol Cancer Res; 13(8); 1185–96. ©2015 AACR.

Introduction

Cell-cycle progression relies on the timely modulation of cyclins that bind and activate members of the cyclin-dependent kinase (CDK) family, driving the cell from G<sub>0</sub>–G<sub>1</sub> to S-phase, through G<sub>2</sub> and finally to initiate cell mitosis. Although the full-length (FL) Cyclin E1 isoform, termed El1, is detected in normal and malignant cells (1), overexpression of up to five low-molecular-weight (LMW) Cyclin E1 isoforms has been reported in malignant tissues (2–4). Amino-terminal truncated isoforms EI2/3 and EI5/6 arise by proteolytic cleavage of the FL protein by Elastase (5) or Calpain 2 (6) proteases. The isoform EI4 is produced from an alternative translation start site (5). LMW Cyclin E1 truncations do not abolish the interaction of Cyclin E1 with cyclin/CDK inhibitors but may instead change the conformation of sites responsible for its inhibition, thus subverting normal cell-cycle control and stimulating cancer cells to progress through the cell cycle more efficiently (7, 8). Importantly, the LMW isoforms of Cyclin E1 are enzymatically hyperactive (5).

Deregulation of Cyclin E1 correlates with poor patient prognosis for those with breast cancer (9), colorectal cancer (3), and inflammatory bowel disease (10). In addition, overexpression of the LMW Cyclin E1 isoform tracks with an increase in CDK2 activity in colorectal cancer (11), a reduction in cell doubling time and an increase in S-phase progression (12, 13). Furthermore, it is argued that proteolysis within the factors themselves or increased transactivation by E2F may result in dysregulated proliferation leading to genomic and chromosomal instability (CIN).

We investigated whether Cyclin E1 (Ccne1) is regulated by the proto-oncogene and transcription factor Myb. We have previously shown that cell-cycle re-entry, progression, and proliferation appear retarded within the colonic crypts of Myb<sup>Hmo</sup>/Plt<sup>þ</sup> hypomorphic mutant mice, which correlated with a significant...
reduction in Ccne1 expression (14, 15). These data suggested that the cell-cycle defects observed in the Myb hypomorphs may be due, in part, to reduced Ccne1 expression. Others have found in prostate carcinoma that cyclin (A1, D1, and E1) expression is induced upon Myb overexpression and decreased upon Myb silencing (16). Furthermore, in Jurkat T cells Myb has been shown to regulate the Ccne1 promoter (17). A functional link between Myb and members of the cell-cycle regulatory apparatus has also been described (18, 19).

Myb overexpression is a feature of colorectal cancer (20) and here we demonstrate that Myb is a transactivator of Ccne1. We observe coexpression of Myb and Cyclin E1 protein within intestinal cells and adenomas and show that Cyclin E1 expression is coupled to Myb function in adenomas and intestinal organoids. Furthermore, we show that Myb can directly engage and regulate the Ccne1 promoter. We found that defective Myb impedes tumorigenesis in mouse models, while the knockdown of Cyclin E1 in established colorectal cancer cells stabilized chromosome ploidy and reduced colorectal cancer cell growth in vitro and tumor formation in mice.

Materials and Methods

Mice

Mybfl/fl hypomorphic mutant mice were generated at the Walter and Eliza Hall Institute (Parkville, VIC, Australia) and their hematopoietic and colon defects described (15, 21). Apcfl/fl, Apcfl/fl+/Mybfl/+ and Apcfl/fl+/Mybfl/fl mice were maintained on a C57BL/6 background under pathogen-free conditions (Supplementary Table S1). Primers used for clon- ing are described elsewhere (Supplementary Table S2). pCMV CAT-Basic-MCSII (4.82 kb of the Ccne1 promoter) were generated by Gateway cloning protocols (22).

Blood cell count

Blood was collected from mice prior to culling by retro-orbital or tail-vein blood collection and blood cell counts determined using CELL-DYN Sapphire Hematology Analyzer.

