BMI1 and MEL18 Promote Colitis-Associated Cancer in Mice via REG3B and STAT3

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BACKGROUND & AIMS: Polycomb group proteins are epigenetic factors that silence gene expression; they are dysregulated in cancer cells and contribute to carcinogenesis by unclear mechanisms. We investigated whether BMI1 proto-oncogene, polycomb ring finger (BMI1), and polycomb group ring finger 2 (PCGF2, also called MEL18) are involved in the initiation and progression of colitis-associated cancer (CAC) in mice. METHODS: We generated mice containing floxed alleles of Bmi1 and/or Mel18 and/or Reg3b using the villin-Cre promoter (called Bmi1ΔIEC, Mel18ΔIEC, DKO, and TKO mice). We also disrupted Bmi1 and/or Mel18 specifically in intestinal epithelial cells (IECs) using the villin-CreERT2-inducible promoter. CAC was induced in cre-negative littermate mice (control) and mice with conditional disruption of Bmi1 and/or Mel18 by intraperitoneal injection of azoxymethane (AOM) followed by addition of dextran sulfate sodium (DSS) to drinking water. Colon tissues were collected from mice and analyzed by histology and immunoblots; IECs were isolated and used in cDNA microarray analyses. RESULTS: Following administration of AOM and DSS, DKO mice developed significantly fewer polyps than control, Bmi1ΔIEC, Mel18ΔIEC, Reg3bΔIEC, or TKO mice. Adenomas in the colons of DKO mice were low-grade dysplasias, whereas adenomas in control, Bmi1ΔIEC, Mel18ΔIEC, Reg3bΔIEC, or TKO mice were high-grade dysplasias with aggressive invasion of the muscularis mucosa. Disruption of Bmi1 and Mel18 (DKO mice) during late stages of carcinogenesis significantly reduced the numbers of large adenomas and the load of total adenomas, reduced proliferation, and increased apoptosis in colon tissues. IECs isolated from DKO mice after AOM and DSS administration had increased expression of Reg3b compared with control, Bmi1ΔIEC, or Mel18ΔIEC mice. Expression of REG3B was sufficient to inhibit cytokine-induced activation of STAT3 in IECs. The human REG3β protein, the functional counterpart of mouse REG3B, inhibited STAT3 activity in human 293T cells, and its expression level in colorectal tumors correlated inversely with pSTAT3 level and survival times of patients. CONCLUSIONS: BMI1 and MEL18 contribute to the development of CAC in mice by promoting proliferation and reducing apoptosis via suppressing expression of Reg3b. REG3B negatively regulates cytokine-induced activation of STAT3 in colon epithelial cells. This pathway might be targeted in patients with colitis to reduce carcinogenesis.

Keywords: PcG; Colon Cancer; Ulcerative Colitis; PAP.

Colitis-associated cancer (CAC) is a subtype of colorectal cancer that is commonly preceded by clinically detectable inflammatory bowel disease, such as Crohn’s disease or ulcerative colitis. CAC is difficult to treat and has high mortality.1,3 The mouse model of CAC has provided important insights into inflammation-associated tumorigenesis. Inflammation is associated with many types of cancers and may play a causative role in tumor initiation and metastasis.3 Several major signaling pathways through which inflammation promotes tumor incidence and size have been identified. IKKβ-dependent activation of NF-κB in pre-malignant epithelial cells prevents apoptosis to facilitate tumor formation.4 Two key cytokines are IL-6 and IL-11, which activate their respective receptors, followed by phosphorylation and activation of STAT3 in malignant cells to promote their proliferation and survival.5–9 Activation of the paracrine or autocrine STAT3 regulatory loop appears to be a major driver in various types of cancers. In pancreatic ductal adenocarcinoma and lung adenocarcinoma, cytokines activate STAT3 in an autocrine manner to promote proliferation and survival of tumor cells.10–12 Therefore, STAT3 may serve as a signaling node that connects autonomous, proto-oncogenic stimuli with environmental inflammatory signals to initiate inflammation-associated tumorigenesis.

Abbreviations used in this paper: AOM, azoxymethane; BMI1, BMI1 proto-oncogene, polycomb ring finger; BrdU, 5-bromo-2-deoxyuridine; CAC, colitis-associated cancer; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; DSS, dextran sulfate sodium; IEC, intestinal epithelial cell; IP, intraperitoneal; JAK, janus kinase; KO, knockout; MEL18, polycomb group ring finger 2; PBS, phosphate-buffered saline; PcG, polycomb group; RT-PCR, reverse-transcription polymerase chain reaction; SEM, standard error of the mean; WT, wild-type.

