Defective Myb Function Ablates Cyclin E1 Expression and Perturbs Intestinal Carcinogenesis

Dane Cheasley\textsuperscript{1,2,3*}, Lloyd Pereira\textsuperscript{1*}, Shienny Sampurno\textsuperscript{1}, Oliver Sieber\textsuperscript{3}, Robert Jorissen\textsuperscript{3}, Huiling Xu\textsuperscript{1,4}, Markus Germann\textsuperscript{1}, Yan Yuqian\textsuperscript{1,4}, Robert G. Ramsay\textsuperscript{1,2,4#} and Jordane Malaterre\textsuperscript{1,4}

Author affiliations: \textsuperscript{1}Sir Peter MacCallum Oncology Department, Differentiation and Transcription Laboratory, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia and the University of Melbourne, VIC 3052, Australia. \textsuperscript{2}Latrobe Institute of Molecular Science, Department of Genetics, Latrobe University, Bundoora, VIC 3083, Australia. \textsuperscript{3}Walter and Elisa Hall Institute, Parkville, VIC 3052, Australia. \textsuperscript{4}Department of Pathology, University of Melbourne, Melbourne, VIC 3052, Australia.

Author notes: *DC and LP share first authorship

Supported by Fellowships (RGR) and Program Grants (JM, LP and RGR, #487900) from the National Health and Medical Research Council of Australia. MG is supported by a Fellowship from the Swiss Science Foundation and the Novartis Foundation, Switzerland.

Corresponding Author: # Robert G. Ramsay. Differentiation and Transcription Laboratory, Peter MacCallum Cancer Centre, East Melbourne, 3002, Victoria, AUSTRALIA, (T) 613 9656 1863, (F) 613 9656 1411, (E) rob.ramsay@petermac.org

Conflicts of interest: None declared.
Abstract

Cyclin E1 is essential for the re-entry of quiescent cells into the cell cycle. When hypomorphic mutant Myb mice (Myb\textsuperscript{Plt4}) 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 (CRC) is responsible for Cyclin E1-driven tumor growth. Here it was found that Myb/MYB and Ccne1/CCNE1 expression 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. By contrast, Myb\textsuperscript{Plt4} served as a dominant negative factor that inhibited wild type Myb and this was not apparently compensated for by the transcription factor E2F1 in intestinal epithelial cells. Myb\textsuperscript{Plt4/Plt4} mice died prematurely on an Apc\textsuperscript{Min/+} background associated with hematopoietic defects including a myelodysplasia, nevertheless Apc\textsuperscript{Min/+} mice were protected from intestinal tumorigenesis when crossed to Myb\textsuperscript{Plt4/+} mice. Knockdown (KD) of Cyclin E1 transcript in murine CRC 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.
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 G0/G1 to S-phase, through G2 and lastly to initiate cell mitosis. Whilst the full length (FL) CyclinE1 isoform, termed E11, is detected in normal and malignant cells (1), over-expression of up to five low molecular weight (LMW) CyclinE1 isoforms has been reported in malignant tissues (2-4). Amino-terminal truncated isoforms E12/3 and E15/6 arise by proteolytic cleavage of the full length protein by Elastase (5) or Calpain 2 (6) proteases. The isoform E14 is produced from an alternative translation start site (5). LMW CyclinE1 truncations do not abolish the interaction of CyclinE1 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 CyclinE1 are enzymatically hyperactive (5).

De-regulation of CyclinE1 correlates with poor patient prognosis for those with breast cancer (9), colorectal cancer (CRC) (3) and inflammatory bowel disease (10). Additionally, over-expression of the LMW CyclinE1 isoform tracks with an increase in CDK2 activity in CRC (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 CyclinE1 (Ccne1) is regulated by the proto-oncogene and transcription factor Myb. We previously showed that cell cycle re-entry, progression and proliferation appear retarded within the colonic crypts of Myb<sup>Plt4/Plt4</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 over-expression and decreases 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 over-expression is a feature of CRC (20) and here we demonstrate that Myb is a transactivator of Ccne1. We observe co-expression of Myb and CyclinE1 protein within intestinal cells and adenomas and show that CyclinE1 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 knock-down (KD) of CyclinE1 in established CRC cells stabilized chromosome ploidy and reduced CRC cell growth in vitro and tumor formation in mice.
Materials and Methods

