INTRODUCTION

Hepatocyte growth factor activator inhibitor-1 (HAI-1), encoded by the SPINT1 gene, is a membrane-associated Kunitz-type serine protease inhibitor that is expressed by most epithelial cells and placental cytotrophoblasts. It was initially identified as a cellular inhibitor of serum hepatocyte growth factor activator and subsequent studies have indicated that HAI-1 is also a critical regulator of epithelial type II transmembrane serine proteases that activate protease-activated receptor-2 (PAR-2). We previously reported that deletion of Spint1 in ApcMin/+ mice resulted in accelerated formation of intestinal tumors, possibly through enhanced nuclear factor-κB signaling. In this study, we examined the role of PAR-2 in accelerating tumor formation in the ApcMin/+ model in the presence or absence of Spint1. We observed that knockout of the F2rl1 gene, encoding PAR-2, not only eliminated the enhanced formation of intestinal tumors caused by Spint1 deletion, but also reduced tumor formation in the presence of Spint1. Exacerbation of anemia and weight loss associated with HAI-1 deficiency was also normalized by compound deficiency of PAR-2. Mechanistically, signaling triggered by deregulated protease activities increased nuclear translocation of RelA/p65, vascular endothelial growth factor expression, and vascular density in ApcMin/+ induced intestinal tumors. These results suggest that serine proteases promote intestinal carcinogenesis through activation of PAR-2, and that HAI-1 plays a critical tumor suppressor role as an inhibitor of matriptase, kallikreins, and other PAR-2 activating proteases.

KEYWORDS
angiogenesis, colon cancer, HAI-1, PAR-2, Spint1
transmembrane serine proteases (TTSPs), particularly matriptase.2,3
Previously, we generated several Spint1 mutant mouse models and
showed that HAI-1 contributes to the integrity of epithelia, including
the intestinal epithelium.2,4 Intestine-specific Spint1 knockout mice
showed increased susceptibility to dextran sulfate sodium-induced
experimental colitis.5 Moreover, the Spint1 deletion led to accelerated
tumor formation in the ApcMin/+ mouse model, indicating that HAI-1 is
a tumor suppressor.5 Indeed, the cell surface immunoreactivity of HAI-1
was markedly reduced in carcinoma cells compared to adjacent normal
enterocytes or adenoma cells in human colon cancers,6,7 and this trend
was confirmed in a murine ApcMin/+ model.5 Consequently, the ratio
of HAI-1 expression relative to its target epithelial protease, matriptase,
was decreased along with the progression of colon cancers.8 Enhanced
tumor formation observed in HAI-1-deficient ApcMin/+ mice was medi-
atred, at least partly, by activation of nuclear factor (NF)-κB signaling9;
however, detailed mechanisms underlying NF-κB activation in the ab-
sence of HAI-1 remain unclear.

Protease-activated receptor-2 (PAR-2) is a 7 transmembrane-span-
ning domain G protein-coupled receptor widely expressed by epithe-
lial, endothelial, and smooth muscle cells, with diverse physiological
and pathological functions.10-13 Specific cleavage by trypsin-like serine
proteases frees an endogenous ligand for interaction with the core of
the receptor, inducing a conformational change that triggers signal transduc-
tion.10,14,15 Type II transmembrane serine proteases are known to acti-
ivate PAR-216-21 and PAR-2 reportedly contributes to tumor progression
through its promotion of invasive growth by cancer cells and by stimu-
lating angiogenesis.11,19,22 Among TTSPs, matriptase is the most potent
PAR-2 activator known in epithelial and tumor tissues.22,23 Transgenic
expression of matriptase in murine keratinocytes induces skin carcino-
genesis24 that is entirely dependent on PAR-2 signaling.25 Activation of
PAR-2 by transgenic matriptase expression led to protumorigenic cyto-
kine expression through activation of NF-κB signaling.25 Previously, we
reported that the loss of intestinal HAI-1/Spint1 led to increased tryp-
sin-like serine protease activity in ApcMin/+ mouse intestine.3 We thus
hypothesize that HAI-1 insufficiency permits the unrestricted activity
of pericellular trypsin-like serine proteases, including matriptase, leading
to activation of PAR-2/NF-κB signaling in colon cancer tissues.

