

Nardilysin inhibits pancreatitis and suppresses pancreatic ductal adenocarcinoma initiation in mice

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ABSTRACT

Objective Nardilysin (NRDC), a zinc peptidase, exhibits multiple localisation-dependent functions including as an enhancer of ectodomain shedding in the extracellular space and a transcriptional coregulator in the nucleus. In this study, we investigated its functional role in exocrine pancreatic development, homeostasis and the formation of pancreatic ductal adenocarcinoma (PDA).

Design We analysed *Ptf1a-Cre; Nrdc^{flox/flox}* mice to investigate the impact of *Nrdc* deletion. Pancreatic acinar cells were isolated from *Nrdc^{flox/flox}* mice and infected with adenovirus expressing Cre recombinase to examine the impact of *Nrdc* inactivation. Global gene expression in *Nrdc-CKO* pancreas was analysed compared with wild-type pancreas by microarray analysis. We also analysed *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* mice to investigate the impact of *Nrdc* deletion in the context of oncogenic *Kras*. A total of 51 human samples of pancreatic intraepithelial lesions (PanIN) and PDA were examined by immunohistochemistry for NRDC.

Results We found that pancreatic deletion of *Nrdc* leads to spontaneous chronic pancreatitis concomitant with acinar-to-ductal conversion, increased apoptosis and atrophic pancreas in mice. Acinar-to-ductal conversion was observed mainly through a non-cell autonomous mechanism, and the expression of several chemokines was significantly increased in *Nrdc*-null pancreatic acinar cells. Furthermore, pancreatic deletion of *Nrdc* dramatically accelerated *Kras^{G12D}*-driven PanIN and subsequent PDA formation in mice. These data demonstrate a previously unappreciated anti-inflammatory and tumour suppressive functions of *Nrdc* in the pancreas in mice. Finally, absence of NRDC expression was observed in a subset of human PanIN and PDA.

Conclusion *Nrdc* inhibits pancreatitis and suppresses PDA initiation in mice.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease.¹ To develop new therapeutic approaches, there is an urgent need to identify the critical factors that control pancreatic tumourigenesis. PDA arises from preneoplastic lesions, among which pancreatic intraepithelial neoplasia (PanIN) is the most common.^{2–3} The most common gene mutation in PDA is an activating mutation of the *Kras* oncogene, which is also the earliest detectable

Significance of this study

What is already known on this subject?

- ▶ Nardilysin (*Nrdc*) exhibits multiple localisation-dependent functions.
- ▶ *Nrdc* promotes inflammation and tumourigenesis in the stomach and liver.
- ▶ Pancreatitis accelerates *Kras^{G12D}*-driven pancreatic tumourigenesis in mice.

What are the new findings?

- ▶ *Nrdc* is dispensable for exocrine pancreatic development but is required for maintenance of the exocrine pancreas in mice.
- ▶ Pancreatic deletion of *Nrdc* leads to spontaneous chronic pancreatitis with increased apoptosis and acinar-to-ductal metaplasia in mice.
- ▶ *Nrdc* inhibits spontaneous pancreatitis and suppresses *Kras^{G12D}*-driven pancreatic tumourigenesis in mice.
- ▶ Nuclear NRDC was absent in a subset of human pancreatic ductal adenocarcinoma (PDA).
- ▶ *Nrdc* has context-dependent oncogenic and tumour suppressive effects.

How might it impact on clinical practice in the foreseeable future?

- ▶ *Nrdc* might constitute a novel gene associated with hereditary pancreatitis in humans.
- ▶ *Nrdc* might represent a therapeutic target for chronic pancreatitis and pancreatitis-associated PDA.

mutation found in PanIN.⁴ Recent studies using mouse models have shown that mutant *Kras* can reprogramme acinar cells to enter the PanIN/PDA lineage through a transdifferentiation process termed acinar-to-ductal metaplasia (ADM).^{5–8} During ADM transition, acinar cells transdifferentiate into duct-like cells that coexpress both acinar and ductal cell markers.⁹ ADM is also induced by several factors including inflammation, epidermal growth factor signalling and Notch signalling.¹⁰ Chronic pancreatitis, characterised by persistent pancreatic inflammation, constitutes a risk factor for PDA in humans,¹¹ and this disease accelerates



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Kras^{G12D}-driven PanIN and PDA formation in mice.^{5,9,12} Thus, further elucidation of the mechanistic link between pancreatitis and pancreatic tumorigenesis might represent a novel approach to understanding the pathological means of PDA formation.

Nardilysin, N-arginine dibasic convertase (NRDC, *Nrdc*), is a zinc peptidase of the M16 family that selectively cleaves dibasic sites.^{13,14} NRDC exhibits widespread expression in various organs with a unique pattern of cellular localisation.¹⁵ We previously identified NRDC on the cell surface as a specific receptor for heparin-binding epidermal growth factor-like growth factor (HB-EGF)¹⁶ and showed that NRDC enhances the ectodomain shedding of multiple membrane proteins including HB-EGF.^{17–21} Moreover, it has been found that NRDC is shuttled between the cytosol and nucleus,^{22,23} and we recently demonstrated that this protein interacts with several nuclear proteins, suggesting a role of NRDC in transcriptional regulation.^{24–26}

In addition, NRDC is highly expressed in gastric, breast and oesophageal cancer cells^{27–29} and promotes cell growth in vitro.²⁷ We previously showed that NRDC maintains intrinsic nuclear factor-kappa B (NF-κB) signalling, and consequently cancer growth, by enhancing ectodomain shedding of tumour necrosis factor-alpha (TNF-α) in gastric cancer cell lines.²⁷ We also recently demonstrated that NRDC is required for maintaining pancreatic β-cell function in mice.²⁶ However, the functional role of NRDC in exocrine pancreatic development, homeostasis and tumorigenesis remains unknown. Therefore, we investigated the role of NRDC in these processes in mice.

MATERIALS AND METHODS

Mice

Nrdc^{flx/flx} mice (accession no. CDB1019K; http://www.clst.riken.jp/arg/mutant_mice_list.html) were generated by gene targeting using TT2 embryonic stem (ES) cells.³⁰ The targeting vector was designed to insert loxP sites upstream and downstream of exon 1 of *Nrdc*. Successful homologous recombination in TT2 ES cells was confirmed by PCR (using 5'-GTA CTG GAT GGA AGC CGG TCT TGT C and TCA TTC AGG CAG GGT TTG AGT CAC C-3' primers) and Southern blotting. The resulting mutant mice were genotyped using tail DNA and PCR (with the primer pair 5'-GCC ATC CCA AGC AGT ACC GGT G and AAA TTG CCC TGG CCG CCT CAC-3'); these mice were crossed with CAG-FLPe transgenic mice to remove the neomycin selection cassette surrounded by the Flp recombinase target sites and were then backcrossed to the C57BL/6J background (>98%). Animals were housed under specific pathogen-free conditions at the Animal Facilities of Kyoto University. The following mouse strains were previously described: *Ptf1a-Cre* (gift from Y Kawaguchi, Kyoto University, Kyoto, Japan)³¹ and *Kras*^{G12D} (gift from David Tuveson, Cold Spring Harbor Laboratory).³² All animal experiments were performed in accordance with institutional guidelines.

Clinical samples

Surgically resected specimens were obtained from 51 patients with pancreatic cancer who had been admitted to Kyoto University Hospital. Written informed consent was obtained from all patients. Surgically resected specimens were routinely processed and immunostained.

Statistics

Data are presented as the means ± SEM. Statistical comparisons between groups were made using the Student's *t* test. *P* values less

than 0.05 were considered statistically significant. All statistical analyses were performed using either GraphPad Prism (V.6.0, GraphPad, San Diego, California, USA) or Microsoft Excel 2016 (Redmond, Washington, USA).

For a detailed description of materials and methods, see supplementary materials and methods. Primer sequences are described in online supplementary table S1.

RESULTS

Nrdc is required for maintenance of the exocrine pancreas in mice

To investigate the expression pattern of *Nrdc* in the exocrine pancreas in mice, we first performed immunohistochemistry. *Nrdc* was expressed in the nucleus of pancreatic acinar cells but not in centroacinar or ductal cells from embryonic development throughout adulthood (figure 1A). Localisation of *Nrdc* was also verified by double immunofluorescence staining for amylase (Amy)/*Nrdc* and pancytokeratin (panCK)/*Nrdc* (online supplementary figure S1A). To investigate the functional requirement for exocrine pancreatic development and maintenance, we generated *Ptf1a-Cre; Nrdc*^{flx/flx} mice, hereafter referred to as *Nrdc*-cKO mice. *Nrdc*-cKO mice were born at the expected Mendelian frequencies and appeared healthy. No gross phenotypes were observed in *Nrdc*-cKO pancreata when compared with wild-type (WT) controls at birth. At 3 weeks of age, *Nrdc*-cKO animals were not significantly different in terms of body weight and pancreata appearance (figure 1B,C and online supplementary figure S1B), whereas the pancreas weight/body weight (PW/BW) ratio of *Nrdc*-cKO mice was significantly less than that of WT controls (figure 1C). Notably, at 8 weeks of age, *Nrdc*-cKO pancreata became progressively atrophic, with the PW/BW ratio of *Nrdc*-cKO mice being even smaller than that of WT controls (figure 1B,C and online supplementary figure S1B). These tendencies were similarly observed in both male and female mice. Quantitative reverse transcriptase-PCR (qRT-PCR) revealed that *Nrdc* expression was markedly reduced in *Nrdc*-cKO pancreata, and immunostaining for *Nrdc* confirmed that *Nrdc* was deleted in pancreatic acinar cells at 3 weeks of age (figure 1D). Histologically, at 3 weeks of age, *Nrdc*-cKO pancreata were indistinguishable from WT pancreata, as indicated by H&E staining (figure 1E). However, at 5 weeks of age, duct-like structures were observed in *Nrdc*-cKO pancreata (figure 1E). Strikingly, at 8 weeks of age, almost all acinar cells were replaced by duct-like cells in *Nrdc*-cKO mice (figure 1E). These data indicated that *Nrdc* is dispensable for exocrine pancreatic development but is required for maintenance of the exocrine pancreas.