Mice

$Myb^{Plt4/Plt4}$ hypomorphic mutant mice were generated at the Walter and Eliza Hall Institute and their hematopoietic and colon defects described (15, 21). $Apc^{Min/+}$, $Apc^{Min/+}:Myb^{+/−}$ and $Apc^{Min/+}:Myb^{Plt4/Plt4}$ mice were maintained on a C57BL/6 background under pathogen-free conditions according to the Peter MacCallum Cancer Centre’s (PMCC) Animal Experimental Ethics Committee guidelines.

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/TRCN0000077777) were purchased from Sigma. Single cell clones were selected in the presence of puromycin, expanded and stable KD (and control vector PLKO.1-PURO) clones reconfirmed by qRT-PCR and western blotting. CT26 tumor cells ($0.5 \times 10^6$) were subcutaneously injected into syngeneic BALB/c mice in the right lower flank. Tumors were measured using an electronic caliper. CRC cell line authenticity was evaluated by RNAseq profiling and their capacity to form tumors in syngeneic mice.

Chromosome Spreads

CT26 cells were seeded overnight at $10^5$ cells. Colchicine (10 µg/ml, Sigma) was added to the media for 1hr and the cells harvested. After KCl (75mM) treatment and fixation with methanol:glacial acetic acid (3:1), cells were dropped onto polylysine slides and allowed to dry
before quick diff staining and mounting. Chromosome numbers were scored using a BX51 Zeiss microscope.

**Western blotting**

Whole cell lysates were generated by incubating primary intestinal crypt cells and cell lines in 0.5% TX-100 lysis buffer followed by nuclei shearing using a 29 gauge needle. Proteins were separated on 4-12% Bis-Tris pre-cast gels (NuPAGE) and transferred to a PVDF membrane. Membranes were blocked in 10% non-fat skim milk powder and probed with antibodies (Supplementary Table 1). Membranes were probed with HRP secondary antibodies (Bio-Rad) and proteins detected using ECL.

**Histology**

Crypts and adenomas were fixed in 10% normal buffered formalin, embedded in paraffin and 2μm 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 PFA4% overnight and the remaining piece frozen down at -80°C for subsequent RNA extraction. All human primary CRC specimens and matched normal tissues were collected at the Royal Melbourne Hospital, Australia. All patients gave informed consent for their de-identified 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 macro-dissected 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 mM Tris-buffer and 1 mM EDTA (pH 8) in a pressure cooker (Biocare Decloker) at 125°C for 3 min. 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 1). Slides were counterstained with hematoxylin dehydrated in ethanol.

**Chromatin immunoprecipitation assays and transfection of NIH3T3 cells**

Protocols have been described in detail previously (14). Primers used in the ChIP analyses are described (Supplementary Table 2).

**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 (Supplementary Table 2). pCMV E2F-1 was a gift of Dr Patrick. Humbert (PMCC). Protocols for CAT reporter assays and q-RT-PCR analysis have been described previously (14). Primers used for qRT-PCR are described (Supplementary Table 2).

**Murine intestinal and adenoma organoid cultures**

Colon organoids were generated using previously established protocols (22).

**Electrophoretic mobility shift assay (EMSA)**

EMSAs were performed with a $^{32}$P-labeled mimA DNA probe and 1μl of HIS-tagged Myb CTL protein (23) in reactions containing 10mM Tris pH 8.0, 50mM NaCl, 2.5% glycerol, 1mM EDTA, 0.25mg/ml BSA and 1μg/ml poly (dl-dC) as previously described (24). Binding reactions were resolved on 0.5 x TBE 6% non-denaturing PAGE (29:1) gels (24). Gels were dried and quantified by PhosphorImager analysis using Image Quant Software.

