



Epithelial Regnase-1 inhibits colorectal tumor growth by regulating IL-17 signaling via degradation of *NFKBIZ* mRNA

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Regnase-1 is a ribonuclease that regulates inflammation in immune cells by degrading cytokine mRNA. *Regnase-1* was identified as one of the frequently mutated genes in the inflamed colorectal epithelium of patients with ulcerative colitis; however, its significance in intestinal epithelial cells during the tumorigenic process remains unknown. Therefore, we developed an *Apc*^{Min/+} mouse model lacking *Regnase-1* in intestinal epithelia. *Regnase-1* deletion significantly enhanced colon tumor growth accompanied by elevated levels of extracellular signal-regulated kinase (ERK) phosphorylation in tumor tissues. Transcriptome analysis of the tumor tissues revealed that *Nfkbiz*, a mediator of the interleukin (IL)-17 signaling pathway, was the primary degradative target of Regnase-1 in enterocytes and that *Regnase-1* deficiency enhanced IL-17 signaling. The treatment with antibiotics or IL-17-neutralizing antibody canceled the proliferative effect of colon tumors due to *Regnase-1* deletion, suggesting the protective role of Regnase-1 against colon tumor growth was dependent on IL-17 signaling triggered by gut microbes. Analysis of the *Nfkbiz* knockout mouse model demonstrated that the tumor-suppressive effect of *Regnase-1* depended on *Nfkbiz* expression. Remarkably, oral treatment of dimethyl fumarate, a potential inhibitor of Regnase-1 protein inactivation, suppressed tumor growth, downregulated *Nfkbiz*, and suppressed ERK activation. Furthermore, TCGA data analysis revealed that low *Regnase-1* expression in colorectal cancer tissue was related to poor prognosis. Therefore, *Regnase-1* represses colon tumor growth by regulating IL-17 signaling via *Nfkbiz* mRNA degradation. *Regnase-1* could be a potential therapeutic target in colon tumors.

colorectal cancer | Regnase-1 | IL-17

Regnase-1, coded by the *ZC3H12A* gene, is an endoribonuclease expressed in immune cells that regulates inflammatory responses by degrading the mRNA of cytokines or chemokines, such as *IL6* and *CXCL1*, by recognizing stem-loop motifs in the 3' untranslated region of target mRNAs (1, 2). Mice lacking *Regnase-1* have shown excessive interleukin (IL)-6 production in macrophages and developed lethal autoimmune inflammation (1). Regnase-1 has a critical role in regulating innate and adaptive immunity and maintaining homeostasis in immune cells (1, 3). However, the single-cell transcriptome of normal tissues shows that epithelial cells in some organs, such as the colon, bronchus, and skin, express *Regnase-1* at a level comparable with immune cells (4). Several reports have discussed the protective role of keratinocyte Regnase-1 against inflammation and skin carcinoma (5, 6) and its antiapoptotic effect in breast cancer cells (7).

Colorectal cancer is the fourth most deadly cancer worldwide, and new therapeutic strategies are urgently needed due to many cases developing resistance against current therapies (8). Whereas most sporadic colorectal cancers arise from adenoma with *APC* gene mutation, colitis-associated cancers specifically develop in inflammatory bowel syndrome cases such as ulcerative colitis (UC). Notably, the significance of Regnase-1 in colorectal epithelial cells has been highlighted in the studies about somatic mutations in the epithelia of UC cases (9, 10), in which *Regnase-1* has been identified as a frequently mutated gene in UC epithelium following analyses of single crypt isolation (9) and organoids (10), independently. Gain-of-function mutations in *Regnase-1* are suspected to enhance its ribonuclease activity and protect nondysplastic epithelium from developing colitis-associated colorectal cancer (9, 10). However, the role of Regnase-1 in intestinal epithelial cells for the development of sporadic colorectal cancer remains unclear.

Despite its physiological and pathological significance, Regnase-1 has never been regarded as a possible therapeutic target until recently. In the first report of a therapeutic approach targeting Regnase-1, antisense oligonucleotides that could elevate the *Regnase-1* mRNA level

Significance

Regnase-1, a messenger RNA (mRNA)-degrading enzyme, plays a critical role in regulating inflammation in immune cells. In the current study, its role in intestinal epithelial cells during tumorigenesis has been revealed. Using an original mouse model, loss of Regnase-1 in the epithelium was demonstrated to promote colon tumor growth via enhanced interleukin (IL)-17 signaling, mediated through *Nfkbiz*, a direct degradative target of Regnase-1. Moreover, a pharmacologic approach using dimethyl fumarate, a potential inhibitor of Regnase-1 protein inactivation, effectively suppressed tumor progression. Clinical data further link low Regnase-1 expression to poor prognosis in colorectal cancer. These findings uncover a protective function of Regnase-1 in the gut epithelium and highlight it as a potential therapeutic target in colorectal tumorigenesis.

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The authors declare no competing interest.

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by inhibiting self-degradation were developed and could be beneficial against inflammatory diseases; however, they have not been tested on humans and can only be treated topically (11).

In this study, we uncovered the noteworthy role that Regnase-1 plays in intestinal epithelial cells in colon tumor growth by analyzing animal models and clinical data. Furthermore, we explored a therapeutic strategy targeting Regnase-1 in colorectal tumors by utilizing a drug approved by U.S. Food and Drug Administration (FDA) currently used for multiple sclerosis and psoriasis. Our study suggests that Regnase-1 could be a potential therapeutic target in cancer biology.

Results

Intestinal-Specific Deletion of *Regnase-1* Enhances Colon Tumor Growth in *Apc*^{Min/+} Mice. To elucidate the role of *Regnase-1* in intestinal tumorigenesis, we generated a mouse model of *Apc*^{Min/+} with *Regnase-1* knockout (KO) specific to intestinal epithelia by crossing *Regnase-1*-floxed mice (3) with *Apc*^{Min/+} and *Villin-Cre*⁺ mice. The abrogation of *Regnase-1* in intestinal epithelial tissues was confirmed by RT-qPCR (Fig. 1A) and immunohistochemical

staining (IHC) (SI Appendix, Fig. S1A). Because *Apc*^{Min/+} mice typically develop multiple tumors in the whole intestine and die from severe anemia at around 20 wk of age, we compared the phenotypes in *Regnase-1*^{fl/fl}; *Apc*^{Min/+} (*Reg1*^{WT}-Min) mice and *Villin-Cre*⁺; *Regnase-1*^{fl/fl}; *Apc*^{Min/+} (*Reg1*^{KO}-Min) mice at the age of 120 d. Body weight at 120 d was significantly lower in *Reg1*^{KO}-Min (Fig. 1B). Remarkably, the colon tumor number and area were significantly larger in *Reg1*^{KO}-Min mice, although no obvious difference was detected in the small intestines (Fig. 1C and D). While the number of small tumors (diameter ≤ 1 mm) was comparable in both genotypes, that of tumors with a diameter > 1 mm was significantly larger in *Reg1*^{KO}-Min (SI Appendix, Fig. S1B). Histologically, inflammation was not observed on non-neoplastic intestinal tissues from either genotype (Fig. 1E). Tumors were histologically tubular adenomas in both genotypes (Fig. 1E). Remarkably, elevated levels of ERK phosphorylation and an increased number of Ki-67-positive cells were observed in *Reg1*^{KO}-Min colon tumors (Fig. 1F and SI Appendix, Fig. S1C and D), but not in *Reg1*^{KO}-Min small intestinal tumors (SI Appendix, Fig. S1E and F), which can be considered to contribute to colon tumor growth. On the other hand, the number of cleaved caspase-3-positive cells in the