Pancreas-specific deletion of *Nrdc* results in acinar-to-ductal reprogramming in mice

To characterise the duct-like structures observed in *Nrdc*-cKO pancreata, we first performed immunostaining for Amy, panCK and Sox9. Although *Nrdc*-cKO pancreata were indistinguishable from WT pancreata at 3 weeks of age, a substantial proportion of Amy-positive acinar cells were replaced by ductal cells that expressed panCK and Sox9 at 5 weeks of age (figure 2A). Notably, at 8 weeks of age, Amy-positive acinar cells were rarely observed, but instead were replaced by panCK-positive and Sox9-positive ductal cells (figure 2A). qRT-PCR analysis showed that the expression of acinar markers including *Amy*, *Cpa1*, *Rbpjl* and ductal markers including *Krt19*, *Krt7* and *Hnf1β* were similar between *Nrdc*-cKO and

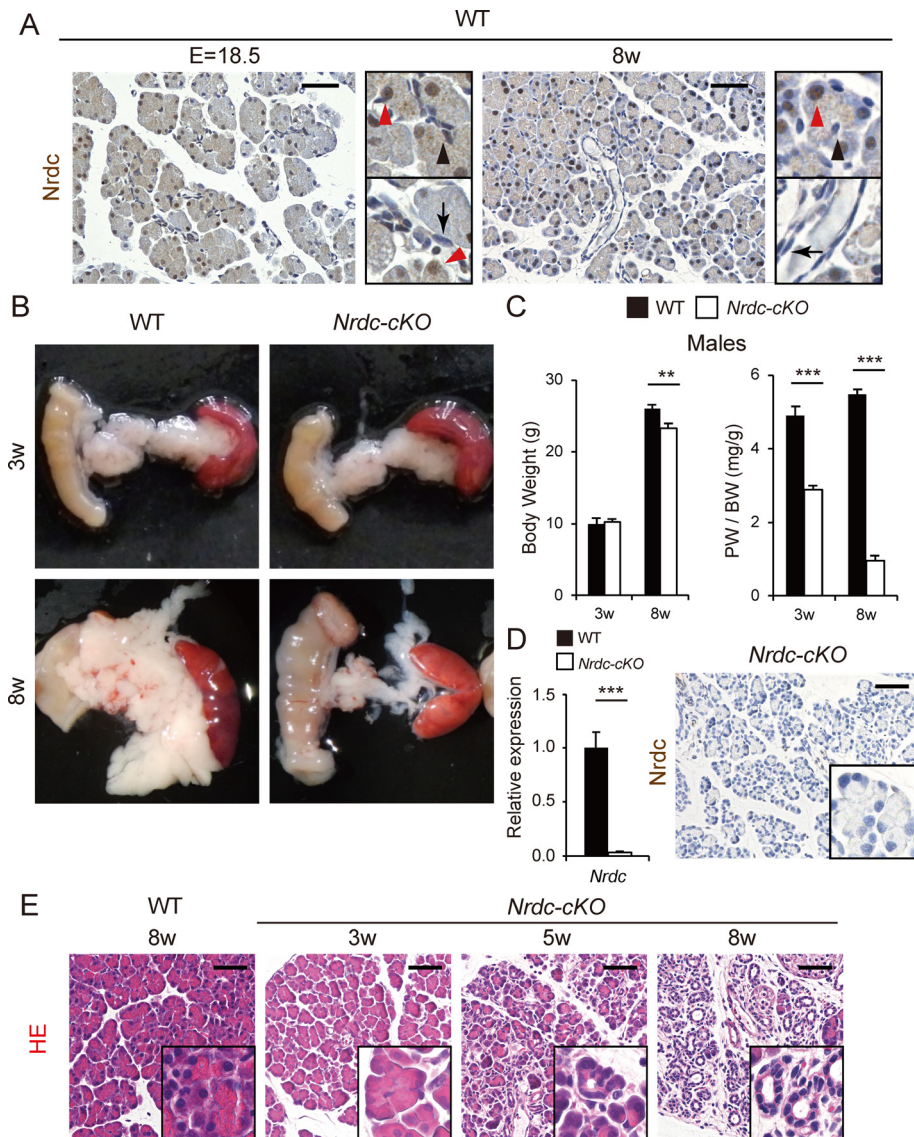


Figure 1 *Nrdc* is expressed in pancreatic acinar cells and is indispensable for the maintenance of the exocrine pancreas in adult mice. (A) Immunostaining for *Nrdc* in WT pancreas at embryonic day (E)18.5 (left) and at 8 weeks of age (right). *Nrdc* was expressed in acinar cells (red arrowhead) but was not expressed in ductal cells (black arrowhead) and centroacinar cells (black arrow). (B) Representative macroscopic view of WT (left) and *Ptf1a-Cre; Nrdc^{flx/flx} Nrdc-cKO* (right) pancreata; tissue was collected when animals were 3 weeks (top) and 8 weeks (bottom) of age. (C) Body weight (left) and pancreas weight/body weight (PW/BW) ratio (right) in male mice at the age of 3 weeks and 8 weeks; $n=5-8$ mice/group. Means $\pm$ SEM are shown. (D) Relative expression levels of *Nrdc* in the whole pancreata at 8 weeks of age, as determined by qRT-PCR; $n=5-6$ mice/group (left). Immunostaining for *Nrdc* in *Nrdc-cKO* pancreata at 8 weeks of age (right). Means $\pm$ SEM are shown. (E) H&E staining of WT pancreata at 8 weeks of age and *Nrdc-cKO* pancreata at 3, 5 and 8 weeks of age. ** $P<0.01$, *** $p<0.005$. Scale bars=50 μ m. *Nrdc*, qRT-PCR, quantitative reverse transcriptase-PCR; WT, wild-type.

WT pancreata at 3 weeks of age; however, the expression level of *Ptf1a* in *Nrdc-cKO* pancreata was approximately half that of WT controls owing to heterozygosity of the *Ptf1a* allele in *Ptf1a-Cre* mice, as reported previously.³³ Moreover, the expression of Sox9 in *Nrdc-cKO* pancreata was significantly upregulated compared with that in WT pancreata (figure 2B). At both 5 weeks and 8 weeks of age, qRT-PCR analysis revealed that the expression of acinar markers including *Amy*, *Cpa1*, *Rbpjl* and *Ptf1a* was markedly downregulated compared with that in WT controls, whereas the expression of ductal markers including *Krt7*, *Hnf1 β* and *Sox9* was significantly upregulated (figure 2B).

To rule out the possibility of the effect of *Ptf1a* gene dosage on the expression of acinar and ductal markers in *Nrdc-cKO*

pancreata, we also performed qRT-PCR for whole pancreata of *Ptf1a-Cre; Nrdc^{+/+}* mice at 3 weeks and 8 weeks of age. At 3 weeks of age, we found that the expression of ductal transcriptional regulators, including *Sox9* and *Hnf1 β* , was upregulated in *Nrdc-cKO* pancreata compared with that in *Ptf1a-Cre* pancreata, although expression of acinar transcriptional regulators including *Ptf1a* and *Rbpjl* was also upregulated in *Nrdc-cKO* pancreata compared with *Ptf1a-Cre* pancreata. At 8 weeks of age, we found downregulated expression of acinar transcriptional regulators, including *Ptf1a* and *Rbpjl*, and upregulated expression of ductal transcriptional regulators, including *Sox9* and *Hnf1b*, in *Nrdc-cKO* pancreata compared with that in *Ptf1a-Cre* pancreata (online supplementary figure S2A).

