Down-regulation of RalGTPase-Activating Protein Promotes Colitis-Associated Cancer via NLRP3 Inflammasome Activation

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SUMMARY

Ral activation impacts interleukin 1β production by nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 inflammasome activation and enhances matrix metalloproteinase-9 and matrix metalloproteinase-13 expression in a newly established mouse colitis-associated cancer model. This study strongly showed that the Ras-like-nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 inflammasome pathway is involved in the mechanism of colitis-associated cancer invasion.

BACKGROUND & AIMS: Ral guanosine triphosphatase–activating protein α2 (RalGAPα2) is the major catalytic subunit of the negative regulators of the small guanosine triphosphatase Ral, a member of the Ras subfamily. Ral regulates tumorigenesis and invasion/metastasis of some cancers; however, the role of Ral in colitis-associated cancer (CAC) has not been investigated. We aimed to elucidate the role of Ral in the mechanism of CAC.

METHODS: We used wild-type (WT) mice and RalGAPα2 knockout (KO) mice that showed Ral activation, and bone marrow chimeric mice were generated as follows: WT to WT, WT to RalGAPα2 KO, RalGAPα2 KO to WT, and RalGAPα2 KO to RalGAPα2 KO mice. CAC was induced in these mice by intraperitoneal injection of azoxymethane followed by dextran sulfate sodium intake. Intestinal epithelial cells were isolated from colon tissues, and we performed complementary DNA microarray analysis. Cytokine expression in normal colon tissues and CAC was analyzed by quantitative polymerase chain reaction.

RESULTS: Bone marrow chimeric mice showed that immune cell function between WT mice and RalGAPα2 KO mice was not significantly different in the CAC mechanism. RalGAPα2 KO mice had a significantly larger tumor number and size and a significantly higher proportion of tumors invading the submucosa than WT mice. Higher expression levels of matrix metalloproteinase-9 and matrix metalloproteinase-13 were observed in RalGAPα2 KO mice than in WT mice. The expression levels of interleukin 1β, NLRP3, apoptosis associated speck-like protein containing a CARD, and caspase-1 were apparently increased in the tumors of RalGAPα2 KO mice compared with WT mice. NLRP3 inhibitor reduced the number of invasive tumors.

CONCLUSIONS: Ral activation participates in the mechanism of CAC development via NLRP3 inflammasome activation. (Cell
Patients with long-standing inflammatory bowel disease (IBD) are at high risk for developing dysplasia and colitis-associated cancer (CAC). In clinical practice, detecting dysplasia and CAC at endoscopy often is difficult. In addition, CAC has a tendency to easily invade intestinal tissues. Therefore, these clinicopathologic features result in the poor prognosis of IBD patients with CACs. Several studies have indicated that sustained intestinal inflammation plays a substantial role in increasing CAC via telomere shortening, DNA damage, and cellular senescence. However, the exact molecular mechanism of CAC remains unclear.

The small guanosine triphosphatase (GTPase) Ras-like (Ral), a member of the Ras subfamily, is known to control various cellular functions by mediating multiple effector molecules, and Ral plays an important role in cell survival by acting through the regulation of TRAF (tumor necrosis factor receptor-associated factor) family member associated NF-κB (nuclear factor-kappa B) activator (TANK)-binding kinase 1, which is the controlling factor of nuclear factor-κB (NF-κB). Thus, Ral also is involved in inflammation. Ral has 2 isoforms: RalA and RalB. RalA and RalB have been shown to play different roles in human cancer: RalA is involved in anchorage-independent growth, and RalB is required for cell survival and migration.

Ral is positively regulated by Ral guanine nucleotide exchange factors, several of which are effector molecules of Ras. Thus, Ral is activated downstream of active Ras. However, Ral is inactivated by RalGTPase-activating proteins (RalGAPs) that we identified previously. RalGAPs are heterodimers of a common β subunit and a catalytic α subunit (RalGAPα1 or RalGAPα2). The heterodimer comprising RalGAPα1 and the RalB β subunit is designated RalGAP1, and the heterodimer comprising RalGAP2 and the RalGAP β subunit is designated RalGAP2. Complex formation is essential for RalGAP activity and protein stability, and loss of either subunit leads to the inactivation of its enzyme activity. Each form of RalGAP regulates both RalA and RalB.

The expression of the α subunit varies among different organ types. We previously reported that RalGAPα2 knockout (KO) mice developed muscle-invasive bladder cancers that were not found in wild-type (WT) mice in chemically induced bladder carcinogenesis models. In addition, we reported that loss of RalGAPα2 promoted local microscopic invasion of prostatic intraepithelial neoplasia in a phagophase and tensin homolog deleted from chromosome 10-deficient mouse model for prostate tumorigenesis in a recent study. In addition, Kodama et al reported a significant reduction in RalGAPα2 expression in hepatocellular carcinoma and a role for RalGAPα2 in the enlargement of hepatocellular carcinoma. These data suggest that RalGAPα2 plays an important role in the development or invasion mechanism in some cancers. However, the precise role of RalGAPα2 in CAC has not been elucidated.

In the present study, we show that RalGAPα2 is highly expressed in the normal colon epithelium and in sporadic colorectal cancer compared with CAC in human samples. In addition, Ral activation greatly enhances tumor invasion, with matrix metalloproteinase (MMP)-9 and MMP-13 expression. Furthermore, Ral activation impacts interleukin (IL)1β production by NLRP3 inflammasome activation. Our data strongly show that the Ral-NLRP3 inflammasome pathway is involved in the mechanism of CAC invasion.

Results

**RalGAPα2 Is Expressed Mainly in Colon Epithelial Cells but Not in Immune Cells**

Western blot (WB) analysis showed that mouse colon tissues strongly expressed the RalGAPα2 subunit but showed little expression of RalGAPα1 (Figure 1A). In addition, both the human and mouse colon cancer cell lines strongly expressed the RalGAPα2 subunit but showed little expression of RalGAPα1 (Figure 1B). Furthermore, WB analysis showed that the expression levels of both RalGAPα1 and α2 were weak in monocytes, T cells, and B cells (Figure 1B).

Knockdown (KD) of RalGAPα2 (Figure 1C) increased the activation of RalA and RalB (Figure 1D and F), and slightly increased the expression levels of total RalA and total RalB (Figure 1E and G) in Colon26 cells. The RalA-GTP/total-RalA and RalB-GTP/total-RalB ratios were significantly higher in the RalGAPα2 KD Colon26 cells than in the controls (Figure 1H).

