

SCIENTIFIC REPORTS

OPEN

Nardilysin regulates inflammation, metaplasia, and tumors in murine stomach

Yuto Kimura¹, Kozo Ikuta¹, Takeshi Kimura², Tsutomu Chiba¹, Hiroko Oshima³, Masanobu Oshima³, Eiichiro Nishi² & Hiroshi Seno¹

Received: 24 October 2016

Accepted: 17 January 2017

Published: 23 February 2017

Chronic inflammation contributes to a wide variety of human disorders. In the stomach, longstanding gastritis often results in structural alterations in the gastric mucosa, including metaplastic changes and gastric cancers. Therefore, it is important to elucidate factors that are involved in gastric inflammation. Nardilysin (N-arginine dibasic convertase; Nrdc) is a metalloendopeptidase of the M16 family that promotes ectodomain shedding of the precursor forms of various growth factors and cytokines by enhancing the protease activities of a disintegrin and metalloproteinase (ADAM) proteins. Here, we have demonstrated that Nrdc crucially regulates gastric inflammation caused by *Helicobacter felis* infection or forced expression of prostaglandin E₂ in K19-C2mE mice. Metaplastic changes following gastric inflammation were suppressed by the deletion of Nrdc. Furthermore, the deletion of Nrdc significantly suppressed N-methyl-N-nitrosourea (MNU)-induced gastric tumorigenesis in the murine stomach. These data may lead to a global therapeutic approach against various gastric disorders by targeting Nrdc.

Chronic inflammation contributes to a wide variety of human disorders. In the stomach, most lifelong chronic gastritis is caused by *Helicobacter pylori* infection¹, although other etiologies, such as non-steroidal anti-inflammatory drugs or autoimmunity, are also prevalent². Longstanding gastritis can result in structural alterations in the gastric mucosa, including metaplastic changes and gastric cancers. Metaplastic changes in the stomach are associated with an increased risk of gastric cancer³. Therefore, it is important to elucidate factors that are involved in gastric inflammation for effective prevention of various gastric diseases, including gastric cancer⁴.

The local microenvironment influences the development of chronic gastritis and metaplastic changes regardless of causative factors, and inflammatory cytokines participate in constructing this environment. There are a number of factors regulating inflammatory cytokines. A disintegrin and metalloproteinase (ADAM) family proteins are involved in ectodomain shedding, and regulate the biological activities of structurally and functionally diverse inflammatory cytokines in a context-dependent manner^{5,6}. Indeed, dysregulation of ectodomain shedding of those factors can be profoundly involved in the pathogenesis of a serial development of gastritis, metaplasia, and gastric cancer⁷.

Nardilysin (N-arginine dibasic convertase; NRDC), a zinc peptidase of the M16 family that selectively cleaves dibasic sites⁸, is diffusely localized in the cytoplasm, and is secreted to the cell surface by undetermined mechanisms⁹. We previously identified NRDC as a specific binding partner of heparin-binding epidermal growth factor-like growth factor (HB-EGF)¹⁰. NRDC also enhances the shedding of tumor necrosis factor- α (TNF- α) through activation of ADAM17^{11,12}. TNF- α is produced as a membrane-anchored protein, shed from the cell surface by proteolytic cleavage, and subsequently activated. The proinflammatory genotype of TNF- α is associated with more than twice the risk of non-cardia gastric cancer¹³. In this respect, we previously demonstrated that NRDC regulates activation of TNF- α and subsequent production of inflammatory cytokines in gastric cancer cells¹⁴. These findings suggest that NRDC regulates chronic inflammation and tumorigenesis in the stomach; however, the *in vivo* role of NRDC in the stomach is still unclear.

¹Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan. ²Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507 Japan. ³Division of Genetics, Cancer Research Institute, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192 Japan. Correspondence and requests for materials should be addressed to E.N. (email: nishi@kuhp.kyoto-u.ac.jp) or H.S. (email: seno@kuhp.kyoto-u.ac.jp)

