INTRODUCTION

Aberrant activation of the WNT pathway (Adenomatous polyposis coli (APC) or β-catenin mutation) is the pre-eminent early event in colorectal cancers (CRC). Nevertheless, parallel activation of other oncogenes including MYB occurs commonly and is reasonably thought to participate in CRC. Furthermore, Myb and β-catenin cooperate to regulate the CRC genes MYC and PTGS2 (COX2) along with the intestinal stem cell (ISC) gene, LGR5. Elevated MYB transcripts are a common feature of CRC and high MYB protein is a poor-prognosis indicator. Others have identified MYB and WNT-target gene, AXIN2 as among nine essential and differentially expressed genes in colon cancer. However, although MYB is on occasion amplified in CRC it is rarely (if ever) found to have mutations in coding regions but rather in transcriptional regulatory regions. Thus, it remains unclear how MYB specifically participates in colorectal carcinogenesis.

The intestine is a rapidly self-renewing tissue maintained by ISCs. The colonic epithelium is composed of the absorptive and secretory lineages continuously renewed by rapidly cycling intestinal progenitor cells (IPC) generated from ISC. ISC are targets for transformation. Two kinds of ISCs have been proposed; crypt basal cells (CBC) and another, radiosensitive +4 ISCs. These share the expression of Musashi-1, Pten and Sox-9 but can be discriminated by specific markers such as Lgr5 and Olfm4 for CBCs or Hopx, Bmi1 and mTert for +4 ISCs. These two populations of ISCs are phylogenetically related and can give rise to each other. IPCs also exhibit a high degree of phenotypic plasticity in emergency situations and can revert to a state of ‘stem-ness’.

Deregulation of ISC signaling pathways are evident in intestinal cancer exemplified in the Wnt-driven ApcMin/+ mouse model, which recapitulates both genetic (Apc mutations) and environmental aspects (inflammation) of human CRC. Importantly, activation of the Wnt pathway in either Lgr5+ or Bmi1+ ISCs but mostly not IPCs has been shown to initiate intestinal cancer. Here we sought to understand why patients with high MYB have worse outcomes and we demonstrate the direct involvement of Myb in activating ISC signaling pathways and loss of the cell cycle regulator p27. Importantly, this work revealed the effect of Myb in the ISC compartment, co-operation with tumor suppressor genes (TSGs) and loss of the cell cycle regulator p27 activates ISC self-renewal, confers constitutive Myb activity throughout the intestinal epithelium, and loss of cell cycle regulator p27.

RESULTS

We show that Myb is frequently elevated in CRC and that those patients with tumors that have the higher level of Myb had...
a commensurate poorer prognosis. This remains the case for early stage CRC (pT3 or pT4N0-M0) tumors (Millet et al., submitted). Loss of Myb function leads to reduced IPC proliferation in the colon of adult mice. To test whether sustained Myb expression is pathogenic we enforced Myb activity, rather than its level of expression, in the gastrointestinal tract by taking advantage of the gastrointestinal tract-epithelial-specific expression pattern conferred by the Gpa33 promoter.20 A Gpa33-driven expression construct that fuses mouse Myb to a mutant ERα ligand-binding domain (Figures 1a and c) was engineered to generate transgenic mice. Transgene DNA sequence integrity was confirmed as well as its tight negative control and sensitive induction by 4-hydroxy-tamoxifen (4OHT) (Supplementary Figure 2). Insertion of the several transgene copies was demonstrated by Southern blotting (Figure 1c), whereas MybER fusion protein expression was confirmed by western blotting with anti-ERα antibody (Figure 1d) as well as anti-Myb antibodies in colonic crypts to levels comparable to untreated mice (Supplementary Figure 2a and c). Of six founders, two lines were used in experiments (Supplementary Figure 2). Insertion of the several transgene copies was demonstrated by Southern blotting (Figure 1c), whereas MybER fusion protein expression was confirmed by western blotting with anti-ERα antibody (Figure 1d) as well as anti-Myb antibodies in colonic crypts to levels comparable to endogenous Myb found in colon cancer cell line, CT26 but is, as expected absent in WT crypts (Figure 1e).

**In vitro** activation of MybER increases IPC function

To understand the cell intrinsic effect of MybER-activation in primary colon cells, IPC were studied in vitro using the organoid assay22 (Figure 1f). MybER-activation led to an increase in organoids formed per plated crypt nest and greater growth as measured by MTT assays (Figure 1g). Then to test whether activated Myb is important for organoid maintenance of superior colon organoid propagation MybER organoids were treated with 4OHT for the entire period of the primary culture before dissociation into single cells. Dissociated organoids were then challenged to grow in the presence or absence of 4OHT to test whether 4OHT-withdrawal affects secondary organoid formation. Figure 1g shows that organoids require sustained MybER-activation to achieve superior growth and that the effect of MybER-activation is reversible. We then tested the promoter occupancy of a well-characterized Myb target gene Lgr5 by MybER fusion protein in colonic crypts isolated from MybER mice exposed to tamoxifen or in untreated mice. Single-cell suspensions were cross-linked and chromatin immunoprecipitation assays were then performed using anti-ERα antibody whereby the binding of MybER to Lgr5 promoter was confirmed and this was further found to be tamoxifen-dependent (Figure 1h).