**RalGAP Does Not Influence T-Helper Cell 1/2/17 or B-Cell Differentiation or Cytokine Production in Macrophages**

Under the steady-state condition, flow cytometry analysis showed no significant difference in T-helper cell (Th)1/Th2/Th17 differentiation of CD4-positive T cells and B-cell differentiation of CD19-positive B cells between WT mice and RalGAPα2 KO mice (Figure 2A and B). In addition, no
significant differences were observed in the cytokines (IL1β, IL12A, or tumor necrosis factor-α [TNF-α]) released from intraperitoneal macrophages collected after intraperitoneal administration of thioglycolate between the WT mice and RalGAPα2 KO mice (Figure 2C). These results did not change under the dextran sulfate sodium (DSS) condition (Figure 2D–F). These findings showed that RalGAP did not influence T-cell or B-cell differentiation or cytokine production of macrophages regardless of the administration of DSS.

**RalGAPα2 Expression Is Decreased in CAC**

We compared the expression levels of RalGAPα2 in human normal colon tissue, sporadic colorectal cancer, and CAC samples by immunohistochemical staining (Figure 3A). The ratios showing strong RalGAPα2 staining in the normal colon tissue, sporadic colorectal cancer, and CAC samples were 85.7% (30 of 35), 80.0% (12 of 15), and 25% (5 of 20), respectively. Thus, the expression of RalGAPα2 in CAC was significantly lower than that in normal colon tissue or sporadic colorectal cancer (Figure 3B). When CAC is divided into 2 groups as noninvasive and invasive, the ratios showing strong RalGAPα2 staining in noninvasive and invasive CAC samples were 80.0% (4 of 5) and 6.7% (1 of 15), respectively (Figure 3C). Therefore, the expression of RalGAPα2 decreased, that is, activation of Ral was observed at a more advanced stage in CAC. In addition, patient information from the human cohort is shown in Figure 3D. There were no significant differences in patients’ backgrounds between patients with sporadic colorectal cancer and those with CAC.
Figure 2. (A–C) The differences in the contributions of immune cells in steady state between WT mice and RalGAPα2 KO mice were analyzed by flow cytometry or enzyme-linked immunosorbent assay. (A) T-cell differentiation. (B) B-cell differentiation. (C) Cytokine expression of macrophages (1-way analysis of variance with the Holm correction). (D–F) The differences in the contributions of the immune cells after dextran sulfate sodium treatment between WT mice and RalGAPα2 KO mice were analyzed by flow cytometry or enzyme-linked immunosorbent assay. (D) T-cell differentiation. (E) B-cell differentiation. (F) Cytokine expression of macrophages (1-way analysis of variance with the Holm correction). Data are either representative or shown as means ± SEM (error bars) of 3 independent experiments. IFN, interferon.

**Bone Marrow Chimeric Mice Showed RalGAPα2 Did Not Affect Immune Cell Function**

To confirm the contribution of RalGAPα2 in immune cells to CAC in vivo, we generated bone marrow chimeric mice and divided them into 4 groups, as follows: (1) WT to WT, (2) WT to RalGAPα2 KO, (3) RalGAPα2 KO to WT, and (4) RalGAPα2 KO to RalGAPα2 KO mice, and CAC was induced in these mice by the AOM/DSS protocol shown in Figure 5A. Tumor occurrence was examined at the distal end of the colon tissues by macroscopic images (Figure 5B) and histopathologic findings (Figure 5C). Compared with the totally WT mice (group 1), the totally RalGAPα2 KO mice (group 4) developed significantly more tumor growth in terms of number (group 1 vs group 4, 3.6 ± 1.6 vs 6.1 ± 2.1) (Figure 5D) and larger tumor sizes (group 1 vs group 4, 2.9 ± 1.4 vs 7.4 ± 2.6 mm) (Figure 5E). Although it was not significant (P = .07), the ratio of mice with SM invasive tumors in group 4 was higher than that in group 1 (group 1 vs group 4, 0% [0 of 10] vs 37.5% [3 of 8]) (Figure 5F). In addition, the number of SM invasive tumors in the cancer specimens in group 4 was significantly higher than that in the specimens in group 1 (group 1 vs group 4, 0% [0 of 20] vs 28.6% [8 of 28]) (Figure 5G). These results were consistent with the results in the mouse CAC model described earlier (Figure 4A–H).

Next, we examined Ral activation in mouse colon tissues. The pull-down analysis showed that the tissues from the RalGAPα2 KO mice (normal colon tissues of RalGAPα2 KO mice [KO-N] and tumors of RalGAPα2 KO mice [KO-T]) showed increased activation of RalA and RalB (Figure 4I and K) and slightly increased expression levels of total RalA and total RalB (Figure 4J and L). The RalA-GTP/total RalA and RalB-GTP/total RalB ratios in the tissues from the RalGAPα2 KO mice were significantly higher than those in the tissues from the WT mice. These ratios were increased markedly in the KO-T mice (Figure 4M). These findings suggest that the down-regulation of RalGAPα2 induced Ral activation, especially in colon tumors.
RalGAPα2 KD Colon26 Cells Show Increased Migratory and Invasive Capacities

The in vitro wound healing (Figure 6A) and cell invasion assays (Figure 6B) showed that significantly more RalGAPα2 KD Colon26 cells than control cells migrated and invaded. In addition, Ral activation correlated with an increase in the cell migratory and invasive capacities.

MMP-9 and MMP-13 Expression Levels Are Up-Regulated in Colon Tumors From RalGAPα2 KO Mice

Phenotypic differences in colonic epithelial cells from both WT mice and RalGAPα2 KO mice were examined. Colonic epithelial cells were isolated from colon tissues, and flow cytometry was performed to determine whether more than 90% of the isolated cells were CD326-positive/CD45-negative epithelial cells (Figure 6C). Then, microarray analysis showed that the gene expression levels of MMP-9 and MMP-13 in colon epithelial cells from the RalGAPα2 KO mice increased to more than twice those of the WT mice. On the other hand, the MMP-2, -3, -7, and -12 gene expression levels in the RalGAPα2 KO mice did not increase compared with those in the WT mice (Figure 6D).