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Polycomb group (PcG) proteins are frequently upregulated in various cancers and are considered proto-oncogenes. It is therefore important to understand the molecular mechanisms underlying this oncogenic function. As a core component of the PRC1 complex, BMI1 is one of the best-studied mammalian PcG proteins. BMI1 was first identified as a proto-oncogene in the initiation of lymphoma in collaboration with Myc. However, in some cases, BMI1 appears to have effects beyond silencing of the Cdkn2a locus. For example, deletion of p16 and p19 only partially rescues neural stem cell maintenance in Bmi1-deficient mice. BMI1 regulates the DNA damage response (DDR) pathway independently of the Ink4a/Arf pathway. Moreover, the requirement of BMI1 in the development of glioma is independent of Ink4a/Arf. These observations suggest that there are multiple pathways downstream of BMI1 that promote stem cell self-renewal and tumorigenesis. MEL18, a homologue of BMI1, has been less well studied. Knock-out studies indicate that Bmi1 and Mel18 have synergistic roles in Hox gene regulation and skeletal patterning. Biochemically, both BMI1 and MEL18 are able to effectively stimulate E3 ubiquitination ligase activity of recombinant Ring1B. In addition, BMI1 and MEL18 constitute mutually exclusive PRC1 complexes, but these complexes all accumulate at H3K27me3-rich regions. These observations indicate that these 2 genes have a similar biological and biochemical function and might be functionally redundant in tissues in which both genes are abundantly expressed.

In this study, we investigated the potential role of Bmi1 and Mel18 in the homeostasis of colonic epithelium and CAC. Bmi1 is expressed in intestinal stem cells in the crypt of the small intestine and is required for intestinal stem cell self-renewal and epithelial homeostasis in the small intestine. However, the contribution of BMI1 to CAC, particularly given its potentially redundant function with MEL18, has not been defined. Here, we generated Bmi1 and Mel18 conditional knockout (KO) mice and specifically depleted their function in the intestinal epithelium. Depletion of Bmi1 or Mel18 alone or simultaneously does not significantly affect colonic homeostasis. However, deletion of both genes, but not alone, was able to significantly inhibit tumor initiation and development in the experimental model of CAC, indicating that BMI1 and MEL18 have redundant roles in colitis-associated tumorigenesis. We determined that BMI1 or MEL18 is required for STAT3 activation in premalignant cells in response to STAT3-activating cytokines released from inflammatory cells and identified REG3B as a novel PcG target whose up-regulation inhibits cytokine signaling and, consequently, tumorigenesis.

**Materials and Methods**

**Experimental Animals**

All animal experiments were approved by the Institutional Animal Care and Use Committee at National Institute of Biological Sciences (Beijing, China) in accordance with China’s Ministry of Health national guidelines for housing and care of laboratory animals. No statistical method was used to pre-determine sample size. Animal numbers were determined to optimize minimum numbers necessary for statistical significance according to the previous literature. All mice were bred and maintained under specific pathogen-free conditions at the animal facility of the National Institute of Biological Sciences, and all experiments were performed using 6- to 8-week-old sex-matched mice. C57BL/6 mice were from Vital River Laboratory Animal Technology Company (Beijing, China) and all mice used in the experiments were backcrossed to C57BL/6 mice for at least 6 generations. Cre-negative littermate mice were used as wild-type (WT) controls. For generation and validation of conditional knockout Bmi1, Mel18, and Reg3b alleles, see Supplementary Materials and Methods.

The following mouse alleles were used: villin-Cre (Jackson Laboratory, Bar Harbor, ME), villin-CreERT^2 (gift of Dr Sylvie Robine, Paris, France), and Cdkn2a^fl/fl (Dr Liang Chen, Beijing, China). For villin-CreERT^2 induction, mice were injected intraperitoneally (IP) with tamoxifen (Sigma-Aldrich, St Louis, MO, #T5648) in sunflower oil at a concentration of 2 mg per 20 g body weight for 5 consecutive days.

**Colitis-Associated Colon Cancer Induction**

The CAC model was performed essentially as previously described. Briefly, 6- to 8-week-old sex-matched mice were IP injected with 10 mg/kg of azoxymethane (AOM; Sigma-Aldrich, St Louis, MO, #A5486). After 5 days, the mice were treated with 2.5% dextran sulfate sodium (DSS; MP Biomedicals, Santa Ana, CA, molecular weight 35–50 kDa, #D216011080) in drinking water for 5 days, which was then followed by 16 days of regular water. This cycle was repeated twice. On day 80 or 100, mice were IP injected with 100 mg/kg 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO, #B9285) and sacrificed 3 hours later. In all experiments, littermate controls were used to enable comparison with mice of the same genetic background. The animals that exhibited health concerns not related to the study conditions were excluded from the analysis.
The animals were blindly monitored daily by weighing and clinical scoring during AOM-DSS treatment. Polyp load was identified as a sum of the diameters of all tumors in a given mouse.25

**Isolation of Intestinal Epithelial Cells**

IECs were isolated from the freshly dissected colon as described previously.3,36 After removal of the Payer’s patches and the adventitial fat, the colons were cut open longitudinally and washed with phosphate-buffered saline (PBS). Colons were cut into 2- to 3-mm pieces and incubated in Hank’s balanced salt solution containing 5 mmol/L EDTA at 37°C, shaking for 20 minutes. The supernatant was collected and centrifuged. The pellet was washed in ice-cold PBS and maintained in RNA stabilization solution or snap frozen in liquid nitrogen for Western blot analysis.