To dissect the effect of MybER-activation on different crypt cell compartments we conducted gene expression analysis in various cell population based on the activity of aldehyde dehydrogenase. Colonic stem cells have been shown to be characterized by high-aldehyde dehydrogenase activity. We analyzed the expression of cell cycle gene (CyclinE1; Ccne1) and ISC gene Lgr5, the TSG Pten and the pro-angiogenic gene Vegfa (Figure 2a) in populations with high (AldeHi), low (AldeLo) and no (AldeNEG) aldehyde dehydrogenase activity. Predicted Myb target genes, Lgr5, Ccne1 and Pten (unpublished data) were elevated in various populations of cells. Notably Lgr5 was elevated in both AldeHi and AldeLo population in contrast to Vegfa and Ccne1, which were elevated in AldeHi population and Pten, which was significantly induced only in the AldeLo population.

To evaluate the role of Myb-activation during tumor initiation and progression we established organoid cultures from early stage adenomas in non-symptomatic MybER:Apcmin/+ mice (Supplementary Figure 2). Our results demonstrate that activated MybER is required at least during the first 48 h of initiation of culture to significantly increase organoid numbers (Supplementary Figure 2a–c). Sustained MybER-activation was also required to achieve optimal growth, whereas 4OHT-withdrawal abrogated the enhanced growth of MybER adenoma organoids (Supplementary Figure 2a and c). These data suggest that Myb is required to support both maximum adenoma initiation and progression and that the effects of MybER-activation are reversible.

By using adenomas from MybER:Apcmin/+ mice we were also able to establish cultures of relatively uniform cells that typically express the Wnt and Myb target gene, Lgr5 and ask whether modulating MybER activity with 4-OHT affects Lgr5 expression. Data in Figure 2b confirm that this strategy shows Lgr5 expression is subject to the ON/OFF control by MybER activity even in the context of activated Wnt signaling.

Activated Myb in vivo does not change IPC homeostasis unless p27 is lost

**In vivo** effects of sustained Myb activity on crypt morphology was investigated along with proliferation. We found that the crypt morphology was largely unaltered following MybER-activation (Supplementary Figure 3a). Furthermore, IPC proliferation was similar to that prior to tamoxifen-treatment (Supplementary Figure 3b). Two weeks of Tamoxifen produced modest but not significant effects on proliferation (Supplementary Figure 3a, d and e) when PCNA+ cells were scored according to crypt location or total number.

In view of such modest effects of activated MybER alone we postulated that homeostatic mechanisms might be restricting the **in vivo** effect of elevated Myb activity particularly in view of the increased Ccne1 expression in the colon of all MybER transgenic lines (Supplementary Figure 3c). As Pten mRNA expression was elevated in the AldeLo progenitor/precursor population (Figure 2a) we decided to explore Pten expression at the protein level in vivo within crypts finding significantly higher expression in MybER mice treated with tamoxifen compared with similarly treated controls (Supplementary Figure 4). These observations are consistent with reduced Pten expression observation in Myb-hypomorphic mutant colons (unpublished observations). As Pten is a negative regulator of the cell cycle and is molecularly considered upstream of p2723,24 and that p27 in turn is a negative regulator of Cdk/CyclinE1 activity25 we hypothesized that WT levels of p27 might mask effects of MybER in vivo. Accordingly, we established compound MybER:p27+/− mice. As expected we found p27 was reduced in both p27+/− and p27−/− MybER colons. We also found that its expression was strongly induced in discrete population of cells located at the bottom of the crypts in MybER colon (Supplementary Figure 5a).

Although crypts of p27−/− mice show increased proliferation, this was not observed in p27+/− mice19 where IPC proliferation was comparable to WT mice (Supplementary Figure 6b, d and e); however, analysis of proliferation in p27−/−:MybER colons using immunohistochemistry (IHC) showed a significant increase in the total number of PCNA-positive cells, more remained in cycle up to crypt positions 14 compared with tamoxifen-treated WT mice (Supplementary Figure 6b, d and e). Thus, we hypothesized that p27 loss may increase the number of CyclinE1-positive cells. To confirm reduced p27 expression in p27−/−:MybER mouse we performed p27 IHC. We found that p27 is prominently expressed in the CPC zone (Supplementary Figure 5a). Quantitation of CyclinE1 IHC (Supplementary Figure 5b) showed that the number of CyclinE1-positive cells was not increased by MybER-activation alone. By contrast, when MybER was activated in p27−/− mice their number was significantly increased.