To test this hypothesis, we generated ApcMin/+ mice with an in-
testine-specific Spint1-deletion with or without the superimposition
of global PAR-2/F2rl1 deletion to analyze the effects of PAR-2 sig-
naling on intestinal carcinogenesis and on the enhanced tumor sus-
ceptibility induced by HAI-1 deficiency. We found that the deletion
of F2rl1 reduced tumor formation both in control and Spint1-deleted
ApcMin/+ mice and decreased the activation of NF-κB and angiogene-
sis in HAI-1 deficient tumors.

2 | MATERIALS AND METHODS

2.1 | Mice

All animal experiments were carried out using protocols approved by
the Institutional Animal Care and Use Committee of the University
of Miyazaki. ApcMin/+ mice and Villin-Cre mice were obtained from
The Jackson Laboratory. ApcMin/+ Spint1Lox/Lox/P excell/Cre mice4,5
were crossed with F2rl1-deficient mice6 to generate ApcMin/+ mice
with an intestine-specific Spint1 deletion and global PAR-2/F2rl1
deletion (Spint1Lox/Lox/P excell/Cre/F2rl1−/−/ApcMin/+). Mice were as-
sessed daily, and body weights were recorded weekly. Blood sam-
ple s were obtained from the right venticile, and EDTA-containing
plasma samples were used for analyzing hemoglobin concentration.

At 15 weeks of age, all mice were killed to evaluate the number
and sizes of intestinal tumors. The tumor size was scored as pre-
viously described.5 For histological analysis, intestinal tissues were
fixed in 4% paraformaldehyde in PBS and embedded in paraffin.
Four-micrometer-thick sections were stained with H&E or processed
for immunohistochemical analysis.

2.2 | Quantitative RT-PCR

Total RNA was prepared with TRIzol (Life Technologies Japan), fol-
lowed by DNase I (Takara Bio) treatment. For RT-PCR, 3 μg total
RNA was reverse-transcribed with a mixture of Oligo (dT)12-18 (Life
Technologies Japan) and random primers (6 mers) (Takara Bio) using
200 units of ReverTra Ace (Toyobo), and 1/30 of the resulting cDNA
was processed for quantitative RT-PCR. Real-time RT-PCR was un-
tertaken in a Thermal Cycler Dice Real Time System II (Takara Bio)
using the SYBR Premix Ex Taq II (Takara Bio). For internal control,
β-actin mRNA was also measured. The following primers were used:
β-actin forward, 5′-TGACAGGATGCAGAAGGAGA, and reverse, 5′-GC
GGAAAGTGACAGGTGAG; Pecam1 (CD31) forward, 5′-GGAAA
GCCAACAGCCATACGG, and reverse, 5′-GAGCCTTCCGTCT
CTTGGTGA; Vegfa forward, 5′-CAGGCCTGCTGTAACGATGAA,
and reverse, 5′-CTGACATTCAACATCTGCTGTG; Bcl2 (Bcl-2) forward,
5′-ACCGTCTGACTTTCCGAG, and reverse, 5′-GGTGTGTCG
ATGCCGGTTCA; and Bad forward, 5′-GCCATAGCTGTGAGAAG,
and reverse, 5′-CAAACTCTGGATCTGGAACA.

2.3 | Immunohistochemical analyses

For immunohistochemistry, tissue sections were processed for an-
tigen retrieval by microwaving for 10 minutes at 96°C in 10 mmol/L
citrate buffer (pH 6.0), followed by treatment with 3% H2O2 in PBS
for 10 minutes. After blocking in 5% normal goat serum (Dako) in
PBS, the sections were incubated with anti-NF-κB p65 (RelA/p65)
rabbit mAb (Cell Signaling Technology) or anti-CD31 rabbit polyclon-
al Ab (Cell Signaling Technology), or antiphosphorylated MET
(Y1235) rabbit polyclonal Ab,27 or anti-β-catenin rabbit polyclonal
Ab (Sigma-Aldrich) or anti-CD45 rat mAb (Wuxi Biosciences) for
16 hours at 4°C and then incubated with Envision labeled polymer
reagents (Dako) for 30 minutes at room temperature. The reactions
were revealed by nickel and cobalt-3,3′-diaminobenzidine (Pierce)
and counterstained with Mayer’s hematoxylin. To quantify RelA/
p65 nuclear translocation and CD31+ vessels, stained sections with
nonneoplastic mucosa and tumor tissues were selected and photographed at 200× magnification. Two independent investigators counted the RelA/p65+ nuclei and CD31+ vessels and the mean number per field was calculated. To evaluate the immunoreactivity of phosphorylated MET, we scored as described by Fukushima et al.27