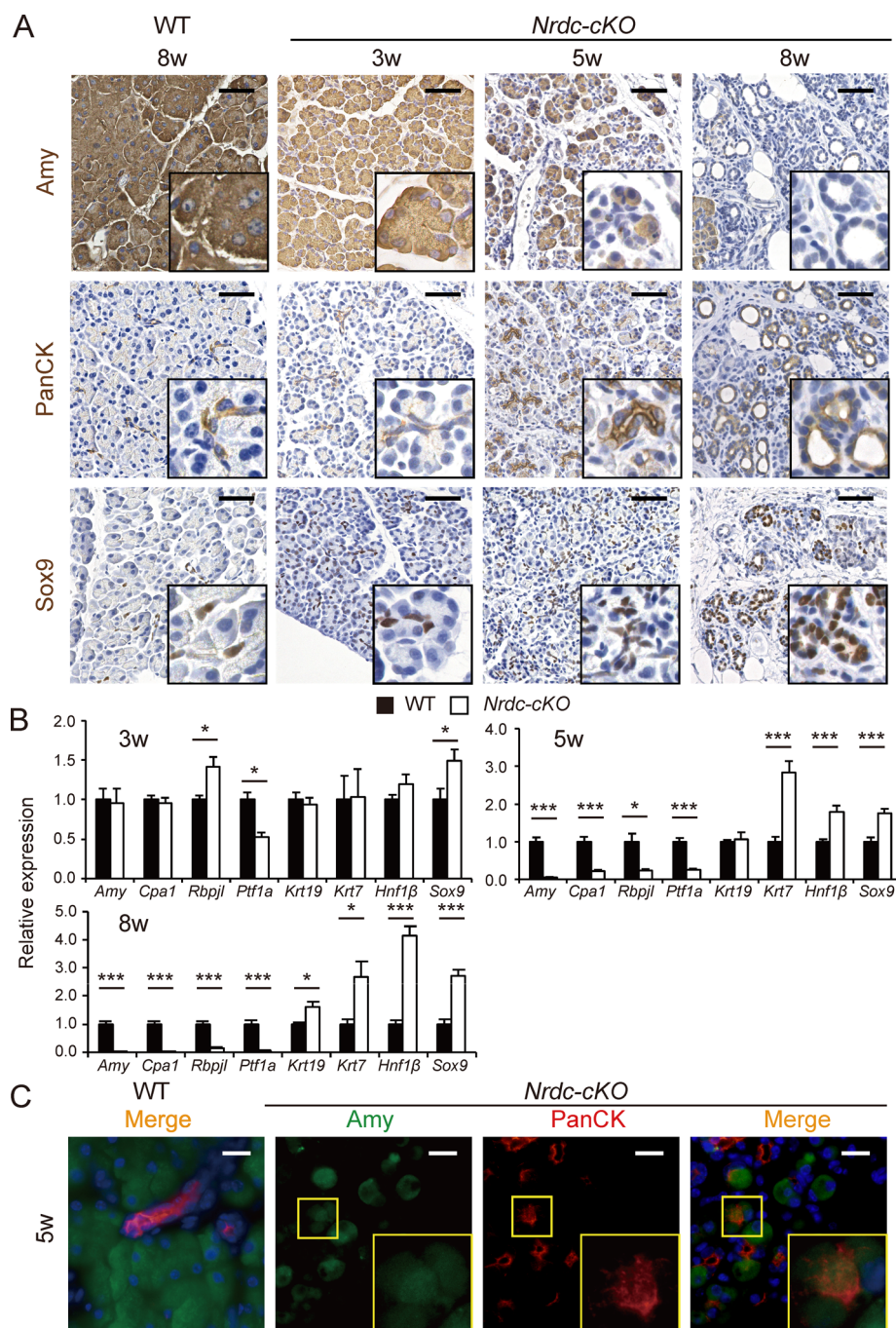


Figure 2 Pancreas-specific deletion of *Nrdc* leads to acinar-to-ductal reprogramming in mice. (A) Immunostaining for amylase (Amy), pancytokeratin (panCK) and Sox9 in WT pancreata at 8 weeks of age and *Nrdc-cKO* pancreata at 3, 5 and 8 weeks of age. (B) Relative expression levels of acinar and ductal markers in WT and *Nrdc-cKO* pancreata at 3, 5 and 8 weeks of age; $n=5-6$ mice/group; means $\pm$ SEM are shown. (C) Amy/panCK/4',6-diamidino-2-phenylindole (DAPI) costaining in WT and *Nrdc-cKO* pancreata at 5 weeks of age. * $P<0.05$, ** $p<0.01$, *** $p<0.005$. Scale bars=50 μ m. WT, wild-type.

To better characterise the ductal cells of *Nrdc-cKO* pancreata, we performed immunostaining for Pdx1, activated Notch1 and Hes1. In both adult WT and *Nrdc-cKO* pancreata, Pdx1 was expressed only in pancreatic islet cells but not in acinar or ductal cells (online supplementary figure S2B). qRT-PCR showed no significant difference in *Pdx1* expression between WT and *Nrdc-cKO* pancreata at all time-points (online supplementary figure S2C). Activated Notch1 was expressed in ductal cells and centroacinar cells in WT pancreata, whereas it was expressed only in ductal cells in *Nrdc-cKO* pancreata.

Hes1 was expressed in a subset of ductal cells and centroacinar cells in WT pancreata, whereas Hes1 expression was slightly upregulated in ductal and centroacinar cells of *Nrdc-cKO* pancreata (online supplementary figure S2B). However, qRT-PCR showed no significant difference in *Hes1* expression between WT and *Nrdc-cKO* pancreata at all time-points (online supplementary figure S2C). Notably, immunostaining revealed Amy-double and PanCK-double positive cells in *Nrdc-cKO* pancreata at 5 weeks of age, whereas these were not observed in WT controls (figure 2C). These data suggested

that Notch signalling is upregulated and that acinar cells are spontaneously and progressively replaced by ductal cells through ADM in *Nrhc-cKO* pancreata.

Pancreas-specific deletion of *Nrhc* results in spontaneous chronic pancreatitis

ADM is induced by several factors including oncogenic *Kras*, epidermal growth factor signalling, Notch signalling and pancreatitis.¹⁰ To investigate whether pancreatitis occurs in *Nrhc-cKO*

mice, we first performed immunostaining for F4/80 and CD45 using WT controls, *Nrhc-cKO* and WT mice with cerulein-induced pancreatitis as a positive control. F4/80-positive and CD45-positive cells were significantly increased in *Nrhc-cKO* pancreata compared with that in WT controls at 5 weeks of age (figure 3A,B). Sirius red staining revealed fibrotic changes in *Nrhc-cKO* pancreata at both 5 weeks and 8 weeks of age, whereas this was rarely observed in WT pancreata (figure 3C). qRT-PCR showed that many genes encoding inflammatory