Quantitative polymerase chain reaction (qPCR) showed that MMP-9 and MMP-13 messenger RNA (mRNA) expression in the KO-T significantly increased compared with that in the tumors of WT mice (WT-T) (Figure 6E). Gelatin zymography analysis showed that activated MMP-9 was increased drastically in the KO-T (Figure 6F). WB analysis showed that MMP-13 expression apparently increased in the KO-T (Figure 6G). In addition, immunohistochemical staining showed an increased number of cells that were positive for MMP-9 (Figure 6H) and MMP-13 (Figure 6I) at the invasive fronts of the tumors in the RalGAPα2 KO group compared with the WT group. These results suggest that MMP-9 and MMP-13 are involved in colon tumor invasion in RalGAPα2 KO mice.

IL1β Expression Is Up-Regulated Significantly in Colon Tumors From RalGAPα2 KO Mice

Next, we examined cytokine levels in mouse tissues by qPCR. IL1β expression in KO-N was significantly
higher than that in normal colon tissues of WT mice (WT-N). In addition, IL1β expression in the KO-T was significantly higher than that in the WT-T or KO-N. The expression levels of IL4 and IL13 were significantly lower in the KO-T than in WT-T. There were no differences in the expression levels of TNF-α, interferon-γ, IL6, IL10, IL12A, IL17, or IL18 (Figure 7A). These data suggest a strong association between IL1β expression and a CAC-promoting inflammatory environment in RalGAPα2 KO mice.

Figure 4. (A) The AOM/DSS administration protocol. We performed this protocol in both WT mice (n = 20) and RalGAPα2 KO mice (n = 20). (B) The endoscopic findings of colon tumors in WT mice and RalGAPα2 KO mice. (C) The macroscopic findings of colon tumors in WT mice and RalGAPα2 KO mice. (D) H&E staining of colon sections of WT mice and RalGAPα2 KO mice on day 63 (original magnification, 100×). Scale bars: 100 μm. (E) Tumor number in the WT mice and RalGAPα2 KO mice. *P < .01, Student t test. (F) Tumor maximum diameter in the WT mice and RalGAPα2 KO mice. *P < .01, Student t test. (G) The ratio of mice with submucosal invasive tumors in the WT mice and RalGAPα2 KO groups. *P < .01, Fisher exact test. (H) The number of submucosal invasive tumors in the cancer specimens of WT mice and RalGAPα2 KO mice. *P < .01, Fisher exact test. (I and J) The expression of RalA-GTP, RalB-GTP, total RalA, and total RalB in the colonic mucosa and CAC of both WT mice and RalGAPα2 KO mice was analyzed by the glutathione-S-transferase–Sec5 pull-down assay. (K and L) Densitometric analysis of the expression levels of RalA-GTP, RalB-GTP, total RalA, and total RalB in the colonic mucosa and CAC of both WT mice and RalGAPα2 KO mice by Western blot. (M) The ratios of the expression of RalA-GTP/total RalA and RalB-GTP/total RalB in the colonic mucosa and CAC of both WT mice and RalGAPα2 KO mice. Data are either representative or shown as the means ± SEM (error bars) of 3 independent experiments. (K–M) *P < .01, **P < .05 by repeated-measures analysis of variance with the Holm correction.
Down-Regulation of RalGAPa2 Induces Activation of the NLRP3 Inflammasome

Next, we focused on the association between Ral activation and the NLRP3 inflammasome as a main inducer of IL1β. WB analysis showed that RalGAPa2 KD increased the expression levels of NLRP3, apoptosis associated speck-like protein containing a CARD (ASC), pro/cleaved caspase-1, and pro/cleaved caspase-11 in Colon26 cells (Figure 7B). In vivo data showed that the expression of NLRP3, ASC, and pro/cleaved caspase-1 tended to be increased in both WT-T and KO-T. These proteins were expressed more strongly in KO-T than in WT-T. Moreover, pro/cleaved caspase-11, NF-κB p65, phosphorylated NF-kappaB p65 (NF-κB p-p65), signal transducer and activator of transcription 3 (STAT3), and phosphorylated STAT3 tended to be increased in KO-N than in WT-N. However, these high expressions were not observed in KO-T (Figure 7C). These data indicate that down-regulation of RalGAPα2 did not intensify the noncanonical NLRP3 inflammasome activation or NF-κB-STAT3 signaling, but intensified the canonical NLRP3 inflammasome activation in CAC.

NLRP3 Inhibitor Administration Suppresses the Invasive Potential of Colon26 Cells and of Tumors in RalGAPα2 KO Mice

To evaluate the role of the inflammasome in CAC development in RalGAPα2 KO mice, we examined the in vitro and in vivo effects of NLRP3 inhibitors on cell invasion. The number of invading Colon26 cells transfected with RalGAPα2 small interfering RNA (siRNA) decreased significantly after administration of the NLRP3 inhibitor (Figure 8).

Next, we investigated the effect of NLRP3 inhibitor on the AOM/DSS mouse model of the following 4 groups: WT + AOM/DSS, WT + AOM/DSS/NLRP3 inhibitor, RalGAPα2 KO + AOM/DSS, and RalGAPα2 KO + AOM/DSS/NLRP3 inhibitor (Figure 9A). Tumor occurrence was examined at the distal end of the colon tissue by endoscopy, macroscopic images, and histopathologic findings, and the representative images of the group of WT + AOM/DSS/NLRP3 inhibitor and RalGAPα2 KO + AOM/DSS/NLRP3 inhibitor are shown in Figure 9B–D. The NLRP3 inhibitor did not affect the number of tumors...
Figure 6. (A) In vitro effect of Ral activation on the migration of RalGAPα2 knockdown Colon26 cells. Scale bars: 200 μm. *P < .01, repeated-measures analysis of variance with the Holm correction. (B) In vitro effect of Ral activation on the invasion of RalGAPα2 knockdown Colon26 cells. Scale bars: 200 μm. *P < .01, 1-way analysis of variance with the Holm correction. (C) Flow cytometry findings for the isolated colon epithelial cells that were CD326-positive/CD45-negative. (D) The ratio of the gene expression levels of colon epithelial cells in RalGAPα2 KO mice/WT mice. (E) The mRNA expression levels of MMP-9 and MMP-13 were analyzed by quantitative polymerase chain reaction. The average expression levels in WT-N were defined as 1. *P < .01, **P < .05, repeated-measures analysis of variance with the Holm correction. (F) Gelatin zymography of activated MMP-9 in colon mucosa and CAC. (G) Western blot of MMP-13 in colon mucosa and CAC. (H and I) Immunohistochemical staining for MMP-9 and MMP-13 in the CAC of WT mice (a) and the CAC of RalGAPα2 KO mice (b). Scale bars: 100 μm. The proportion of cells that are positive for MMP-9 and MMP-13 at the invasive fronts of tumors from WT mice and RalGAPα2 KO mice (n = 10 per group). **P < .05, Fisher exact test. Data are either representative or shown as the means ± SEM (error bars) of 3 independent experiments.
in either the AOM/DSS-treated WT group or the RalGAPα2 KO group (Figure 9E). However, the mean size of the tumors in the RalGAPα2 KO group treated with NLRP3 inhibitor was decreased significantly in comparison with that in the group without NLRP3 inhibitor treatment (RalGAPα2 KO with NLRP3 inhibitor vs RalGAPα2 KO without NLRP3 inhibitor, 7.0 ± 2.1 vs 5.1 ± 2.0 mm) (Figure 9F). With regard to the tendency toward tumor invasion, the ratio of mice with SM invasive tumors in the RalGAPα2 KO group treated with NLRP3 inhibitor was decreased significantly compared with that in the group without NLRP3 inhibitor treatment (RalGAPα2 KO with NLRP3 inhibitor vs RalGAPα2 KO without NLRP3 inhibitor, 45% [9 of 20] vs 10.0% [2 of 20]) (Figure 9G). In addition, the number of SM invasive tumors in the RalGAPα2 KO group treated with NLRP3 inhibitor was decreased significantly compared with that in the group without NLRP3 inhibitor treatment (RalGAPα2 KO with NLRP3 inhibitor vs RalGAPα2 KO without NLRP3 inhibitor, 20% [24 of 120] vs 8.8% [6 of 68]) (Figure 9H). In addition, the expression of NLRP3, ASC, pro/cleaved caspase-1, MMP-9, and MMP-13 in KO-T were decreased when they were treated with NLRP3 inhibitor (Figure 9I–K). These results suggest that the Ral-NLRP3 inflammasome pathway with up-regulation of MMPs is involved in the cell invasion phenotype of CAC.