Activated Myb accelerates AOM-driven tumorigenesis

In view of this association between elevated Myb and CRC patient outcome we treated MybER mice with tamoxifen for an extended period of time (7 months) and at this point started to detect sick mice that once examined revealed elevated aberrant crypt foci (ACF) and a modest increase in colon tumors
The cooperation we had previously found between Myb and activated β-catenin in driving Myc, Cox-2 and Lgr5 expression in the GI prompted us to generate compound MybER:Apcmin/+ mice. Cohorts of these mice were exposed to tamoxifen but their disease-free survival was not different to Apcmin/+ mice alone on tamoxifen (Figure 3c). As we have found that inflammation appears to be responsible for an increase Myb expression during early events of CRC, for example, ACF and adenoma formation (Pereira et al., in press) prior to detectable mutated Myb regulatory sequences we decided to activate Myb in the initiation phase of CRC. MybER mice were treated with tamoxifen over a period of 8 weeks, six of which also employed weekly colon carcinogen azoxymethane (AOM) injections26 (Figure 3d). Hence, MybER was activated for 2 weeks prior to AOM-treatment to allow the expansion of the ISC pool. To demonstrate this we have employed the Aldefluor assay
following tamoxifen-treatment for 2 weeks, to identify colonic ISC. Such cells have been shown to act as tumor-initiating precursor cells in the colon and to be responsible for the transition from colitis to cancer.\textsuperscript{27} As predicted, we found that this population was expanded (Figure 3e).

Subsequent exposure of tamoxifen-treated mice to AOM markedly accelerated colon tumorigenesis in MybER mice on either WT or \(\text{Apc}^{\text{Min/}+}\) backgrounds (Figure 3f) and led to more frequent, as well as larger tumors, on an \(\text{Apc}^{\text{Min/}+}\) background (Figure 3g). As Myb overexpression or \(\text{Apc}\) mutation both drive proliferation and block cyto-differentiation in colon cells,\textsuperscript{28} we evaluated PCNA staining and indirectly explored the state of \(\text{Myb}\) transgenic compared with non-transgenic mice on an \(\text{Apc}\text{Min/}+\text{Myc}\) background.

Frequent, as well as larger tumors, on an \(\text{Apc}\text{Min/}+\) background resulted in a larger class of \(\text{ACF}\) compared with \(\text{ACF}\) with \(\text{Myb}\text{Min/}+\) alone (Supplementary Figure 7b), confirming cooperation between \(\text{Myb}\) and the Wnt pathway in CRC initiation. The number and size of tumors were also assessed in these animals (Supplementary Figure 7d and e). Although changes were not significant the size of tumors generated on an \(\text{Apc}\text{Min/}+\) background following \(\text{Myb}\) activation trended to being larger.

Activated \(\text{Myb}\) in \textit{vivo} reproduces human CRC clinical symptoms In view of the increased morbidity of the \(\text{Myb}\text{ER}:\text{Apc}^{\text{Min/}+}\) and \(\text{Myb}\text{ER}\) mice compared with their \(\text{Apc}^{\text{Min/}+}\) and WT counterparts we decided to take a CRC-clinical perspective in evaluating symptoms.

**Figure 2.** Molecular analysis of WT and \textit{MybER} AldeHi, AldeLo and AldeNEG colonic crypt cell populations shows differential intestinal stem cell gene expression increased following \(\text{Myb}\) activation and is reversible in adenomas. (a) Gene expression analyses were performed using qRT-PCR on RNA extracted from WT and \textit{MybER} FACS sorted colonic crypt cells based on ALDH activity. \textit{Myb} target gene \(\text{Lgr5}\) mRNA expression was elevated in both \(\text{AldeHi}\) and \(\text{AldeLo}\) population. By contrast, \(\text{mRNA}\) of predicted target genes \(\text{Vegfa}\) and \(\text{Ccnd1}\), which were only significantly elevated in \(\text{AldeHi}\) population while \(\text{Pten}\) mRNA, was significantly elevated in the \(\text{AldeLo}\) population. (b) ON/OFF control of \textit{Myb} target \(\text{Lgr5}\) by \textit{MybER} following addition and withdrawal of 4OHT in \textit{MybER}:\textit{Apc}\text{Min/}+\text{C} derived adenoma organoids cultured for 6 days were treated with 4OHT for 24 h before harvesting or were washed twice in DMEM/F12 and re-incubated in 4OHT-free adenoma media for 48 h before harvesting. Data presented for individual samples (\(n>4\)) are mean ± s.e.m.; \(*P<0.05\) two-sided \(t\)-test.