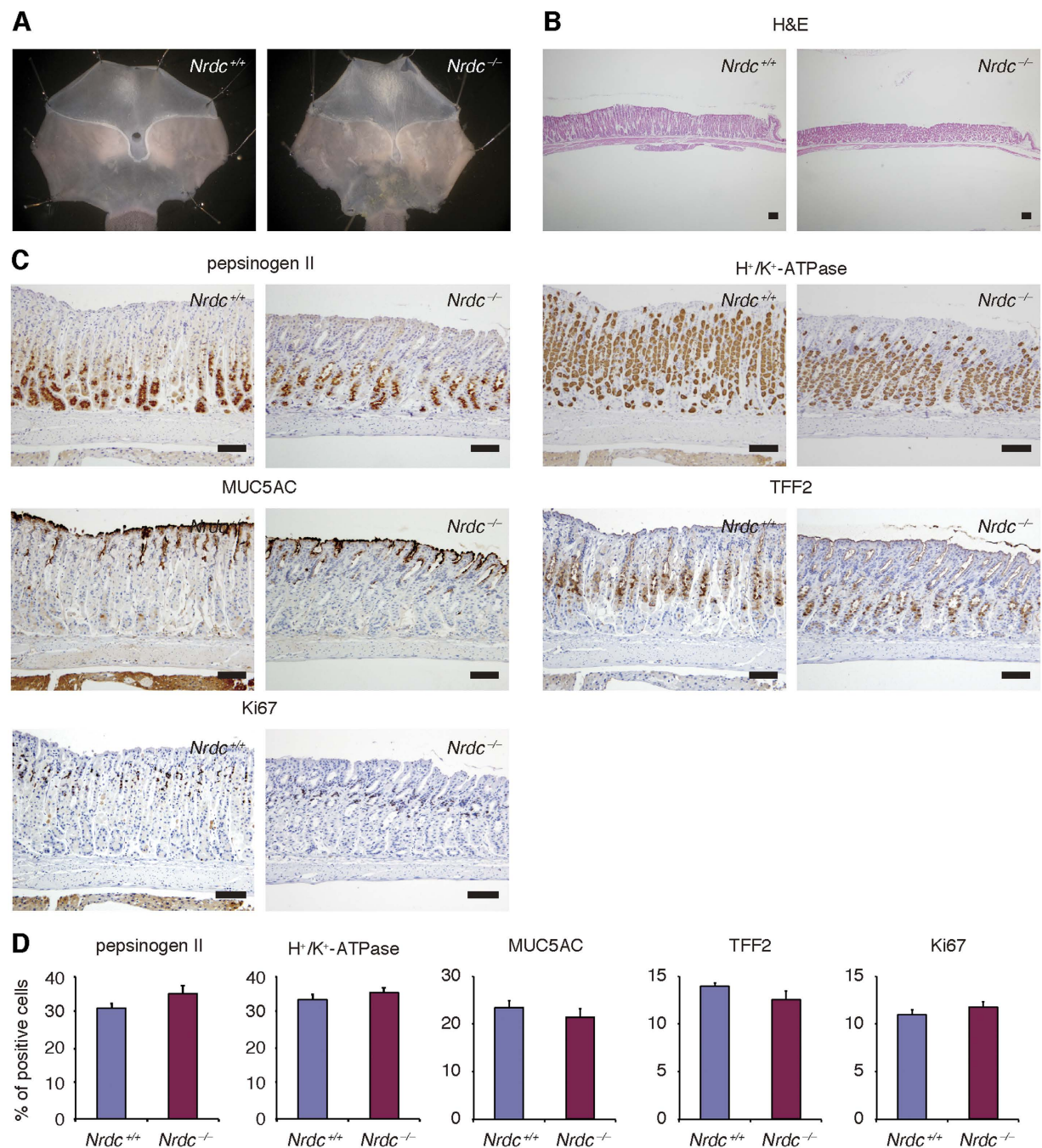


Figure 1. Stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice under physiological conditions. (A) Representative macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. (B) H&E staining of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs. Bars = 100 μ m. (C) Immunohistochemistry for pepsinogen II, H⁺/K⁺-ATPase, Muc5ac, TFF2, and Ki67 in *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. Bars = 100 μ m. (D) Percentages of epithelial cells immunostained with pepsinogen II, H⁺/K⁺-ATPase, Muc5ac, TFF2, and Ki67 in *Nrdc*^{+/+} and *Nrdc*^{-/-} mice.

In this study, we examined the role of *Nrdc* during the development of chronic gastritis and metaplastic changes in the stomach using *Nrdc* knockout mice. We also investigated the effect of *Nrdc* in chemically-induced gastric tumorigenesis.

Results

Nrdc did not alter the differentiation status of gastric mucosa under physiological conditions.

We first examined the effects of *Nrdc* deletion in the stomach under physiological conditions. *Nrdc*^{-/-} mice tend to maintain lower body weights than *Nrdc*^{+/+} mice¹⁵. Therefore, the mean stomach size of *Nrdc*^{-/-} mice was slightly smaller than that of *Nrdc*^{+/+} mice (Fig. 1A). Other than the stomach size, there were no macroscopic differences between *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. Although the gastric mucosa was slightly thinner in *Nrdc*^{-/-} mice

(Fig. 1B), there were no significant differences in the differentiation status of gastric mucosal cells (Fig. 1C). Immunohistochemistry showed that the percentages of pepsinogen II-positive cells within gastric glands were not significantly different between *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. This indicated that chief cell differentiation was not affected by *Nrdc* status (Fig. 1C and D). Further, immunostainings for H⁺/K⁺-ATPase, Muc5ac, and TFF2 did not show significant differences between *Nrdc*^{+/+} and *Nrdc*^{-/-} mice, indicating that differentiation of parietal, pit, and mucous neck cells was not regulated by *Nrdc* under physiological conditions, respectively (Fig. 1C and D). Furthermore, the proportion of Ki67-positive cells in the gastric glands of *Nrdc*^{-/-} mice was similar to that of *Nrdc*^{+/+} mice. Therefore, deletion of *Nrdc* did not alter epithelial cell proliferation under physiological conditions (Fig. 1C and D).

Gastritis caused by *Helicobacter felis* infection was attenuated by *Nrdc* deletion. *Helicobacter felis* infection is a well-characterized mouse model that mimics chronic *Helicobacter pylori* infection in the human stomach¹⁶. Based on a previous report¹⁶, we administered suspending water containing *Helicobacter felis* to *Nrdc*^{+/+} and *Nrdc*^{-/-} mice for three days. Mice were sacrificed 20 weeks after the end of administration. Regardless of *Nrdc* gene status, *Helicobacter felis* was infected successfully into the gastric mucosae (Fig. 2A and B). The thickness of the mucosa of gastric corpus in *Nrdc*^{+/+} mice with *Helicobacter felis* infection was significantly different from mice without the infection, but was not significantly different from *Nrdc*^{-/-} mice regardless of *Helicobacter felis* infection (Fig. 2C). Of note, formation of lymphoid follicles, a characteristic of *Helicobacter felis* infection, was scarce in *Nrdc*^{-/-} mice (Fig. 2D), and the inflammation score was more severe in *Nrdc*^{+/+} than in *Nrdc*^{-/-} mice with *Helicobacter felis* infection (Fig. 2E). Consistent with these findings, infiltration of Gr1-positive neutrophils was not prominent in *Nrdc*^{-/-} mice, and that of F4/80-positive macrophages was significantly decreased compared to *Nrdc*^{+/+} mice (Fig. 2F and G). The mRNA expression of Cxcl1 and Ccl2, factors that recruit neutrophils and macrophages, respectively, was also decreased in *Nrdc*^{-/-} mice (Fig. 2H). Notably, mRNA levels of interleukin (IL)-1 α , IL-1 β , IL-6, and IL-12, that can contribute to chronic gastritis in humans^{13–17}, were not significantly increased in *Nrdc*^{-/-} mice (Fig. 2I). Thus, the deletion of *Nrdc* attenuates gastric inflammation caused by *Helicobacter* infection.