Figure 7. (A) The gene expression levels of IL1β, TNF-α, IFN-γ, IL4, IL6, IL10, IL12A, IL13, IL17, and IL18 were examined in the colon tissues of WT mice and RalGAPα2 KO mice by quantitative polymerase chain reaction. The average expression level in the WT-N was defined as 1. *P < .01, **P < .05, repeated-measures analysis of variance with the Holm correction. (B) The differences in the expression levels of NLRP3, ASC, pro/cleaved caspase-1, and pro/cleaved caspase-11 in the controls and RalGAPα2 knockdown Colon26 cells were analyzed by Western blot. (C) The differences in the expression levels of NLRP3, ASC, pro/cleaved caspase-1, pro/cleaved caspase-11, NF-κB p65, NF-κB p-p65, STAT3, and phosphorylated STAT3 in colon mucosa and CAC were analyzed by Western blot. Data are either representative or shown as the means ± SEM (error bars) of 3 independent experiments.
Figure 8. The effect of NLRP3 inhibitor on the invasion of RalGAPα2 knockdown Colon26 cells and the controls. *P < .01, 1-way analysis of variance with the Holm correction. Data are either representative or shown as the means ± SEM (error bars) of 3 independent experiments. HPF, high-power field.

**RalGAPα2 KD Leads to NLRP3 Transcription via AP-1**

The luciferase reporter analysis showed that RalGAPα2 KD Colon26 cells led to mouse NLRP3 transcription when the plasmids did not extend 1.0 kb upstream from exon 1, but when the plasmids extended 1.6 kb (Figure 10A and B). We focused on some transcriptional factors (Nfatc2, Sox6, AP-1, and Gata1) between this 0.6-kb sequence. The results did not change with the 1.6-kb plasmid except for the sequences of Sox6, Nfatc2, and Gata1. However, the expression of NLRP3 was reduced with the plasmid except for the sequence of AP-1 (Figure 10C). These results show that RalGAPα2 KD leads to NLRP3 transcription via AP-1.

**Discussion**

This study showed a critical role for the down-regulation of RalGAPα2, the major inhibitory regulator of the small GTPase Ral in the colon, in the invasive tumorigenesis of CAC with up-regulation of MMP-9 and MMP-13. In addition, we showed that RalGAPα2 down-regulation induced IL1β expression via NLRP3 inflammasome. Notably, the NLRP3 inhibitor significantly suppressed tumor growth with an invasive phenotype during CAC tumorigenesis, indicating the involvement of decreased RalGAPα2 expression in the progression of CAC via NLRP3 activation with up-regulation of MMPs.

First, we found that RalGAPα2 expression in human CAC decreased significantly compared with that in human sporadic colorectal cancer. Ral activation has been reported to contribute to tumor development in some cancers. In gastrointestinal cancers, some reports have been found on the association with sporadic colorectal cancer. Martin et al reported that activation of RaIα is necessary for the anchorage-independent growth of sporadic colorectal cancer. Furthermore, Singh et al reported that Ral guanine nucleotide exchange factor activation plays an important role as an effector in sporadic colorectal cancer cell lines. Ohta et al reported that the decreased expression of the RasGTPase activating protein is associated with colorectal tumor progression. However, the roles of RalGAP in sporadic colorectal cancer and CAC have not been investigated. In the present study, immunohistochemical analysis indicated that the mechanism of Ral activation in CAC might be different from that in sporadic colorectal cancer. In addition, down-regulation of RalGAPα2 was observed at a more advanced stage in CAC. Therefore, we used RalGAPα2 KO mice with the AOM/DSS model to investigate the role of Ral activation in the development of CAC and compared the histopathologic features of CAC in RalGAPα2 KO mice with those in WT mice. Our data showed that the RalGAPα2 KO mice with the AOM/DSS model showed significantly larger CAC sizes and more invasive phenotypes than the WT mice. We showed significant increases in the RalA/B-GTP and total Ral A/B ratios of CACs in the colon tumor tissue of RalGAPα2 KO mice compared with those in the colon tumor tissues of the WT mice in the AOM/DSS CAC model. Taken together, these data show that Ral activation contributes to the development and invasion mechanism of CAC.

Second, we focused on the contribution of RalGAPα2 on immune cells. WB analysis showed that the expression levels of RalGAPα2 were weak in monocytes, T cells, and B cells (Figure 1B). Flow cytometry showed that RalGAPα2 did not influence T-cell or B-cell differentiation or cytokine production of macrophages regardless of the administration of DSS (Figure 2). Furthermore, data on bone marrow chimeric mice showed that RalGAPα2 did not affect immune cell function in the CAC mechanism (Figure 5).