**Statistical Analysis**

Data were presented as mean ± standard error of the mean (SEM). Graphical analyses, statistical analysis, and nonlinear regression analysis of the data were performed using GraphPad Prism Software (La Jolla, CA). Differences between groups were determined using 1-way analysis of variance (ANOVA), 2-way ANOVA, unpaired Student’s 2-tailed t-test, or Fisher’s exact test. Kaplan–Meier method and log-rank test were used for survival analyses. A P value <.05 indicated statistical significance for all data sets.

**Results**

**BMI1 and MEL18 Are Required for the Initiation and Progression of CAC Tumorigenesis**

A germline KO of both the Bmi1 and Mel18 alleles was generated and described previously.3,36 Analysis of these doubly deficient mice has revealed that BMI1 and MEL18 act in synergy and in a dose-dependent manner to repress Hox genes and mediate survival of the developing embryos, suggesting that these 2 genes have overlapping roles in these processes.24 Because the doubly deficient mice die at 9.5 dpc, to investigate the function of these genes in adult tissues, we generated floxed alleles of Bmi1 (Supplementary Figure 1A-C) and Mel18 (Supplementary Figure 1D-F), respectively. Without excision, the homozygous mice with the floxed alleles were fertile and healthy and had no apparent abnormalities.

To examine the function of these genes in CAC development, we generated mice containing floxed alleles of Bmi1 and/or Mel18 and a transgene expressing Cre recombinase under the control of the villin gene promoter (villin-Cre), an intestine-specific promoter.12 These mice are hereafter referred to as Bmi1ΔIEC, Mel18ΔIEC, and DKO mice (Supplementary Figure 2A and B), respectively. Interestingly, IEC-specific depletion of Bmi1 and/or Mel18 did not have an overt effect on epithelial homeostasis in the colon (Supplementary Figure 2C), and the baseline rates of cell proliferation and apoptosis were also indistinguishable between WT and single or DKO mice (Supplementary Figure 2D and E). Furthermore, Bmi1ΔIEC, Mel18ΔIEC, and DKO mice were fertile and healthy and did not exhibit any overt phenotype. These data suggest that Bmi1 and Mel18 are largely dispensable in the epithelium for colonic development and homeostasis.

Next, we examined whether these genes are required for tumor development in the CAC mouse model. In this model, mutation and chronic inflammation in mice of appropriate genotypes was triggered by injection with the colonotropic mutagen AOM, followed by 3 cycles of treatment with the luminal toxin DSS (Figure 1A), as previously described.4 After the AOM-DSS treatment, upon gross inspection, the WT mice gradually developed intestinal polyps. Similarly, Bmi1ΔIEC or Mel18ΔIEC mice gradually developed polyps. However, DKO mice exhibited significantly fewer polyps compared with WT, Bmi1ΔIEC, or Mel18ΔIEC mice (Figure 1B). Histologic analysis revealed that the DKO mice had markedly decreased polyp multiplicity and polyp load (Figure 1C). In addition, adenomas observed in the colons of DKO mice generally displayed only low-grade dysplasias (Figure 1B), but adenomas with high-grade dysplasia characterized by aggressive invasion of the muscularis mucosa were frequently observed in WT, Bmi1ΔIEC, or Mel18ΔIEC colons (Figure 1B). Immunohistochemistry with Ki-67 and BrdU, both markers of cellular proliferation, revealed significant reduction in polyp cell proliferation in the DKO mice compared with WT mice (Supplementary Figure 3A and B). Active caspase-3 and TUNEL analysis revealed an increase in the apoptotic response of the colonic mucosa in DKO mice (Supplementary Figure 3C). The reciprocal decrease in proliferation and increase in cell death occurred specifically in the polyp tissues, but not in adjacent normal epithelial cells in DKO mice (Supplementary Figure 3A-C). The above data demonstrate that Bmi1 and Mel18 are required for CAC tumorigenesis.

To define the stage at which the loss of Bmi1 and Mel18 impacts tumorigenesis, we generated mice containing the floxed alleles of Bmi1 and Mel18 and the villin-Cre-ER12 driver,33 which allows controlled gene ablation in IECs upon tamoxifen treatment. Similar to the above results with the non-inducible villin-Cre, conditional deletion of both genes during the earliest stages of CAC resulted in a significant decrease in polyp multiplicity and polyp load (Supplementary Figure 4A and B). To determine whether Bmi1 and Mel18 are continuously required for tumor progression after tumor initiation, we administered tamoxifen to the DKO mice with villin-Cre-ER12 after the last DSS treatment (Figure 1D). Remarkably, conditional depletion of Bmi1 and Mel18 during late stages of CAC significantly reduced the multiplicity of large adenomas and the load of total adenomas (Figure 1E and F, Supplementary Figure 4C). Although a similar tendency was observed for the total number of polyps, it did not reach statistical significance (Figure 1F). Consistent with the profound effect on polyp size, there was a significant reduction in cell proliferation in the DKO mice and a reciprocal increase in the epithelial apoptosis (Supplementary Figure 4D and E). Taken together, these data suggest that Bmi1 and MEL18 are required for both initiation and progression of colorectal neoplasia in the mouse model of CAC.