Gastritis caused by forced expression of prostaglandin (PGE)₂ was attenuated by *Nrdc* deletion. We examined the role of *Nrdc* in *K19-C2mE* mice, another mouse model that expresses PGE₂ abundantly in the gastric mucosa and mimics human gastritis¹⁶. Similar to *Helicobacter felis*-induced gastritis, mucosae of the gastric corpus of *Nrdc*^{+/+} mice were macroscopically thicker compared to *Nrdc*^{-/-} mice at 30 weeks of age (Fig. 3A). Histologically, the mucosae of gastric corpus were remarkably hyperplastic in *Nrdc*^{+/+} mice, consistent with a previous report¹⁶. However, this hyperplastic change was not prominent in *Nrdc*^{-/-} mice (Fig. 3B). Consequently, mucosae were significantly thinner in *Nrdc*^{-/-} mice (Fig. 3C). Infiltration of Gr1-positive neutrophils and F4/80-positive macrophages was significantly decreased in *Nrdc*^{-/-} mice compared to *Nrdc*^{+/+} mice (Fig. 3D and E). The expression of Cxcl1 and IL-1 β mRNA was significantly decreased in *Nrdc*^{-/-} mice compared to *Nrdc*^{+/+} mice (Fig. 3F), although we could not demonstrate alterations of Ccl2, IL-1 α , IL-6, and IL-12 mRNA between *Nrdc*^{+/+} and *Nrdc*^{-/-} mice in the presence of *K19-C2mE* alleles. Thus, like *Helicobacter felis*-induced gastritis, deletion of *Nrdc* attenuates gastric inflammation and hyperplasty caused by the forced expression of PGE₂.

Metaplastic changes were attenuated by *Nrdc* deletion. We next investigated whether *Nrdc* plays a role in metaplastic changes following gastritis. Staining for TFF2 and/or staining with Alcian blue are widely used to detect metaplastic changes in the gastric corpus¹⁸. Upon *Helicobacter felis* infection for 20 weeks, the percentage of TFF2-stained cells was significantly lower in *Nrdc*^{-/-} mice than in *Nrdc*^{+/+} mice (Fig. 4A and B). The percentage of Alcian blue-stained cells was also reduced in *Nrdc*^{-/-} mice compared to *Nrdc*^{+/+} mice at 26 weeks of *Helicobacter felis* infection (Fig. 4C and D).

In *K19-C2mE* mice at the age of 30 weeks, Alcian blue staining showed that the development of metaplastic changes was significantly less prominent in *Nrdc*^{-/-} mice compared with *Nrdc*^{+/+} mice (Fig. 4E and F). Together, these data indicate that the deletion of *Nrdc* leads to the suppression of metaplastic changes in the stomach.

Formation of gastric tumors was suppressed by *Nrdc* deletion. Chronic gastritis and metaplastic changes are associated with the development of gastric cancers. In both *Helicobacter felis* infection and the *K19-C2mE* chronic gastritis mouse models, we noticed that the increase in the number of Ki67-positive cells in the gastric glands of *Nrdc*^{+/+} mice was not remarkable in *Nrdc*^{-/-} mice (Fig. 5A). Based on this finding, we hypothesized that the formation of gastric tumors may also be suppressed in *Nrdc*^{-/-} mice. Because it requires a long period to develop tumors in *Helicobacter felis*-infected mouse stomach, we administered a chemical carcinogen to rapidly induce mouse gastric tumors. Administration of N-methyl-N-nitrosourea (MNU) causes polypoid tumors in the gastric antrum with inflammatory reactions in the stroma^{19–21}. In *Nrdc*^{+/+} mice, MNU treatment resulted in polyp formation in the gastric antrum (Fig. 5B and C). In *Nrdc*^{-/-} mice, the number and burden of gastric tumors was dramatically reduced compared with that in *Nrdc*^{+/+} mice (Fig. 5B,D and E). Thus, deletion of *Nrdc* attenuates gastric tumorigenesis induced by MNU.

Discussion

In the present study, we demonstrated that *Nrdc* crucially regulates gastric inflammation caused by *Helicobacter felis* infection or forced expression of PGE₂. Metaplastic changes following gastric inflammation were suppressed by the deletion of *Nrdc*. The deletion of *Nrdc* significantly suppressed chemically-induced tumorigenesis of the stomach.

Helicobacter pylori-induced gastritis is a primary inflammatory disorder of the human stomach, affecting about half of the global population¹. Recent improvements in the hygienic environment have reduced the rate of *Helicobacter pylori* infection; however, other causative factors such as non-steroidal anti-inflammatory drugs