Cell lines, tumor cell injections, and shRNA

The propagation and derivation of CT26, MC38, YAMC, and NIH3T3 cell lines have been described in detail elsewhere (14). Lentiviral small hairpin RNA particles (CyclinE1; NM007633/NIH3T3 cell lines have been described in detail elsewhere (14). Chromatin immunoprecipitation assays and transfection of NIH3T3 cells

Crypts and adenomas were fixed in 10% normal buffered formalin, embedded in paraffin, and 2-μm consecutive sections generated to capture same cell sectioning. Sections were stained with hematoxylin and eosin (H&E). CT26 subcutaneous tumors were halved and fixed in paraformaldehyde (PFA) 4% overnight and the remaining piece frozen down at −80°C for subsequent RNA extraction. All human primary colorectal cancer specimens and matched normal tissues were collected at the Royal Melbourne Hospital (Parkville, VIC, Australia). All patients gave informed consent for their deidentified samples and clinical data to be used in human ethics approved research. Tissue specimens were embedded in optimal cutting temperature compound and H&E-stained. Tumors were macrodis- sected to greater than 70% neoplastic cell content before total RNA was extracted.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using Envision kit (Dako). Antigen retrieval was performed in 10 mmol/L Tris- buffer and 1 mmol/L EDTA (pH 8) in a pressure cooker (Biocare Decloker) at 125°C for 3 minutes. Slides were allowed to cool and washed with dH2O, followed by rinsing in Tris-buffered saline plus 0.01% Tween20 (pH 7.6) before addition of primary antibody (Supplementary Table S1). Slides were counterstained with hematoxylin dehydrated in ethanol.

Chloramphenicol reporter assays and qRT-PCR analyses

pCyclinE3.2CAT-Basic-MCSII (3.2 kb of the Ccne1 promoter) and pCyclinE4.8CAT-Basic-MCSII (4.82 kb of the Ccne1 promoter) were generated by Gateway cloning Ccne1 promoter PCR fragments into pCAT-Basic-MCSII. Primers used for cloning are described elsewhere (Supplementary Table S2). pCMV E2F-1 was a gift of Dr Patrick Humbert (PMCC). Protocols for CAT reporter assays and qRT-PCR analysis have been described previously (14). Primers used for qRT-PCR are described (Supplementary Table S2).

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Electrophoretic mobility shift assay
Electrophoretic mobility shift assays (EMSA) were performed with a \( ^{32}P \)-labeled mimA DNA probe and 1 µL of HIS-tagged Myb CTL protein (23) in reactions containing 10 mmol/L Tris pH 8.0, 50 mmol/L NaCl, 2.5% glycerol, 1 mmol/L EDTA, 0.25 mg/mL BSA, and 1 µg/mL poly (dl-dC) as previously described (24). Binding reactions were resolved on 0.5 × TBE 6% nondenaturing PAGE (29:1) gels (24). Gels were dried and quantified by PhosphorImager analysis using Image Quant Software.

Quantitation and statistical analysis
Statistical analysis was calculated using GraphPad Prism version 5.0 (GraphPad Software Inc.). Assays were repeated in triplicate to calculate the mean ± SEM. Student t tests or Mann–Whitney tests were used to compare groups. One-way ANOVA tests were used for multiple group analyses.

Results
Myb and Cyclin E1 are coexpressed in intestinal cells and adenomas
To explore the relationship between Myb and Cyclin E1 (Ccne1), qRT-PCR analysis was performed on mouse colorectal cancer cell lines. Myb expression was high in the MC38 cells compared with the immortalized colon epithelial cell line YAMC, and this corresponded with the high levels of Ccne1 mRNA (Fig. 1A and B). Cyclin E1 protein can be expressed as different isoforms (Fig. 1C; refs. 2–4); however, the predominantly expressed isoform of Cyclin E1 in MC38 and YAMC extracts was an approximately 25- to 30-kDa LMW isoform (Fig. 1D).