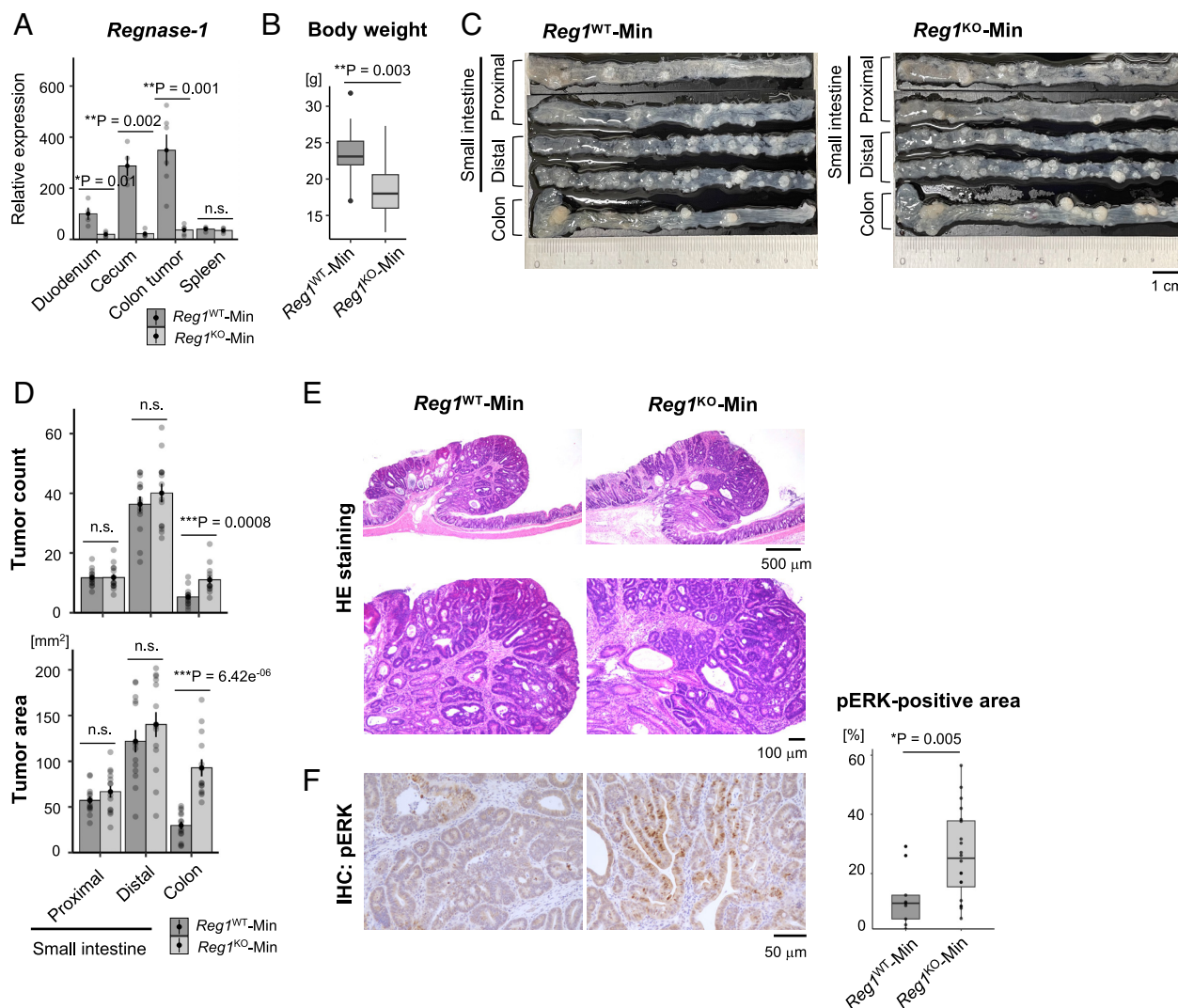


Fig. 1. Phenotypes of *Apc*^{Min/+} mice with intestine-specific *Regnase-1* deletion (*Reg1*^{KO}-Min) compared to control *Apc*^{Min/+} mice (*Reg1*^{WT}-Min). (A) *Regnase-1* mRNA levels in intestinal or splenic tissues from 120-d-old *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (N = 4). (B) Body weight of 120-d-old *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (N = 14). (C) Representative intestines at 120 d old. (D) Tumor number and area in the proximal half, distal half of small intestines, and colons of 120-d-old *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (N = 14). (E) Representative hematoxylin and eosin (HE) staining of colon tumors at 120 d old. (F) Representative IHC staining of pERK in colon tumor tissues. Quantification of the pERK-positive area (nine tumors from four *Reg1*^{WT}-Min mice and 20 tumors from four *Reg1*^{KO}-Min mice). Data are presented as mean ± SE.

tumors of the colon and small intestine was comparable between *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (SI Appendix, Fig. S1 C–F). Ki-67-positive cells also seem to be increased in the normal colon and small intestinal tissues of *Reg1*^{KO}-Min mice than those of *Reg1*^{WT}-Min mice, although the difference between the genotypes was not significant (SI Appendix, Fig. S1 G and H). Thus, we observed an interesting phenotype that colon tumor growth in *Apc*^{Min/+} mice was promoted by *Regnase-1* deletion in epithelial cells.

Regnase-1 Deletion Causes Upregulation of Antimicrobial Peptides. To explore the change of the gene expression profile in the intestines of *Reg1*^{KO}-Min mice, we performed transcriptome analysis of colon tumor tissues isolated from *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice by RNAsequencing (RNA-seq) (12). Although the *Regnase-1* gene (*Zc3h12a*) itself was not a differentially expressed gene (DEG) (SI Appendix, Table S1) as its deletion site is restricted to exons 4 to 6 (3), the deletion of those exons by the Cre-LoxP system was confirmed by the expunction of reads on this site (SI Appendix, Fig. S1I). Among 242 DEGs (false detection ratio < 0.2), 197 genes were upregulated and 45 genes were downregulated (Fig. 2A). Gene set enrichment analysis (GSEA) using Mouse MSigDB (M2: CP, Reactome) (Fig. 2B) and gene ontology analysis of DEGs (SI Appendix, Fig. S1J) indicated that the genes encoding antimicrobial peptides, mainly α -defensin, were the most significantly enriched DEGs.

Antimicrobial peptides play a key role in intestinal barrier defense by repressing commensal and/or pathogenic microbes (13). To learn whether the elevated expression of antimicrobial peptides leads to gut microbe alteration, we measured the level of total bacteria and predominant bacterial species in mouse feces by qPCR of fecal DNA (14, 15). The load of bacteria was not significantly reduced in *Reg1*^{KO}-Min, except *Bacteroidetes* (SI Appendix, Fig. S2 A and B). Next, we evaluated taxonomy abundance by total bacteria in each sample detected by qPCR and the proportion of each phylum determined by 16S rRNA gene meta-sequencing (16). This analysis revealed the same tendency, although reduction in *Reg1*^{KO}-Min was significant only in *Proteobacteria* (SI Appendix, Fig. S2C), so further studies are required to determine its pathological significance. Taken together, antimicrobial peptide upregulation caused by *Regnase-1* deletion might influence the composition of intestinal microbiota, but did not affect the bacterial load.

Regnase-1 Deletion in Epithelial Cells Leads to Altered Infiltration of Immune Cells. Because the GSEA results (Fig. 2B) indicated that gene sets involving immunity, including PHOSPHORYLATION_OF_CD3_AND_TCR_ZETA_CHAINS, CHEMOKINE_RECEPTORS_BIND_CHEMOKINES, and PD_1_SIGNALING, were downregulated in *Reg1*^{KO}-Min tumor tissues, we estimated the population of infiltrating cells by RNA-seq data using mMCP counter (17, 18). Neutrophil infiltration was amplified, and T cell infiltration was suppressed both significantly in *Reg1*^{KO}-Min tumor tissues (Fig. 2C). Therefore, we quantified the infiltration of T cells and neutrophils using immunohistochemistry. Augmented neutrophil infiltration was observed indeed in *Reg1*^{KO}-Min tumor tissues (Fig. 2 D and E). Although CD8⁺ T cells were scarcely detected in tumors in both *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (SI Appendix, Fig. S3A), the number of CD4⁺ T cells in tumor tissues was significantly lower in *Reg1*^{KO}-Min mice (Fig. 2 D and E). The suppression of CD4⁺ T cell infiltration in *Reg1*^{KO}-Min mice was also observed in nontumor colon tissues (SI Appendix, Fig. S3B). These phenotypes could be associated with the altered chemokine expression profile observed in *Reg1*^{KO}-Min mice, including upregulation of *Cxcl1/2*, which recruits neutrophils (19, 20), and downregulation of *Cxcl10*, which recruits T cells (21) (Fig. 2F and SI Appendix, Fig. S3

C and D). To investigate the potential role of altered immune cell infiltration in tumor growth, we treated our mouse models with Reparixin, a CXCR2 inhibitor targeting the Cxcl1/2 receptor (22). In the colon of Reparixin-treated mice, downstream chemokines were downregulated (SI Appendix, Fig. S4A) and infiltration of Ly6G⁺ cells in tumor tissues was suppressed (SI Appendix, Fig. S4B). Despite this suppression, colon tumor growth remained unaffected (SI Appendix, Fig. S4 C and D), suggesting that altered immune cell infiltration may not play a major role in tumor growth in our model.