**BMI1 and MEL18 Regulate the Proliferation and Survival of Premalignant Cells Independently of Ink4a/Arf**

Because inflammation plays a critical role in tumorigenesis,6,7 we hypothesized that the reduced adenoma multiplicity
and load in DKO mice could be because of decreased intestinal inflammation. To this end, we investigated the acute colitis model that uses a single 5-day course of DSS. Unexpectedly, after AOM-DSS treatment, the DKO mice exhibited greater body weight loss than WT or single KO mice (Figure 2A). Histologically, these mice exhibited a significantly higher degree of mucosa damage and an increased incidence of ulcerations (Figure 2A and B). These are typical phenotypes of hyper-inflammatory response. In agreement with this notion, proinflammatory genes, including IL-6, IL-1β, IL-11, TNFα, and Cox-2, were significantly elevated in the colonic mucosa of DKO mice (Figure 2C). Thus, the reduced polyp multiplicity and load in DKO mice may not be because of reduced inflammation, it is possible the DKO causes the disruption of downstream pathways that are important for the inflammation-induced tumorigenic function.

In contrast to the increased inflammatory phenotypes, immunohistochemical analysis of Ki-67 revealed impaired

Figure 1. Double deficiency of Bmi1 and Mel18 decreases adenoma formation and growth in the CAC model. (A) A schematic overview of the CAC model. (B) Representative colon sections of WT, Bmi1ΔIEC, Mel18ΔIEC, and DKO mice at the end of the CAC challenge stained with H&E. (C) Polyp multiplicity and average polyp load were analyzed in WT, Bmi1ΔIEC, Mel18ΔIEC, and DKO mice. (D) Scheme of Bmi1 and Mel18 deletion during the late stage of CAC growth. (E) H&E-stained of representative colon sections of WT and DKO mice at the end of the CAC challenge showing the polyp reduction in DKO mice. (F) Polyp number, number of polyps (polyp size >2 mm), and polyp load were analyzed in WT and DKO mice. Scale bars = 100 μm. Data are presented as the mean ± SEM (n = 6-30 mice per genotype). ns, not significant; *P < .05; ***P < .001; by 1-way ANOVA (C) or 2-tailed, unpaired t-test (F).
Figure 2. Colonic inflammation is increased in the DKO mice through dampened STAT3 activation during DSS-induced colitis. (A) The body mass of DKO mice decreased dramatically compared with WT littermates during acute DSS colitis (upper panel). Ulcer number and histologic damage at day 15 in WT and DKO mice treated with 3.5% DSS (lower panel). (B) H&E-stained of colons from WT and DKO mice at day 15. (C) Relative cytokine mRNA levels in whole colonic mucosa from WT and DKO mice at day 15. (D) Immunohistochemical analysis of Ki-67 and cleaved caspase-3 in colons from WT and DKO mice at day 15 of the CAC model. (E) Polyp numbers and polyp load in WT, Cdkn2aΔEC, DKO, and BM/CΔEC mice. (F) Colonic lysates were prepared at the indicated times, and the expression and phosphorylation of the indicated proteins were analyzed. β-actin was used as loading controls. Scale bars = 100 μm. Data are presented as the mean ± SEM (n = 3-15 mice per group). ns, not significant; *P < .05; **P < .01; ***P < .001; by 2-tailed, unpaired t-test (A,D), 2-way ANOVA (C), or 1-way ANOVA (E). All data shown are representative of at least 3 independent experiments.
proliferation of the colon epithelium of DKO mice compared with WT (Figure 2D). Staining for active caspase-3 revealed a reciprocal increase in apoptosis of IECs in DKO mice (Figure 2D). In contrast, we did not observe any differences in mass loss (Supplementary Figure 5A and B) or histopathologic morphology (Supplementary Figure 5C) between Bmi1ΔIEC or Mel18ΔIEC and WT mice. Therefore, simultaneous depletion of Bmi1 and Mel18, but not either alone, reduces cell proliferation and increases cell death in IECs during CAC tumorigenesis, resulting in tumor suppression. Collectively, these results indicate that the reduced polypl multiplicity and load in DKO mice is because of impaired epithelial cell proliferation and decreased epithelial cell survival, despite the accompanying increase in inflammatory response, a paradoxical disconnection that has been observed previously.4,6,7