Western blot analysis was also performed on small intestinal crypt lysates derived from wild-type (WT) and Myb\(^{Plt4/Plt4}\) hypomorphic mutant mice that we had previously reported to express reduced expression that corresponded to reduced Myb functional target of Myb, an in silico analysis was performed on the mouse (Supplementary Fig. S1) and human (Supplementary Fig. S2) Ccne1/CCNE1 promoters to identify potential Myb-binding elements (MBE; ref. 26). Multiple MBEs were observed throughout the Ccne1/CCNE1 promoters and these clustered within a 2-kb proximal and a 4-kb distal region upstream of the transcription start sites (Fig. 2A).

To confirm Myb occupancy on the Cyclin E1 promoter, we performed ChiP assays on colon crypts using a chicken IgY antibody raised against the mouse GST–Myb fusion protein with the DNA-binding domain deleted. As a control, we used an IgY antibody directed against glutathione S-transferase (GST). Both were affinity purified on antigen columns and have been characterized previously (14). Binding of Myb correlated with active chromatin (AcH3K27 mark) in WT crypts (Fig. 2B and C). Myb occupancy and the AcH3K27 mark were decreased in the crypts harboring the Myb\(^{Plt4}\) mutation (Fig. 2B and C). Functional cooperation between Myb and β-catenin has been documented previously (14) and we noted several potential high-affinity β-catenin/TCF-4 binding motifs within the murine Ccne1 promoter (Fig. 2A and Supplementary Fig. S1). However, we did not observe significant β-catenin occupancy on the Ccne1 promoter (Fig. 2B and C).

We next examined whether Myb had a direct functional effect on the mouse Ccne1 promoter activity. CAT reporters containing a 3.2 or 4.8-kbp region of the Ccne1 promoter (pCyclinE3.2CAT and pCyclinE4.8CAT) were cotransfected with FL, Flag-tagged Myb expression plasmid into NIH3T3 cells and assessed for reporter activity. Historically, Ccne1 regulation has been ascribed to the transcription factor E2F1 (27). We confirmed this relationship with both the pCyclinE3.2CAT and pCyclinE4.8CAT reporters showing that exogenous E2F1 can robustly activate these promoters (Fig. 2D and E).

Cotransfection of Myb with either of the Ccne1 reporters produced a characteristic Myb response (14, 28) whereby maximal Myb-induced CAT activity was achieved with 1 to 2 µg of Myb plasmid expression. In contrast, the Myb\(^{Plt4}\) hypomorphic mutant at best modestly transactivated these Ccne1 constructs (Fig. 2F and G). Using incremental amounts of Myb\(^{WT}\), an apparent parabolic response with the pCyclinE4.8CAT promoter construct became evident with the peak at a dose 10-fold lower than that observed with WT Myb (Fig. 2F). Collectively, the reporter and ChIP analyses suggested that Ccne1 is a direct and positively regulated target of Myb.

Myb\(^{Plt4}\) shows defective DNA binding and inhibits Myb\(^{WT}\)
We then investigated the mechanism impairing Myb\(^{Plt4}\) promoter binding and activation of Ccne1. Recombinant HIS-tagged Myb\(^{WT}\)–CTL and Myb\(^{Plt4}\)–CTL Myb proteins were expressed (29) and purified (Fig. 3A and B). The CTL-truncated Myb\(^{WT}\) protein retains interaction functions associated with the leucine-rich domain (LZ) and shows specific DNA-binding activity (29). The Myb\(^{WT}\) and Myb\(^{Plt4}\)–CTL Myb proteins were assessed for DNA activity in EMSA containing the mimA DNA probe (Fig. 3C and D; ref. 30). Consistent with previous studies (24), robust binding of Myb\(^{Plt4}\)–CTL to the mimA sequence was observed. However, the DNA-binding efficiency of the Myb\(^{Plt4}\)–CTL mutant was decreased by approximately 40% at the highest concentration used (Fig. 3C and D). These data suggest that the impaired Myb\(^{Plt4}\) transcriptional activity observed in
Figure 1.
Ccne1/CCNE1 expression is increased with MYB/Myb in murine and human intestinal cells and in colorectal cancer. qRT-PCR for (A) Myb and (B) Ccne1 mRNA using total RNA from the colon adenocarcinoma MC38 and immortalized intestinal YAMC cell lines. C, diagram of various Cyclin E1 isoforms reported to be expressed in tumor cells. The approximately 50kDa FL Cyclin E1 protein (E1) is cleaved by Elastase or Calpain 2 proteases to generate the LMW isoforms E12/3 (Trunk 1) and E15/6 (Trunk 2). The P21 and P27 (Cip/Kip) interaction domain and the centrosome localization sequence (CLS) are noted. D, Western blot analysis on whole cell lysates from MC38 cells, YAMC cells, NIH3T3 cells, WT small intestinal, MybPlt4/Plt4 small intestinal, and small intestinal adenomas from ApcMin/þ mice. LMW truncated Cyclin E1 is the predominant form expressed. Error bars represent mean ± SEM; *, P < 0.05; ***, P < 0.001. E and F, consecutive sections of human colon crypts and adenomas were subjected to IHC staining for Cyclin E1 and MYB. Boxed regions highlight nuclear staining for MYB and cytoplasmic and nuclear staining for Cyclin E1. G, MYB and (H) CCNE1 mRNA expression within human adenomas and matched normal crypts. Mean ± SEM; *, P < 0.05; ***, P < 0.001; ****, P < 0.0001.**
vivo (Fig. 2B, C, F, and G) may reflect a defect in DNA-binding function.