Third, we focused on the mechanism of tumor invasion in RalGAPα2 KO mice. In the AOM/DSS CAC model, colon tumors in RalGAPα2 KO mice showed an invasive phenotype compared with those in WT mice, similar to our previous report with bladder cancer and prostate cancer, which indicated that tumors harboring down-regulated RalGAPα2 were predisposed to tumor invasion. However, in those studies, we did not address the molecular mechanism underlying the invasive and metastatic phenotypes.

Here, we attempted to elucidate the mechanism underlying the acquisition of the invasive phenotype in Ral-mediated CACs. First, we performed microarray analysis with colon epithelial cells isolated from both WT and RalGAPα2 KO mice. We found that the gene expression levels of MMP-9 (gelatinase B) and MMP-13 (collagenase 3) in the colon epithelial cells of the RalGAPα2 KO mice were more than twice the levels of those in WT mice. Strikingly, we found that the expression levels of MMP-9 and MMP-13 were increased drastically in the CAC tissues of the RalGAPα2 mice. There have been many reports regarding the...
critical role of MMPs in cancer invasion and metastasis.17,18 Our in vitro and in vivo analyses suggested that aberrant Ral activation could contribute to the invasive phenotype of CACs via the induction of MMPs.

Next, we focused on the role of inflammation in CAC. Although various types of cytokines, such as TNF-α, IL1β, IL6, IL10, and IL12/23, are involved in the pathophysiology of IBD,19 our study yielded the unexpected findings of extremely high IL1β expression in the colon tumors of RalGAPα2 KO mice in comparison with other cytokines, such as Th1, Th2, and Th17 cytokines. Several reports have addressed the association between IL1β and CAC. Neutrophils were found to produce IL1 in experimentally induced colitis and in patients with CAC.20 Blockade of IL1β activity was shown to reduce tumorigenesis in mice by impairing macrophage-dependent IL6 secretion.21 Moreover, it was reported that IL1β up-regulates MMP gene expression.22-24 Taken together, the data in our study indicate that the levels of IL1β produced by cancer cells with down-regulated RalGAPα2 contributed to tumor invasion through MMPs.

When considering IL1β expression in the tumors of RalGAPα2 KO mice, we focused on the association between Ral and the NLRP3 inflammasome. To assess how Ral was involved in NLRP3 inflammasome complex activation, we examined NLRP3 complex activation in Ral-activated colon cancer cell lines and the Ral-activated mouse CAC model.

Figure 9. (A) The protocol for AOM/DSS/NLRP3 inhibitor administration. (B) Endoscopic findings of CAC occurrence with NLRP3 inhibitor administration in WT mice and RalGAPα2 KO mice. (C) Macroscopic findings of CAC occurrence with NLRP3 inhibitor administration in WT mice and RalGAPα2 KO mice. (D) Histopathologic findings of CAC occurrence with NLRP3 inhibitor administration in WT mice and RalGAPα2 KO mice. (E–H) These 4 groups were compared for (E) tumor number (*P < .01, 1-way analysis of variance with the Holm correction), (F) tumor maximum diameter (*P < .01, 1-way analysis of variance with the Holm correction), (G) the ratio of mice with submucosal invasive tumors (**P < .05, Fisher exact test with the Holm correction), and (H) the number of submucosal invasive tumors in the cancer specimens (**P < .05, Fisher exact test with the Holm correction). M, mucosal tumors. (I) The expression of NLRP3, ASC, and pro/cleaved caspase-1 in colon mucosa and CAC with or without NLRP3 inhibitor administration were analyzed by Western blot. (J and K) The mRNA expression of IL1β, IL18, MMP-9, and MMP-13 in colon mucosa and CAC with or without NLRP3 inhibitor administration were analyzed by quantitative polymerase chain reaction. Data are either representative or shown as the means ± SEM (error bars) of 3 independent experiments. The average expression level in the WT-N without NLRP3 inhibitor was defined as 1. *P < .01, **P < .05, repeated-measures analysis of variance with the Holm correction.
Intriguingly, we found that down-regulation of RalGAPα2 led to NLRP3 in flammosome activation and that NLRP3 inhibition led to decreasing invasive phenotype. However, NLRP3 inhibition did not affect the tumor number but affected its invasive phenotype in vivo. Although a relatively small sample size may have been affected, it was considered that NLRP3 inhibition affected the Ral-NLRP3–MMP pathway and reduced tumor invasion in comparison with tumor enlargement.

Our study showed a significant role for the Ral-activated NLRP3 in flammosome with up-regulation of MMPs in CAC. To date, the role of the in flammosome in CAC progression remains controversial. Several studies have indicated that in flammasome components provide protection against tumorigenesis in CAC.25,26 In contrast, recent studies have shown that inflammasomes can promote tumor development in CAC.27,28 In addition, we identified the NLRP3–AP-1 axis under a Ral-activated state. There have been a few reports on the relationship between Ral and AP-1,29 and between NLRP3 and AP-1.30,31 However, no reports on the association among Ral, NLRP3, and AP-1 in CAC have been reported. Given our in vitro and in vivo results, the Ral-NLRP3 in flammosome interaction provides new insight into the elucidation of the CAC mechanism.

In conclusion, our study showed that RalGAPα2 down-regulation was involved in the mechanism of inflammation-induced colon tumor development. In addition, we identified a possible link between Ral and the NLRP3 in flammosome. Hence, the Ral-NLRP3 pathway can be a potential future therapeutic target for limiting CAC progression.

Materials and Methods

Ethics

This study was performed in accordance with the relevant guidelines and regulations of the animal experiment committee of Sapporo Medical University (Sapporo, Japan). The protocol was approved by the Institutional Committee for Animal Research and the Gene Recombination Experiment Safety Committee at Sapporo Medical University. With regard to human tissues, we used human sporadic colorectal cancer and CAC samples that were obtained surgically at Sapporo Medical University and Hyogo College of Medicine (Nishinomiya, Japan) between 2005 and 2015 under a protocol approved by the institutional review board.

Cell Culture and Chemicals

Human cancer cell lines (lung, A549; breast, MCF7; colon, Colo320; prostate, DU145; and liver, Hep3B), human immune cell lines (monocyte, THP-1; T cell, SupT1; and B cell, Daudi), and a mouse cancer cell line (colon, Colon26) were kindly provided by the laboratory of the National Institutes of Biomedical Innovation, Health and Nutrition, Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were maintained in RPMI-1640 medium (cat. R8758; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (cat. 26140079; Thermo Fisher Scientific, Waltham, MA) and penicillin plus streptomycin (100 U/mL + 100 μg/mL, respectively).
transfection reagent (cat. 13778075; Thermo Fisher Scientific) were transfected with siRNA at 10 nmol/L using RNAiMAX control no. 1 siRNA; Thermo Fisher Scientific) used as a negative control (cat. 4390843, silencer negative control previously. They were housed in plastic cages (4–6 mice/cage) under controlled conditions of humidity (50% ± 10%), light (12/12-hour light/dark cycle), and temperature (24°C ± 2°C) in specific pathogen-free conditions. Drinking water and a pelleted basal diet were available ad libitum.