2.4 | Cell culture

The Caco2 cell line was obtained from the Riken BRC Cell Bank. Cells were cultured in DMEM containing 10% FBS. For transient silencing of F2RL1, 2 kinds of siRNA were used. One (PAR-2 siRNA #1) was an siRNA pool (ON-TARGETplus SMARTpool siRNA; Thermo Fisher Scientific) used with the siGENOME Non-Targeting siRNA pool as a control. The other (PAR-2 siRNA #2) was Stealth siRNA (Invitrogen) and the sequence was 5′-UCACACUGUAAAGACCCUAUUG-3′. Transfection was carried out using Lipofectamine RNAiMax reagent (Invitrogen) followed by cultivation in DMEM supplemented with 10% FBS for 24 hours. The cells were treated with or without 10 µmol/L PAR-2 agonist, Ser-Leu-Ile-Gly-Arg (SLIGR)-NH₂, or 10 µmol/L PAR-2 selective antagonist, Phe-Ser-Leu-Leu-Arg-Tyr (FSLLRY)-NH₂ (Peptides International).28

2.5 | Immunoblot analysis

Mouse intestinal tumors were homogenized on ice in CelLytic MT (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 100 mmol/L NaF, and 1 mmol/L Na₃VO₄. The extracts were centrifuged at 20 000 g for 15 minutes at 4°C, and the resulting supernatants were used for experiments. Cellular proteins were extracted with CelLytic M (Sigma-Aldrich) supplemented with protease inhibitor cocktail, 100 mmol/L NaF, and 1 mmol/L Na₃VO₄, centrifuged 15 000 g for 15 minutes, and the supernatants were collected. Equal amounts of total proteins were separated by SDS-PAGE under reducing conditions using 4%–12% gradient gels and transferred onto an Immobilon-P membrane (Millipore). After blocking with 5% nonfat milk in 25 mmol/L TBS with 0.1% Tween-20, pH 7.6 (TBS-T), the membranes were incubated overnight at 4°C with primary Ab, followed by washing with TBS-T and incubation with HRP-conjugated secondary Ab diluted in TBS-T with 1% BSA for 1 hour at room temperature. The labeled proteins were visualized with a chemiluminescence reagent (PerkinElmer Life Science). The following primary Abs were used: anti-β-actin mouse mAb (Sigma-Aldrich), anti-PAR-2 rabbit mAb, anti-phospho-NF-κB p65 (Ser536) rabbit mAb and anti-NF-κB p65 rabbit mAb (Cell Signaling Technology Japan).

2.6 | Enzyme-linked immunosorbent assay

Serum vascular endothelial growth factor (VEGF)-A levels of mice and human VEGF-A levels in culture supernatants of Caco2 were measured with a Quantikine VEGF ELISA kit (R&D Systems) according to the manufacturer’s instructions.

2.7 | Statistical analysis

Statistical analysis was carried out using StatView 5.0 (SAS). Comparison between 2 unpaired groups was made with repeated-measure of variance or the Mann-Whitney U test. Significance was set at P < .05.

3 | RESULTS

3.1 | Deficiency in PAR-2 alleviates increased tumor formation in Spint1-deleted ApcMin/+ mice

Consistent with the previously reported increase in tumor burden,5 Spint1-deleted ApcMin/+ (Spint1loxP/loxP/Villin-Cre/ApcMin/+ mice) gained significantly less weight with age than ApcMin/+ control mice. Strikingly, this phenotype was completely reversed by concomitant deletion of F2rl1 (Spint1loxP/loxP/Villin-Cre/F2rl1−/−/ApcMin/+; Figure 1A). Profound anemia of Spint1-deleted ApcMin/+ mice was also normalized to ApcMin/+ control levels with compound PAR-2 deficiency, although hematocrits remained lower than in naïve mice (Figure 1B). Fifteen weeks after birth, the number of intestinal tumors was significantly (P = .0001) increased in Spint1loxP/loxP/Villin-Cre/ApcMin/+ (146.6 ± 4.5, n = 10) compared with control Spint1loxP/loxP/ApcMin/+ mice (85.4 ± 5.7, n = 11) (Figure 2A,B). Again, concomitant deletion of F2rl1 in Spint1loxP/loxP/Villin-Cre/ApcMin/+ mice significantly (P = .0003) reduced the number of intestinal tumors (92.9 ± 7.3, n = 11) (Figure 2B). Intriguingly, deletion of F2rl1 also reduced the number of tumors formed in ApcMin/+ mice in the presence of Spint1 (Spint1loxP/loxP/F2rl1−/−/ApcMin/+ (P = .0486). F2rl1 deletion also significantly decreased the size of tumors in both the control (P = .0046) and Spint1loxP/loxP/Villin-Cre/ApcMin/+ mice (P = .0092) (Figures 2B and S1). However, tumor histology was not visibly altered by the deletion of F2rl1 (Figure 2C).