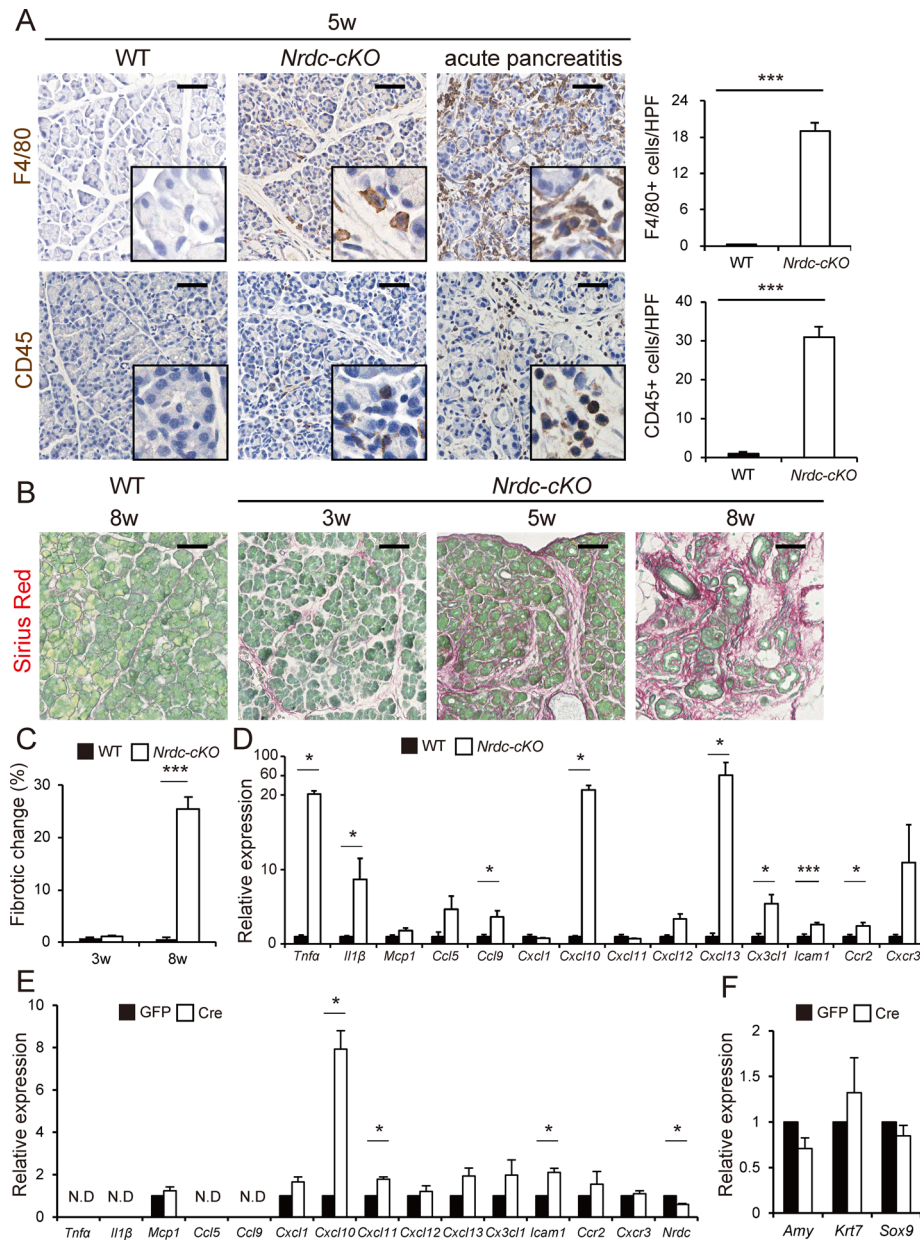


Figure 3 Pancreas-specific deletion of *Nrhc* results in spontaneous chronic pancreatitis in mice. (A) Immunostaining for F4/80 (top) and CD45 (bottom) in WT and *Nrhc-cKO* mice and animals with cerulein-induced pancreatitis, at 5 weeks of age. Scale bar=50 μ m. Numbers of F4/80-positive or CD45-positive cells in WT and *Nrhc-cKO* pancreata; n=6 mice/group. Means $\pm$ SEM are shown. (B) Sirius red staining (red) in WT pancreas at 8 weeks of age and *Nrhc-cKO* pancreata at 3, 5 and 8 weeks of age. Means $\pm$ SEM are shown. (C) Quantification of fibrotic areas in WT and *Nrhc-cKO* pancreata at 3 weeks and 8 weeks of age; n=5–7 mice/group. Means $\pm$ SEM are shown. (D) Relative expression levels of inflammatory cytokines, chemokines and chemokine receptors in WT and *Nrhc-cKO* pancreata at 5 weeks of age, as determined by qRT-PCR; n=5–6 mice/group. Means $\pm$ SEM are shown. (E) Relative expression levels of inflammatory cytokines, chemokines and chemokine receptors in acinar cells isolated from *Nrhc^{flx/flx}* mice infected with adenovirus expressing either GFP or Cre recombinase, as determined by qRT-PCR; n=3 mice/group. Means $\pm$ SEM are shown. (F) Relative expression levels of *Amy*, *Krt7* and *Sox9* in acinar cells isolated from *Nrhc^{flx/flx}* mice infected with adenovirus expressing either GFP or Cre recombinase, as determined by qRT-PCR; n=3 mice/group. * P <0.05, *** p <0.005. Scale bars=50 μ m. qRT-PCR, quantitative reverse transcriptase-PCR; WT, wild-type.

cytokines, chemokines and cytokine receptors, including *Tnfa*, *Il1 β* , *Mcp1*, *Ccl5*, *Ccl9*, *Cxcl10*, *Cxcl13*, *Cx3cl1*, *Icam1*, *Ccr2* and *Cxcr3*, were upregulated in *Nrhc-cKO* pancreata compared with expression in WT controls (figure 3D).

To better characterise the immune cell populations infiltrating *Nrhc-cKO* pancreata, we additionally performed immunostaining for CD3 (T cell marker), CD11b (macrophage and granulocyte marker) and Gr1 (neutrophil marker). We found that a small number of CD3+ T cells and a substantial number of CD11b+/Gr1– macrophages were infiltrated in *Nrhc-cKO* pancreata, whereas Gr1+ neutrophils were not observed in *Nrhc-cKO* pancreata. Conversely, immune cells in cerulein-induced acute pancreatitis included F4/80+ macrophages and Gr1+ neutrophils but not CD3+ T cells (online supplementary figure S3A). As previous reports showed that T cells and macrophages comprise the predominant immune cells to infiltrate in chronic pancreatitis,³⁴ these data indicated that pancreatic deletion of *Nrhc* leads to spontaneous chronic pancreatitis with fibrotic changes. In contrast, serum amylase levels were comparable between WT and *Nrhc-cKO* mice at 3 weeks and 8 weeks of age (online supplementary figure S3B).

Next, to investigate whether chronic pancreatitis persists in older *Nrhc-cKO* mice, we analysed *Nrhc-cKO* pancreata at 22–24 weeks of age. Histological analysis revealed that *Nrhc-cKO* pancreata became severely atrophic and moderately fibrotic at 22–24 weeks of age and that no tumours developed in any *Nrhc-cKO* pancreata at that age (online supplementary figure S4A,B). In addition, the expression of *Nrhc* and *Amy* was downregulated and that of *Tnfa* and *Il1 β* was upregulated in *Nrhc-cKO* pancreata compared with those in controls at that age (online supplementary figure S4C). Taken together, these results suggested that chronic pancreatitis persists in *Nrhc-cKO* mice at least until 22–24 weeks of age.

To investigate the effect of *Nrhc* deletion on pancreatic acinar cells with respect to pancreatitis, we isolated acinar cells from WT and *Nrhc-cKO* mice at 3 weeks of age, at which point the immune cell infiltration was not yet observed. We first verified the purity of the freshly isolated acinar cells, as determined by immunostaining for *Amy*, panCK, F4/80 and CD45 (online supplementary figure S5A). qRT-PCR analysis revealed that the expression of chemokines, including *Mcp1*, *Ccl9*, *Cx3cl1* and *Icam1*, were upregulated in isolated *Nrhc-cKO* acinar cells compared with that in control acinar cells (online supplementary figure S5B). We next isolated acinar cells from *Nrhc^{flx/flx}* mice and infected them with an adenovirus expressing GFP (Ad-GFP) or Cre recombinase (Ad-CreGFP). In pancreatic acinar cells from *Nrhc^{flx/flx}* mice, Ad-CreGFP initiates genetic recombination of the loxP sites to delete *Nrhc*. After infecting these cells with adenoviruses for 3 hours, we cultured them for 2 days in conditioned media. qRT-PCR analyses revealed that the expression of genes encoding chemoattractants for macrophages, neutrophils and activated T cells, including *Icam1*, *Cxcl10* and *Cxcl11*, were significantly upregulated in *Nrhc*-deleted acinar cell explants compared with the expression in controls (figure 3E).

Conversely, qRT-PCR analysis revealed no significant changes in *Amy*, *Krt7* or *Sox9* expression in *Nrhc*-deleted acinar cell explants (figure 3F), suggesting that acinar-to-ductal conversion mainly occurs through a non-cell autonomous mechanism in *Nrhc-cKO* mice. To further confirm such a mechanism, we examined the effect of transforming growth factor alpha (TGF α) on acinar-to-ductal conversion using acinar cells isolated from WT and *Nrhc-cKO* mice at 3 weeks of age. Acinar cell clusters isolated from WT pancreata rarely changed into duct-like structures without TGF α treatment, whereas most changed

into these structures with TGF α treatment, as reported previously.^{35,36} The same results were obtained from acinar cell clusters isolated from *Nrhc-cKO* pancreata (supplementary figure S6). These results support the conclusion that ADM formation in *Nrhc-cKO* pancreata mainly occurs through non-cell autonomous mechanisms.

Collectively, our data indicated that the expression of chemoattractant genes is upregulated in *Nrhc*-null pancreatic acinar cells, resulting in chronic pancreatitis with ADM formation.

Pancreas-specific deletion of *Nrhc* leads to activation of NF- κ B signalling and Erk signalling

To further determine the changes in gene expression in *Nrhc-cKO* pancreata, we performed global gene expression analyses on WT and *Nrhc-cKO* pancreata from 5-week-old animals. Gene ontology and pathway analysis using the Database for Annotation, Visualization and Integrated discovery functional annotation tool revealed that immune response and chemotaxis were significantly increased in *Nrhc-cKO* pancreata compared with those in WT controls (figure 4A). Given the fact that chronic pancreatitis occurs in *Nrhc-cKO* mice, we focused on chemotaxis, the NF- κ B pathway and the Erk1/2 pathway, which are known to be associated with acute and chronic pancreatitis in humans and mice.^{37,38} First, we calculated the false discovery rate (FDR) using the Benjamini-Hochberg method. Our results showed that the FDRs of positive regulation of chemotaxis (GO:0006935), positive regulation of NF- κ B transcription factor activity (GO:0051092) and positive regulation ERK1 and ERK2 cascade (GO:0070374) were upregulated at 1.51E–12, 6.81E–06, and 7.90E–10, respectively (figure 4B). Heat maps focused on the expression of genes belonging to these groups revealed that these pathways were significantly upregulated in *Nrhc-cKO* pancreata compared with that in WT controls (figure 4B). qRT-PCR analyses confirmed the upregulation of components of NF- κ B signalling, including *Tlr3*, *Tnfr11a* and *Traf1*, as well as Erk1/2 signalling, including *Trem2*, *Pla2g5* and *Bmpr*, in *Nrhc-cKO* pancreata (figure 4C). Furthermore, we performed immunostaining for RelA/p65, which is a nuclear factor of the NF- κ B subunit and pErk1/2. RelA/p65 and pErk1/2 were not expressed in acinar cells of WT pancreata, whereas both were expressed in a subset of ductal cells in *Nrhc-cKO* pancreata (figure 4D). These results indicated that pancreatic deletion of *Nrhc* leads to activation of NF- κ B signalling and Erk signalling in mice.

Pancreas-specific deletion of *Nrhc* increases both apoptosis and cell proliferation

In addition to acinar-to-ductal conversion, the pancreata were severely atrophic in *Nrhc-cKO* mice at 8 weeks of age. To investigate the underlying mechanisms leading to this massive atrophy, we evaluated apoptosis and cell proliferation. First, we assessed apoptosis by performing cleaved caspase 3 immunostaining and TUNEL staining. Quantification revealed a marked increase in apoptotic cells in *Nrhc-cKO* pancreata relative to that in WT controls at 5 weeks of age (figure 5A,B). We next assessed cell proliferation by immunostaining for Ki67 and phospho-histone H3 (pHH3). We observed very few Ki67-positive and pHH3-positive cells in WT pancreata. In contrast, more Ki67-positive and pHH3-positive cells were observed in *Nrhc-cKO* pancreata than WT controls (figure 5C,D). Thus, pancreatic deletion of *Nrhc* increases the numbers of both apoptotic and proliferative cells in the pancreas. This finding suggested that the balance between apoptosis and cell proliferation is skewed towards apoptosis; thus, the pancreatic parenchyma becomes progressively atrophic in *Nrhc-cKO* mice.