**Quantitation and Statistics**
Statistical analysis was calculated using Graphpad Prism version 5.0 (Graphpad Software Inc, USA). 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 employed for multiple group analyses.
Results

Myb and CyclinE1 are co-expressed in intestinal cells and adenomas

To explore the relationship between Myb and CyclinE1 (Ccne1), qRT-PCR analysis was performed on mouse CRC cell lines. Myb expression was high in the MC38 cells compared to the immortalised colon epithelial cell line YAMC, and this corresponded with the high levels of Ccne1 mRNA (Fig. 1A and B). CyclinE1 protein can be expressed as different isoforms (2-4) (Fig. 1C) however, the predominantly expressed isoform of CyclinE1 in MC38 and YAMC extracts was the ~25-30kDa LMW isoform (Fig. 1D).

Western blot analysis was also performed on small intestinal (SI) crypt lysates derived from WT and MybPlt4/Plt4 hypomorphic mutant mice that we had previously reported to express reduced Ccne1 mRNA levels and display colonic crypt defects (15). The efficacy of the antibody in detecting both the FL and LMW forms of CyclinE1 was confirmed in NIH3T3 cells (Fig. 1D). Intestinal epithelial cells showed a lower level of CyclinE1 expression that corresponded to reduced Myb function in MybPlt4/Plt4 derived SI crypts. Again the LMW CyclinE1 was the predominant isoform expressed. Western blotting on ApcMin/+ intestinal adenomas (transformed epithelial cells) derived from 4 different ApcMin/+ mice showed a concomitant increase in Myb and LMW CyclinE1 expression (Fig. 1D).

IHC was employed to confirm the presence of MYB and CyclinE1 in normal human colon at the protein level. We observed that MYB expression was predominantly nuclear as expected, while CyclinE1 expression was mostly cytoplasmic as well as nuclear (Fig. 1E). This subcellular localization was recapitulated in human adenomas (Fig. 1F) and is consistent with subcellular expression patterns reported for other tumor cells (25). This analysis was then extended to a larger set of primary human samples where we focused on cancer precursors, colonic adenomas.
and found that MYB and CCNE1 mRNA levels were elevated compared to matched-normal crypts (Fig. 1G and H).

**Myb activates the Ccne1 promoter**

The above observations suggested that Myb might regulate Ccne1 expression. To test whether Ccne1 was a direct transcriptional target of Myb, an *in silico* analysis was performed on the mouse (Supplementary Fig. 1) and human (Supplementary Fig. 2) Ccne1/CCNE1 promoters to identify potential Myb binding elements (MBEs) (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 CyclinE1 promoter we performed ChIP assays on colonic crypts using a chicken IgY antibody raised against the mouse GST-Myb fusion protein with the DNA binding domain deleted. As a control we employed a IgY antibody directed against 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 harbouring the MybPlt4 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. 1). 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 3.2kbp or 4.8kbp region of the Ccne1 promoter (pCyclinE3.2CAT and pCyclinE4.8CAT) were co-transfected with full length, 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).

Co-transfection of Myb with either of the Ccne1 reporters produced a characteristic Myb response (14, 28) whereby maximal Myb-induced CAT activity was achieved with 1-2µg of Myb plasmid expression. By contrast, the Mybₚₛ hypomorphic mutant at best modestly transactivated these Ccne1 constructs (Fig. 2F and G). Using incremental amounts of Mybₚ₄ 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.

**Mybₚ₄ shows defective DNA binding and inhibits Myb⁸WT**

We then investigated the mechanism impairing Mybₚ₄ promoter binding and activation of Ccne1. Recombinant HIS-tagged Myb⁸WT-CTL and Mybₚ₄-CTL Myb proteins were expressed (29) and purified (Fig. 3A and B). The CTL truncated Myb⁸WT protein retains interaction functions associated with the leucine-rich domain (LZ) and shows specific DNA binding activity (29). The Myb⁸WT and Mybₚ₄ CTL-Myb proteins were assessed for DNA activity in electrophoretic mobility shift assay (EMSA) containing the mimA DNA probe (30) (Fig. 3C and D). Consistent with previous studies (24) robust binding of Myb⁸WT-CTL to the mimA sequence was observed. However, the DNA binding efficiency of the Mybₚ₄-CTL mutant was decreased by ~40% at the highest concentration used (Fig. 3C and D). These data suggest that the impaired Mybₚ₄ transcriptional activity observed *in 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 Myb WT to achieve maximal auto-regulation of its own promoter in the presence of MybPlt4 (Fig. 3E). A similar inhibitory effect was also observed by MybPlt4 on the 4.8kb Ccne1 promoter (Fig. 3F).