**Figure 3.** \textit{Myb}\text{ER}-activation accelerates CRC initiation and progression of AOM-driven CRC. (a) ACF in tamoxifen-treated \textit{MybER} mice compared with WT mice on Tamoxifen at 7 months (\(n=3\)). (b) Tumor number is slightly increased in \textit{MybER} mice (\(n=8\)). (c) Activation of \textit{MybER} in mice on an \(\text{Apc}^{\text{Min/}+}\) background does not influence survival. (d) Diagram depicting the pre-treatment and continual access to tamoxifen in chow (8 weeks) and 2 weeks later the initiation of weekly AOM injections used to test the effect of \textit{Myb}-induction in the initiation phase of CRC. (e) Pre-treatment by tamoxifen increases the percentage of cells with high aldehyde dehydrogenase activity identified by FACS (\(n>5\)). (f) Survival experiments were conducted to determine whether \textit{MybER}-activation on a WT or \(\text{Apc}^{\text{Min/}+}\) (\(n>10\)) background translates into poorer survival outcome owing to AOM-induced CRC. Individual mice were harvested when they reached ethical end points defined by either bleeding from the anus, anaemia (pale feet), hunching, severe diarrhea, prolapsed anus or body weight loss > 20%. Survival analysis revealed that \textit{MybER}-activation on a WT and \(\text{Apc}^{\text{Min/}+}\) backgrounds significantly accelerated the initiation and progression of disease and reduced the life expectancy of mice after treatment. (g) Tumors generated on an \(\text{Apc}^{\text{Min/}+}\) background in presence of \textit{MybER} were significantly larger compared with tumors arising in \(\text{Apc}^{\text{Min/}+}\) mice and \textit{MybER}-activation did not alter the number of tumors. (h) Tumors derived from all groups were sectioned and analyzed by IHC for PCNA to assess cell proliferation and for goblet cell differentiation (I–J) using periodic acid staining (PAS). Data presented for individual samples with means ± s.e.m.; \(*P<0.05\), \(**P<0.01\). Survival curves were analyzed using log-rank (Mantel–Cox) Test.
that collectively precipitate the culling of mice following AOM-induced carcinogenesis. In both comparisons, the presence of MybER was associated with a more severe morbidity (Figures 5a and b) except when MybER was activated on an Apc\textsuperscript{Min/-} background where the symptom of anus bleeding shifted toward another symptom, hunching. Hunching relates to
intestinal occlusion by tumors and this is globally increased on the ApcMin/+ background given that tumors also developed in the small intestines (SI) and are more numerous. Hunching was exacerbated by MybER-activation in ApcMin/+:MybER mice probably owing to the fact that there is a significant increase in tumor size in the colon in these mice (Figure 3g). There was no change of distribution between colon and SI in ApcMin/+:MybER compared with ApcMin/+ mice. Mice on an ApcMin/+ background also showed increased anaemia. As mice suffering anemia commonly initiate splenomegaly we measured spleen weights of all mice at the time of cull and found they were significantly larger in MybER mice on an ApcMin/+ background (Figure 5c).

Because of the more severe anemia and anal bleeding in MybER mice we examined colon lesions for evidence of increased vascularization. We consistently observed adenomas with excessive hemorrhage in MybER mice and hypoxia (as shown by hypoxia inducible factor-1α IHC in Figure 5f and Supplementary Figure 8a and elevated expression of Myb and hypoxia inducible factor-1α target gene Vegfa31,32 (Figure 5g). The amount of CD31-positive endothelium and PCNA staining were not increased (Supplementary Figure 8). When the abundance of red blood cells was evaluated in adenomas/carcinomas from MybER mice they were significantly larger in MybER mice on an ApcMin/+ background (Figure 5c).

Because of the more severe anemia and anal bleeding in MybER mice we examined colon lesions for evidence of increased vascularization. We consistently observed adenomas with excessive hemorrhage in MybER mice and hypoxia (as shown by hypoxia inducible factor-1α IHC in Figure 5f and Supplementary Figure 8a and elevated expression of Myb and hypoxia inducible factor-1α target gene Vegfa31,32 (Figure 5g). The amount of CD31-positive endothelium and PCNA staining were not increased (Supplementary Figure 8). When the abundance of red blood cells was evaluated in adenomas/carcinomas from MybER mice they were significantly larger compared with WT mice (Figures 5h and i). These data suggest that Myb-activation influences blood vessel leakage, leading to the clinical symptoms that precipitate culling of mice in AOM studies. By contrast, hypoxia-inducible factor-1α and red blood cell leakage in ApcMin/+ and ApcMin/+:MybER tumors was not significantly different.

**DISCUSSION**

High Myb expression is a robust predictor of CRC patient outcome and by employing a novel gastrointestinal tract transgenic mouse model, we have been able to reveal a parallel malignant role of elevated Myb observed in human CRC2,4,5 not only intrinsic to cancer cells but also in the tumor microenvironment promoting hypoxia and leakiness of the tumor vasculature.

The malignant potential of MYB in CRC has been ascribed previously1 but has not been formally demonstrated. With the very recent revelation that MYB-NFIB fusion genes are pathogenic in adenoid cystic carcinoma33–36 often in the context of few other promalignant genomic changes, expands the spectrum of solid tumors where MYB shows oncogenic association. Our transgenic mice used a fusion construct that essentially truncates the Myb protein. This was necessary to allow the ER ligand-binding domain to be tightly modulated by Tamoxifen. With specific reference to CRC recent insights into how this oncogenic potential might be driven, and in what cells, come from studies such as those that show that loss of Myb function decreased ISC activity and proliferation plus improved survival of ApcMin/+ mice.2,3 Although loss of Myb function was effective in instilling a loss of ISC activity by itself this is the first occasion where the effect of Myb hyperactivation has been evaluated. First, the full effects of hyperactivated-Myb on ISC function under homeostasis was only achieved, when at the same time, one allele of CyclinE1-Cdk inhibiting p27 gene was lost.25 This was consistent with our previous work suggesting that p27 complete loss resulted in increased proliferation in colonic crypt IPCs.19 We also suggest that the effect of p27 haploinsufficiency on intestinal homeostasis