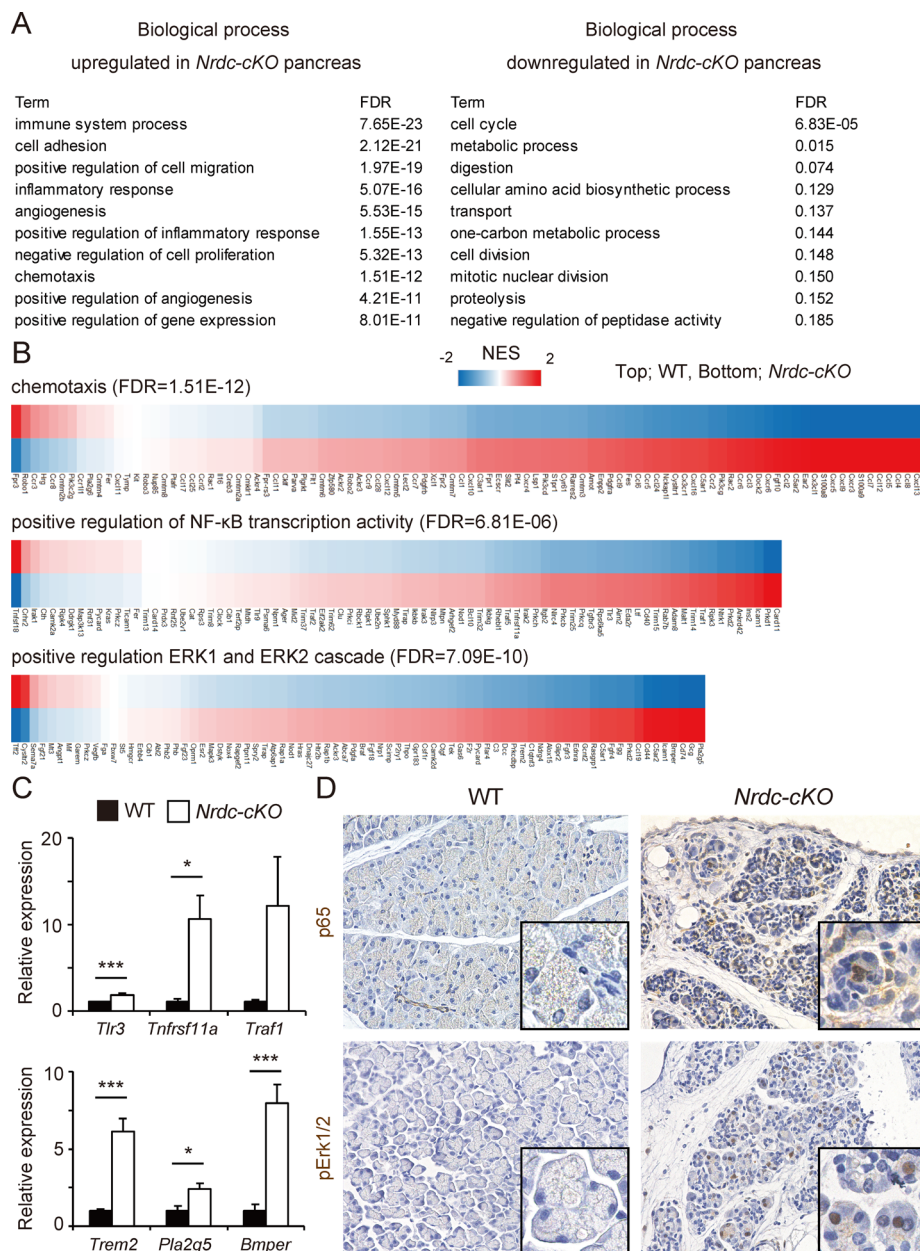


Figure 4 Microarray analysis of *Nrhc-cKO* pancreata. (A) Pathway enrichment shows genes transcriptionally upregulated (left) and repressed (right) in the *Nrhc-cKO* pancreas at 5 weeks of age. Functional categorisations were analysed by Gene Ontology Biological Process (GO_BP) with the DAVID algorithm. (B) Heatmaps showing expression of genes associated with the indicated pathways, comparing WT and *Nrhc-cKO* pancreata at 5 weeks of age. (C) Relative expression levels of *Tlr3*, *Tnfrsf11a*, *Traf1*, *Trem2*, *Pla2g5* and *Bmper* in WT and *Nrhc-cKO* pancreata at 5 weeks of age, as determined by qRT-PCR; n=5–6 mice/group. Means±SEM are shown. (D) Immunostaining for p65 (top) and pErk1/2 (bottom) in WT and *Nrhc-cKO* pancreata at 5 weeks of age. *P<0.05, ***p<0.005. Scale bars=50 μ m. DAVID, Database for Annotation, Visualization and Integrated discovery; FDR, false discovery rate; qRT-PCR, quantitative reverse transcriptase-PCR; WT, wild-type.

Pancreas-specific deletion of *Nrhc* markedly accelerates *Kras*^{G12D}-driven pancreatic tumourigenesis

Given that pancreatic deletion of *Nrhc* leads to spontaneous chronic pancreatitis, we next investigated the impact of pancreatic *Nrhc* deletion on *Kras*-induced pancreatic tumourigenesis. We first determined the expression pattern of *Nrhc* in mouse PDA and precursor lesions including ADM and PanIN. Immunohistochemistry for *Nrhc* revealed that this protein is expressed in ADM, PanIN and PDA in *Ptf1a-Cre; Kras*^{G12D} or *Ptf1a-Cre; Kras*^{G12D}; *Trp53*^{R172H} mice (figure 6A). Next, we generated *Ptf1a-Cre; Kras*^{G12D}; *Nrhc*^{flox/flox} mice and compared them with control *Ptf1a-Cre; Kras*^{G12D}; *Nrhc*^{flox/+} mice. We first

assessed PanIN formation at 4 weeks of age, when this condition was barely observed in control *Ptf1a-Cre; Kras*^{G12D} mice. In *Ptf1a-Cre; Kras*^{G12D}; *Nrhc*^{flox/+} mice, an Alcian blue-positive PanIN area was hardly detected, similar to that observed in *Ptf1a-Cre; Kras*^{G12D} mice. In contrast, the Alcian blue-positive area was dramatically increased in *Ptf1a-Cre; Kras*^{G12D}; *Nrhc*^{flox/flox} mice at the same age (figure 6B,C). Sirius red staining showed much more severe fibrotic changes in *Ptf1a-Cre; Kras*^{G12D}; *Nrhc*^{flox/flox} pancreata compared with that in controls (figure 6B). Immunostaining for F4/80 revealed enhanced macrophage infiltration in *Ptf1a-Cre; Kras*^{G12D}; *Nrhc*^{flox/flox} pancreata compared with that in controls (figure 6B,D). Immunostaining for pErk1/2