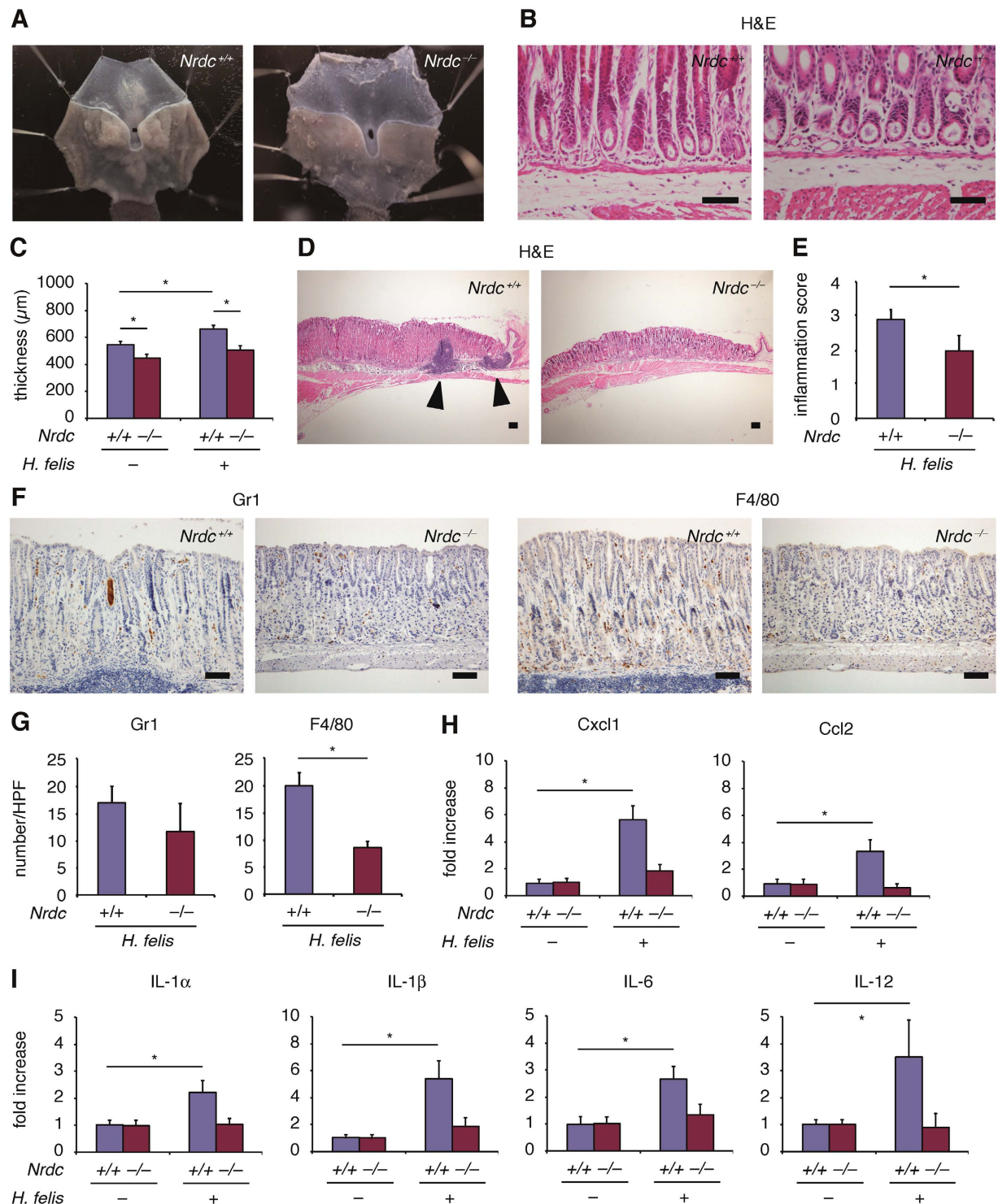


Figure 2. Stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice infected with *Helicobacter felis*. (A) Representative macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice infected with *Helicobacter felis*. (B) Detection of *Helicobacter felis* in the mucosae of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. Note that *Helicobacter felis* is detected in gastric glands of both *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. Bars = 100 μ m. (C) Mucosal thickness of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice with or without *Helicobacter felis* infection. (D) Formation of lymphoid follicle (arrowheads) in *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse gastric mucosae. Bars = 100 μ m. (E) Inflammation scores of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse gastric mucosae. **P* < 0.05. (F) Immunohistochemistry for Gr1 and F4/80 in *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. Bars = 100 μ m. (G) Number of epithelial cells immunostained for Gr1 and F4/80 in *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. **P* < 0.05. (H) mRNA expression of Cxcl1 and Ccl2 in the gastric mucosae of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs. (I) mRNA expression of IL-1 α , IL-1 β , IL-6, and IL-12 in the gastric mucosae of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs. **P* < 0.05.