**Defective Myb function perturbs colon organoid formation and reduces CyclinE1 expression in colon adenomas**

Previously, we have shown that reduced Ccne1 expression in MybPlt4/Plt4 SI organoids correlated with defects in cell cycle re-entry, 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 CyclinE1 specifically in the colon because this is the most clinically relevant to CRC in patients, we sought to assess the impact of Myb on Ccne1 expression and colon organoid cultures (22). MybPlt4/Plt4 colon organoid cultures showed both a significant reduction in Ccne1 expression and a significant reduction in forming capacity and growth (Fig. 4A, B and C).

Because we found that defective Myb function impeded colonic organoid growth and formation, concordant with reduced Ccne1 expression, we decided to examine whether Myb-mediated regulation of Ccne1 expression might play a role in CRC development. We placed an emphasis on assessing whether the Myb-CyclinE1 axis regulated intestinal epithelial cells during the transition from normal to pre-malignant transformation as we had previously observed that the rate of adenoma formation in Apc\textsuperscript{Min/+} mice was significantly reduced on a Myb+/- background (32). To do this the levels of CyclinE1/Ccne1 expression in adenomas formed in Apc\textsuperscript{Min/+}:Myb+/- mice were investigated. While CyclinE1/Ccne1 expression was observed in adenomas from the Apc\textsuperscript{Min/+} mouse (Fig. 4D), in the context of the Myb heterozygous KO...
background (ApCMin+/+;Myb+/−) reduced CyclinE1 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 pre-malignant stage of CRC development.

**CyclinE1 KD effects on CRC cell proliferation and tumor growth**

The above data prompted us to examine the Myb-CyclinE1 axis further with the view that Ccne1 may also play a role in regulating proliferation in established CRC tumor cells. To this end we sought to assess the effects of CyclinE1 knockdown (KD) in CT26 CRC cells. CT26 expresses robust levels of CyclinE1 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). KD 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 CyclinE1 KD clones were then assessed for their capacity to form tumors when injected subcutaneously into BALB/c mice (33). CyclinE1 KD 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.

**CyclinE1 KD effects on chromosome stability**

One of the pleiotropic consequences of CyclinE1 over-expression in cancer cells is the promotion of chromosomal instability. Therefore we performed karyotype analysis of CT26 cells and found that parental CT26 cells showed highly variable ploidy (Fig. 6A). By contrast, we found that cells with CyclinE1 KD 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).

**Myb\(^{Plt4}\) accelerates illness in Apc\(^{Min/+}\) 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 Apc\(^{Min/+}\) mice. There were three reasons to do this. Firstly, we had shown that heterozygous loss of Myb slowed tumor formation in Apc\(^{Min/+}\) mice (32). Second, because Myb\(^{Plt4/Plt4}\) 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 Myb\(^{Plt4}\) had an inhibitory effect on WT Myb function (Fig. 3F).

It was therefore completely unexpected that Apc\(^{Min/+}\) mice on a Myb\(^{Plt4/Plt4}\) background showed significantly shorter times to illness than on either an Apc\(^{Min/+}\) or Apc\(^{Min/+}:Myb^{Plt4/+}\) background (Fig. 7A). Furthermore, while both genders of Apc\(^{Min/+}\) mice were equally affected (Fig. 7B) male Apc\(^{Min/+}:Myb^{Plt4/Plt4}\) 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 SI of Apc\(^{Min/+}:Myb^{Plt4/Plt4}\) mice was significantly reduced (Fig. 7D and E). However, this was only associated with mice being culled by 120 days compared to the other cohorts lasting to 170 days. This difference remained the case when the adenoma data for Apc\(^{Min/+}:Myb^{Plt4/Plt4}\) and Apc\(^{Min/+}:Myb^{Plt4/+}\) mice were subcategorised into distal and proximal regions of the colon and SI (Fig. 7F). Thus the basis for their premature culling being due to adenomas alone seemed remote. Collectively, these data suggest that while Apc\(^{Min/+}:Myb^{Plt4/+}\) mice die from adenoma-associated symptoms similar to Apc\(^{Min/+}\) mice,
Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/Plt4} mice must be succumbing to additional pathologies associated with the combination of Apc and Myb functional loss.