Murine Care

Male C57BL/6J mice (age, 7 wk) were purchased from Sankyo Labo Service Co (Tokyo, Japan). RalGAPα2 KO mice (C57BL/6J strain) were originally generated as described previously. They were housed in plastic cages (4–6 mice/cage) under controlled conditions of humidity (50% ± 10%), light (12/12-hour light/dark cycle), and temperature (24°C ± 2°C) in specific pathogen-free conditions. Drinking water and a pelleted basal diet were available ad libitum.

Generation of Bone Marrow Chimeric Mice

Cell suspensions from WT or RalGAPα2 KO mice bone marrow were prepared from femurs and tibias, filtered, and counted. Eight- to 12-week-old male WT or RalGAPα2 KO mice received a single intravenous injection of 5.0 × 10⁶ bone marrow cells after total body irradiation using a gamma-ray unit (Gammacell 40; Marubeni Utility Service, Tokyo, Japan). Total body irradiation was administered to recipients in 2 doses (5 Gy + 5 Gy) with a 3-hour interval between the 2 procedures to reduce intestinal damage. Mice then were followed up clinically for 4 weeks. The following groups of chimeric mice were generated: (1) WT to WT, (2) WT to RalGAPα2 KO, (3) RalGAPα2 KO to WT, and (4) RalGAPα2 KO to RalGAPα2 KO mice (Figure 5A).

Mouse CAC Model

With regard to the induction of CAC, we injected male mice intraperitoneally (age, 8 wk), and after bone marrow transfection male mice (ages, 12–16 wk) were given 12 μg/g/body weight AOM (cat. A5486; Sigma-Aldrich) on day 0. Mice were given 2.0% DSS (cat. MFD000081551, molecular weight 36–50 kilodaltons; MP Biomedicals, Irvine, CA) in water ad libitum for 5 days followed by 16 days of regular drinking water. Then, mice were subjected to an additional 2 DSS cycles. Furthermore, we intraperitoneally injected glyburide (NLRP3 inhibitor) (cat. NBP2-30141; Novus Biologicals, Littleton, CO) at 50 mg/kg into some male mice daily from tumor occurrence to death according to the AOM/DSS protocol described earlier. The occurrence of CAC was evaluated by endoscopy once a week.

Endoscopic Procedure and Sample Collection

A colonoscopic examination was performed using a microscope system (AVS, Tokyo, Japan), which included a video system (AVS) and a video monitor (Sony, Tokyo, Japan). Mice were anesthetized with isoflurane (cat. 4548995008299; Fujifilm Wako Pure Chemical Cooperation, Osaka, Japan) before endoscopy without preparations, such as fasting or laxatives. Mice were examined endoscopically with respect to inflammation and tumor development in the colon. Colonoscopic examination was performed once a week from day 7 to the end of the study, namely, the 63rd day. Mice were killed on the 63rd day of the experiment.

WT-N, WT-T, KO-N, and KO-T were collected. With regard to histopathologic analysis for colon cancer, the tumor number, tumor maximum diameter, percentage of mice with SM invasion, and percentage of colon cancers with SM invasion among all colon cancers were investigated in our experiments. Tumor number and size were evaluated by visual inspection. In addition, histopathologic examinations of the tumor depth were performed using H&E staining.

Differentiation of T Cells

After the spleen and leg bones were extracted from each mouse at age 8 weeks, the spleen was treated, and CD4+ CD62⁺ T cells were isolated using a magnetic cell sorting (MACS) system (cat. 130-106-643; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. These cells were incubated in a 96-well T-cell activation plate (anti-mouse CD3 antibody) (cat. 354720; Corning, Inc, Bedford, MA) for 48 hours, and T-cell differentiation was analyzed by flow cytometry (BD FACSaria II; BD Biosciences, San Jose, CA) using 7-aminoactinomycin D (cat. 420403; BioLegend, San Diego, CA) and a Mouse Th1/Th2/Th17 Phenotyping Kit (cat. 560758; BD Biosciences), including rabbit anti-mouse fluorescein isothiocyanate–interferon-γ, rabbit anti-mouse allopurinoycin–IL4, and rabbit anti-mouse phycoerythrin–IL17A, according to the manufacturer's instructions. These experiments also were performed after 5 days of DSS administration and after 5 days of water drinking.

Differentiation of B Cells

After spleens and leg bones were extracted from each mouse at age 8 weeks, they were treated and CD19⁺ B cells were isolated using a magnetic cell sorting system (cat. 130-121-301; Miltenyi Biotec) according to the manufacturer's instructions. The differentiation of these cells was investigated by flow cytometry (BD FACSaria II) by using 7-aminoactinomycin D (cat. 420403; BioLegend) and antibodies as follows: rat anti-mouse fluorescein isothiocyanate–IgM (cat. 11-4011-85; Thermo Fisher Scientific) and rat anti-mouse allopurinoycin–IgD (cat. 17-5993-82; Thermo Fisher Scientific).
probes and primer sequences used in this study are shown.