The Cdkn2a locus, which encodes the p16Ink4a and p19Arf tumor suppressors, is targeted by BMI1.19 Significant up-regulation of p16 mRNA levels has also been observed in Mel18−/− mouse embryonic fibroblasts.19 Consistent with those observations, our quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis revealed that the transcription of p16Ink4a and p19Arf was already significantly increased in Bmi1ΔIEC or Mel18ΔIEC single KO mice (Supplementary Figure 6A). Therefore, the increased cell death by apoptosis in Bmi1ΔIEC/Mel18ΔIEC double KO cannot be explained simply by increased transcription of p16Ink4a and p19Arf. To functionally test this hypothesis, we generated villin-Cre; Cdkn2aΔIEC/mice (termed Cdkn2aΔIEC), Bmi1ΔIEC/Cdkn2aΔIEC, and Mel18ΔIEC/Cdkn2aΔIEC double-deficient mice, and Bmi1ΔIEC/Mel18ΔIEC/Cdkn2aΔIEC triple-deficient mice (hereafter referred to as B/M/CΔIEC). We confirmed the absence of the p16 and p19 proteins in the colon epithelium of Cdkn2aΔIEC mice by immunoblot analysis of isolated epithelial cells (Supplementary Figure 6B). As expected, after AOM-DSS treatment, there was no significant alteration in polypl multiplicity or polypl load between B/M/CΔIEC and DKO mice (Figure 2E), between Bmi1ΔIEC and Bmi1ΔIEC/Cdkn2aΔIEC double-deficient mice, or between Mel18ΔIEC and Mel18ΔIEC/Cdkn2aΔIEC double-deficient mice (Supplementary Figure 6C). In addition, when chronic DSS colitis was induced by 3 cycles of DSS without AOM injection, no significant difference in the intensity of epithelial damage was observed between B/M/CΔIEC and DKO mice (Supplementary Figure 6D). These data suggest that tumor suppression by Bmi1 and Mel18 ablation is not caused by activation of the Ink4a/Arf locus.

**BMI1 and MEL18 are Required for STAT3 Activation in Premalignant Cells**

The observed up-regulation of IL-6 and IL-11, which are major STAT3 activators in the colon,6,7 suggested that STAT3 might be highly activated in premalignant IECs in the DKO mice. However, using immunoblot analysis with antibodies against STAT3 phosphorylated at tyrosine residue 705 (pSTAT3, the activated STAT3), we instead observed dampened STAT3 activation during 8–16 days of induced colitis in IECs of DKO mice, a time period that is considered critical for the initiation of tumorigenesis (Figure 2F). By contrast, activation of several other signaling pathway effectors, such as S6 kinase (S6K) and extracellular signal-regulated kinase (ERK), remained largely unchanged, and the STAT3 negative feedback regulator SOCS3 was not changed (Figure 2F). Reduced pSTAT3 staining in IECs in sectioned tissues from DKO mice was also observed (Supplementary Figure 7A). Again, the reduction in the pSTAT3 level was observed only in DKO mice and not Bmi1ΔIEC or Mel18ΔIEC mice (Supplementary Figure 7B and C), indicating that BMI1 and MEL18 have redundant roles in facilitating STAT3 activation in premalignant IECs.

Inflammation via IL-6/STAT3 signaling induces the expression of cell cycle-related regulators and anti-apoptotic genes to promote the survival and proliferation of premalignant IECs.6,7 Consistent with a reduction in STAT3 activity in DKO mice, RT-PCR analysis revealed that many STAT3-regulated genes, including cyclins (cyclin B1, D1, D2 and E), c-Myc, and cdc2, were moderately or significantly down-regulated (Supplementary Figure 7D). In addition, the proapoptotic gene Bcl10 and p21 (cell-cycle inhibitor) were significantly up-regulated in DKO mice compared with control mice (Supplementary Figure 7D). This alteration in STAT3 activation provides a molecular explanation for the tumor suppression effect of Bmi1 and Mel18 ablation during CAC tumorigenesis.

**Identification of Reg3b and Reg3g as Novel PcG Target Genes in IECs**

To elucidate the molecular mechanisms of BMI1 and MEL18 in STAT3 regulation and, consequently, CAC tumorigenesis, we performed cDNA microarray analysis of premalignant IECs isolated from WT, Bmi1ΔIEC, Mel18ΔIEC, and DKO mice on day 12 after AOM-DSS treatment. As predicted, the Ink4a/Arf (Cdkn2a) locus was among the top up-regulated genes (Supplementary Table 1). Interestingly, 2 similar genes, Reg3b and Reg3g, were also at the top in the list of genes that were specifically up-regulated in DKO IECs (Figure 3A and Supplementary Table 1). Reg3b and Reg3g encode 2 small proteins that belong to a lectin family of secreted factors. Among the 7 family members of the Reg genes in the mouse genome, Reg3b and Reg3g exhibited pronounced up-regulation (>35 fold), Reg4 exhibited much less pronounced up-regulation (about 4-fold), and other Reg genes remained unaltered in DKO mice (Figure 3A and B). The up-regulation of Reg3b and Reg3g was further confirmed by quantitative RT-PCR analysis (Figure 3B), immunoblot and immunohistochemical staining for REG3B (Figure 3C and D).