**Hematopoietic defects in Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/Plt4} hypomorphic mice**

To investigate why Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/Plt4} mice showed reduced survival associated with reduced adenoma burden the hematopoietic system was examined. Mice suffering hematopoietic stress commonly develop extra-medullary hematopoiesis manifested by enlarged spleens (30). However, all mice on an Apc\textsuperscript{Min/+} background had enlarged spleens and this effect was not modified in Myb\textsuperscript{Plt4/Plt4} mutant, Apc\textsuperscript{Min/+} or Myb\textsuperscript{Plt4/+} mice (Supplementary Fig. 3A). Myb\textsuperscript{Plt4/Plt4} mutant mice were found to have elevated platelet numbers on a mutant Mpl\textsuperscript{KO} background (36) and this phenotype was recapitulated on a pure C57BL/6 background. This thrombocytosis was maintained on an Apc\textsuperscript{Min/+} background but not in Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/+} mice (Supplementary Fig. 3B). As blood parameters in the Myb\textsuperscript{Plt4/+} mice are indistinguishable from WT mice (data not shown) it can be implied that under homeostasis that Myb\textsuperscript{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\textsuperscript{Min/+} and Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/Plt4} mice noting that all Apc\textsuperscript{Min/+} mice remained significantly anemic by several measures such as RBC, hemoglobin (HGB) and hematocrit (HCT) levels (Supplementary Fig. 3D, E and F). This blood profile is consistent with macrocytic anemia as reported in Apc mutant mice previously (39) but is significantly exacerbated in Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/Plt4} mice (Supplementary Fig. 3I) where mean cell volumes were significantly evaluated. Collectively, these blood analyses suggest that while Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/+} mice require culling due to intestinal tumor-associated symptoms, Apc\textsuperscript{Min/+}:Myb\textsuperscript{Plt4/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 CyclinE1 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 Myb in CRC (40) may be responsible for promoting Ccne1/CyclinE1 expression that drives cell proliferation and tumor growth. When Myb was elevated in mouse and human CRC cells and adenomas Ccne1 expression was increased. We also observed that in the highly proliferative cells of the intestinal crypts from the SI and colon, that the LMW CyclinE1 isoform was the predominant form expressed. These data suggest that LMW CyclinE1 expression is not restricted to transformed cells.

Promoter analysis showed that the Ccne1 promoter contained several MBEs, suggesting that Ccne1 might be a direct Myb target. Our results support this view with Myb transactivating Ccne1-CAT reporters and ChIPs showing enrichment of Myb at the Ccne1 promoter. The Myb
\textsuperscript{Plt4} mutation reduced Myb transactivation of Ccne1-CAT reporters and its binding at the Ccne1 promoter. The increase in acetylated Histone H3K27 in WT crypts compared to Myb
\textsuperscript{Plt4/Plt4} crypts is also consistent with increased Ccne1 promoter activity in the presence of functional Myb. Wnt activation, via Apc loss, is central to CRC and Myb co-operates with activated β-catenin to drive some CRC genes (14). To determine if this occurs with Ccne1 we examined the Ccne1 promoter for the presence of β-catenin, but found that Myb regulated Ccne1 independently of canonical Wnt signaling. In view of these data we propose that Myb is responsible for Ccne1 regulation in the intestine. Furthermore the E2F1 transcription factor does not appear to compensate for defective Myb function in intestinal epithelial cells of Myb
\textsuperscript{Plt4/Plt4} crypts under homeostasis as well as under severe stress (14, 15).
We have also shown the DNA binding activity of Myb<sup>Plt4</sup> protein to be defective. It has been proposed that the Plt4 mutation, that substitutes an aspartic acid for a valine, changes the leucine rich region-mediated interaction between two Myb proteins, causing effects similar to that observed in the Myb DBD (Plt3) and TAD mutant (M303V) mice (15). This finding coupled with our observations that Myb<sup>Plt4</sup> is inhibitory when at high concentrations in vitro suggests a mechanism where Myb<sup>Plt4</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>Plt4</sup> may thus have a proclivity to form inactive homodimers that exacerbate the recovery process. Indeed this situation is evident in Myb<sup>Plt4/Plt4</sup> and Myb<sup>Plt4/+</sup> heterozygous mice following ionizing radiation (14). With radiation treatment the response of Myb<sup>Plt4/Plt4</sup> mutant mice (~40% function) is indeed worse than that observed in heterozygous KO mutant mice (~50% function) (14). On this point we have explored whether the Myb<sup>Plt4/KO</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 (dys-)function is incompatible with life which is consistent with other reports (41).