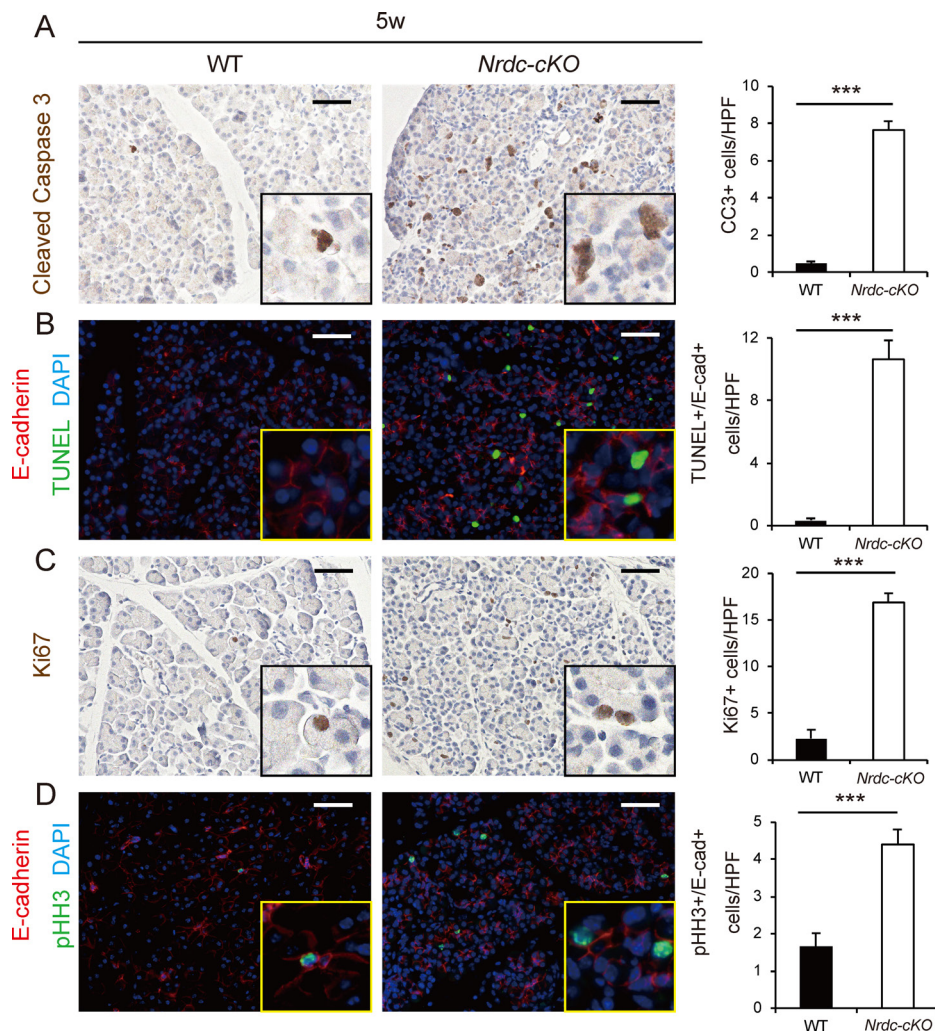


Figure 5 Pancreas-specific deletion of *Nr4c* leads to increased apoptosis and cell proliferation. (A) Immunostaining for cleaved caspase 3 in WT and *Nr4c-cKO* pancreata at 5 weeks of age (left) and numbers of cleaved caspase 3-positive cells (right); *n*=6 mice/group. (B) Coimmunostaining of E-cadherin and TUNEL in WT and *Nr4c-cKO* pancreata at 5 weeks of age (left) and numbers of E-cadherin/tunel double positive cells (right); *n*=6 mice/group. (C) Immunostaining for Ki67 in WT and *Nr4c-cKO* pancreata at 5 weeks of age (left) and numbers of Ki67-positive cells (right); *n*=6 mice/group. (D) Coimmunostaining for E-cadherin and pHH3 in WT and *Nr4c-cKO* pancreata at 5 weeks of age (left) and numbers of E-cadherin/pHH3 double positive cells (right); *n*=6 mice/group. **P*<0.05, ****p*<0.005. Scale bars=50 μm. HPF, high power field; pHH3, phospho-histone H3; WT, wild-type.

and Claudin18 confirmed that the Alcian blue-positive area comprised PanIN lesions in *Ptf1a-Cre; Kras^{G12D}; Nr4c^{flx/flx}* mice (figure 6B). At 8 weeks of age, PanIN lesions with fibrotic changes and macrophage infiltration were even more prominent in *Ptf1a-Cre; Kras^{G12D}; Nr4c^{flx/flx}* mice compared with those in control *Ptf1a-Cre; Kras^{G12D}; Nr4c^{flx/+}* mice (online supplementary figure S7A–C). Furthermore, invasive and metastatic ductal adenocarcinoma spontaneously developed in 1 out of 10 *Ptf1a-Cre; Kras^{G12D}; Nr4c^{flx/flx}* mice at 19–24 weeks of age (online supplementary figure S8). Given that in *Ptf1a-Cre; Kras^{G12D}* mice greater than 6 months of age, invasive and metastatic PDA occurs at a very low (less than 10%) frequency,³² these data suggested that *Nr4c* inhibits PanIN and subsequent PDA formation in the presence of oncogenic *Kras*.

Nuclear NRDC expression is absent in a subset of human PanIN and PDA

We next performed immunostaining for NRDC on 51 human PDA samples. All PDA samples also contained distinguishable ADM and PanINs. Immunohistochemical analysis revealed that NRDC was expressed in pancreatic acinar nuclei in all 43 out of

51 samples that were identified as containing pancreatic acinar cells (with no such cells detected in the remaining eight samples). We found that NRDC expression was absent in subsets of ADMs 43% (22 out of 51 samples), PanINs 51% (26 out of 51 samples) and PDAs 53% (27 out of 51 samples) (figure 7A,B). In addition, NRDC-negative ADMs, PanINs and PDAs were always observed together in the same sample, suggesting that the absence of NRDC is an early event during pancreatic tumourigenesis.

DISCUSSION

In the present study, we demonstrated that pancreatic deletion of *Nr4c* leads to spontaneous chronic pancreatitis concomitant with infiltrated inflammatory cells, acinar-to-ductal conversion, increased apoptosis and atrophic pancreas in mice. Furthermore, we revealed that pancreas-specific deletion of *Nr4c* dramatically accelerates *Kras*-driven pancreatic tumourigenesis, demonstrating a previously unappreciated tumour suppressive function of *Nr4c* in the pancreas.

Regarding the mechanism by which the loss of *Nr4c* induces chronic pancreatitis, we found that the expression of several chemokines was upregulated in *Nr4c-cKO* acinar cells. It is

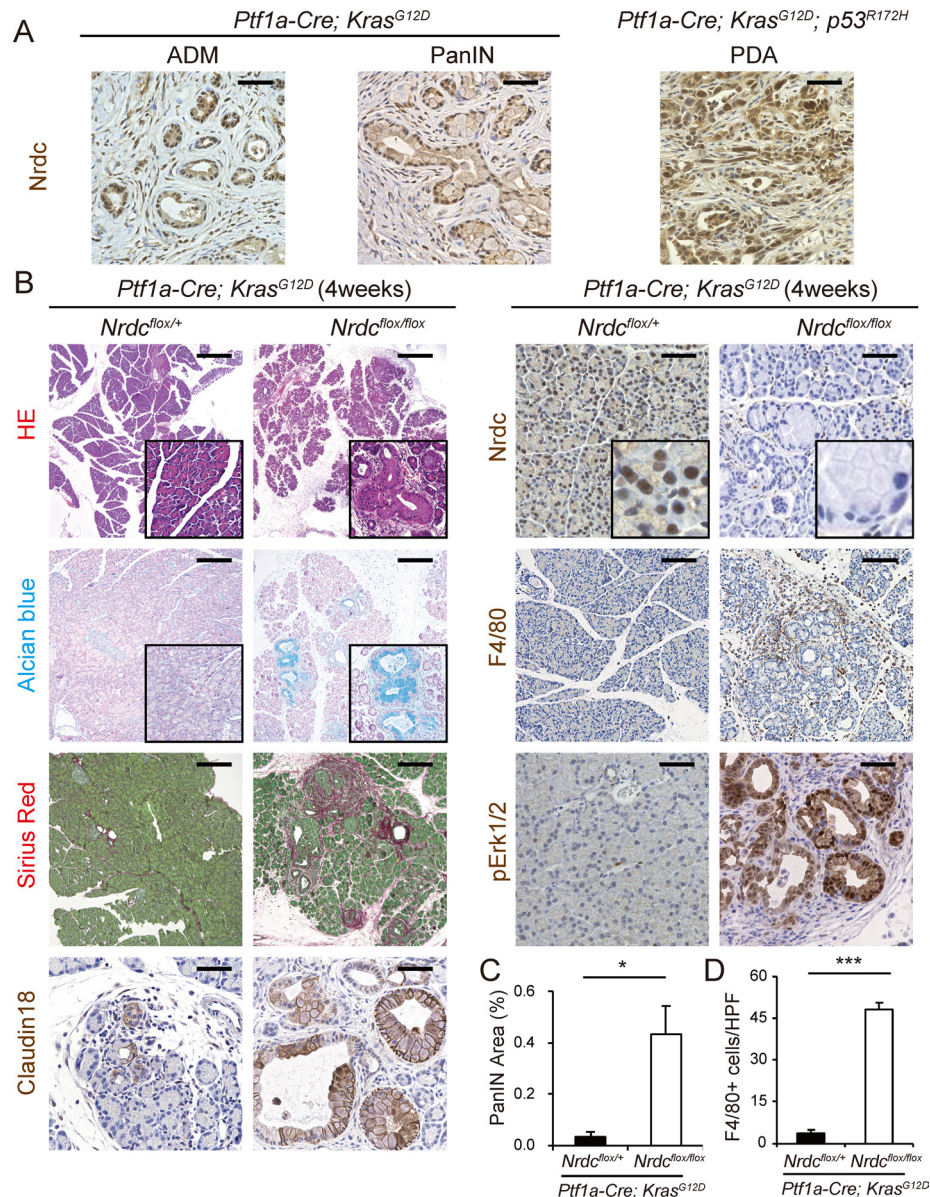


Figure 6 Pancreas-specific deletion of *Nrdc* concomitant with mutant *Kras* dramatically accelerates pancreatic intraepithelial neoplasia (PanIN) formation. (A) Immunostaining for *Nrdc* in acinar-to-ductal metaplasia (ADM) and PanIN of *Ptf1a-Cre; Kras^{G12D}* mice and in PDA of *Ptf1a-Cre; Kras^{G12D}; p53^{R172H}* mice. Scale bar=50 μ m. (B) H&E staining, Alcian blue staining, Sirius red staining and immunostaining for *Nrdc*, F4/80, pErk1/2 and Claudin18 in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/+}* (control) and *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* mice at 4 weeks of age. Scale bars: H&E, 500 μ m; Alcian blue, 200 μ m; F4/80, 100 μ m; Sirius red, 200 μ m; pErk1/2 and Claudin 18, 50 μ m. (C) Quantification of Alcian blue-positive PanIN lesions in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/+}* and *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* mice at 4 weeks of age; n=5 mice/group. Means $\pm$ SEM are shown. (D) Numbers of F4/80-positive cells in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/+}* and *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* pancreata at 4 weeks of age; n=5 mice/group. Means $\pm$ SEM are shown. * P <0.05, *** P <0.005. PDA, pancreatic ductal adenocarcinoma.

known that inflammation and fibrosis in chronic pancreatitis are likely orchestrated via signalling of soluble cytokines and chemokines.³⁹ We found that the expression of chemokines including *Mcp1*, *Ccl5*, *Cx3cl1* and *Icam1* was significantly increased in isolated *Nrdc*-cKO acinar cells at 3 weeks of age, when immune cell infiltration was not yet observed and that the expression of *Cxcl10* was markedly increased in *Nrdc*-deleted acinar cells in ex vivo culture experiments. In previous reports, islet-specific overexpression of *Cxcl10* leads to spontaneous infiltration of inflammatory cells in murine islets.⁴⁰ Therefore, it is possible that the upregulated expression of chemokines contributes to recruitment of inflammatory cells and thereby leads to persistent chronic pancreatitis in *Nrdc*-cKO mice.