As Myb positively auto-regulates its own promoter (14, 31), reporter assays can be performed on the Myb promoter. These revealed an inability of full-length MybWT to achieve maximal auto-regulation of its own promoter in the presence of MybPlt4 mutant colonic crypts. D–G, transactivation of the Ccne1 promoter. NIH3T3 cells were cotransfected with pCyclinE3.2CAT-basic-MCSII (proximal promoter) or pCyclinE4.8CAT-basic-MCSII (distal plus proximal promoter) and pE2F-1, pEFMybFLAG or pEFMybPlt4FLAG. Error bars, mean ± SEM; *, P < 0.05.

Defective Myb function perturbs colon organoid formation and reduces Cyclin E1 expression in colon adenomas.

Previously, we have shown that reduced Ccne1 expression in MybPlt4/Plt4 small intestinal organoids correlated with defects in cell-cycle reentry, progression, and proliferation (14). We also described defects in cell-cycle progression in the colonic crypts of MybPlt4/Plt4 hypomorphic mice (15). To further our understanding of this relationship between Myb and Cyclin E1 specifically in the
colon because this is the most clinically relevant to colorectal cancer in patients, we sought to assess the impact of Myb on \( \text{Ccne1} \) expression and colonic organoid cultures (22). Myb\(^{-} \) colon organoid cultures showed both a significant reduction in \( \text{Ccne1} \) expression and a significant reduction in forming capacity and growth (Fig. 4A–C).

Because we found that defective Myb function impeded colonic organoid growth and formation, concordant with reduced \( \text{Ccne1} \) expression, we decided to examine whether Myb-mediated regulation of \( \text{Ccne1} \) expression might play a role in colorectal cancer development. We placed an emphasis on assessing whether the Myb–Cyclin E1 axis regulated intestinal epithelial cells during the transition from normal to premalignant transformation as we had previously observed that the rate of adenoma formation in \( \text{Apc}^{\text{Min/+}} \) mice was significantly reduced on a \( \text{Myb}^{+/-} \) background (32).

To do this, the levels of Cyclin E1/\( \text{Ccne1} \) expression in adenomas formed in \( \text{Apc}^{\text{Min/+}; \text{Myb}^{+/-}} \) mice were investigated. Although Cyclin E1/\( \text{Ccne1} \) expression was observed in adenomas from the \( \text{Apc}^{\text{Min/+}} \) mouse (Fig. 4D), in the context of the Myb heterozygous knockout (KO) background (\( \text{Apc}^{\text{Min/+}; \text{Myb}^{+/-}} \))
reduced Cyclin E1 IHC staining was observed (Fig. 4D). This was also confirmed at the mRNA level (Fig. 4E). Collectively, the above data underscore the regulatory relationship between Myb and Ccne1 in the colon as well as in this premalignant stage of colorectal cancer development.