Myb<sup>Plt4/Plt4</sup> colon organoid cultures showed impeded growth and formation concordant with reduced Ccne1 expression in mice (15) and in small intestinal organoid cultures (14). These observations led us to examine Myb regulated Ccne1 in the pre-malignant adenomatogenesis stage of CRC development. IHC and qRT-PCR analyses highlighted that when mice expressed heterozygous levels of Myb within Apc<sup>Min/+</sup> adenomas there was a significant reduction in CyclinE1/Ccne1. These data suggest that in the context of adenomatogenesis over-expression and/or de-regulation of hyperactive CyclinE1-associated functions may impact on early tumor progression. These functions include uncontrolled cell cycle entry, the induction of aneuploidy
(42), chromosomal instability (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 CRC with elevated Myb expression (46). Indeed activated CyclinE1 through its promotion of CIN accelerates LOH (47) with $Apc^{wt}$ LOH having been shown to be a key event in adenoma formation in the $Apc^{Min/+}$ mouse (48). Indeed, we have recently shown that CIN begins immediately following the loss of the WT allele in $Apc^{Min/+}$ organoid cultures (49). Furthermore, we observed a decrease in chromosomal abnormalities in CT26 cells, most notably the degree of tetraploidy with CyclinE1 KD. Our examination of the regulatory relationship between Myb and CyclinE1 has underscored the role of Myb function in the biology of $Apc^{Min/+}$ 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 GI tract in $Apc^{Min/+}:Myb^{Plt4/Plt4}$ versus $Apc^{Min/+}:Myb^{Plt4/+}$ mice. Our observations suggest the combined defects $Apc^{Min/+}$ and $Myb^{Plt4/Plt4}$ compound to produce a severe deficit in hematopoiesis manifested more by thrombocytosis and reduced erythropoiesis than accelerated intestinal carcinogenesis. Indeed, $Apc^{Min/+}$ mice succumb to high rates of anaemia which leads to culling of mice in the absence of bowel obstructions; these blood effects are more severe in $Apc^{Min/+}:Myb^{Plt4/Plt4}$ mice. Additionally, $Apc^{Min/+}$ mice progressively develop myelodysplasia (50). Thus the reduced intestinal tumor burden in $Apc^{Min/+}:Myb^{Plt4/Plt4}$ mutant mice at the time of cull may simply be due to their shortening lifespan and insufficient time to develop adenomas. By, contrast, $Apc^{Min/+}:Myb^{Plt4/+}$ mutant mice display a reduced intestinal tumor burden in the absence of any identifiable fatal hematopoietic failure beyond those evident in $Apc^{Min/+}$ 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 $Apc^{Min/+}:Myb^{Plt4/+}$ mice and highlight the role of Myb in early events driving adenoma formation. These experiments also revealed a gender differences in that male $Apc^{Min/+}:Myb^{Plt4/Plt4}$ mice had shorter life expectancy compared to female $Apc^{Min/+}:Myb^{Plt4/Plt4}$ mice which warrant future attention.