Nfkbiz and Its Downstream Genes Are Upregulated in the Large Intestine Tissue by Regnase-1 Deletion. We speculated that the above-described transitional expression profile patterns by *Regnase-1* deletion would have resulted from the altered expression of some key immune mediator because *Regnase-1* regulates immune responses by degrading specific transcripts including various immune mediators (1, 2). Among 242 DEGs, *Nfkbiz* was detected as the only immune mediator which has been reported to be a target of degradation by *Regnase-1* (2) (Fig. 2A and SI Appendix, Table S2) rather than *Il6*, which is a principal degradative target of *Regnase-1* in immune cells (1). *Nfkbiz* is a key regulator of the IL-17 signaling pathway and is posttranscriptionally regulated through mRNA degradation by *Regnase-1* (2, 23) (Fig. 3A). I κ B ζ protein, transcribed from the *Nfkbiz* gene, was confirmed to be highly expressed in *Reg1*^{KO}-Min tumor tissues by immunohistochemistry (Fig. 3B).

Accordingly, GSEA revealed a significant upregulation of *Nfkbiz*-dependent genes (24–27) (Fig. 3C), whereas the expression levels of *Il17alf*, *Il17ralc*, *Il23a*, and *Rorc*, all of which work upstream of *Nfkbiz*, were not substantially changed (Fig. 3D and SI Appendix, Fig. S5). ROR γ immunohistochemistry showed that Th17 cell infiltration was comparable in tumor tissues of both genotypes (Fig. 3E). These data positively demonstrate that *Nfkbiz*-downstream gene upregulation in *Reg1*^{KO}-Min tumors was caused by the impaired degradation of *Nfkbiz* mRNA by *Regnase-1*. *Nfkbiz* mRNA expression was significantly upregulated in non-neoplastic cecal tissues of *Reg1*^{KO}-Min mice, whereas *Il6* mRNA expression was upregulated in the duodenum, not in the cecum (Fig. 3F). Therefore, *Nfkbiz* mRNA could be the primary degradative target of *Regnase-1* in the large intestinal epithelium.

IL-17 signaling has a key role in controlling infection, particularly on mucosal barriers. It induces host defense molecules, such as α -defensins (28, 29), promotes neutrophil infiltration (30), and inhibits T cell infiltration (21) by altering the chemokine expression profile. While *Nfkbiz* is induced by several inflammatory signals including IL-1 β , IL-36, IL-17, and JAK-STAT (31), the GSEA results discussed earlier suggested that in the large intestinal epithelium, *Nfkbiz* upregulated by *Regnase-1* deletion will be closely related to IL-17-related signaling.

Gut Microbes Contribute to Colon Tumor Growth under IL-17 Signaling. The question remains as to why the exacerbation of tumor growth by *Regnase-1* deletion occurs only in colons. Previous studies have pointed out that the impairment of the epithelial barrier on colorectal adenoma lesions results in invasion of microbial products, triggering IL-17-mediated tumor growth (32); thus, we were motivated to explore the role of gut microbes in IL-17-mediated tumor growth in *Apc*^{Min/+} mouse intestines. We treated mice with a combination of four antibiotics, ampicillin (500 mg/L), neomycin (500 mg/L), metronidazole (500 mg/L), and vancomycin (250 mg/L) in drinking water. Although half dose of that in the reported protocol (33, 34) was exploited to avoid toxicity at an early age, sufficient bacteria eradication except *Betaproteobacteria* was confirmed in fecal samples, obtained after 9 wk of treatment, by

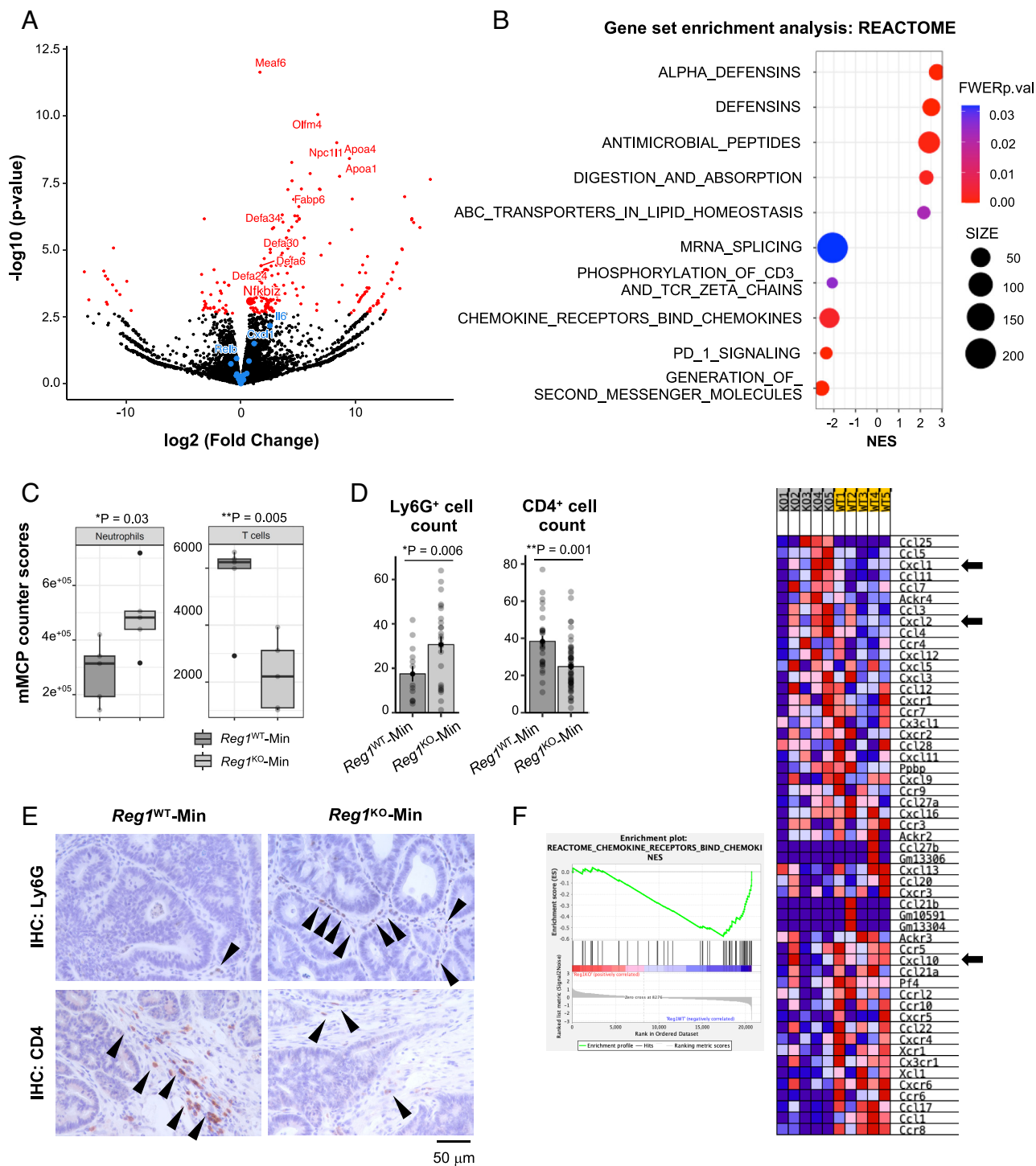


Fig. 2. Transcriptome analysis of colon tumor tissues from *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice. (A) Volcano plot presenting the result of RNA-seq performed in colon tumor tissues of *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (N = 5). Differentially expressed genes are shown in red. Genes previously reported as a degradative target of Regnase-1, except *Nfkbiz*, are shown in blue. (B) Enriched gene sets detected by GSEA of RNA-seq results using Mouse M5igDB (M2: CP, Reactome). (C) Estimated level of intratumoral immune cell infiltration calculated using mMCP counter. (D) Quantification of immune cells per sight field in colon tumor tissues by IHC of Ly6G (13 tumors from five *Reg1*^{WT}-Min mice and 25 tumors from six *Reg1*^{KO}-Min mice) and CD4 (18 tumors from nine *Reg1*^{WT}-Min mice and 42 tumors from ten *Reg1*^{KO}-Min mice). (E) Representative IHC of Ly6G and CD4 in colon tumor tissues. Arrowheads indicate positively stained cells. (F) Enrichment plot for the gene set REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES. Black arrows indicate *Cxcl1*, *Cxcl2*, and *Cxcl10*. Data are presented as mean ± SE.

qPCR (14, 15) (Fig. 4A and SI Appendix, Fig. S6A). When *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice underwent this gut sterilization protocol from weaning age until 120 d old, the tumor development in the colons was significantly repressed (Fig. 4B and C and SI Appendix, Fig. S6B and C); however, this phenotype was not seen in the small intestines (SI Appendix, Fig. S6D). Under antibiotics treatment, *Il17a* and *Il17f* expression was considerably decreased either with

or without *Regnase-1* deletion both at the mRNA level and at the protein level (SI Appendix, Fig. S6E and F).