Cyclin E1 knockdown effects on colorectal cancer cell proliferation and tumor growth

The above data prompted us to examine the Myb–Cyclin E1 axis further with the view that Ccne1 may also play a role in regulating proliferation in established colorectal cancer tumor cells. To this end, we sought to assess the effects of Cyclin E1 knockdown in CT26 colorectal cancer cells. CT26 expresses robust levels of Cyclin E1 and following transduction of shRNAs (KD4) directed against Ccne1 mRNA we were successful in reducing protein expression in bulk culture (data not shown) and in two single cell clones (Fig. 5A). Knockdown confirmation was determined in replicate Western blotting experiments (Fig. 5B). When these cells were propagated under growth limiting conditions (0.2% FCS) modest effects on cell growth using the MTT assay at day 4 were observed (Fig. 5C). The two CT26 Cyclin E1 knockdown clones were then assessed for their capacity to form tumors when injected subcutaneously into BALB/c mice (33). Cyclin E1 knockdown significantly reduced tumor formation particularly in the case of clone #1 (Fig. 5D). Furthermore, in clone#1, but not clone#10, spleen weights in tumor-bearing mice were also reduced (Fig. 5E and F). This is consistent with the systemic effects of high CT26 tumor burden on splenomegaly in mice.

Cyclin E1 knockdown effects on chromosome stability

One of the pleiotropic consequences of Cyclin E1 overexpression in cancer cells is the promotion of CIN. Therefore, we performed karyotype analysis of CT26 cells and found that parental CT26 cells showed highly variable ploidy (Fig. 6A). In contrast, we found that cells with Cyclin E1 knockdown had significantly stabilized cell karyotypes (Fig. 6A–C). This finding is consistent with other reports that have shown Ccne1 levels affect karyotype stability (34, 35).

MybPlt4 accelerates illness in ApcMin/+ mice but reduces intestinal adenomagenesis

We next focused our attention on investigating the consequences of defective Myb function on the initiation and progression of adenomagenesis in ApcMin/+ mice. There were three...
reasons to do this. First, we had shown that heterozygous loss of Myb slowed tumor formation in ApcMin/+ mice (32). Second, because MybPlt4/Plt4 mice are on the threshold of viability, we anticipated that this minimal level of Myb (≈40% WT function) would protect mice from developing tumors. Third, our transcriptional transactivation studies showed that MybPlt4 had an inhibitory effect on WT Myb function (Fig. 3F). It was therefore completely unexpected that ApcMin/+ mice on a MybPlt4/Plt4 background showed significantly shorter times to illness than on either an ApcMin/+ or ApcMin/+:MybPlt4/+ background (Fig. 7A). Furthermore, while both genders of ApcMin/+ mice were equally affected (Fig. 7C) male ApcMin/+:MybPlt4/Plt4 mice were significantly more affected in terms of reduced survival (Fig. 7C). As it was assumed that the basis for culling mice would be due to the consequences of adenoma formation with associated intestinal obstruction and blood loss, it was therefore surprising to find adenoma burden in the colon and small intestinal of ApcMin/+:MybPlt4/Plt4 mice was significantly reduced (Fig. 7D and E). However, this was only associated with mice being culled by 120 days compared with the other cohorts lasting to 170 days. This difference remained the case when the adenoma data for ApcMin/+:MybPlt4/Plt4 and ApcMin/+:MybPlt4/Plt4 mice were subcategorized into distal and proximal regions of the colon and small intestinal (Fig. 7F). Thus, the basis for their premature culling being due to adenomas alone seemed remote. Collectively, these data suggest that while ApcMin/+:MybPlt4/+ mice die from adenoma-associated symptoms similar to ApcMin/+ mice, ApcMin/+:MybPlt4/Plt4 mice must be succumbing to additional pathologies associated with the combination of adenomatous polyposis coli (Apc) and Myb functional loss.