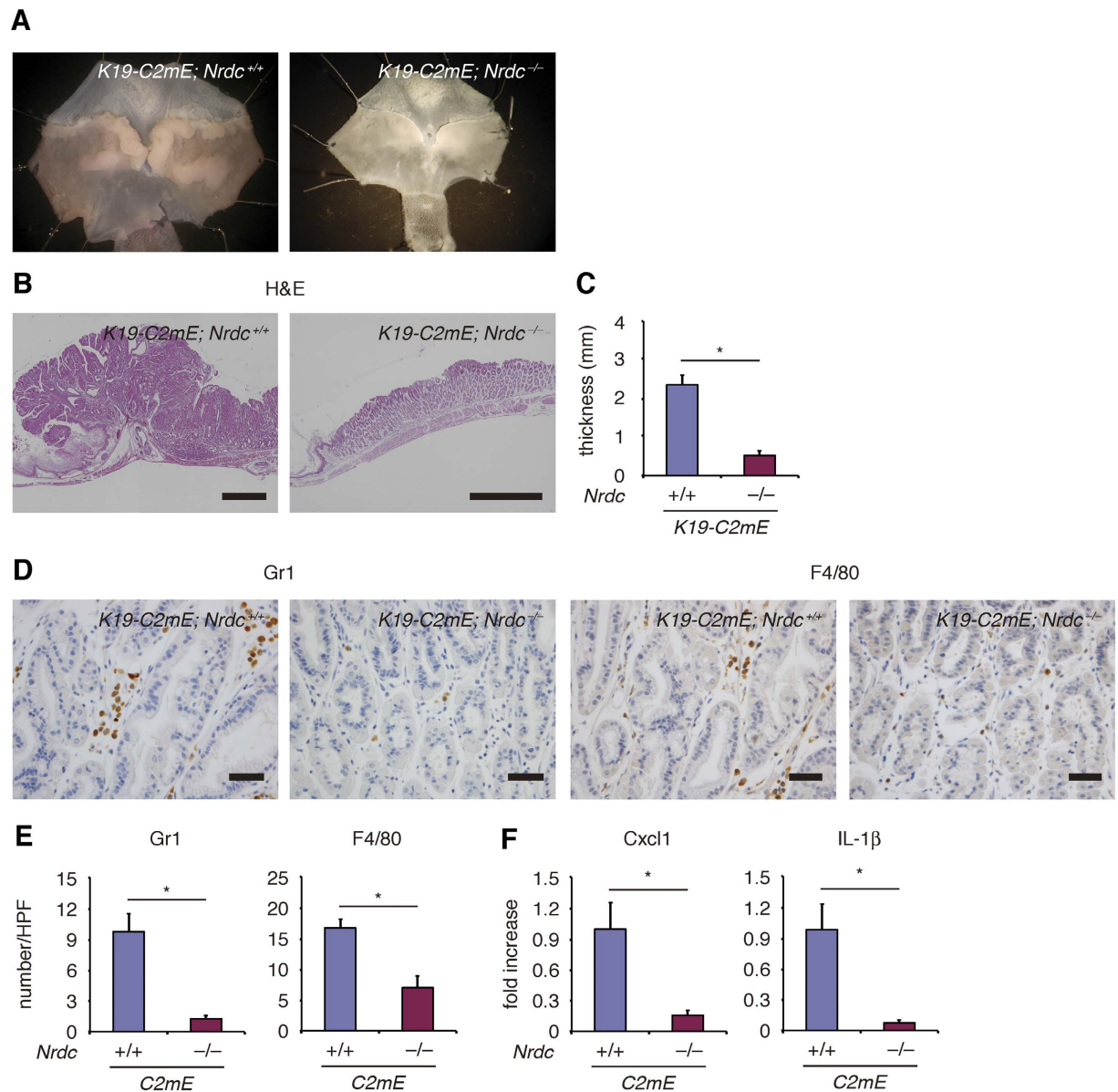


Figure 3. Gastritis caused by forced expression of prostaglandin E₂. (A) Representative macroscopic views of the stomachs of *Nrdc^{+/+}* and *Nrdc^{-/-}* mice with forced expression of PGE₂ by the insertion of *K19-C2mE* alleles. (B) H&E staining of *Nrdc^{+/+}* and *Nrdc^{-/-}* mouse stomachs with forced expression of PGE₂. Bars = 1000 μm. (C) Mucosal thickness of the *Nrdc^{+/+}* and *Nrdc^{-/-}* mice with forced PGE₂ expression. **P* < 0.05. (D) Immunohistochemistry for Gr1 and F4/80 in *Nrdc^{+/+}* and *Nrdc^{-/-}* mice. Bars = 100 μm. (E) Numbers of epithelial cells immunostained for Gr1 and F4/80 in *Nrdc^{+/+}* and *Nrdc^{-/-}* mice. **P* < 0.05. (F) mRNA expression of Cxcl1 and IL-1β in the gastric mucosae of *Nrdc^{+/+}* and *Nrdc^{-/-}* mouse stomachs. **P* < 0.05.

or autoimmunity also contribute to the development of acute and chronic gastritis²². Regardless of the causative factors, chronic inflammation of the gastric mucosa can slowly progress to mucosal atrophy, induce metaplastic changes, and finally cause gastric cancer. In addition to the treatment of acute gastritis that may result in hemorrhagic complications, detection and therapeutic intervention in the early stages of the developmental cascade from chronic gastritis to gastric cancer is important. Therefore, it would be helpful to understand the molecular basis of chronic inflammation in the stomach.

Chronic inflammation is regulated by a feedback loop consisting of diverse inflammatory cytokines, and is closely associated with the recruitment of inflammatory cells in tissues. We previously demonstrated that the deletion of *Nrdc* critically suppresses ectodomain shedding, activation of TNF-α, and the production of other inflammatory cytokines in gastric cancer cells¹⁴. We also showed that the deletion of *Nrdc* significantly suppresses mouse steatohepatitis with attenuated inflammatory cytokine production and reduced infiltration of inflammatory cells²³. Therefore, in this study, we examined the undetermined *in vivo* role of *Nrdc* during the developmental process of chronic gastritis, metaplastic changes, and gastric tumors.