To determine whether BMI1 and MEL18 directly or indirectly regulate Reg3b and Reg3g expression, we conducted chromatin immunoprecipitation (ChIP) assays with BMI1 and MEL18 across the promoter regions of Reg3b and Reg3g loci in mouse colon epithelial tissues and CT26.WT cells, a mouse colon carcinoma cell line. The results revealed that BMI1 and MEL18 were able to bind to promoter regions for Reg3b and Reg3g and that this binding activity was accompanied by H2A ubiquitination (Figure 3E and F, and Supplementary Figure 8A-D). In contrast, ChIP assays
Figure 3. Identification of reg3b as a novel PcG target. (A) Heat-map of the microarray results shown significant up-regulation of the Reg3b and Reg3g genes in IECs of the Bmi1 and Mel18 DKO compared with control mice. Red, up-regulated; green, down-regulated; black, no change. Results are representative of 2 independent experiments. (B) Quantitative RT-PCR analysis of mouse Reg family genes in IEC samples from DKO and WT mice. (C) Immunoblot analysis of the REG3B protein in isolated colonic enterocytes from WT, Bmi1ΔIEC, Mel18ΔIEC, and DKO mice. β-actin was used as loading controls. (D) Immunohistochemical analysis of the REG3B protein in colonic tissues from WT and DKO mice. (E and F) Schematic representation of the Reg3b locus, with black squares marked 1, 2 indicating the amplified regions in the ChIP studies (upper panel). ChIP analysis of the binding of the BMI1 (E) and MEL18 (F) antibodies at the Reg3b different loci in mouse colonic epithelial tissues (lower panel). IgG was used as a control. Scale bars = 100 μm. Data are presented as the mean ± SEM (n = 3-6 mice per group). *P < .05; **P < .01; by 2-tailed, unpaired t-test (E,F). All data shown are representative of at least 3 independent experiments.
did not detect any significant binding activity for BMI1 or MEL18 in the promoter regions of other Reg family genes (Supplementary Figure 8E). These observations are consistent with the results obtained by microarray analysis (Figure 3A) and indicate that the Reg3b and Reg3g loci are specifically targeted by BMI1 and MEL18 in the colon epithelium. Interestingly, many PcG genes, especially M33 and Cbx8, were significantly up-regulated during CAC tumor development (Supplementary Figure 8F). Along with the observation that H2A ubiquitination was enriched at Reg3b promoter, it is possible that the canonical PRC1 complexes are involved in the regulation of REG3B expression.

REG3B Inhibits IL-6/IL-11–Mediated STAT3 Activation

To determine whether REG3B/REG3G can negatively regulate STAT3 signaling, we expressed full-length Reg3b and Reg3g in 293T cells and examined the effect on STAT3 activation in response to IL-6 or IL-11. Normally, cellular STAT3 is strongly phosphorylated upon IL-6 stimulation. Remarkably, in response to IL-6, STAT3 phosphorylation was considerably inhibited in Reg3b-transfected cells but not Reg3g-transfected cells (Supplementary Figure 9A), and overexpression of REG3B was able to inhibit STAT3 phosphorylation during the entire period of IL-6 treatment in CT26.WT cells (Figure 4A). Similarly, IL-11 treatment induced moderate and prolonged STAT3 activation in CT26.WT cells, and overexpression of REG3B also significantly inhibited IL-11–induced STAT3 phosphorylation (Figure 4B). These data demonstrate that expression of REG3B, but not REG3G, effectively interferes with cytokine-mediated STAT3 activation in cultured cells.

Mechanisms of IL-6/IL-11-mediated STAT3 activation are well understood. The binding of extracellular IL-6/IL-11 to the cell surface receptor complex (IL-6R/GP130 or IL-11R/GP130) triggers activation of the receptor complex, which then activates the associated Janus kinase (JAK) via their mutual phosphorylation. The activated JAK then phosphorylates cytoplasmic STAT3 and induces their dimerization and activation. We observed that expression of REG3B reduced JAK phosphorylation (Figure 4C), as well as GP130 phosphorylation (Figure 4D), indicating that REG3B interferes with STAT3 signaling above JAK phosphorylation, possibly at the level of the receptor complex. Interestingly, REG3B was able to interact with IL-6R and IL-11R, respectively, in both CT26.WT cell extract (Supplementary Figure 9B and C) and colonic epithelial tissue extract (Figure 4E). However, we failed to detect any interactions of REG3B with IL-6 or GP130 by immunoprecipitation (Figure 4F and Supplementary Figure 9B). Moreover, pre-incubation of cells with recombinant REG3B protein inhibited IL-6–induced STAT3 activation in a dose-dependent manner (Supplementary Figure 9D). In contrast, addition of REG3B protein after IL-6 treatment failed to inhibit STAT3 activation (Supplementary Figure 9E). Taken together, these observations suggest that REG3B interferes with STAT3 signaling extracellularly by binding to the receptor complex and inhibiting its activation by IL-6 or IL-11.