In our acinar cell culture experiments, we also found that the expression of *Sox9*, which plays a critical role in ADM formation, is upregulated in *Nrdc*-deleted acinar cells. Although ADM formation is mediated mainly through a non-cell autonomous mechanism in *Nrdc*-cKO mice, it is possible that increased expression of *Sox9* in acinar cells might also contribute to ADM formation in these mice.

In this study, we showed that NF- κ B and Erk signalling is activated in *Nrdc*-cKO pancreata. It has been shown that both pathways play important roles in the development of pancreatitis.^{38 41 42} Pancreatic acinar cells overexpressing NF- κ B/p105 or those treated with TGF α , which activates Erk1/2 signalling, undergo ADM formation in vitro.^{35 36 43} Moreover, TNF- α , a

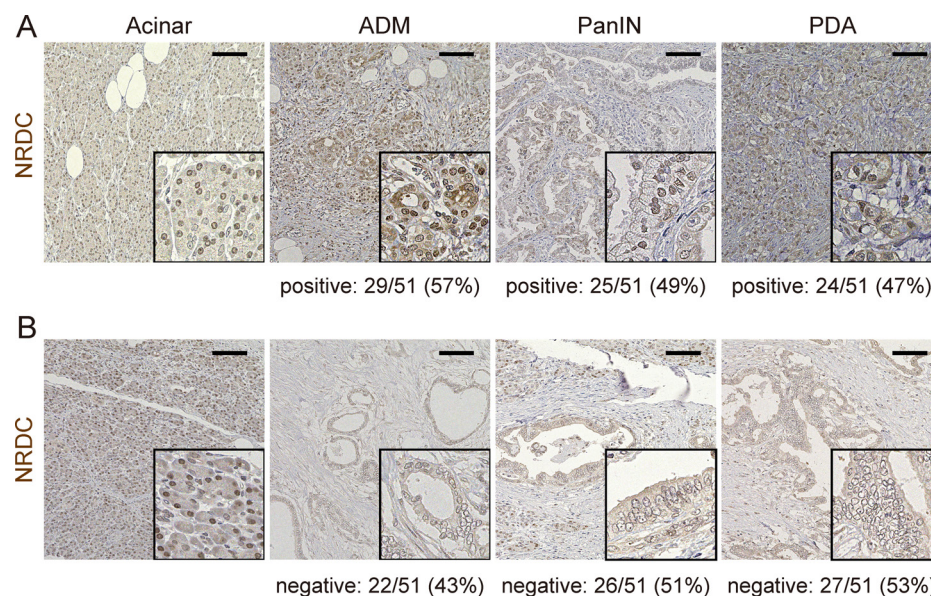


Figure 7 Absence of nuclear NRDC expression in a subset of human acinar-to-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDA). (A and B). Immunostaining for NRDC in human acinar cells, ADM, PanIN and PDA. (A) Nuclear NRDC was expressed in subsets of ADM, PanIN and PDA samples. (B) Nuclear NRDC was absent in subsets of ADM, PanIN and PDA samples. Scale bars: 100 µm. NRDC, Nardilysin.

crucial inflammatory mediator associated with pancreatitis,⁴⁴ has been shown to enhance inflammatory cytokine expression and proliferation via NF-κB and Erk1/2 signalling.⁴⁵ We showed that the expression of *Tnfa* is upregulated in *Nrdc*-cKO pancreata, whereas deletion of *Nrdc* in pancreatic acinar cells did not result in upregulation of *Tnfa* expression in a cell autonomous manner. Together, these data suggested that inflammatory cells recruited by chemoattractants secrete inflammatory mediators including TNFα, and activate NF-κB and Erk1/2 signalling, resulting in chronic pancreatitis in *Nrdc*-cKO mice.

Our study revealed that pancreas-specific deletion of *Nrdc* dramatically accelerates PanIN and subsequent PDA formation in the presence of *Kras*^{G12D}. Chronic pancreatitis constitutes a risk factor for PDA in humans, and pancreatic inflammation accelerates *Kras*-driven PDA formation in mice. Given that pancreatic *Nrdc* inhibits spontaneous chronic pancreatitis, it is conceivable that *Nrdc* functions as a tumour suppressor in *Kras*-driven PDA formation by inhibiting pancreatitis.

In our previous reports, *Nrdc* was shown to enhance inflammation and carcinogenesis through its extracellular function on the cell surface.^{27,46} Moreover, we demonstrated that the production of inflammatory cytokines and the recruitment of inflammatory cells are significantly suppressed concomitant with the suppression of TNFα release on *Nrdc* deletion in a mouse steatohepatitis model.⁴⁶ In contrast, in the present study, we showed that *Nrdc* is expressed in the nucleus of pancreatic acinar cells and that pancreatic *Nrdc* acts as a tumour suppressor for *Kras*^{G12D}-driven PanIN and PDA formation most likely through blocking pancreatitis in mice. Although it remains unknown as to why *Nrdc* plays opposing roles in inflammation and tumourigenesis in different organs, we speculate that the function of *Nrdc* depends on its cellular localisation. Future studies are required to clarify this issue.

Finally, our data showed that NRDC is absent in a subset of human PanIN and PDA samples, whereas NRDC was expressed in the nucleus of human pancreatic acinar cells. This finding suggests the possibility that the absence of nuclear NRDC in PanIN and PDA is also associated with accelerated pancreatic

tumourigenesis in humans. Furthermore, given the existence of a number of familial or hereditary pancreatitis disorders in humans with unknown genetic aetiology, it would also be worthwhile to determine whether *Nrdc* might represent a potential novel gene for familial or hereditary pancreatitis in humans.

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Contributors KI and AF conceived and designed the study. SO, KM, NG, HS, MT, YoK, YM, YuK, TM and KK performed the experiments and analysed the data. KT, SU, ST, TC and EN. contributed reagents/materials/analysis tools. KI wrote the manuscript, and AF, EN and HS revised it.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval The protocol was approved by the Ethics Committee of Kyoto University.

Provenance and peer review Not commissioned; externally peer reviewed.

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Supplementary Data

Nardilysin inhibits pancreatitis and suppresses pancreatic ductal adenocarcinoma initiation in mice

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Supplementary table S1

Supplementary materials and methods

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Whole pancreata were collected in RNAlater (Ambion, Austin, TX) and subsequently total RNA was extracted using the RNA mini Kit (Qiagen, Hilden, Germany). Single-strand complementary DNA (cDNA) was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Basel, Switzerland). qRT-PCR was performed using SYBR Green I Master (Roche Applied Science) and a Light Cycler 480 (Roche Applied Science). The results were standardized by comparing to levels of *GAPDH* mRNA for tissues in each sample. Primer sequences are described in supplementary table S1.

Histological analyses and immunostaining

The pancreata were resected at various time points, fixed with 4% buffered paraformaldehyde solution, embedded in paraffin, and sectioned to a thickness of 5 μm . For immunohistochemistry, antigen retrieval was achieved by heating in citric acid buffer (pH 6.0) or EDTA buffer (pH 8.0). Blocking was performed using blocking solution (Dako, Santa Clara, CA). Sections were incubated with primary

antibodies overnight at 4 °C and with biotinylated secondary antibody for 1 h at room temperature; immunoperoxidase labeling was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and sections were colored with diaminobenzidine (DAB) substrate (Dako, Santa Clara, CA) and counterstained with hematoxylin. For immunofluorescence, sections were incubated with primary antibodies overnight at 4 °C and with fluorophore-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Primary antibodies used in this study were obtained from the indicated suppliers as follows: mouse anti-human NRDC (raised against recombinant human NRDC in our laboratory, clone #102), rat anti-mouse Nrhc (raised against recombinant mouse Nrhc in our laboratory, clone #135), rabbit anti-Amylase (Abcam, Cambridge, MA, ab21156), rabbit anti-Pan-cytokeratin (Abcam, ab9377), mouse anti-Cytokeratin (Dako, M3515), rabbit anti-Sox9 (Millipore, San Diego, CA, AB5535), rabbit anti-cleaved caspase3 (Cell Signaling, Danvers, MA, 9664), rat anti-Ki67 (Dako, M7249), rabbit anti-phospho-Histone H3 (Millipore, 06-570), guinea pig anti-Pdx1 (Abcam, ab47308), rabbit anti-activated Notch1 (Abcam, ab8277), rabbit anti-Hes1 (gift from Dr. Sudo) ¹, mouse anti-E-cadherin (BD Transduction Laboratories, San Jose, CA, 610182), rabbit anti-E-cadherin (Cell