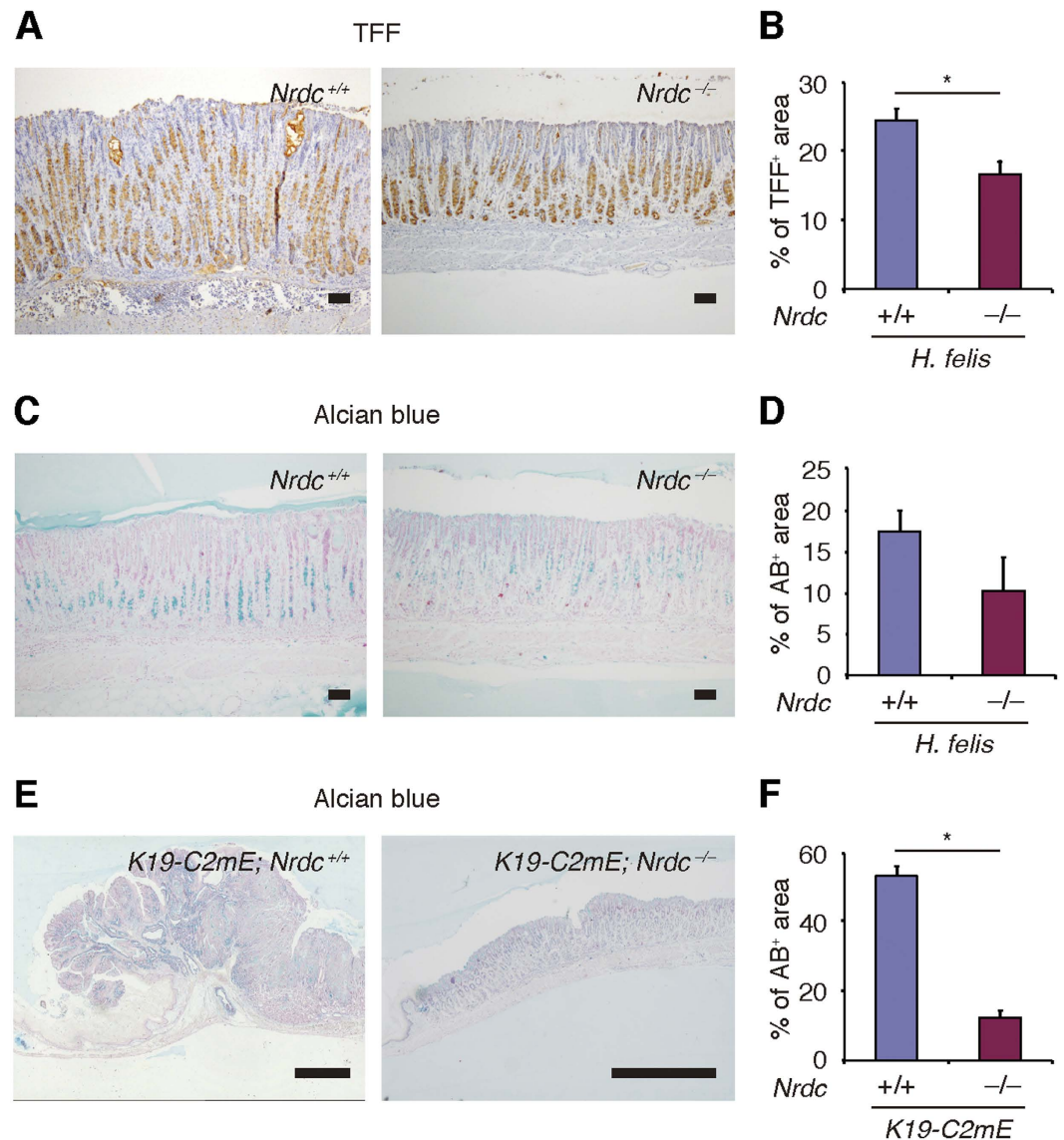


Figure 4. Metaplastic changes in *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs. (A) Immunohistochemistry for TFF2 in *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs with *Helicobacter felis* infection. Bars = 100 μ m. (B) Areas stained for TFF2 in *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs with *Helicobacter felis* infection. **P* < 0.05. (C) Alcian blue staining of *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs with *Helicobacter felis* infection. Bars = 100 μ m. (D) Areas stained with Alcian blue in *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs with *Helicobacter felis* infection. **P* < 0.05. (E) Alcian blue staining of *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs with PGE₂ expression. Bars = 1000 μ m. (F) Areas stained with Alcian blue in *Nrhc*^{+/+} and *Nrhc*^{-/-} mouse stomachs with forced PGE₂ expression. **P* < 0.05.

To mimic chronic infection and inflammation in the human stomach caused by *Helicobacter pylori*, we used a *Helicobacter felis*-infected mouse model. Mucosae of the gastric corpus were thinner in *Nrhc*^{-/-} than in *Nrhc*^{+/+} mice, and the inflammation score was less severe in *Nrhc*^{-/-} mice. Infiltration of neutrophils and macrophages was decreased in *Nrhc*^{-/-} mice concomitantly with attenuated expression of inflammatory cytokines. Although infiltration of inflammatory cells such as neutrophils may not be critical for the development of *Helicobacter felis*-induced gastritis²⁴, this pattern of cytokine expression is consistent with our previous data obtained from a human gastric cancer cell line¹⁴. Notably, levels of IL-1 α , IL-1 β , IL-6, and IL-12, which are the main contributors to human chronic gastritis, were decreased in *Nrhc*^{-/-} mice. Thus, the deletion of *Nrhc* attenuates gastric inflammation, indicating that *Nrhc* also plays a role in the intravital stomach. It is also important that the deletion of *Nrhc* attenuated inflammation caused by the forced expression of PGE₂. This indicated that the deletion of *Nrhc* attenuates gastric inflammation regardless of the cause of inflammation, and a global therapeutic approach may be possible by targeting *Nrhc*.

We next questioned whether *Nrhc* plays a role in metaplastic changes and tumorigenesis. In *Helicobacter felis*-infected mice, metaplastic changes develop in the gastric corpus, and progress to mucous metaplasia that can be stained with Alcian blue^{25,26}. In the present study, metaplastic changes induced by *Helicobacter felis* infection, or forced expression of PGE₂, were significantly attenuated by the deletion of *Nrhc*. More importantly, gastric

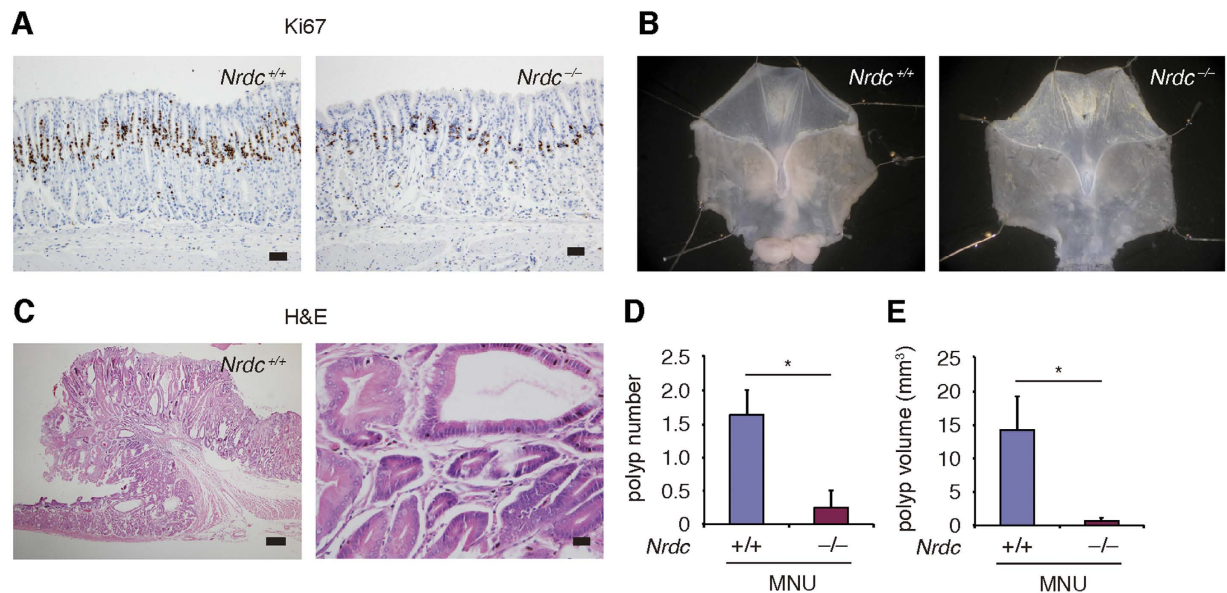


Figure 5. Formation of gastric tumors in *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs. (A) Ki67 immunostaining in *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs with the forced PGE₂ expression. Bars = 50 μ m. (B) Macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice treated with N-methyl-N-nitrosourea (MNU). (C) H&E staining of gastric tumor in *Nrdc*^{+/+} mouse. Bars = 200 μ m (left) and 20 μ m (right). (D) Polyp number in the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice treated with MNU. **P* < 0.05. (E) Polyp volume of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse gastric tumors established by MNU treatment. **P* < 0.05.

tumor formation was remarkably suppressed by the same genetic change. It is now becoming clear that the tumor microenvironment, which is orchestrated by inflammatory cells, is an indispensable participant during a wide-range of tumor developmental processes²⁷.