**Figure 4.** MybER-activation alters the molecular expression profile of tumors. qRT-PCR was used to analyse expression of MybER, CcneE1, Cond1, Myc, Lgr5, Olfm4, Bmi1, Aldh1 and Pten in tumors. Data presented for individual samples (n = 6) with means ± s.e.m.; *P < 0.05, **P < 0.01; ANOVA.
is achieved by allowing CyclinE1 to exert its pro-proliferative action on IPCs. Although MybER-induced tumorigenicity in mice by itself required extended periods of time (~6–7 months), it is markedly increased with AOM-treatment and on an Apcmin/+ background. These collective observations clearly demonstrate that Myb needs to act in concert with other factors in keeping with the generally accepted view that CRC requires multiple oncogenic ‘hits’.37

Apcmin/+:MybER compound mice were not without complexities as the Min mutation does progressively compromise the hematological and immunological competency, which is very likely to accelerate the progressive decline of mice carrying tumors evident in 80-day-old mice and to modulate the aetiology of clinical symptoms such as blood loss and anaemia.38–40

Accordingly, the use of the intestinal-specific activation of Myb alone in the tumor induction phase (first ~60 days) in the presence of AOM-treatment and with the knowledge that induced-MybER expands the ISC compartment may explain the shortened time to cull of MybER mice compared with WT littermates (160 vs 273 days, P < 0.01). By contrast in mice on an

**Figure 5.** MybER-activation promotes CRC-associated clinical symptoms. (a) AOM-treated mice on a WT or (b) Apcmin/+ background were euthanized owing to a spectrum of symptoms. (c) Splenomegaly was significantly higher in Apcmin/+:MybER mice (grey zone indicates normal spleen weights in age-matched WT mice). (d) The distribution of tumors between small intestines and colon was similar in AOM treated Apcmin/+:MybER and Apcmin/+ mice. (e) Tumors arising on a MybER background were consistently redder indicative of an increase in red blood cells (RBC) (red arrows show red tumors). (f) The tumors were also significantly more hypoxic as shown by HIF1α IHC and (g) Vegfa mRNA expression was significantly higher in mice on a MybER background. (h–i) Although vascularization was not found to increase in MybER mice (see Supplementary Figure 8) the amount of RBCs in tumors on a WT background was most apparently increased (red arrows). Data presented for individual samples (n > 5) with means ± s.e.m.; *P < 0.05, two-sided t-test.
Apc\(^{min/+}\) background, mice when treated with AOM require culling on average at 95 days and only slightly (but significantly) earlier, when MybER is activated (average at 90 days).

Interestingly, some mice required euthanizing much earlier in the Apc\(^{min/+}\)-MybER cohort than the Apc\(^{min/+}\) alone cohort. The comorbidities that arise because of systemic effects of the Min mutation highlight the need to investigate ACF and tumor burden at specific times following AOM-treatment particularly when the presence of SI adenomas inherent to this mouse genotype compromise the health of Apc\(^{min/+}\) mice.

Using combinations of mice with TSG defects we observed that the Pten/p27 axis might be responsible for keeping Myb activity in-check presumably via CyclinE1/Cdk2 inhibition. However, although p27 monoallelic-loss increases ISC proliferation as seen in extended PCNA staining in colonic crypts, this did not influence MybER oncogenic potential. Thus, it is rather unlikely that Pten/p27 influences the ability of Myb to promote ISC properties in adenomas. On the other hand, Myb clearly acts in concert with Apc\(^{min/+}\) to enhance ISC activity and gene expression in normal and tumor tissue, a property ultimately responsible for adenoma formation.31

In this study, we found that Myb-activation co-operates with mutational events induced by AOM to faithfully reproduce human clinical symptoms of CRC better than found in Apc\(^{min/+}\) mice. Myb-hyperactivation also leads to an increase in VEGFA expression associated with an increase in hemorrhaging tumors as well as hypoxia and red blood cell leakage into surrounding stroma. This leakage we speculate is due to VEGFα-driven vessel permeabilization.3,27,42,43 In this context VEGFα was positively correlated with MybER-induction and this observation was consistent with RNAseq data obtained from human CRC correlated with MybER-induction and this observation was consistent with VEGFA in CRC metastasis augments clinical management.46

Permeablization.3,27,42,43 In this context hypoxia and red blood cell leakage into surrounding stroma. Further gene expression changes and function consequences are mapped-out in Figure 6. A working model of MybER in inducing genes and initiating ACFs plus subsequent progression to AOM-induced adenoma is mapped-out in Figure 6.

In conclusion, we report the generation of the Apc\(^{min/+}\)-MybER transgenic mice that exacerbates CRC initiation and reproduces aspects of human CRC pathology. Myb co-operates with the loss of TSG Apc where both alleles need to be affected, whereas our studies demonstrate another instance where p27 haploinsufficiency unleashes aspects of the pro-proliferative potential of Myb. This is consistent with the manner in which p27 seems to act as a tumor progression rheostat47 but this alone is not sufficient for accelerating CRC. Here we demonstrate that Myb confers a malignant phenotype to cancer cells and we provide understanding why high MYB equates to poor prognosis and discriminates patients who are likely to do poorly and relapse from patients who fare better. These data are in accordance with our previous reporting that elevated Myb is a feature of metastatic CRC.46 Several MYB target genes serve as therapeutic targets, for example, BCL-2, VEGFA, COX-2 and GRP-78, placing Myb at the hub for the regulation of a range of pro-malignant genes. Thus, MYB represents an ‘Achilles heel’ in CRC and we have shown that Myb can be targeted immunologically in the setting of mouse CRC.48,49 raising the prospect of considering this strategy in the human disease.