Next, we tried to investigate the relationship between *Regnase-1*-mediated tumor suppression and IL-17 by weekly intraperitoneal injections of an IL17A-neutralizing antibody or isotype control (35) starting at 30 d of age until killing of animals. Upon treatment with the anti-IL17A antibody, colon tumor growth was

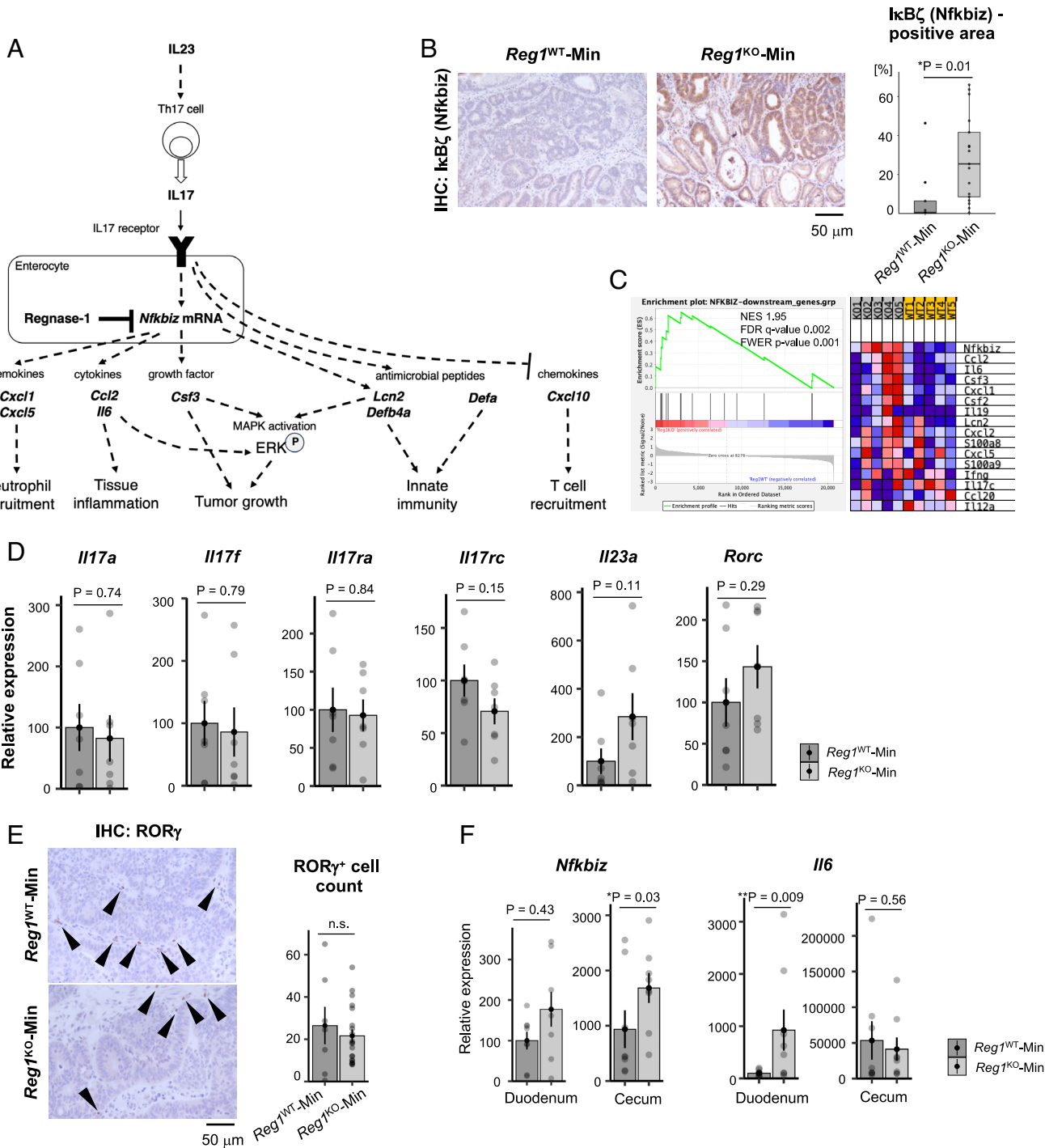


Fig. 3. Upregulation of *Nfkbiz* in *Reg1*^{KO}-Min intestinal tissues. (A) IL-17 signaling pathway schema. (B) Representative IHC of IκBζ in colon tumor tissues. Quantification of the IκBζ-positive area (nine tumors from four *Reg1*^{WT}-Min mice and 17 tumors from four *Reg1*^{KO}-Min mice). (C) Enrichment plot for the gene set of *Nfkbiz*-downstream genes obtained from RNA-seq performed in Fig. 2. (D) mRNA expression levels of *Il17a*, *Il17f*, *Il17ra*, *Il17rc*, *Il23a*, and *Rorc* in colon tumors from 120-d-old *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (N = 7). (E) Representative IHC of RORγ in colon tumor tissues. Arrowheads indicate positively stained cells. Quantification of Th17 cells per sight field in colon tumor tissues (seven tumors from four *Reg1*^{WT}-Min mice and 20 tumors from four *Reg1*^{KO}-Min mice) by IHC of RORγ. (F) mRNA levels of *Nfkbiz* and *Il6* in nontumor intestinal tissues from 120-d-old *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (N = 8). Data are presented as mean ± SE.

repressed to comparable levels in *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice (Fig. 4 D and E and *SI Appendix*, Fig. S7 A and B), suggesting that IL-17 signaling is required for *Regnase-1*-mediated tumor suppression. The effect of the anti-IL17A antibody was not obvious in the small intestines (*SI Appendix*, Fig. S7C).

Taken together, these results suggest that the protective role of *Regnase-1* against colon tumor growth could be dependent on IL-17 signaling triggered by microbes.

***Nfkbiz* Is Required in the Enhancement of Colon Tumor Growth by *Regnase-1* Deletion.** It has been suggested that the IL-17 signaling pathway possibly promotes intestinal tumor development (35), and its activity correlates with poor prognosis in patients with colon cancer (36). The above transcriptomic finding led us to hypothesize that *Regnase-1* might function protectively against the tumor-promotive role of IL-17 signaling by degrading *Nfkbiz* mRNA. Indeed, the knockdown of *Regnase-1* considerably