Inflammation plays decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis²⁸. We previously reported that NRDC is highly expressed in the epithelium of human gastric cancer tissues¹⁴. Knockdown of NRDC attenuates gastric cancer cell growth both *in vitro* and in a xenograft model. These previous data showed that TNF- α secreted from gastric cancer cells themselves initiates a feedback loop to enhance inflammatory cytokine expression.

As for the pivotal role of *Nrdc* *in vivo*, we previously showed in a mouse steatohepatitis model that the production of inflammatory cytokines and recruitment of inflammatory cells are significantly suppressed concomitantly with the suppression of TNF- α release by the deletion of *Nrdc*²³. Although we did not again examined the shedding status of TNF- α in mouse stomach in the present study, the remarkable suppressive effect of *Nrdc* deletion on inflammation may lead to the suppression of metaplastic changes and tumor formation. In this respect, it is worth noting that metaplastic changes were detected in *Nrdc*^{-/-} mice, albeit milder than in *Nrdc*^{+/+} mice, and that gastric tumor formation was almost completely blocked in *Nrdc*^{-/-} mice. This discrepancy indicates a possibility that formation of gastric tumors by MNU treatment requires inflammatory responses, and that metaplastic changes may be regulated at least partly by factors other than *Nrdc*. To determine the exact mechanisms underlying how *Nrdc* coordinates inflammation, metaplastic changes, and tumor formation, further studies are required. However, the findings obtained in available studies, including the current work, suggest that a global therapeutic approach against various gastric disorders may be possible by targeting NRDC.

Methods

Animal models. Generation of the *Nrdc* knockout mouse with a CBA background was described previously¹⁵. Animals were housed under specific pathogen-free conditions at the Animal Facilities of Kyoto University. All animal experiments were performed in accordance with institutional guidelines. The Review Board of Kyoto University granted ethical permission for this study, and The Kyoto University Animal Experimentation Committee approved the experimental protocol. For the infection by *Helicobacter felis*, suspending water containing *Helicobacter felis* was gavaged to *Nrdc*^{+/+} and *Nrdc*^{-/-} mice for three days according to the previous report¹⁶. K19-C2mE mice were generated as previously described¹⁶. To analyze chemically-induced gastric tumorigenesis, mice at the age of 6 weeks were administered MNU (Sigma-Aldrich, St. Louis, MO, USA) in drinking water at 240 ppm on alternate weeks for five weeks as described previously¹⁹.

Histological and immunohistochemical analyses. Mouse stomach was resected, fixed in 4% buffered paraformaldehyde solution, embedded in paraffin, and cut into sections 5- μ m thick. For immunostaining, the sections were incubated overnight with the primary antibodies at 4 $^{\circ}$ C, after which the secondary antibodies were added. The primary antibodies used were rat anti-F4/80 (Abcam, Cambridge, MA, USA), rat anti-Gr-1 (eBioscience, San Diego, CA, USA), sheep anti-pepsinogen II (Abcam), mouse anti-H⁺/K⁺-ATPase α subunit (MBL,

Nagoya, Japan), mouse anti-Muc5AC (Abcam), mouse-anti-spasmolytic polypeptide (TFF2) (R&D Systems, Minneapolis, MN, USA), and rat anti-Ki67 (Dako, Glostrup, Denmark). All immunohistochemical analyses were performed with immunoglobulin isotype controls. For Alcian blue staining, deparaffinized sections were incubated with Alcian blue solution for 30 minutes, followed by counterstaining with Nuclear Fast Red. To determine the differentiation status of the gastric mucosa under physiological conditions, stained cells were counted in 10 randomly selected gastric glands per mouse in 6 *Nrdc*^{+/+} and 4 *Nrdc*^{-/-} mice. In *Helicobacter felis* infection experiments, we used 12 *Nrdc*^{+/+} and 6 *Nrdc*^{-/-} mice. In addition, 6 *Nrdc*^{+/+}; K19-C2mE and 3 *Nrdc*^{-/-}; K19-C2mE mouse samples were subjected to the analyses. Using these mice, to analyze histology and count inflammatory cells, eight high power field sections from each mouse were selected randomly. To investigate gastric tumorigenesis, 12 *Nrdc*^{+/+} and 6 *Nrdc*^{-/-} mice were treated with MNU. Inflammation scores were determined according to the previous report²⁴.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA, USA). Single-strand complementary DNA was synthesized using a First Strand SYBR Green Master Mix (Roche Applied Science, Basel, Switzerland). qRT-PCR was performed using FastStart SYBR Green Master (Roche Applied Science) and the LightCycler 480 system (Roche Applied Science). Values are expressed as arbitrary units relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The primer sets used were: interleukin (IL)-1 α -forward, CTGATGAAGCTCGTCAGGCAG; IL-1 α -reverse, TGGTGCTGAGATAGTGTTCGTC; IL-1 β -forward, GCAACTGTTCTGAACTCAACT; IL-1 β -reverse, ATCTTTTGGGGTCCGTCAACT; IL-6-forward, TAGTCCTTCCTACCCCAATTTCC; IL-6-reverse, TTGGTCCTTAGCCACTCCTTC; IL-12-forward, ACTCTGCGCCAGAAACCTC; IL-12-reverse, CACCCTGTTGATGGTCACGAC; Cxcl1-forward, CTGGGATTCACCTCAAGAACATC; Cxcl1-reverse, CAGGGTCAAGGCAAGCCTC; Ccl2-forward, ATCCACGGCATACTATCAACATC; Ccl2-reverse, CAAGGCTCACCATCATCGTAG; Gapdh-forward, AGGTCGGTGTGAACGGATTTCG; and Gapdh-reverse, TGTAGACCATGTAGTTGAGGTCA. In each experiment, 3–6 samples were subjected to the reactions.

Statistical analyses. Results are expressed as means $\pm$ standard error unless stated otherwise. Differences between treatments, groups, and strains were analyzed by the two-tailed Student's *t*-test.