**MATERIALS AND METHODS**

**Generation of MybER transgenic mice**

Complementary DNA encoding the MybERT2 fusion protein was inserted in to a described expression construct encompassing a 4.1 kb promoter sequence of the murine Gpa33 gene.23 The Gpa33-MybERT2 fragment was excised and microinjected into the male pronuclei of fertilized mouse oocytes (CBA/CAH/C57BL/6). Injected embryos were transferred to pseudo-pregnant mice. The transgenic lines obtained were intercrossed with C57BL/6 females.

**Southern blot analysis and PCR genotyping**

Ten μg of genomic DNA was extracted from MybER or WT liver using DNA easy Kit (Qiagen, Chadstone, Victoria, Australia) and was digested using EcoR I. Following precipitation the DNA was suspended in 10 μl resolved on a 0.8% agarose gel and capillary transferred onto Gene screen+ hybridization transfer paper. Membranes were prehybridized for 30 min at 42 °C in ULTRAhyb buffer (AMBIOn, Scoursesby, Victoria, Australia) and hybridized overnight at 42 °C with [32P] labeled probes. Probes were generated from restriction enzyme digestion of the MybERT2 plasmid; gel purified and labeled using the DECAprime II kit (AMBIOn). For routine genotyping 50 ng of genomic tail DNA was obtained and the MybER transgene was amplified using HotStar-Taq DNA polymerase (Qiagen). MybER primers for genotyping were:- forward 5'ggagctggtggtctgtgcttga tgc-3' and reverse 5'catcgaagctctactgaagggtctgg-3'.

**Western blotting**

Soluble protein was extracted from mouse colon and colon adenomas using radioimmunoprecipitation assay buffer (25 mM HEPES pH8, 300 mM KCl, 1 mM EDTA, 0.015% sodium dodecyl sulphate, 0.015% sodium deoxycholate, 1% Triton X, Complete Protease Inhibitor cocktail). Lysates were incubated on ice for 30 min and then mechanically lysed using a dounce homogenizer. Lysates were subsequently centrifuged at 14 k rpm, 4°C for 30 min and the soluble protein extracts were resolved on 4–12% NuPAGE MOPS gels (Invitrogen, Scorsesby, Victoria, Australia) and transferred to polyvinylidene difluoride membrane. Membranes were probed with anti-estrogen receptor antibody (TE111.5D11; Thermo Scientific, Scorsesby, Victoria, Australia) and anti-Myc, p27, Mab 6.2.50 Membranes were probed with mouse horseradish peroxidase secondary antibodies (Bio-Rad, Gladesville, New South Wales, Australia) and developed by enhanced chemiluminescence or with alkaline phosphatase secondary antibody plus Nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt substrate (Roche, Castle Hill, New South Wales, Australia). Chromatin immunoprecipitation assays

Mice were treated with Tamoxifen chow for a week prior to crypt extraction and cross linking. Chromatin immunoprecipitation assays were performed on the Lgr5 promoter as described previously.5 Anti-estrogen receptor antibody (TE111.5D11; Thermo Scientific) was used to chromatin immunoprecipitation the MybER fusion protein.

**Figure 6.** Model describing the effects of MybER-activation on crypt biology and adenoma gene expression as initiating events for CRC. Pink cells identify differentiated goblet cells that are reduced during aberrant crypt focus (ACF) formation following MybER-activation. Further gene expression changes and function consequences are established following progression from ACFs to adenomas.
Oligonucleotide sequences for quantitative real-time PCR
Quantitative real-time PCR reactions were conducted using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Scoresby, Victoria, Australia). For one reaction, 8 μl complementary DNA (1:10 dilution) was combined with 10 μl SYBR Green PCR Master Mix (Applied Biosystems) and 200 ng primers (Geneworks, Thebarton, South Australia, Australia) and amplified using temperatures of 50 °C for 2 min and 95 °C for 15 min. These initial steps were followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. RNA was extracted using Trizol (Invitrogen) and treated with RNasea-free DNaseI (1 U μl−1; Promega, Madison, WI, USA). Superscript III Reverse Transcriptase (Invitrogen) was used for reverse transcription and mRNA synthesis. Gene expression was normalized to β2-microglobulin. Primers sequences were designed against the complementary DNA sequence of target genes and spanned exon–exon junctions.