Hematopoietic defects in ApcMin/+:MybPlt4/Plt4 hypomorphic mice

To investigate why ApcMin/+:MybPlt4/Plt4 mice showed reduced survival associated with reduced adenoma burden the hematopoietic system was examined. Mice suffering from hematopoietic stress commonly develop extra-medullary hematopoiesis manifested by enlarged spleens (30). However, all mice on an ApcMin/+ background had enlarged spleens and this effect was not modified in MybPlt4/Plt4 mutant, ApcMin/+:MybPlt4/Plt4 or MybPlt4/Plt4 mice (Supplementary Fig. S3A). MybPlt4/Plt4-mutant mice were found to have elevated platelet numbers on a mutant MplKO background (36) and this phenotype was recapitulated on a pure C57BL/6 background. This thrombocytosis was maintained on an ApcMin/+ background but not in ApcMin/+:MybPlt4/Plt4 mice (Supplementary Fig. S3B). As blood parameters in the MybPlt4/Plt4 mice are indistinguishable from WT mice (data not shown), it can be implied that under homeostasis that MybPlt4/Plt4 mice are essentially normal.
Red blood cell parameters in adult the WT C57BL/6 mice were consistent with other reports (37, 38). However, the defect of thrombocytosis was the most overt blood profile difference between Apc<sup>Min</sup>−/− and Apc<sup>Min</sup>−/−:Myb<sup>P44/T4</sup> mice noting that all Apc<sup>Min</sup>−/− mice remained significantly anemic by several measures such as RBC, hemoglobin (HGB), and hematocrit (HCT) levels (Supplementary Fig. S3D–S3F). This blood profile is consistent with macrocytic anemia as reported in Apc-mutant mice previously (39) but is significantly exacerbated in Apc<sup>Min</sup>−/−:Myb<sup>P44/T4</sup> mice (Supplementary Fig. S3I) where mean cell volumes were significantly evaluated. Collectively, these blood analyses suggest that while Apc<sup>Min</sup>−/−:Myb<sup>P44/T4</sup> mice require culling due to intestinal tumorigenesis, Apc<sup>Min</sup>−/−:Myb<sup>P44/T4</sup>/Plt4−/− mice rather succumb to hematopoietic failure before adenomas become a substantial clinical issue.

Overall, in the specific case of intestinal epithelial cells, Myb is clearly required for the regulation of Cyclin E1 expression in normal and transformed cells, and in turn Cyclin E1 levels are central to chromosome (in)stability. Finally, these studies make it apparent that intestinal tumorigenesis depends upon Myb-regulated Cyclin E1 expression.

**Discussion**

We have previously observed that hypomorphic Myb-mutant mouse intestinal and colon crypts showed reduced *Ccne1* expression (14, 15). These data prompted us to investigate whether Myb directly regulated *Ccne1* and to explore the concept that elevated *Ccne1* expression is central to chromosome (in)stability. Indeed this situation is evident in mice lacking Myb (15). This finding coupled with our observations that Myb<sup>P44/T4</sup> is inhibitory when at high concentrations in vitro suggests a mechanism where Myb<sup>P44/T4</sup> protein forms inactive homodimers that mask its DNA-binding activity and thus impair its transactivation function (23). Under conditions where Myb expression is rapidly induced as part of the crypt recovery process, mutant Myb<sup>P44/T4</sup> may thus have a proclivity to form inactive homodimers that exacerbate the recovery process. Indeed this situation is evident in Myb<sup>P44/T4</sup> and Myb<sup>M303V</sup> heterozygous mice following ionizing radiation (14). With radiation treatment, the response of Myb<sup>P44/T4</sup> mutant mice (~40% function) is indeed worse than that observed in heterozygous knockout mutant mice (~50% function; ref. 14). On this point, we have explored whether the Myb<sup>P44/T4</sup> mice might show a more severe blood or intestinal phenotype but have not been able to breed such mice (unpublished observations), suggesting that 20% Myb (dynein) function is incompatible with life that is consistent with other reports (41).
Myb<sup>Plt4/Plt4</sup> colon organoid cultures showed impeded growth and formation concordant with reduced Ccn1 expression in mice (15) and in small intestinal organoid cultures (14). These observations led us to examine Myb-regulated Ccn1 in the premalignant adenomatogenesis stage of colorectal cancer development. IHC and qRT-PCR analyses highlighted that when mice expressed heterozygous levels of Myb within Ap<sup>Min/+</sup> adenomas, there was a significant reduction in Cyclin E1/Ccn1. These data suggest that in the context of adenomatogenesis overexpression and/or deregulation of hyperactive Cyclin E1-associated functions may affect early tumor progression. These functions include uncontrolled cell-cycle entry, the induction of aneuploidy (42), CIN (43) and defects in the assembly of the mini chromosome maintenance complex resulting in premature mitosis entry (44, 45).