References

- Pounder, R. E. & Ng, D. The prevalence of *Helicobacter pylori* infection in different countries. *Aliment Pharmacol Ther.* **9**, Suppl 2, 33–39 (1995).
- Nishiura, H. *et al.* Interleukin-21 and tumor necrosis factor- α are critical for the development of autoimmune gastritis in mice. *J Gastroenterol Hepatol.* **28**, 982–991 (2013).
- Choi, E., Hendley, A. M., Bailey, J. M., Leach, S. D. & Goldenring, J. R. Expression of Activated Ras in Gastric Chief Cells of Mice Leads to the Full Spectrum of Metaplastic Lineage Transitions. *Gastroenterology* **150**, 918–930 (2016).
- Fox, J. G. & Wang, T. C. Inflammation, atrophy, and gastric cancer. *J Clin Invest.* **117**, 60–69 (2007).
- Blobel, C. P. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol.* **6**, 32–43 (2005).
- Seals, D. F. & Courtneidge, S. A. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev.* **17**, 7–30 (2003).
- Murphy, G. The ADAMs: signalling scissors in the tumour microenvironment. *Nat Rev Cancer.* **8**, 929–941 (2008).
- Pierotti, A. R. *et al.* N-arginine dibasic convertase, a metalloendopeptidase as a prototype of a class of processing enzymes. *Proc Natl Acad Sci USA* **91**, 6078–6082 (1994).
- Hospital, V. *et al.* N-arginine dibasic convertase (nardilysin) isoforms are soluble dibasic-specific metalloendopeptidases that localize in the cytoplasm and at the cell surface. *Biochem J.* **349**, 587–597 (2000).
- Nishi, E., Prat, A., Hospital, V., Elenius, K. & Klagsbrun, M. N-arginine dibasic convertase is a specific receptor for heparin-binding EGF-like growth factor that mediates cell migration. *EMBO J.* **20**, 3342–3350 (2001).
- Nishi, E., Hiraoka, Y., Yoshida, K., Okawa, K. & Kita, T. Nardilysin enhances ectodomain shedding of heparin-binding epidermal growth factor-like growth factor through activation of tumor necrosis factor- α -converting enzyme. *J Biol Chem.* **281**, 31164–31172 (2006).
- Hiraoka, Y. *et al.* Ectodomain shedding of TNF- α is enhanced by nardilysin via activation of ADAM proteases. *Biochem Biophys Res Commun.* **370**, 154–158 (2008).
- El-Omar, E. M. *et al.* Increased risk of noncardia gastric cancer associated with roinflammatory cytokine gene polymorphisms. *Gastroenterology* **124**, 1193–1201 (2003).
- Kanda, K. *et al.* Nardilysin and ADAM proteases promote gastric cancer cell growth by activating intrinsic cytokine signalling via enhanced ectodomain shedding of TNF- α . *EMBO Mol Med.* **4**, 396–411 (2012).
- Ohno, M. *et al.* Nardilysin regulates axonal maturation and myelination in the central and peripheral nervous system. *Nat Neurosci.* **12**, 1506–1513 (2009).
- Oshima, H. *et al.* Prostaglandin E₂ signaling and bacterial infection recruit tumor-promoting macrophages to mouse gastric tumors. *Gastroenterology* **140**, 596–607 (2011).
- Yuzhalin, A. The role of interleukin DNA polymorphisms in gastric cancer. *Hum Immunol.* **72**, 1128–1136 (2011).
- Hoffmann, W. TFF2, a MUC6-binding lectin stabilizing the gastric mucus barrier and more. *Int J Oncol.* **47**, 806–816 (2015).
- Yamachika, T. *et al.* N-methyl-N-nitrosourea concentration-dependent, rather than total intake-dependent, induction of adenocarcinomas in the glandular stomach of BALB/c mice. *Jpn J Cancer Res.* **89**, 385–91 (1998).
- Tsukamoto, T., Mizoshita, T. & Tatematsu, M. Animal models of stomach carcinogenesis. *Toxicol Pathol.* **35**, 636–648 (2007).
- Leung, W. K. *et al.* Transgenic cyclooxygenase-2 expression and high salt enhanced susceptibility to chemical-induced gastric cancer development in mice. *Carcinogenesis* **29**, 1648–1654 (2008).
- Hirschowitz, B. I. Nonsteroidal antiinflammatory drugs and the gastrointestinal tract. *Gastroenterologist.* **2**, 207–223 (1994).
- Ishizu-Higashi, S. *et al.* Deletion of nardilysin prevents the development of steatohepatitis and liver fibrotic changes. *PLoS One* **29**, e98017 (2014).
- Rogers, A. B. Histologic scoring of gastritis and gastric cancer in mouse models. *Methods Mol Biol.* **921**, 189–203 (2012).
- Fox, J. G. *et al.* Hypertrophic gastropathy in *Helicobacter felis*-infected wild-type C57BL/6 mice and p53 hemizygous transgenic mice. *Gastroenterology* **110**, 155–166 (1996).
- Wang, T. C. *et al.* Mice lacking secretory phospholipase A2 show altered apoptosis and differentiation with *Helicobacter felis* infection. *Gastroenterology* **114**, 675–689 (1998).
- Coussens, L. M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860–867 (2002).
- Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell.* **140**, 883–899 (2010).

Acknowledgements

This work was supported in part by Grants-in-Aid KAKENHI (25112707, 26293068, 26293173, 26116715, 16K09394, 16K15216, 16K15427, 16K15216) and a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) from the Ministry of Education, Culture, Sports, Science and Technology/the Japan Society for the Promotion of Science. It was also supported by Health Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare (The development of innovative therapeutic drug for the intractable inflammatory bowel disease/Comprehensive Research on Lifestyle-Related Diseases including Cardiovascular Diseases and Diabetes Mellitus); the Kobayashi Foundation for Cancer Research; the Naito Foundation; Princess Takamatsu Cancer Research Fund 13-24514; the Takeda Science Foundation; the Uehara Memorial Foundation.

Author Contributions

Y.K., E.N., and H.S. designed the studies and wrote the manuscript; Y.K. and K.I. performed experiments and were involved in data analysis; H.O. and M.O. provided essential materials; T.K. and T.C. supervised all studies.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kimura, Y. *et al.* Nardilysin regulates inflammation, metaplasia, and tumors in murine stomach. *Sci. Rep.* 7, 43052; doi: 10.1038/srep43052 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2017