REG3B Treatment Suppresses CAC Tumorigenesis

To determine whether REG3B expression is sufficient to suppress CAC tumorigenesis, we treated 2 groups of mice separately with recombinant REG3B protein. One group was treated at “early stages” and another group was treated at “late stages” of CAC, as illustrated in Figure 5A and Figure 5C, respectively. Strikingly, both early and late REG3B treatment effectively reduced polyp multiplicity and polyp load compared with PBS-treated mice (Figure 5B and D). Immunohistochemical staining revealed that REG3B treatment significantly reduced cell proliferation and induced apoptosis in epithelial cells (Supplementary Figure 10A-D). In addition, it markedly reduced pSTAT3 levels in infiltrating inflammatory cells and IECs of CAC adenomas (Supplementary Figure 10A and C). Meanwhile, we did not observe any altered expression of BMI1, MEL18, p16, and p19 proteins (Supplementary Figure 11A) between control and REG3B treatment mice. These data suggest that REG3B treatment is sufficient to interfere with STAT3 activation in vivo and is effective in preventing both tumor initiation and growth during CAC tumorigenesis.

The BMI1/MEL18-REG3B-STAT3 Signaling Axis in CAC Tumorigenesis

The above results imply that excessive production of REG3B following Bmi1 and MEL18 ablation interferes with cytokine-mediated STAT3 activation in premalignant cells and consequently suppresses tumorigenesis. Therefore, PcG proteins may promote CAC tumorigenesis through a novel BMI1/MEL18-REG3B-STAT3 signaling axis. To further test the existence of this regulatory pathway, especially to evaluate the contribution of REG3B in STAT3 inhibition followed by the loss of BMI1 and MEL18, we generated Bmi1, Mel18, and Reg3b single, double, or triple mutant 3T3 cells, a mouse embryonic fibroblast cell line, using the CASPR/Cas9 system (Supplementary Figure 11B, C) and tested their responsiveness to IL-6– or IL-11–induced STAT3 activation. Normally, treatment with IL-6 or IL-11 in 3T3 cells was able to induce robust STAT3 activation (Figure 6A). Interestingly, the levels of STAT3 activation were significantly reduced when both Bmi1 and Mel18, but not either alone, were mutated (Figure 6A). Interestingly, up-regulation of Reg3b was observed in either Bmi1 or Mel18 KO cells, but a significantly higher magnitude of up-regulation was found in the Bmi1<sup>−/−</sup>/Mel18<sup>−/−</sup> (termed B<sup>−/−</sup>/M<sup>−/−</sup>) cells (Supplementary Figure 11C), indicating that BMI1 and MEL18 have a partially redundant function in regulating REG3B in these cells as well. Importantly, additional mutation of Reg3b could rescue STAT3 activity in the Bmi1<sup>−/−</sup>/Mel18<sup>−/−</sup>/Reg3b<sup>−/−</sup> (termed B<sup>−/−</sup>/M<sup>−/−</sup>/R<sup>−/−</sup>) cells back to control levels (Figure 6A), suggesting that the inhibitory effect on STAT3 following the loss of BMI1 and MEL18 is mainly because of the activation of REG3B.

To determine the contribution of this regulatory axis, especially the REG3B component, to CAC tumorigenesis in vivo, we generated conditional Reg3b-deficient mice by CRISPR-Cas9-mediated targeting (Supplementary Figure 12A), and studied its role in the tumor suppressive
effect following Bmi1 and Mel18 depletion. The villin-Cre; Reg3b^{+/+} deficient mice (termed Reg3b^{AIEC}), which appeared phenotypically normal, were almost completely devoid of REG3B protein in IECs (Supplementary Figure 12B). We then engineered the Bmi1^{AIEC}/Mel18^{AIEC}/Reg3b^{AIEC} triple-deficient mice (referred to as TKO hereafter), and studied the consequences in the AOM-DSS model. Strikingly, TKO mice showed efficient development of adenomas, with
The up-regulation of pSTAT3 expression in TKO adenoma cells (Figure 6E). Therefore, the tumor inhibitory effect caused by Bmi1 and Mel18 depletion can be completely eliminated by depleting Reg3b, demonstrating genetically that Reg3b is the major downstream effector of Bmi1 and Mel18 in regulating CAC tumor development. Taken together, these observations strongly support a linear regulatory relationship among Bmi1/Mel18, REG3B, and STAT3, which may constitute an important regulatory pathway to control the initiation and development of CAC.

**REG3β in Human Colorectal Cancer**

Next we assessed the potential role of the Reg family genes in human colorectal cancers. There are 5 Reg family genes in the human genome that are aligned contiguously in a single cluster: REG1a, REG1b, REG3β, REG3γ, and REG4. We first expressed each of these genes in 293T cells using lentiviral-based vectors and examined the effect on IL-6-mediated STAT3 signaling. Interestingly, among these 5 genes, only REG3β exhibited robust STAT3 inhibition (Figure 7A). Coincidently, REG3β is the most similar to mouse REG3b and is considered the human ortholog of mouse REG3B. To determine whether REG3β expression is sufficient to suppress CAC tumorigenesis, we treated mice with recombinant REG3β protein at late stage of CAC. As illustrated in Figure 7B, REG3β treatment significantly reduced polyp load and marginally reduced polyp multiplicity (statistically not significant) compared with PBS treatment (Figure 7B).