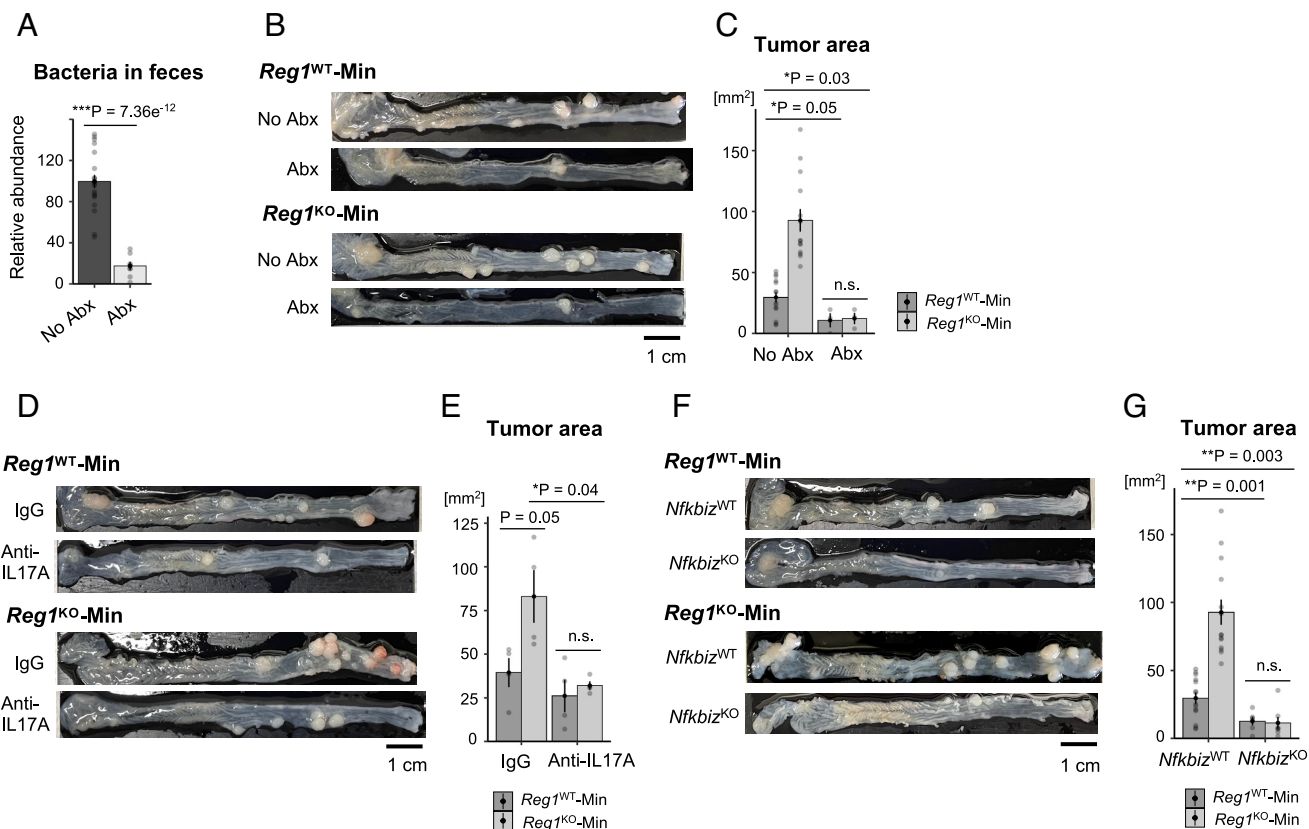


Fig. 4. Phenotypes of *Reg1*^{WT}-Min mice and *Reg1*^{KO}-Min mice under antibiotics treatment or intestinal-specific *Nfkbiz* deletion. (A) Quantification by qPCR of bacteria in feces from *Apc*^{Min/+} mice with or without oral antibiotics (Abx) treatment for 9 wk (N = 20 for control and N = 8 for Abx-treated). (B) Representative colon from mice with or without oral Abx treatment since weaning until 120 d old. (C) Tumor area in colons of mice without Abx treatment (N = 14 for *Reg1*^{WT}-Min and *Reg1*^{KO}-Min each) and mice with oral Abx treatment (N = 3 for *Reg1*^{WT}-Min and *Reg1*^{KO}-Min each) since weaning until 120 d old. (D) Representative colon from mice intraperitoneally injected weekly with isotype control (IgG) or anti-IL17A antibody from 30 d old until 120 d old. (E) Tumor area in colons of mice treated with IgG (N = 4 for both *Reg1*^{WT}-Min and *Reg1*^{KO}-Min) and those treated with anti-IL17A antibody (N = 4 for both *Reg1*^{WT}-Min and *Reg1*^{KO}-Min) from 30 d old until 120 d old. (F) Representative colon from mice with or without *Nfkbiz*^{KO} at 120 d old. (G) Tumor area in colons of mice without *Nfkbiz*^{KO} (N = 14 for *Reg1*^{WT}-Min and *Reg1*^{KO}-Min each) and mice with *Nfkbiz*^{KO} (N = 6 for *Reg1*^{WT}-Min and N = 7 for *Reg1*^{KO}-Min) at 120 d old. Data are presented as mean ± SE.

elevated *Nfkbiz* mRNA levels in the human colon cancer cell line, HT29, regardless of IL-17A treatment (SI Appendix, Fig. S8A). Similarly, IL-17A treatment upregulated *Nfkbiz* in the organoids derived from a colon tumor of both *Reg1*^{WT}-Min and *Reg1*^{KO}-Min mice, and the expression level of *Nfkbiz* was substantially higher in the organoids from a *Reg1*^{KO}-Min tumor than those from a *Reg1*^{WT}-Min tumor (SI Appendix, Fig. S8B). To clarify whether *Nfkbiz* is one of the IL-17 mediators pivotal in accelerating tumor growth, we generated a mouse model of *Apc*^{Min/+} with *Regnase-1* and/or *Nfkbiz* KO, specific to intestinal epithelia, by crossing *Nfkbiz*-floxed mice (37) with *Reg1*^{WT}-Min or *Reg1*^{KO}-Min mice. The deletion of *Nfkbiz* in intestinal tissues was confirmed by RT-qPCR (SI Appendix, Fig. S8C). Remarkably, at 120 d old, both *Villin-Cre⁺; Nfkbiz^{fl/fl}; Apc^{Min/+} (Reg1^{WT}-Nfkbiz^{KO}-Min)* mice and *Villin-Cre⁺; Regnase-1^{fl/fl}; Nfkbiz^{fl/fl}; Apc^{Min/+} (Reg1^{KO}-Nfkbiz^{KO}-Min)* mice were found to have significantly smaller tumors than *Reg1*^{WT}-Min or *Reg1*^{KO}-Min mice in the colons (Fig. 4 F and G and SI Appendix, Fig. S8 D and E); however, this tendency was not observed in the small intestines again (SI Appendix, Fig. S8F). Tumor development was comparable in the colons of *Reg1*^{WT}-*Nfkbiz*^{KO}-Min and *Reg1*^{KO}-*Nfkbiz*^{KO}-Min mice (Fig. 4 F and G). This result indicates that *Nfkbiz* is needed for colon tumor growth in *Apc*^{Min/+} mice and that colon tumor growth augmentation by *Regnase-1* deletion does not arise without *Nfkbiz* expression. In summary, the protective role of *Regnase-1* against tumor growth is exerted via *Nfkbiz* expression.

Dimethyl Fumarate (DMF) Inhibits the Activation of IL-17-Mediated Proliferative Signal in the Human Colon Cancer Cell Line.

Given the protective role of Regnase-1 against *Nfkbiz*-mediated tumor growth, we considered that augmentation of Regnase-1 could impede the growth of colon tumors. DMF is an FDA-approved drug used for the treatment of multiple sclerosis or psoriasis. Although DMF has been supposed to have anti-inflammatory function by activating *NRF2*, an antioxidative transcription factor (38), it has recently been discovered that DMF has another therapeutic mechanism independent of *NRF2* specifically via suppressing phosphorylation of Regnase-1 protein, leading to the prevention of its ribonuclease inactivation (39). Phosphorylation of the Regnase-1 protein by the IκB kinase (IKK) complex has been shown to induce its degradation by a ubiquitin–proteasome–dependent mechanism under IL-1β stimulation (40). To confirm whether a similar mechanism operates in colon epithelial cells under IL-17 stimulation, we used HT29 cells transfected with a Myc-Regnase-1 expression vector. While Myc-Regnase-1 WT levels diminished after IL-17A treatment (50 ng/mL), Myc-Regnase-1 S438L, a mutant at a phosphorylation site, did not diminish (SI Appendix, Fig. S9A). This degradation was inhibited by treatment with the IKK inhibitor TPCA-1 (SI Appendix, Fig. S9B), indicating that IL-17 stimulation leads Regnase-1 protein to phosphorylation-mediated degradation depending on IKK. Thus, DMF could possibly be used to augment Regnase-1.

Once HT29 cells were treated with IL-17A (50 ng/mL), endogenous Regnase-1 protein instantly diminished, and IL-17 signaling could start downstream gene transcription (Fig. 5A), as previously reported (41). Due to the decrease in Regnase-1, ERK was phosphorylated, indicating the activation of IL-17-mediated proliferative signal. However, when cells were pretreated with DMF (25 μ M) for 18 h, IL-17-mediated degradation of Regnase-1 and phosphorylation of ERK was inhibited (Fig. 5A). This phenomenon persisted 3 h after IL-17A stimulation (Fig. 5B). Reasonably, DMF treatment suppressed IL-17-mediated upregulation of *NFKBIZ* and *Regnase-1* mRNA, degradation targets by Regnase-1 protein, and *LCN2* mRNA, a downstream gene of IL-17 signaling, in a dose-dependent manner (Fig. 5C). Furthermore, IL-17A treatment to *RegI*^{WT}-Min organoids promoted ERK phosphorylation accompanied with decreased of Regnase-1 expression, and DMF inhibited the degradation of Regnase-1 and ERK phosphorylation (SI Appendix, Fig. S9C). On the other hand, while phosphorylated ERK (pERK) was also induced by IL-17A in *RegI*^{KO}-Min organoids, ERK phosphorylation was not efficiently inhibited by DMF treatment (SI Appendix, Fig. S9D). Accordingly, DMF can inhibit the proliferative signals caused by IL-17 signaling in colon cancer cells via preventing Regnase-1 protein inactivation.