| Primers (mouse) | Reverse | Forward |
|-----------------|---------|---------|
| Beta2-microglobulin | 5′-GTCCTGGCCGTGGCC-3′ | 5′-TACCCCAACCTGACAGACT-3′ |
| MybER | 5′-TTGATAAGCTTGTACGA | 5′-AGGAGCCATTACCAACACAG-3′ |
| Cyclin E1 | 5′-GGCGACCATCATCACTC | 5′-TTTCTGACCATATCATCT-3′ |
| Bmi1 | 5′-TCTCCTCCTCCTGTGCTCAATC | 5′-AATTAGTCCCCAGGGTCTC |
| Pten | 5′-CTCTGTCTTGGGAAATT | 5′-GAGCAGCTGTGATTAAGTTCAGTGTC-3′ |
| Aldh1 | 5′-AAAGAACCTTCCCTACACCAT | 5′-GAGCAGCTTCCCTGCAGG |
| Lgr5 | 5′-TATCTGGCCGGGGTGGAT | 5′-CAAGGAGCTATGCTCAGCTG |
| Cyclin D1 | 5′-GAGCTGTGAGAGGAGGA | 5′-GCTGTGTAATTCGAGGAGAGCA |
| Myc | 5′-CTGGAGACAGACAGCTC | 5′-GCTGTGTAATTCGAGGAGAGCA |
| Olfactomedin4 | 5′-GAGCTCTCTCTCTC | 5′-GCCCCCTTCCCTAC-3′ |
| Hif1a | 5′-GCTTCACAGACAAATG | 5′-GAATTAGTCCCCAGGGTCTC |
| Vegfa | 5′-AGGCTGTGTAACACAGAT | 5′-GTGCTGGCTTTGGTGA |

IHCs

Mice were culled and the colons were cut open in cold phosphate-buffered saline (PBS) and fixed overnight in neutral buffered formalin at ambient temperature. For CD31 (Pharmingen, North Ryde, New South Wales, Australia) staining samples were fixed in 4% paraformaldehyde at 4 °C overnight. Colons were processed for paraffin embedding and sectioning at 4 μm. Periodic Acid Schiff (PAS) reagent stain and IHC for PCNA (1:1000 dilution) were performed using high power magnification. PCNA, Periodic Acid Schiff’s reagent stain and CD31 staining on tumor samples were quantified using Metamorph software (Universal Imaging, Sunnydale, CA, USA). Statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Quantitation and statistical analysis

The number and location of PCNA+ cells were recorded in 10–20 crypts under high power magnification. PCNA, Periodic Acid Schiff’s reagent stain and CD31 staining on tumor samples were quantified using Metamorph software (Universal Imaging, Sunnydale, CA, USA). Statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank the Peter MacCallum Histology and Microscopy departments and animal facility as well as the NHMRC Senior Research Fellowship Scheme (RR, ME) and Program Grant scheme (RGR, JM and ME).

REFERENCES

1. Ramsay RG, Gonda TJ. MYB function in normal and cancer cells. Nat Rev Cancer 2008; 8: 523–534.
2. Ciznadija D, Tothill R, Waterman ML, Zhao L, Huynh D, Yu RM et al. Intestinal adenoma formation and MYC activation are regulated by cooperation between MYB and Wnt signaling. Cell Death Differ 2009; 16: 1530–1538.
3. Cheasley D, Pereira L, Lightowler S, Vincan E, Malaterre J, Ramsay RG. Myb controls intestinal stem cell genes and self-renewal. Stem Cells 2011; 29: 2042–2050.
4. Hugo H, Cures A, Saraweeha N, Drabsch Y, Purcell D, Mantamadiotis T et al. Mutations in the MYB intron I regulatory sequence increase transcription in colon cancers. Genes Chromosomes Cancer 2006; 45: 1143–1154.
5. Thompson MA, Flegg R, Westin EH, Ramsay RG. Microsatellite deletions in the c-myb transcriptional attenuator region associated with over-expression in colon tumour cell lines. Oncogene 1997; 14: 1715–1723.
6. Bicocchi A, Benassi B, D’Agnano I, D’Angelo C, Buglioni S, Mottolese M et al. c-Myc and Bcl-x overexpression predicts poor prognosis in colorectal cancer: clinical and experimental findings. Am J Pathol 2001; 158: 1289–1299.
7. Cheung HW, Cowley G3, Weir BA, Boehm JS, Rusin S, Scott JA et al. Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. Proc Natl Acad Sci USA 2011; 108: 12372–12377.
8. Rothenberg ME, Nusse Y, Kalisky T, Lee JJ, Dolan C, van den Born M et al. Identification of a CRIT+ c-KIT+ colon cancer cell population that supports tumor growth. Cell 2012; 149: 1195–1205 e1196.
9. Barker N, Ridgway RA, van ES, van den Wetering M, Begthel H, van den Born M et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 2009; 457: 608–611.
10. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. Ill. Entero-endocrine cells. Am J Anat 1974; 141: 503–519.
11. Potten CS, Kovacs L, Hamilton E. Continuous labelling studies on mouse skin and intestine. Cell Tissue Kinet 1974; 7: 271–283.
12. Potten CS. Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. Nature 1977; 269: 518–521.
Myb promotes intestinal cancer
J Malaterre et al