Previously, we reported a parallel increase in aneuploidy in primary and metastatic colorectal cancer with elevated Myb expression (46). Indeed activated Cyclin E1 through its promotion of CIN accelerates LOH (47) with Ap<sup>Min/+</sup> LOH having been shown to be a key event in adenoma formation in the Ap<sup>Min/+</sup> mouse (48). Indeed, we have recently shown that CIN begins immediately following the loss of the WT allele in Ap<sup>Min/+</sup> organoid cultures (49). Furthermore, we observed a decrease in chromosomal abnormalities in CT26 cells, most notably the degree of tetraploidy with Cyclin E1 knockdown. Our examination of the regulatory relationship between Myb and Cyclin E1 has
underscored the role of Myb function in the biology of Apc\textsuperscript{Min/+} mouse, and on the initiation and progression of adenomagenesis. We were intrigued by the dichotomous effect of shortened survival but reduced tumor burden throughout the gastrointestinal (GI) tract in Apc\textsuperscript{Min/+}:Myb\textsuperscript{P4/+} versus Apc\textsuperscript{Min/+}:Myb\textsuperscript{P4/-} mice. Our observations suggest the combined defects Apc\textsuperscript{Min/+} and Myb\textsuperscript{P4/+} compound to produce a severe deficit in hematopoiesis manifested more by thrombocytopenia and reduced erythropoiesis than accelerated intestinal carcinogenesis. Indeed, Apc\textsuperscript{Min/+} mice succumb to high rates of anemia that leads to culling of mice in the absence of bowel obstructions; these blood effects are more severe in Apc\textsuperscript{Min/-}:Myb\textsuperscript{P4/+} mice. In addition, Apc\textsuperscript{Min/+} mice progressively develop myelodysplasia (50). Thus, the reduced intestinal tumor burden in Apc\textsuperscript{Min/-}:Myb\textsuperscript{P4/+} mutant mice at the time of cull may simply be due to their shortening lifespan and insufficient time to develop adenomas. In contrast, Apc\textsuperscript{Min/+}:Myb\textsuperscript{P4/-} mutant mice display a reduced intestinal tumor burden in the absence of any identifiable fatal hematopoietic failure beyond those evident in Apc\textsuperscript{Min/-} mice. These data suggest that reduced intestinal adenoma formation can be attributed to the heterozygous loss of Myb function (and perhaps when Myb expression is induced—a dominant negative effect) in the intestine of Apc\textsuperscript{Min/-}:Myb\textsuperscript{P4/+} mice and highlight the role of Myb in early events driving adenoma formation. These experiments also revealed a gender difference in that male Apc\textsuperscript{Min/-}:Myb\textsuperscript{P4/-} mice had shorter life expectancy compared with female Apc\textsuperscript{Min/-}:Myb\textsuperscript{P4/+} mice that warrant future attention.

In summary, we have shown that Myb regulates Cyclin E1 expression in the GI Tract. Defective Myb impairs Cyclin E1 expression in primary epithelial cells and delays intestinal tumorigenesis. In the knowledge that Cyclin E1 drives CIN and aneuploidy, this Myb target gene may aid and abet evolutionary changes that advantage colorectal cancer. Finally, these data contribute to understanding why colorectal cancer with the highest levels of Myb are associated with the poorest patient outcomes (46).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Pereira, R.G. Ramsay, J. Malaterre
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