3.2 | Protease-activated receptor-2 drives NF-κB activation induced by Spint1 deletion

We previously reported that NF-κB signaling is activated in HAI-1-deficient ApcMin/+ tumors. Moreover, an NF-κB inhibitor suppressed the HAI-1 loss-mediated enhancement of tumorigenicity in ApcMin/+ mice.9 Protease-activated receptor-2 is known to activate NF-κB signaling in various human cancers, including colon cancer,25,29-31 and HAI-1 regulates PAR-2-activating TTSPs.22 Thus, the activation of NF-κB in HAI-1-deficient intestine might result from the excessive activation of PAR-2. To test this hypothesis, we evaluated the effect of F2rl1 deletion on the nuclear translocation of the NF-κB subunit RelA/p65. The

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ablation of PAR-2/F2rl1 abrogated the enhanced nuclear translocation (Figure 3A,B) and phosphorylation of RelA/p65 resulting from the loss of HAI-1 (Figure 3C) in intestinal tumors. The enhanced nuclear translocation of RelA/p65 was also observed in stroma cells adjacent to the tumor cells in HAI-1-deficient intestine, which was alleviated by the concomitant deletion of F2rl1 (Figure S2A). To support this, the number of CD45+ cells increased in the HAI-1-deficient intestine (Figure S2B). These results indicate that increased NF-κB activation in Spint1-deleted ApcMin/+ mice is driven by PAR-2 signaling.

3.3 | Protease-activated receptor-2 signaling increases VEGF-A expression and vascular density

Next, we explored the mechanism by which PAR-2/NF-κB signaling increased mean intestinal tumor size and number in ApcMin/+ mice. Protease-activated receptor-2 deficiency did not affect the Ki-67 labeling index or mRNA levels of Bcl2 and Bad genes in the intestinal tumors (Figure S3), indicating that cell proliferation rate and apoptotic signals were not altered. We then examined the expression of VEGF-A, a key regulator of angiogenesis, in mouse tissues and serum. Intriguingly, there was a highly significant increase in serum VEGF-A protein levels in Spint1loxP/loxP/Villin-Cre/F2rl1−/−/ApcMin/+ mice that was abolished with compound deficiency of PAR-2 (Figure 4A). Accordingly, HAI-1 deficiency was associated with a nonsignificant increase in Vegfa gene expression in the ApcMin/+ tumors that was significantly (P = .035) decreased with the compound deficiency of PAR-2 (Figure 4B).

Consistent with PAR-2-dependent VEGF expression, capillary density was significantly (P = .0282) decreased in tumors from Spint1loxP/loxP/Villin-Cre/F2rl1−/−/ApcMin/+ compared with those from Spint1loxP/loxP/Villin-Cre/ApcMin/+ mice, although Spint1 deficiency did not by itself significantly increase vascular density in tumors or adjacent mucosa (Figure 5A). Quantitative RT-PCR confirmed decreased mRNA levels for the Pecam1 gene, which encodes the endothelial marker CD31, after F2rl1 deletion in both tumors and adjacent mucosa (Figure 5B). These results suggest a PAR-2-dependent effect of local HAI-1 deficiency on local and systemic VEGF-A levels that in turn impacts vascular density and tumor growth.