Target molecules were normalized to serum albumin as the standard. The supernatants were determined with the Bradford method using bovine serum albumin as the standard. The supernatants were boiled in sample buffer (0.05 mol/L Tris-HCl, 2% sodium dodecyl sulfate, 6% β-mercaptoethanol, 10% glycerol, and 1.25% bromphenol blue), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gels), and transferred onto polyvinylidene fluoride membranes (cat. Sny0720; Pall Corporation, Pensacola, FL). The membranes were blocked with blocking buffer (Tris-buffered saline with 0.5% Tween-20) containing 5% milk powder and then incubated with primary antibodies overnight at 4°C. Rabbit anti-RalGAPα1-α2 and -β polyclonal antibodies were generated and provided by the author (H.H.). Other primary antibodies that we used were as follows: rabbit anti-RalA (cat. Ab96759; Abcam), rabbit anti-RalB (cat. Ab129077; Abcam), rabbit anti-MMP-13 (cat. Ab39012; Abcam), rabbit anti-NLRP3 (cat. Ab214185; Abcam), rabbit anti-ASC (cat. Sc-22514-R; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-caspase-1 (cat. Ab138483; Abcam), rabbit anti-caspase-11 (cat. Ab180673; Abcam), rabbit anti-NF-κB p65 (cat. Ab16502; Abcam), rabbit anti-NF-κB p65 (phospho S536) (cat. Ab68299; Abcam), rabbit anti-STAT3 (cat. Ab68153; Abcam), rabbit anti-STAT3 (phospho S727) (cat. Ab30647; Abcam), and mouse anti-β-actin conjugate (cat. O17-24573; Fujifilm Wako Pure Chemical Cooperation). We used horseradish-peroxidase-labeled anti-rabbit IgG as a secondary antibody (cat. 7074P2; Cell Signaling Technology, Inc, Danvers, MA). The membranes were incubated with luminescence detection reagent (cat. 32106; Thermo Fisher Scientific), and we visualized luminescence using an analyzer (LAS-3000; Fujifilm Co, Tokyo, Japan). Densitometric analysis was performed using ImageJ software version 1.52h (National Institutes of Health, Bethesda, MD, available: http://rsbweb.nih.gov/ij/).

In addition, MMP-9 expression was analyzed by gelatin zymography, which was performed using a gelatin zymography kit (cat. PMC-AK47-COS; Cosmo Bio Co, Tokyo, Japan) according to the manufacturer's instructions. The gel was imaged with the detector system (AE-9000 E-graph; ATTO Inc, Danvers, MA).

Cytokines Released From Intraperitoneal Macrophages

Macrophages were collected from both WT and RalGAPα2 KO mice on day 4 after intraperitoneal administration of 5.0% thioglycolate (cat. T0632; Sigma-Aldrich). They were seeded at 1.0 × 10^6 cells/well in a 6-well culture plate and incubated overnight in medium containing 40 ng/mL recombinant murine macrophage colony-stimulating factor (cat. 315-02; Peprotech, Rocky Hill, NJ). We compared the cytokine levels of the WT mice and RalGAPα2 KO mice by enzyme-linked immunosorbent assay after collecting the culture supernatants of macrophages. These experiments also were performed after 5 days of DSS administration and after 5 days of water drinking.

qPCR, WB, Gelatin Zymography, and Enzyme-Linked Immunosorbent Assay

Total RNA was prepared and reverse-transcribed using a SuperScript VILO complementary DNA Synthesis Kit (cat. 11754050; Thermo Fisher Scientific). The mRNA expression of Mmp-9 and Mmp-13 was analyzed using TaqMan Gene Expression Assays (Thermo Fisher Scientific). Furthermore, the mRNA expression of cytokines derived from mouse colon tissues was analyzed using Power SYBR Green PCR Master Mix (cat. 4367659; Thermo Fisher Scientific). The probes and primer sequences used in this study are shown in Tables 1 and 2, respectively. The gene expression levels of target molecules were normalized to β-actin expression.

For the WB analyses, cells and tissues were lysed in RIPA buffer (cat. R0278; Sigma-Aldrich) with protease inhibitor (cat. S8820; Sigma-Aldrich). Protein concentrations were determined with the Bradford method using bovine serum albumin as the standard. The supernatants were precipitated in sample buffer (0.05 mol/L Tris-HCl, 2% sodium dodecyl sulfate, 6% β-mercaptoethanol, 10% glycerol, and 1.25% bromophenol blue), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gels), and transferred onto polyvinylidene fluoride membranes (cat. Sny0720; Pall Corporation, Pensacola, FL). The membranes were blocked with blocking buffer (Tris-buffered saline with 0.5% Tween-20) containing 5% milk powder and then incubated with primary antibodies overnight at 4°C. Rabbit anti-RalGAPα1-α2 and -β polyclonal antibodies were generated and provided by the author (H.H.). Other primary antibodies that we used were as follows: rabbit anti-RalA (cat. Ab96759; Abcam), rabbit anti-RalB (cat. Ab129077; Abcam), rabbit anti-MMP-13 (cat. Ab39012; Abcam), rabbit anti-NLRP3 (cat. Ab214185; Abcam), rabbit anti-ASC (cat. Sc-22514-R; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-caspase-1 (cat. Ab138483; Abcam), rabbit anti-caspase-11 (cat. Ab180673; Abcam), rabbit anti-NF-κB p65 (cat. Ab16502; Abcam), rabbit anti-NF-κB p65 (phospho S536) (cat. Ab68299; Abcam), rabbit anti-STAT3 (cat. Ab68153; Abcam), rabbit anti-STAT3 (phospho S727) (cat. Ab30647; Abcam), and mouse anti-β-actin conjugate (cat. O17-24573; Fujifilm Wako Pure Chemical Cooperation). We used horseradish-peroxidase-labeled anti-rabbit IgG as a secondary antibody (cat. 7074P2; Cell Signaling Technology, Inc, Danvers, MA). The membranes were incubated with luminescence detection reagent (cat. 32106; Thermo Fisher Scientific), and we visualized luminescence using an analyzer (LAS-3000; Fujifilm Co, Tokyo, Japan). Densitometric analysis was performed using ImageJ software version 1.52h (National Institutes of Health, Bethesda, MD, available: http://rsbweb.nih.gov/ij/).

In addition, MMP-9 expression was analyzed by gelatin zymography, which was performed using a gelatin zymography kit (cat. PMC-AK47-COS; Cosmo Bio Co, Tokyo, Japan) according to the manufacturer's instructions. The gel was imaged with the detector system (AE-9000 E-graph; ATTO Co, Tokyo, Japan).