To assess the clinical relevance of REG3β in colorectal cancer, we examined REG3β expression in a tumor tissue microarray consisting of 87 colorectal cancer specimens, and found that patients with higher expression of REG3β in the tumor tissue had a better 5-year survival rate than patients with moderate or lower expression of REG3β (P < .01 and P < .0001, respectively) (Figure 7C). To evaluate the relationships between REG3β expression and STAT3 activity, we examined REG3β and pSTAT3 expression by immunostaining in tumor tissue microarray consisting of 92 colorectal cancer specimens. We found that REG3β was more frequently expressed in tumor tissues with low pSTAT3 than with high pSTAT3 levels (Figure 7D and E). Taken together, these data suggest that the expression level of REG3β inversely correlates with pSTAT3 activity and disease prognosis, and indicate that REG3β could be a tumor suppressor in CAC development by inhibiting STAT3 activity.

**Discussion**

We have identified a novel and critical role for Bmi1 and its homologous protein MEL18 in the initiation and progression of CAC tumorigenesis by regulating cellular response to cytokine signaling. We have identified Reg3b as a new tumor suppressor gene targeted by Bmi1/MEL18 in the experimental model of CAC. Gene expression profiling identified significant up-regulation of Reg3b and Reg3g expression when Bmi1 and Mel18 are depleted. The results of ChIP assays suggest that Bmi1 and MEL18 directly target
the promoter regions of both Reg3b and Reg3g. Interestingly, although most Reg genes are aligned in a single cluster in the genome, BMI1 and MEL18 seem to specifically target Reg3b and Reg3g, at least in colonic epithelial cells, indicating that this gene cluster is under sophisticated regulatory control. Importantly, our triple conditional KO mice revealed that Reg3b is responsible for the tumor suppressive effect following the ablation of Bmi1 and Mel18. Consistent with the genetic relationships, administration of recombinant REG3B significantly suppresses tumor growth.
initiation and growth during CAC tumorigenesis, and the effect is largely similar to that caused by ablation of Bmi1 and Mel18. The importance of Reg3b repression by BMI1 and MEL18 for tumorigenesis suggests that Reg3b represents another major tumor suppressor locus targeted by PcG proteins.
Our in vitro and in vivo studies strongly suggest that the tumor suppressive effect of REG3B in the CAC model is, at least in part, because of its anti-STAT3 activity. REG3B belongs to a family of small, secreted proteins that contain a single C-type calcium-dependent lectin domain. Reg proteins, also called pancreatitis-associated proteins, were initially discovered as proteins that are strongly induced by pancreatitis or during islet regeneration. Subsequent studies revealed that Reg proteins are also expressed in a number of physiologic or pathologic processes. For instance, it has been suggested that Reg3g functions as a carbohydrate-binding bactericidal lectin to maintain microbial integrity in the gut. Prior to this report, few studies have implicated Reg proteins in regulating STAT3 activity. In Reg3b KO mice, STAT3 activity is enhanced during liver regeneration. By contrast, STAT activation is reduced in the small intestine but not the colon. It is possible that inversely correlated with the prognosis of colorectal cancer.

Reg family proteins share a common lectin domain, they may have distinct functions that are cell type-specific or context-dependent. Indeed, although Reg3b and Reg3g are targeted by BMI1 and MEL18, only Reg3b displays robust anti-STAT3 activity. We observed that the addition of recombinant REG3B protein to the medium inhibited IL-6-mediated STAT3 activation, suggesting that REG3B acts extracellularly to inhibit STAT3 signaling. Moreover, we found that REG3B physically interacts with the receptor complex but not the ligand. These observations indicate that REG3B interferes with STAT3 signaling by interfering with the activation of the IL-6R/GP130 receptor complex (Figure 7F). Further molecular and structural studies are needed to determine whether REG3B exerts its effect via interference with the ligand-receptor interaction or through other mechanisms. Because REG3B also possesses bactericidal activity, in addition to the direct role in interfering STAT3 signaling in tumor cells, it could also indirectly impact tumor progression by modulating microbial composition in the gut, especially during late stages of tumorigenesis, which warrants further investigation in the future.

Consistent with a tumor-suppressive role for REG3B/Reg3g in the CAC model, the expression of REG3b is inversely correlated with the prognosis of colorectal cancers. Interestingly, REG3B/Reg3g is highly expressed in the small intestine but not the colon. It is possible that the expression of these proteins and other potential tumor suppressors in the small intestine provides a tumor-suppressive environment, in agreement with the strikingly distinct incidences of carcinomas in the small intestine and colon. Identification of REG3B as a polycomb target also indicates a potential connection among polycomb function, gut microbiota, and cancer. Further investigation of the Reg family proteins as potential tumor suppressors and their regulation under physiologic and pathologic conditions may facilitate the development of effective therapeutics for colorectal cancers or other inflammation-associated diseases.

**Supplementary Material**

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2017.07.044.

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Conflicts of interest
The authors disclose no conflicts.

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