3.4 | Protease-activated receptor-2 signaling activates NF-κB signaling and enhances VEGF-A expression in Caco2 human colon cancer cell line

Finally, to examine the role of PAR-2 in NF-κB activation and VEGF-A expression of colon cancer cells, we analyzed the effects of F2RL1 silencing, PAR-2 antagonist, or PAR-2 agonist on the Caco2 human colon carcinoma cell line. Knockdown of F2RL1 or treatment of the cells with PAR-2 antagonist suppressed the phosphorylation of RelA/p65 (Figure 6A). In contrast, PAR-2 agonist enhanced the phosphorylation of RelA/p65 (Figure 6A). Next, we examined the effects of PAR-2 agonist and antagonist on the VEGF-A expression by Caco2 cells. As shown in Figure 6B, incubation of the cells with PAR-2 agonist enhanced VEGF-A mRNA levels. On the contrary,
PAR-2 antagonist suppressed the VEGF-A expression. The released VEGF-A proteins tended to be increased by the PAR-2 agonist treatment, but the difference was not statistically significant (Figure 6B).

4 | DISCUSSION

In this study, we show that ablation of PAR-2/F2rl1 nearly eliminates the enhancement of NF-κB signaling and tumor formation caused by the loss of HAI-1/Spint1 in Apc\textsuperscript{Min/+} mice, providing evidence that PAR-2 is critically involved in NF-κB activation and the susceptibility to carcinogenesis caused by HAI-1 insufficiency. The significance of PAR-2 on NF-κB signaling was also confirmed in the Caco2 human colon carcinoma cell line. Protease-activated receptor-2 deficiency also reduced tumor size and number, even in the presence of Spint1. These results suggest that protease signaling is also spontaneously deregulated in the Apc\textsuperscript{Min} model, contributing significantly to tumor growth.

The detailed molecular mechanisms linking activated NF-κB signaling to enhanced tumor formation remain unclear. Protease-activated receptor-2-dependent VEGF-A expression and angiogenesis induced by HAI-1 deficiency could play a role. However, it remains to be determined whether the increased VEGF-A level in Spint1\textsuperscript{LoxP/LoxP}/Villin-Cre/Apc\textsuperscript{Min/+} mice was a direct effect of HAI-1 deficiency-induced PAR-2 activation or an epiphenomenon of the increased tumor burden. In this regard, the mean tumor size in Spint1\textsuperscript{LoxP/LoxP}/Villin-Cre/Apc\textsuperscript{Min/+} mice is also of great interest.
Villin-Cre/F2rl1−/−/ApcMin/+ mice was larger than that of Spint1−/−/ApcMin/+ mice, but the serum VEGF-A levels and VEGF-A mRNA levels were similar between these 2 groups. Thus, the increased serum VEGF-A proteins did not simply rely on the secondary effect of increased tumor burden. As PAR-2 activation in fact enhanced the VEGF-A production in Spint1−/−/F2rl1−/−/ApcMin/+ mice by 2-fold compared to Spint1−/−/ApcMin/+ mice, we hypothesized that both factors (increased tumor burden and HAI-1 deficiency-induced PAR-2 activation) account for the enhanced VEGF-A production in Spint1−/−/F2rl1−/−/ApcMin/+ mice.
Villin-Cre/Apc\(^{Min/+}\) mice. Importantly, nuclear translocation of β-catenin and activation of the hepatocyte growth factor-MET axis, which were enhanced in Spint1-deleted Apc\(^{Min/+}\) mice,\(^5\) were not significantly altered by compound PAR-2/F2rl1 deletion (Figure S4).

Nuclear factor-κB signaling has important roles in cancer progression, primarily through its stimulation of cell proliferation, survival, and angiogenesis.\(^{32-34}\) In this regard, activation of PAR-2 by an extracellular trypsin-like serine protease transduces NF-κB signaling.\(^{22,29,35}\) Although several in vitro studies have reported that PAR-2 promotes the proliferation of human colon cancer cell lines,\(^{36-38}\) the role of PAR-2 in colorectal cancer has not been addressed in vivo. Here, we report for the first time that PAR-2 promotes tumor formation and growth in mouse intestine harboring the Apc mutation. Protease-activated receptor-2 is expressed not only in epithelial cells but also leukocytes, endothelial cells, and smooth muscle cells.\(^{19,39,40}\)