DMF Hinders Colon Tumor Growth by Preventing Inactivation of Regnase-1 Protein. We treated *RegI*^{fl/fl} (*RegI*^{WT}) mice and *Villin-Cre*⁺; *RegI*^{fl/fl} (*RegI*^{KO}) mice with vehicle or 0.05% DMF in drinking water for 5 wk. DMF treatment did not cause any systemic adverse effects, including body weight loss (SI Appendix, Fig. S10A) or any change in intestinal histology (SI Appendix, Fig. S10B) in either genotype. Immunoblotting using lysates from cecum tissues showed that DMF treatment increased the protein level of Regnase-1 (Fig. 6A). Consistent with this result, the mRNA level of Regnase-1 target genes, *Nfkbiz* and *Regnase-1* itself, were observed to be significantly lowered by DMF treatment, whereas *Regnase-1* deletion eliminated this effect (Fig. 6B), indicating that *Nfkbiz* suppression by DMF is dependent on increased Regnase-1 protein level, as expected. pERK in cecum tissues was identified to

be decreased by DMF treatment (Fig. 6A). These results suggest that oral DMF treatment can considerably affect the activity level of Regnase-1 protein without apparent toxicity.

Next, we treated *RegI*^{WT}-Min mice and *RegI*^{KO}-Min mice with vehicle or 0.05% DMF in drinking water continuously from weaning to 120 d old. Again, no systemic adverse effect was detected by DMF treatment (SI Appendix, Fig. S10C). Remarkably, DMF treatment significantly reduced the size of colon tumors in *RegI*^{WT}-Min mice, but not in *RegI*^{KO}-Min mice (Fig. 6C and D and SI Appendix, Fig. S10D–F). These data indicate that the therapeutic effect of DMF depends on Regnase-1. A decreased number of Ki-67-positive cells and a reduced pERK-positive area were observed in DMF-treated *RegI*^{WT}-Min mice (SI Appendix, Fig. S10G). Notably, DMF treatment changed the profile of infiltrating immune cells in the opposite direction of that seen under *Regnase-1* deletion. Explicitly, neutrophil and CD8⁺ T cell recruitment to tumor tissues appeared to be suppressed and enhanced, respectively (Fig. 6E). This effect was not evident in *RegI*^{KO}-Min mice. Although CD4⁺ T cell recruitment was not elevated even higher in tumor tissues, it tended to be increased in nontumor tissues (SI Appendix, Fig. S10H). Collectively, oral DMF treatment can hinder *Nfkbiz*-mediated tumor growth in colons by Regnase-1 augmentation.

Colorectal Cancer Cases with Low Regnase-1 Expression Have Poorer Prognosis. Next, we evaluated the expression levels of the Regnase-1 and NFKBIZ (also known as I κ B ζ) proteins in human colon tumor tissues using immunohistochemistry. In most of the 11 cases that we tested (SI Appendix, Table S3), ranging from tubular adenoma to moderately differentiated adenocarcinoma, Regnase-1 protein was found to be highly expressed in tumor tissues compared to that in nontumor tissues [Fig. 7A (Cases 4, 6), Upper, Fig. 7B], and a small percentage of tumors had low levels of Regnase-1 expression [Fig. 7A (Case 10), Upper, Fig. 7B]. Although the expression of the NFKBIZ protein seemed to be suppressed by Regnase-1 protein in tumor tissues [Fig. 7A (Case 4, 6), Lower], a few cancer cases with low Regnase-1 expression had high levels

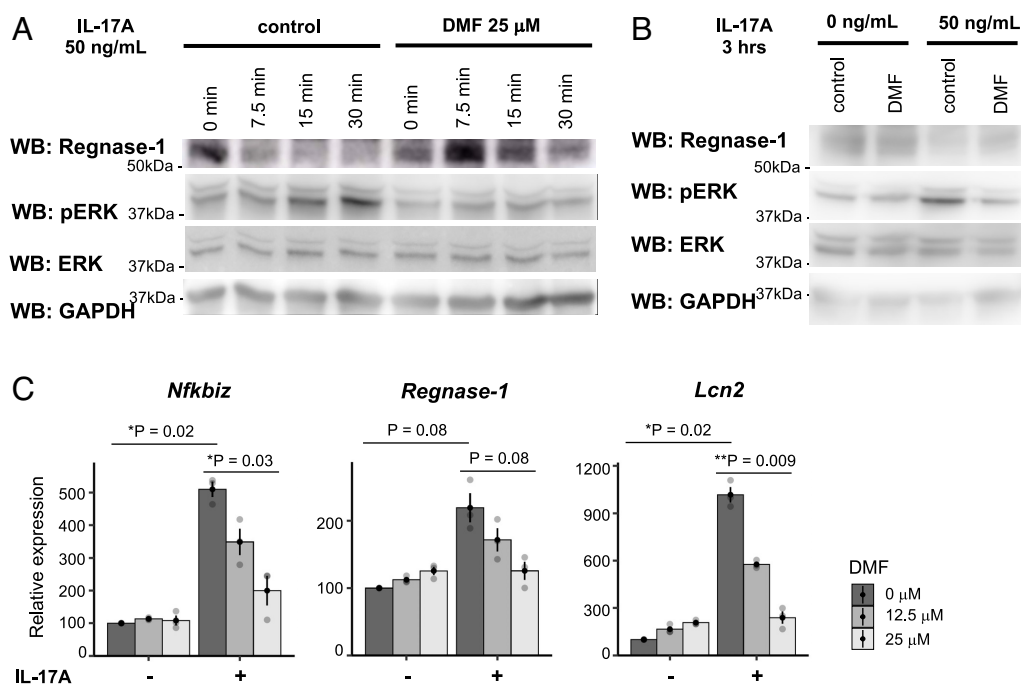


Fig. 5. Effect of DMF on human colon cancer cell line HT29. (A) Immunoblotting using lysates of HT29 cells after IL-17A (50 ng/mL) treatment with or without DMF pretreatment. (B) Immunoblotting using lysates of HT29 cells after 3 h of IL-17A treatment with or without DMF pretreatment. (C) mRNA levels of *Nfkbiz*, *Regnase-1*, and *Lcn2* in HT29 cells 3 h after IL-17A (50 ng/mL) treatment with DMF pretreatment. Data are presented as mean $\pm$ SE.