In summary, we have shown that Myb regulates CyclinE1 expression in the GI and during tumorigenesis. These observations underscore the regulatory relationship between $Myb$ and $CyclinE1$ in the GI Tract. Defective Myb impairs CyclinE1 expression in primary epithelial cells and delays intestinal tumorigenesis. In the knowledge that CyclinE1 drives CIN and aneuploidy, this Myb target gene may aid and abet evolutionary changes that advantage CRC. Finally, these data contribute to understanding why CRC with the highest levels of Myb are associated with the poorest patient outcomes (46).
Acknowledgments

This work has been supported by the National Health and Medical Research Council of Australia including a Fellowship to RGR. The authors wish to thank Michael Christie for assistance with the processing of human CRC specimens, the PMCC microscopy team and animal facilities staff. We are particularly grateful to Dr Ryan Cross for carefully reading and advice in the preparation of the manuscript.
**Figure Legends**

**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 CyclinE1 isoforms reported to be expressed in tumor cells. The ~50kDA FL CyclinE1 protein (El) is cleaved by Elastase or Calpain 2 proteases to generate the LMW isoforms El2/3 (Trunk 1) and El5/6 (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 SI, Myb<sup>Plt4/Plt4</sup> SI and SI adenomas from *Apc<sup>Min</sup>/+* mice. LMW truncated CyclinE1 is the predominant form expressed. Error bars represent mean ±SEM, ***, P <0.001; * P <0.05. (E and F) Consecutive sections of human colon crypts and adenomas were subjected to IHC staining for CyclinE1 and MYB. Boxed regions highlight nuclear staining for MYB and cytoplasmic and nuclear staining for CyclinE1. (G) *MYB* and (H) *CCNE1* mRNA expression within human adenomas and matched normal crypts. Mean ± SEM; P-*, <0.05; ***< 0.001; **** <0.0001).

**Figure 2.** Myb binds and activates the *Ccne1* promoter. (A) Schematic of the *Ccne1* promoter depicting putative Myb, TCF-4, and E2F-1 binding consensus motifs. Primers sets used for ChIP (black arrows). (B and C) ChIP of the *Ccne1* promoter in Myb<sup>WT</sup> and Myb<sup>Plt4/Plt4</sup> mutant colonic crypts. (D-G) Transactivation of the *Ccne1* promoter. NIH3T3 cells were co-transfected with pCyclinE3.2CAT-basic-MCSII (proximal promoter) or pCyclinE4.8CAT-basic-MCSII (distal plus proximal promoter) and pE2F-1, pEFMybFLAG or pEFMybPlt4FLAG. Error bars represent mean ± SEM, * P, <0.05.
**Figure 3.** Myb$^{Plt4}$ is defective for DNA binding. (A) Structure of full-length WT Myb and truncated CTL-Myb proteins. The Plt4 mutation (#) substitutes an aspartic acid (D) for a valine (V) residue at amino acid position 384 within the leucine zipper (LZ); DBD, DNA binding domain; NRD, Negative regulatory domain; R1-3, Repeats. (B) WTCTL-Myb or CTL-Myb$^{Plt4}$ were expressed in *E. coli* as previously described (23), Talon resin-purified and assessed by Coomassie staining. (C) CTL proteins were employed in EMSA reactions with increasing amounts of protein and a stable amount of *mimA* probe (D) Relative binding was evaluated across triplicate EMSAs by Phosphor-Image analysis. CTL-Myb$^{Plt4}$ exhibits decreased binding activity. (E) Myb-mediated transactivation of the *Myb* promoter is impeded by Myb$^{Plt4}$. NIH3T3 cells were transfected with pMybCAT-basic-MCSII and ratios of 1:0, 1:3, 1:1, 3:1 and 0:1 Myb$^{Plt4}$: Myb$^{WT}$. (F) Reporter assays were performed on the *Ccne1* promoter using varying ratios of Myb$^{Plt4}$: Myb$^{WT}$ as in (E). Error bars represent mean ± SEM, ** P, <0.01; ***, P < 0.001.