An enzyme-linked immunosorbent assay was performed using the IL1β Assay it (cat. MLB00C; R&D Systems,

### Table 2. Primer Sequences

| Gene     | Forward              | Reverse              |
|----------|----------------------|----------------------|
| IL1β     | 5'-CAACCCACAAAGTGATTTCCATCGA-3' | 5'-GATCCACACTCTCCAGCTGCA-3' |
| IL4      | 5'-CAACCCCCCCAGTCTGTTGCA-3'   | 5'-TGTTGGTTCTTGCCTTGGC-3'   |
| IL6      | 5'-CTGCAGACATCCACTCAGCT-3'   | 5'-TCAGAGGTTCATCCCCAGAACCA-3' |
| IL10     | 5'-CTATGCTTCTGCTTCTTACTG-3'  | 5'-AGCAGATGTGGTCACGACTG-3'  |
| IL12A    | 5'-TGAGCTCTACCTGTCTGCA-3'    | 5'-CCTTGAGCCCTTCAAGGCA-3'   |
| IL13     | 5'-CACCTAGGCTTCACAGCCTCC-3'  | 5'-CCAGGGATGGTCTCTTCACT-3'  |
| IL17     | 5'-TCTCTGGTCTTTGTCTGCT-3'    | 5'-CCTGGAACGCTGTAOGATGT-3'  |
| IL18     | 5'-GACAGCCTGTTCCAGGATGAT-3'  | 5'-GGTGACACGGCAGCTTCTAATA-3' |
| TNF-α    | 5'-CATGCACCACACTCAAGGAC-3'   | 5'-GGGCCTAGATCTTATCCAGGAC-3' |
| IFN-γ    | 5'-CAGCTCCCTCCCTGCTGTTGTT-3' | 5'-ATCTGCTGTCGCTGACAGATTTTT-3' |
| β-actin  | 5'-GACCCTGAAGTACCACCCATGAA-3' | 5'-AAGGTTGTTGTCGACGATTTCT-3' |
Glutathione-S-Transferase–Sec5 Pull-Down Assay

Lysates of Colon26 cells or mouse tissues were centrifuged at 20,900 × g for 10 minutes. Supernatants containing 200 µg of proteins were incubated at 4°C for 30 minutes with glutathione beads (cat. 17075601, Glutathione Sepharose 4B; GE Healthcare, Uppsala, Sweden) coated with 20 µg of glutathione-S-transferase–Sec5 Ral-binding domain.4 After washing the beads, the bead-associated GTP-RalA and GTP-RalB were analyzed by WB as described earlier.

Cell Invasion Assay

Cell invasion assays were performed using Colon26 cells. In addition, the group was divided into 2 groups according to the presence or absence of 5 µg glyburide (NLRP3 inhibitor) (cat. NBP2-30141; Novus Biologicals) administration. Cell invasion through the Matrigel membrane was quantified using a commercially available cell invasion kit (cat. 354481; Corning, Inc). Colon26 cells (5 × 10^5 cells/mL) were added to the upper compartments of the chambers and incubated for 22 hours with serum-free RPMI-1640 medium (cat. R8758; Sigma-Aldrich). The cells in the upper chambers were removed, and the cells that had invaded through the Matrigel matrix were stained with May-Giems for 2 hours. The extent of infiltration was measured by counting cells.

Microarray Analysis

Phenotypic differences in colonic epithelial cells from both WT mice and RalGAPα2 KO mice were examined. Colonic epithelial cells were isolated from colon tissues, and after flow cytometry using BD FACSaria II (BD Biosciences) was performed to determine whether more than 90% of the isolated cells were CD326-positive/CD45-negative epithelial cells, microarray analysis was performed using a 3D-Gene Human Oligo chip 25k (Toray Industries, Inc, Tokyo, Japan). Microarray slides were scanned using a 3D-Gene Scanner (Toray Industries, Inc) and processed with 3D-Gene Extraction software (Toray Industries, Inc).

 Luciferase Reporter Assay

The NLRP3 promoter was inserted into pGL4.10 plasmids containing luciferase sequences (cat. E6651; Promega, Madison, WI) downstream of the promoter, and the plasmids whose concentrations were adjusted to 1 µg/well were transfected into RalGAPα2 KD Colon26 cells using Screen Fect A (cat. 299-73203; Fujifilm Wako Pure Chemical Cooperation). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (cat. E1910; Promega) with a microplate reader (Infinite M1000 Pro; Tecan, Männedorf, Switzerland), and Renilla luciferase activity was used as the internal control. These experiments were performed with plasmids, except for the Nfatc2, Sox6, AP-1, and Gata1 sequences.

Immunohistochemistry

Immunohistochemistry was performed on 5-µm, formalin-fixed, paraffin-embedded sections. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide, and the sections were incubated overnight at 4°C with primary antibody. The primary antibodies used were as follows: rabbit anti-RalGAPα2, rabbit anti–MMP-9 (cat. ab38898; Abcam), and rabbit anti–MMP-13 (cat. ab39012; Abcam). A subsequent reaction was performed using a horseradish-peroxidase, enzyme-labeled polymer of the Real EnVision detection system (cat. K5007; Agilent, Santa Clara, CA). When a positive reaction was visualized using a diaminobenzidine solution, counterstaining was performed using Mayer’s hematoxylin.

For the human samples, normal colon tissues were defined as the distal healthy part with sporadic colorectal cancers. Moreover, CACs were divided into 2 groups: noninvasive (in situ or T1) or invasive (T2-4). The RalGAPα2 expression level of each human sample was graded as strong staining or weak staining. For the mouse samples, the MMP-9 and MMP-13 expression levels of each mouse tissue were graded as strong staining or weak staining at the invasive front of the tumors. These classifications were conducted independently by 2 observers in a blind fashion. Although 80% of the judgments coincided, 20% did not. When the results of the evaluations differed between the 2 observers, the discrepancies were resolved by discussion.

Patient Cohort

We examined patient information regarding age, sex, underlying disease (ulcerative colitis or Crohn's disease), colitis range (left or total), disease duration, and TNM classification in patients with sporadic colorectal cancer or CAC.

Statistical Analysis

To compare groups, categoric variables were compared using the Fisher exact test. Two-group comparisons of mean...
values, multigroup comparisons of repeatedly measured samples using ratios relative to the control samples, and multigroup comparisons of repeatedly measured samples using measured values relative to control values were analyzed using the Student t test, repeated-measures analysis of variance, and 1-way analysis of variance, respectively. Multiple comparisons were corrected with the Holm method. A difference was considered significant when \( P < .05 \). All tests were 2-tailed.

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Author contributions
Tomoya Iida, Daisuke Hirayama, Naoki Minami, and Hiroshi Nakase designed the study; Hiroki Ikeuchi and Seiichi Hirota provided human samples; Ryutaro Shirakawa and Hisanori Horiiichi provided knockout mice; Tomoya Iida, Daisuke Hirayama, Naoki Minami, Minoru Matsuura, Kohei Wagatsuma, Kentaro Kawakami, and Kanna Nagaiishi performed the research; Tomoya Iida and Masanori Nojima analyzed the data; Tomoya Iida, Hisanori Horiiichi, and Hiroshi Nakase wrote the manuscript; and Hiroshi Nakase directed the research. All authors helped to perform the research and approved the final draft of the manuscript.

Conflicts of interest
The authors disclose no conflicts.

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