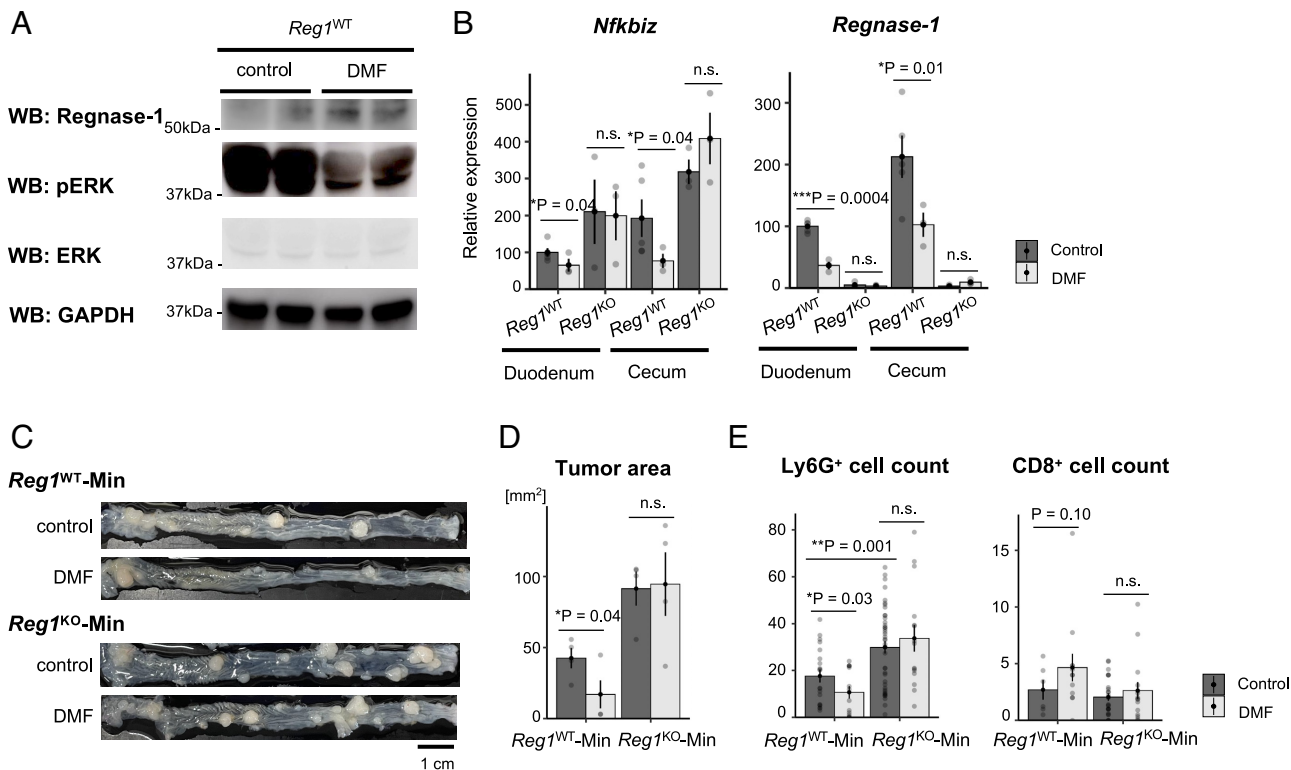


Fig. 6. Effect of oral DMF treatment on mice. (A) Immunoblotting using lysates of nontumor cecum tissues from *Reg1*^{WT} mice treated with vehicle or DMF for 5 wk. (B) mRNA levels of *Nfkbiz* and *Regnase-1* in nontumor cecum tissues from *Reg1*^{WT} and *Reg1*^{KO} mice (N = 4) treated with vehicle or DMF for 5 wk. (C) Representative colon from mice treated with vehicle or DMF since weaning age until 120 d old. (D) Tumor area in colons of 120-d-old mice treated with vehicle or DMF (N = 4 for *Reg1*^{WT}-Min and *Reg1*^{KO}-Min each). (E) Quantification of neutrophils and CD8⁺ T cells per sight field in colon tumor tissues of mice treated with or without DMF by IHC of Ly6G (20, 14, 42, 15 tumors from each group, respectively) and CD8 (6, 12, 25, 15 tumors from each group, respectively), respectively. Data are presented as mean $\pm$ SE.

of NFKBIZ expression [Fig. 7A (Case 10), Lower], suggesting an inverse correlation between the expression level of Regnase-1 and NFKBIZ in tumor tissues (Fig. 7C). Intriguingly, several cancers had scattered areas with depleted Regnase-1 expression, and these areas had high NFKBIZ expression (Fig. 7D), which strongly supports there being a close relationship between Regnase-1 and NFKBIZ. We also found that tumor cells positive for NFKBIZ showed elevated pERK expression, but cells without NFKBIZ upregulation did not express pERK (SI Appendix, Fig. S11 A and B), compatible with the in vitro and in vivo experimental data.

Finally, we analyzed the relationship between the Regnase-1 expression level and prognosis in colorectal adenocarcinoma cases using The Cancer Genome Atlas Program (TCGA) Firehose Legacy dataset on the cBioPortal platform (42, 43). Among 379 cases that had RNA-seq data of colon cancer tissues, 68 cases had a higher-than-average expression of both *IL17A* and *IL17F*. We divided these cases into two groups depending on whether their expression levels of *Regnase-1* mRNA in colorectal cancer tissues were higher or lower than the average. Both overall survival and disease-free survival were significantly shorter in the group with low Regnase-1 expression (Fig. 7 E and F). This result indicates the protective role of Regnase-1 in human colorectal cancers.

Discussion

In an *Apc*^{Min/+} mouse model of intestinal epithelium-specific *Regnase-1* deletion (*Reg1*^{KO}-Min), we observed an intriguing phenotype that tumor growth was accelerated by *Regnase-1* loss only in the colon. Here, *Nfkbiz* was identified as a sole immune mediator and degradative target of Regnase-1 (2) by transcriptome analysis of colon tumor tissues. *Nfkbiz* is a known transcriptional

regulator pivotal in the IL-17 signaling pathway that plays a key role in the pathogenesis of autoimmune diseases, such as psoriasis (26, 27) and experimental autoimmune encephalomyelitis (44), and homeostasis in the intestines (45). *Nfkbiz* upregulates multiple key inflammatory mediators such as LCN2, IL-6, G-CSF, and CCL2 (24, 25), each of which is known to activate ERK signaling and contribute to cell proliferation (46–49), although the mechanism of ERK activation under IL-17 stimulation is not fully elucidated (50). In our transcriptome results, genes related to IL-17 signaling were differentially expressed, including antimicrobial peptides (28, 29) and lipid metabolism (51), in the condition of *Regnase-1* deletion. While *Nfkbiz* is induced by several inflammatory signals including IL-1 β , IL-36, IL-17, and JAK-STAT (31), these results indicate that in intestinal epithelia, *Nfkbiz* upregulated by *Regnase-1* deletion will be closely related to IL-17 signaling. IL-17 signaling is also reported to promote the growth of colorectal tumors both in the animal model and in the human and be related to poor prognosis (35, 36). Ye et al. previously demonstrated that Regnase-1 has a tumor-suppressive role in colorectal cancer by inhibiting the NF- κ B signaling pathway (52). They showed that Regnase-1 inhibited K63-linked ubiquitylation of TRAF6, which leads to NF- κ B activation. Our findings are consistent with this previous report in that Regnase-1 suppresses colorectal tumor development by regulating the NF- κ B signaling pathway. However, our current study further uncovers a molecular mechanism by which Regnase-1 suppresses colorectal tumor growth: through repression of IL-17 signaling via degradation of *Nfkbiz* mRNA.

The reason why Regnase-1 has protective effects against tumor growth only in the colon could be related to the localization of gut microbes involved in IL-17 signaling. First, IL-17 signaling

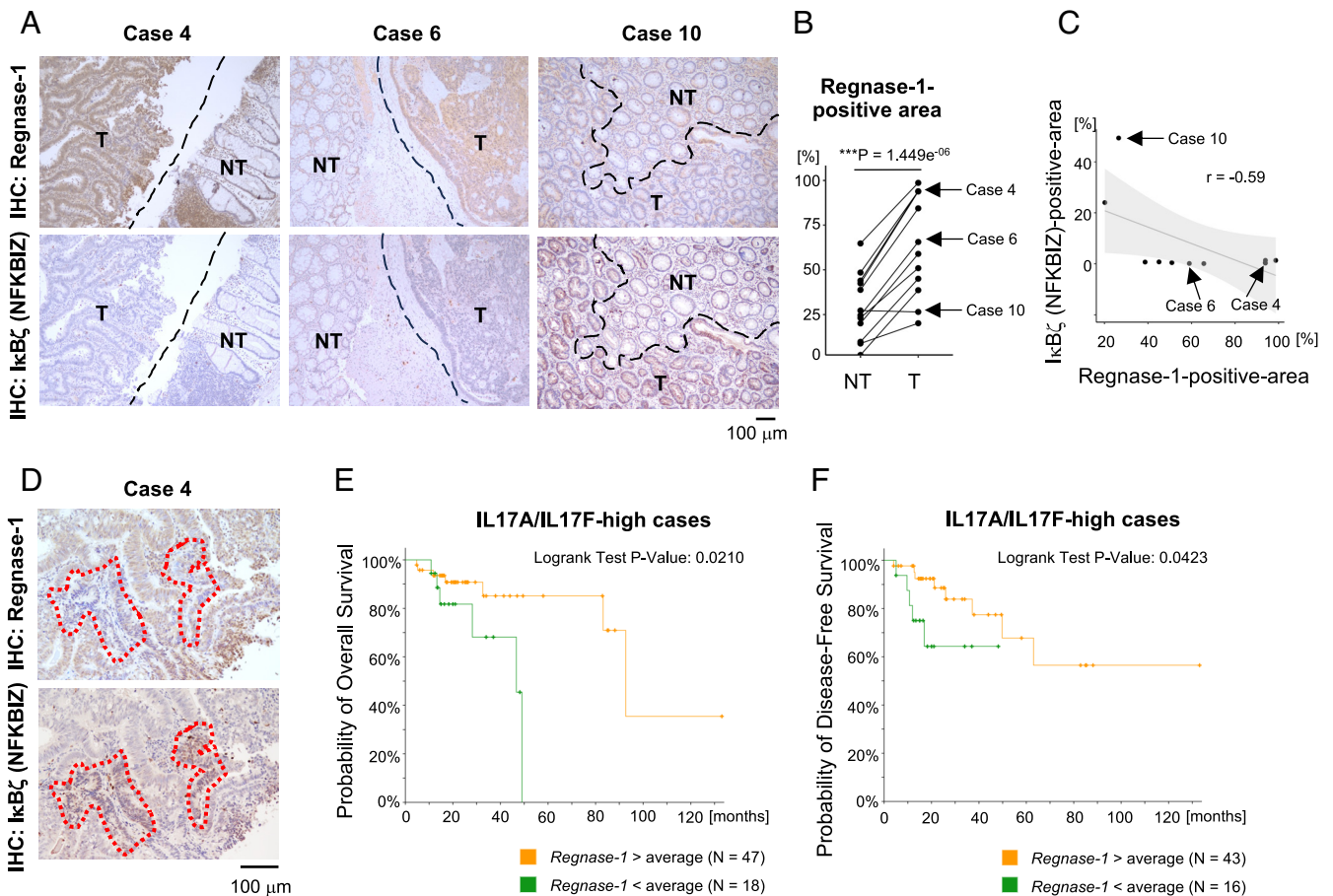


Fig. 7. Expression of *Regnase-1* in human colorectal cancer cases. (A) Representative IHC of *Regnase-1* and *IκBζ* (NFKBIZ) proteins in colorectal tumor tissues. T and NT represent tumor and nontumor tissues, respectively. (B) Quantification of the *Regnase-1*-positive area in tumor and nontumor tissues. (C) Correlation between *Regnase-1*-positive area and *IκBζ*-positive area rate in tumor tissues. (D) Representative image showing spots with depleted *Regnase-1* expression and elevated NFKBIZ expression. (E) Overall survival and (F) disease-free survival of colorectal cancer cases with IL17A/IL17F expression higher than the average of each from the TCGA Firehose Legacy dataset.