**Figure 4.** Normal Myb function is required for *Ccne1* expression within intestinal adenomas and colon organoid cultures. (A) Total RNA was extracted from colon organoids (*WT* and *Myb$^{Plt4/Plt4}$*) at day 10 and subjected to *Ccne1* qRT-PCR. (B and C) Organoids were counted at day 10 and expressed as a percentage of organoids formed per crypt nest. (D) Consecutive sections of *Apc$^{Min/+}$* and *Apc$^{Min/+}$:Myb$^{+/+}$* adenomas were IHC stained for CyclinE1 and Myb. (E) qRT-PCR performed on total RNA extracted from *WT* crypts, *Apc$^{Min/+}$* adenomas and *Apc$^{Min/+}$:Myb$^{+/+}$* adenomas shows that *Ccne1* expression is increased in adenomas compared to WT crypts and is reduced in the presence of 50% WT Myb.
**Figure 5.** CyclinE1 KD effects CRC tumor growth. (A) Clones of transduced CT26 cells were assessed for KD of CyclinE1 by Western blot. CyclinE1 KD was most substantial in shRNA clones #1 and #10. (B) Multiple western blots were performed to ensure stable CyclinE1 KD. (C) CT26 clones #1 and #10 were assessed *in vitro* for growth by MTT assay compared to CT26 cells transduced with the vector control. (D) CT26 cells clones #1 and #10 as well as vector control were injected sub-cutaneously into the flanks of mice. Cohorts of mice were scored over time. (E) The average spleen weights of tumor bearing mice were assessed. (F) Spleens and tumors from mice injected with clone #1or vector control at time of cull are shown. Mean ± SEM, * P, <0.05; ***< 0.001; **** <0.0001).

**Figure 6.** Stabilization of ploidy in CRC cells following CyclinE1 KD. (A) CT26 cells show highly variable chromosome numbers which are partially and significantly stabilised in CyclinE1 KD #clone 1, and clone #10 respectively. (B) Hyper tetraploidy is significantly reduced in CyclinE1 KD #clone 1 (C) and partially reduced in CyclinE1 KD clone #10. *P > 0.05; ** p >0.01, Fisher’s exact test.

**Figure 7.** Myb\(^{Plt4}\) accelerates illness in Apc\(^{Min/+}\) mice but reduces intestinal adenomagenesis. (A) Survival experiments were conducted to assess the consequences of defective Myb function on the initiation and progression of adenomatogenesis in Apc\(^{Min/+}\) mice. Analysis revealed that Apc\(^{Min/+}\) :Myb\(^{Plt4/Plt4}\) mutant mice had a significantly reduced life expectancy. ****, P < 0.0001. (B) Survival experiments showed that life expectancy is equally affected for male and female Apc\(^{Min/+}\) mice and (C) that Apc\(^{Min/+}\) :Myb\(^{Plt4/Plt4}\) male mice were extraordinarily affected. *, P = 0.0263. Quantitation of tumor burden in the GI tract of Myb\(^{Plt4}\) mice on an Apc\(^{Min/+}\) background.
(D); **, P = 0.0028; ***, P = 0.0009. (E); **, P = 0.0028; ****, P < 0.0001. (F) Subcategorization of tumor burden in the distal and proximal regions of the colon versus small intestine (SI) in $Apc^{Min/+}:Myb^{Plt4/+}$ and $Apc^{Min/+}:Myb^{Plt4/Plt4}$ mice.
References
16. Srivastava SK, Bhardwaj A, Singh S, Arora S, McClellan S, Grizzle WE, et al. Myb overexpression overrides androgen depletion-induced cell cycle arrest and apoptosis in


### Molecular Cancer Research

#### Defective Myb Function Ablates Cyclin E1 Expression and Perturbs Intestinal Carcinogenesis

Dane Cheasley, Lloyd Pereira, Shienny Sampurno, et al.

*Mol Cancer Res* Published OnlineFirst May 1, 2015.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-15-0014</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mcr.aacrjournals.org/content/suppl/2015/05/02/1541-7786.MCR-15-0014.DC1">http://mcr.aacrjournals.org/content/suppl/2015/05/02/1541-7786.MCR-15-0014.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

---

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.