Although PAR-2 is hardly detectable in normal fibroblasts, activated fibroblasts express PAR-2, as do cancer-associated fibroblasts.\(^{28,41}\) Protease-activated receptor-2 is also expressed by most cancer cells of epithelial origin, including colorectal cancer cells.\(^{42}\) In this study, we did not address which cells were responsible for PAR-2-induced tumor promotion and VEGF-A expression. In a mouse model of matriptase-induced skin carcinogenesis, the excess activation of keratinocyte PAR-2 was crucial for tumor promotion.\(^{25}\) As HAI-1 and TTSP are both membrane anchored with mostly local actions, it is likely that epithelial PAR-2 of the intestine is also responsible for the increased frequency of tumor formation in the current study. To clarify this question, further studies using mice with intestinal epithelium-specific F2rl1-deletion will be required.
This study did not identify the protease(s) responsible for the presumed excess activation of PAR-2 in the Spint1-deleted ApcMin/+ intestine. Protease-activated receptor-2 is activated by various trypsin-like serine proteases. Among them, the main candidate protease is matriptase, a HAI-1 regulated protease that is widely expressed in epithelial cells, including enterocytes. The matriptase-PAR-2 axis has been implicated in development, inflammation, and cancer. The ST14 gene, encoding matriptase, was originally proposed as a suppressor of colon cancer. Indeed, ablation of St14/matriptase in the intestinal epithelium impaired the barrier function and induced mucosal inflammation, eventually resulting in the formation of colon adenocarcinoma in mice. Excess matriptase activity also leads to the disturbance of epithelial integrity in the intestine. Moreover, matriptase is known to be upregulated in various human cancers and deregulated activities of matriptase contribute to tumor progression. In colorectal cancers, the ratio of matriptase / HAI-1 mRNA is increased during the early stages of carcinogenesis. These lines of evidence indicate that normal, tightly regulated matriptase activity is critically required for the integrity of intestinal epithelium, and dysregulation of matriptase contributes to neoplastic progression of the intestinal epithelium. Protease-activated receptor-2 can also be activated by the tissue factor (TF)/factor VIIa (FVIIa) complex, coagulation factor Xa (FXa), and the ternary TF/FVIIa/FXa complex. Like matriptase and PAR-2, TF is frequently expressed in various cancers, including colorectal cancer. A recent study reported that TF-dependent coagulation initiation on epithelia triggers matriptase activation, which in turn activates PAR-2, suggesting that cancer-associated loss of vascular integrity might trigger TTSP activation secondary to activation of the coagulation cascade. In a previous study of TF expression in human colorectal carcinoma cell lines, most lines expressed TF with augmented expression in a highly metastatic subline. In colorectal cancers, high TF expression in tumor cells also correlated with poor patient prognosis. Kallikrein-related peptidases (KLKs) are also candidate PAR-2 activators in this context. Matriptase is an activator of KLK5, which is also directly inhibited by HAI-1. Moreover, Kallikrein-related peptidase 5 is trypsin-like serine protease that can efficiently activate PAR-2 as well as KLK14, another PAR-2 activating KLK. Kallikrein-related peptidase 7 is known to be expressed by human colon cancer cell lines in vitro and colon cancer tissues in vivo.
In summary, we showed that a protease-activated G-protein-coupled receptor, PAR-2, contributes to intestinal carcinogenesis in the murine ApcMin model, and accounts, at least partly, for the increased tumor susceptibility associated with genetically induced deficiency of the TTSP inhibitor HAI-1 in intestinal epithelial cells. Mechanistically, PAR2 signaling promotes NF-κB activation and tumor angiogenesis. This study illustrates the importance of tight regulation of pericellular serine protease activities for epithelial homeostasis and points to serine proteases and protease-activated receptors as therapeutic targets in epithelial carcinogenesis.

ACKNOWLEDGMENTS
We thank Ms Yukari Torisu and Junko Kurogi for their excellent technical assistance. This work was supported by Japan Society for the Promotion of Science KAKENHI 15K08311 (MK), 16H05175 (HK), and 17K08764 (TF) and by the French National Research Agency (ANR-15-CE14-0009: EC).

DISCLOSURE
The authors declare that there is no conflict of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Kawaguchi M, Yamamoto K, Kataoka H, et al. Protease-activated receptor-2 accelerates intestinal tumor formation through activation of nuclear factor-κB signaling and tumor angiogenesis in ApcMin/+ mice. Cancer Sci. 2020;111:1193–1202. [https://doi.org/10.1111/cas.14335](https://doi.org/10.1111/cas.14335)