on the surface of intestinal tumors is triggered by the effect of microbial products on a defected mucosal barrier and promotes tumor growth (32, 53). Second, colonization of enterotoxigenic *Bacteroides fragilis*, a bacterium that has been linked to colorectal tumorigenesis epidemiologically, enhances tumor formation only in the colon due to its localizing niche (53). Although a specific bacterium influencing tumor growth in our mouse model was not identified, we speculate that IL-17 signaling is triggered by microbes and, therefore, the protective role of *Regnase-1* is prominent in the colon where abundant bacteria reside. The data in this study showing that both oral treatment of antibiotics and intraperitoneal injection of anti-IL17A antibody canceled the effect of *Regnase-1* on colon tumor growth rate corroborated the relationship between tumor growth, IL-17 signaling, and bacterial flora in the colon. On the other hand, this phenotype might be specifically observed in *Apc*^{Min/+} mice because *Apc* gene mutation is a cause of colorectal adenomas which may possess carcinogenic potential. Different effects of *Regnase-1* on cancer development might be observed if other oncogenic mouse models are used. The mechanism of the tumor growth in the small intestine needs to be elucidated for the future.

The immune profile shift caused by *Regnase-1* depletion was characteristic of IL-17 signaling (21, 30). In *Reg1*^{KO}-Min colon tumors, infiltration of neutrophils and CD4⁺ T cells was observed to be enhanced and repressed, respectively, indicating that *Regnase-1* in epithelial cells could modify immunity against tumors, likely due to the alteration of the chemokine expression

profile. IL-17 signaling is critical in neutrophil recruitment (54), which is related to cell proliferation, migration, and invasion by forming extracellular traps (55), resulting in poor prognosis in colorectal cancer (56). In our mouse adenoma model, however, immune cell infiltration activated by chemokines such as Cxcl1 and Cxcl2 may not play a major role in tumor growth, as reducing neutrophil infiltration through CXCR2 inhibitor treatment did not affect colon tumor development. Recently, IL-17 has also been reported to inhibit CXCL10 and result in decreased recruitment of T cells in colorectal tumors (21). Although CD8⁺ T cells were too scarce to be lowered by *Regnase-1* deletion in our mouse tumor tissues, likely because they were adenomas, not carcinomas, CD4⁺ T cell infiltration in *Reg1*^{KO}-Min colon tumors was repressed. Whether this could be associated with the decrease of antitumoral immunity (57) needs further investigation in the future.

The level of impact that IL-17 signaling gives on colorectal cancers would vary in each case depending on the gut microbiome or the expression of IL-17 receptors. In a tumor with a low degree of IL-17-mediated inflammation, *Regnase-1* mRNA will stay self-degraded by *Regnase-1* protein. However, with activated IL-17 signaling, *Regnase-1* protein would be inactivated through phosphorylation (41), leading to an elevated *Regnase-1* mRNA level due to cessation of self-degradation. However, in some of the colorectal cancer cases on TCGA, *Regnase-1* mRNA was relatively low even with activated IL-17 signaling, and these cases had poorer prognoses than the cases with higher levels of *Regnase-1* mRNA expression. This result strongly supports the

protective role of Regnase-1 against tumor growth. Although the reason for insufficient *Regnase-1* mRNA expression with activated IL-17 signaling is not yet identified, it might be due to DNA methylation of the *Regnase-1* gene promoter or the alteration of IL-17 receptors and/or signaling mediators. The mechanism of patchy Regnase-1 protein loss observed in cancer tissues could also be caused by tumor heterogeneity associated with such Regnase-1 insufficiency.

We hypothesized that Regnase-1 could be a potential therapeutic target if it does play a key role in tumor biology; thus, we explored a therapeutic strategy of colorectal tumors targeting Regnase-1. We utilized DMF, an FDA-approved drug already used safely for multiple sclerosis and psoriasis, both of which are autoimmune diseases involved with IL-17 signaling in their pathogenesis (26, 58). While the pharmacological mechanism of DMF was not precisely proved except for the antioxidative effect by transcriptional activation of *NRF2*, Ohgakiuchi et al. recently reported a novel function of DMF to stabilize Regnase-1 protein by inhibiting its phosphorylation and to elevate its activity at the protein level, independent of *NRF2* (39). Because Regnase-1 is known to be posttranscriptionally regulated especially when responding to IL-17 stimulus (41), it will be advantageous to augment Regnase-1 at the protein level. Although DMF is not a drug that specifically targets Regnase-1, the antitumor effect of DMF observed in our animal experiment is considered to depend on Regnase-1 in epithelial cells because the effect disappeared in the intestinal epithelium-specific *Regnase-1* KO model. Thus, DMF could possibly be a promising and practical approach to control colorectal tumor growth by augmenting Regnase-1 protein. Recently, we reported a therapeutic strategy aiming Regnase-1 by developing antisense oligonucleotides that raise the expression of *Regnase-1* mRNA by blocking its stem-loop motifs used for self-degradation (11). It might also be available for the treatment of colorectal cancer if the antisense oligonucleotides are safely delivered to the tumor cells in vivo.

One of the limitations of our study is that we could not analyze the relationship between the expression level of Regnase-1 protein, level of IL-17 signaling, and prognosis in a colorectal cancer cohort, due to the lack of public proteome data that included Regnase-1 expression. Furthermore, neutrophil recruitment to human colorectal tumors might not be precisely recapitulated in a mouse model because mice do not have the *IL8/CXCL8* gene (59), which is a main inducer of neutrophil chemotaxis in humans.

Collectively, this study revealed that Regnase-1 expressed in colorectal epithelium regulates IL-17 signaling via *NFKBIZ* mRNA degradation and contributes to the suppression of tumor growth. Regnase-1 was first shown to be a possible target of therapy of colorectal tumors.

Materials and Methods

Mice. The preparation of Regnase-1-floxed and Villin-Cre transgenic mice has been described previously (3, 60). *Apc*^{Min/+} mice were obtained from The Jackson Laboratory (Bar Harbor, MN). *Nfkbiz*-floxed mice (RBRC06410)(37) were obtained from Riken BRC (Tsukuba, Japan). All mice were maintained in a specific pathogen-free facility at the Kyoto University Faculty of Medicine. Primers for genotyping PCR are listed on *SI Appendix, Table S4*. Both male and female animals were equally examined in the experiments.

Cell and Organoid Culture. HT29 cells (RRID: CVCL_0320) were cultured in Dulbecco's modified eagle medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ incubator. 500,000 cells were seeded per well on a 6-well plate 24 h before the experiment. The colon tumor organoids derived from *Reg1*^{WT}-Min mice and *Reg1*^{KO}-Min mice were generated and cultured as previously described (61, 62). The detailed protocol of analyses is described in SI Appendix, Supporting Information Text.

Statistics. Data are expressed as mean ± SE. Statistical analysis was performed using Student's *t* test for differences between two groups. *P* < 0.05 was considered significant. All graphs were plotted using R (v.4.2.2).

Study Approval. All animal experiments were approved by the Ethics Committee for Animal Experiments and performed in accordance with the Kyoto University Guidelines for Animal Experiments. This research conformed to the provisions of the Declaration of Helsinki. The study protocol was approved by the ethics committee of Kyoto University, and written informed consent was obtained from each patient.

Data, Materials, and Software Availability. Sequencing datasets are available at the DDBJ Sequence Read Archive (DRA, <https://www.ddbj.nig.ac.jp/dra/index-e.html>) under accession numbers **DRA017138** and **DRA017139** (12, 16).

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