13. Uma S. Intestinal stem cells. Current Gastroenterol Rep 2010; 12: 340–348.
14. Lin SA, Barker N. Gastrointestinal stem cells in self-renewal and cancer. J Gastroenterol 2011; 46: 1039–1055.
15. Takeda N, Jain R, LeBoeuf MR, Wang Q, Lu MM, Epstein JA. Interconversion between intestinal stem cell populations in distinct niches. Science 2011; 334: 1420–1424.
16. van Es JH, Sato T, van de Wetering M, Lyubimova A, Yee Nee AN, Gregoireff A et al. Dil1(+) secretory progenitor cells revert to stem cells upon crypt damage. Nat Cell Biol 2012; 14: 1099–1104.
17. Ramsay RG, Barton AL, Gonda TJ. Targeting c-Myb expression in human disease. Expert Opin Ther Targets 2003; 7: 235–248.
18. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature 2012; 491: 254–258.
19. Malaterre J, Carpinelli M, Ernst M, Alexander W, Cooke M, Sutton S et al. c-Myb is required for progenitor cell homeostasis in colonic crypts. Proc Natl Acad Sci USA 2007; 104: 3829–3834.
20. Flentjar N, Chu PY, Ng AY, Johnstone CN, Heath JK, Ernst M et al. TGF-betaR1 rescues development of small intestinal epithelial cells in Eif3-deficient mice. Gastroenterology 2007; 132: 1410–1419.
21. Corbett TH, Griswold Jr DF, Roberts BJ, Peckham JC, Schabel Jr FM. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res 1975; 35: 2434–2439.
22. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett’s epithelium. Gastroenterology 2011; 141: 1762–1772.
23. Gottschalk AR, Basila D, Wong M, Dean NM, Brandts CH, Stokoe D et al. p27kip1 is required for PTEN-induced G1 growth arrest. Cancer Res 2001; 61: 2105–2111.
24. Tsutsui S, Iinoue H, Yasuda K, Suzuki K, Tahara K, Higashi H et al. Inactivation of PTEN is associated with a low p27kip1 protein expression in breast cancer. Cancer 2005; 104: 2048–2053.
25. Tshilais J, Kapusta L, Slingerland J. The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. Annu Rev Med 1999; 50: 401–423.
26. Altemani A, Costa AF, Montalli VA, Mosqueda-Taylor A, Paes de Almeida O, Ramsay RG, Ciznadija D, Sicurella C, Reyes N, Mitchelhill K, Darcy PK et al. The prognostic significance of Myb protein and downstream target genes in salivary adenoid cystic carcinoma. Cancer Biother Radiopharm 2009; 24: 227–236.
27. Bell D, Roberts D, Karpowicz M, Hanna EY, Weber RS, El-Naggar AK. Clinical significance of Myb protein and downstream target genes in salivary adenoid cystic carcinoma. Cancer Biother Radiopharm 2011; 12: 569–573.
28. You S, Ohmori M, Pena MM, Nassri B, Quilton J, Al-Assad ZA et al. Developmental abnormalities in multiple proliferative tissues of ApcMin/+ mice. Int J Exp Pathol 2006; 87: 227–236.
29. Coletta PL, Muller AM, Jones EA, Muhl B, Holwess S, Clarke D et al. Lymphodepletion in the ApcMin/+ mouse model of intestinal tumorigenesis. Blood 2004; 103: 1050–1058.
30. von Holstein SL, Fehr A, Persson M, Therkildsen MJ, Prause JU, Heegaard S et al. Adenoid cystic carcinoma of the lacrimal gland: MYB gene activation, genomic imbalances, and clinical characteristics. Ophthalmol 2013.
31. Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. Vascul Pharmacol 2002; 39: 225–237.
32. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF. Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis 2008; 11: 109–119.
33. Ouyang DL, Chen JJ, Getzenberg RH, Schoen RE. Noninvasive testing for colonic cancer. J Clin Gastroenterol 2005; 39: S21–S35.
34. Mitani Y, Li J, Rao PH, Zhao YJ, Bell D, Lippman SM et al. A clinical signature of altered cyclin-dependent kinase (CDK) and truncated forms of murine c-myb proteins. Oncogene Res 2001; 10: 437–444.
Minerva Access is the Institutional Repository of The University of Melbourne

Author(s):
Malaterre, J; Pereira, L; Putoczki, T; Millen, R; Paquet-Fifield, S; Germann, M; Liu, J; Cheasley, D; Sampurno, S; Stacker, SA; Achen, MG; Ward, RL; Waring, P; Mantamadiotis, T; Ernst, M; Ramsay, RG

Title:
Intestinal-specific activatable Myb initiates colon tumorigenesis in mice

Date:
2016-05-12

Citation:
Malaterre, J., Pereira, L., Putoczki, T., Millen, R., Paquet-Fifield, S., Germann, M., Liu, J., Cheasley, D., Sampurno, S., Stacker, S. A., Achen, M. G., Ward, R. L., Waring, P., Mantamadiotis, T., Ernst, M. & Ramsay, R. G. (2016). Intestinal-specific activatable Myb initiates colon tumorigenesis in mice. ONCOGENE, 35 (19), pp.2475-2484. https://doi.org/10.1038/onc.2015.305.

Persistent Link:
http://hdl.handle.net/11343/127982

File Description:
Published version

License:
CC BY-NC-SA