signaling, 24E10), rabbit anti-F4/80 (Abcam, ab6640), rat anti-CD45 (BD Pharmingen, San Jose, CA, 550539), rabbit anti-CD3 (Abcam, ab5690), rabbit anti-CD11b (Abcam, ab133357), rat anti-Gr1 (Invitrogen, Waltham, MA, 14-5931-82), rabbit anti-NF- κ B p65 (Cell Signaling, 8242), rabbit anti-p44/p42-MAPK (Cell Signaling, 4370), and rabbit anti-Claudin18 (Invitrogen, 700178). TUNEL staining (In Situ Death Detection Kit; Roche, 11 684 795 910) was performed to assess apoptotic cells. Alcian blue staining was performed to assess the PanIN area. Sirius red staining (saturated picric acid containing 0.1% Direct Red 80 and 0.1% Fast Green FCF; Sigma-Aldrich, St. Louis, MO) was performed to visualize fibrotic areas. For quantitative analysis of PanIN or fibrotic areas, the measurement of Alcian blue-positive PanIN or Sirius red-positive areas was performed using ImageJ (National Institutes of Health, Bethesda, MD). For quantitative analysis of cell numbers, cell counting was performed using at least five random high-power fields from three animals for each genotype. Whole pancreatic areas were measured using a BZ-X Analyzer (Keyence, Osaka, Japan).

Measurement of serum amylase

Serum amylase was measured by using Spotchem D AMY (amylase) (Arkray, Tokyo, Japan)

Acinar cell isolation and culture

Mouse pancreata were minced into small pieces and digested with collagenase P (0.2 mg/ml) for 10 min. After two washes with HBSS buffer containing 5% FBS, the tissue suspension was filtered through a 100- μ m cell strainer. The flow-through was layered on HBSS plus 30% FBS solution and centrifuged. The cell pellet was centrifuged and re-suspended in a 1:1 ratio of acinar culture medium to rat-tail collagen Type I solution, at a final concentration of 1 mg/ml, and plated on a rat-tail collagen Type I-coated plate. Acinar culture medium consisted of Waymouth's media with 10% FBS, 1% penicillin/streptomycin, 100 μ g /ml trypsin inhibitor, and 1 μ g/ml dexamethasone ²³.

Adenovirus infection

Following acinar cell isolation, the cell pellet was re-suspended in acinar culture medium, infected with 200–300 MOI Ad-Cre-GFP (Vector Biolabs, Malvern, PA, 1700) or Ad-GFP (Vector Biolabs, 1060) and incubated for 2–3 h.

Subsequently, the cells were pelleted and re-suspended in acinar culture medium.

Microarray gene expression analysis

Total RNA from WT and *Nr4c1*-cKO pancreata was examined using the SurePrint G3 Mouse GE v2 8x60K Microarray (Agilent). Processed signal intensities were normalized by the global scaling method. A trimmed mean probe intensity was determined by removing 2% of the lower and the higher end of the probe intensities to calculate the scaling factor. Normalized signal intensities were then calculated from the target intensity on each array using this factor, such that the trimmed mean target intensity of each array was arbitrarily set to 2500. Data were analyzed to identify gene probes that showed more than a two-fold change. GO analysis was then performed by uploading the microarray data to DAVID. The complete microarray data set is available from GEO (GSE99438).

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Supplementary figures

Supplementary figure S1

Localization of *Nrdc* in WT pancreas and pancreatic weight/body weight (PW/BW) ratio of *Nrdc*-cKO female mice.

A. Double immunostaining for amylase/*Nrdc* (top) and PanCK/*Nrdc* (bottom) in WT pancreata at 8 weeks of age.

B. Body weight (left) and pancreas weight/body weight ratio (right) in female mice at 3 and 8 weeks of age; $n = 4\text{--}5$ mice/group. Means $\pm$ SEM are shown.

Supplementary figure S2

Molecular characterization in *Nrdc*-cKO pancreata.

A. Relative expression levels of acinar markers, ductal markers, and *Nrdc* in whole *Ptf1a-Cre*; *Nrdc*^{+/+} and *Nrdc*-cKO pancreata at the indicated ages, as determined by qRT-PCR; $n = 3\text{--}6$ mice/group. Means $\pm$ SEM are shown. * $P < .05$, ** $P < .01$, *** $P < .005$.

B. Immunostaining for Pdx1, activated Notch1, and Hes1 in WT pancreata at 8 weeks of age and *Nrdc*-cKO pancreata at 3, 5 and 8 weeks of age. Scale bars = 50 μm . Arrowheads point to ductal cells and arrows point to centroacinar cells.

C. Relative expression levels of Pdx1 and Hes1 in whole WT and *Nrhc-cKO* pancreata at the indicated weeks, as determined by qRT-PCR; n = 5–6 mice/group. Means $\pm$ SEM are shown.

Supplementary figure S3

Characteristics of chronic pancreatitis induced by deletion of pancreatic *Nrhc* in mice.

A. Immunostaining for CD3, CD11b, and Gr1 in WT and *Nrhc-cKO* pancreata at 5 weeks of age. Scale bars = 50 μ m.

B. Serum amylase of WT and *Nrhc-cKO* mice at 3 and 8 weeks of age; n = 4–5 mice/group. Means $\pm$ SEM are shown.

Supplementary figure S4

Chronic pancreatitis persists in *Nrhc-cKO* mice at 22–24 weeks of age.

A. H&E staining, Alcian blue staining, and Sirius red staining in WT and *Nrhc-cKO* mice at 24 weeks of age. Scale bar = 200 μ m.

B. Pancreas weight/body weight (PW/BW) ratio in mice at the age of 22–24 weeks; n = 4 mice/group. Means $\pm$ SEM are shown.

C. Relative expression levels of *Nrhc*, *Amy*, and *Krt7* (left), and *Tnfa* and *Il1 β* (right) in the whole pancreata at 22–24 weeks of age, as determined by qRT-PCR; n = 4 mice/group. * $P < .05$, *** $P < .005$. Means $\pm$ SEM are shown.

Supplementary figure S5

Expression of several chemokines and Sox9 is upregulated in acinar cells isolated from *Nrhc-cKO* pancreata at 3 weeks of age.

A. H&E staining and immunostaining for Amy and PanCK in acinar cells isolated from WT and *Nrhc-cKO* pancreas at 3 weeks of age. Scale bars = 20 μ m.

B. Relative expression levels of *Amy*, *Sox9*, *Hes1*, and *Pdx1*, inflammatory chemokines and *Nrhc*, as determined by qRT-PCR; n = 4 mice/group. * $P < .05$, ** $P < .01$, *** $P < .005$. Means $\pm$ SEM are shown.

Supplementary figure S6

***Nrhc*-null pancreatic acinar cells do not transdifferentiate into ductal cells in a cell autonomous manner.**

Brightfield images of acinar cell clusters from WT and *Nrhc-cKO* mice at 3 weeks of age after 1 or 5 d of culture in collagen gel with or without TGF α . Scale bars =

500 μm .

Supplementary figure S7

Pancreas-specific deletion of *Nrdc* concomitant with mutant *Kras* accelerates pancreatic intraepithelial neoplasia (PanIN) formation with fibrotic changes at 8 weeks of age.

A. H&E staining, Alcian blue staining, Sirius red staining, and immunostaining for *Nrdc*, F4/80, pErk1/2, and Claudin 18 in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/+}* and *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* mice at 8 weeks of age. Scale bars: H&E, 500 μm ; *Nrdc*, 50 μm ; Alcian blue, 200 μm ; F4/80, 100 μm ; Sirius red, 200 μm ; pErk1/2 and Claudin 18, 50 μm .

B. Quantification of Alcian blue-positive PanIN lesions in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/+}* and *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* pancreata at 8 weeks of age; n = 5–6 mice/group. Means $\pm$ SEM are shown.

C. Quantification of fibrotic areas in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/+}* and *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* pancreata at 8 weeks of age; n = 5–6 mice/group. Means $\pm$ SEM are shown. * $P < 0.05$, *** $P < 0.005$.

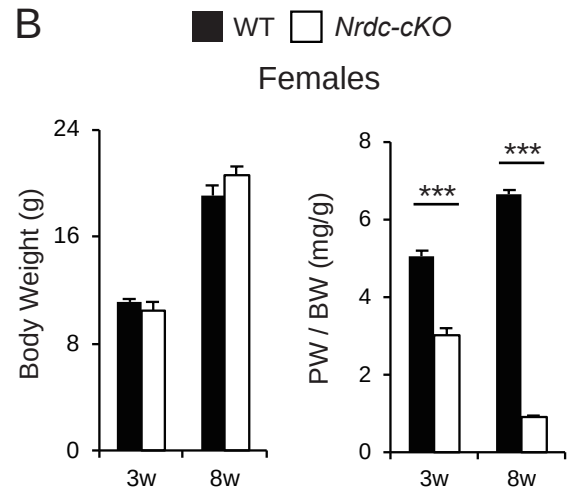
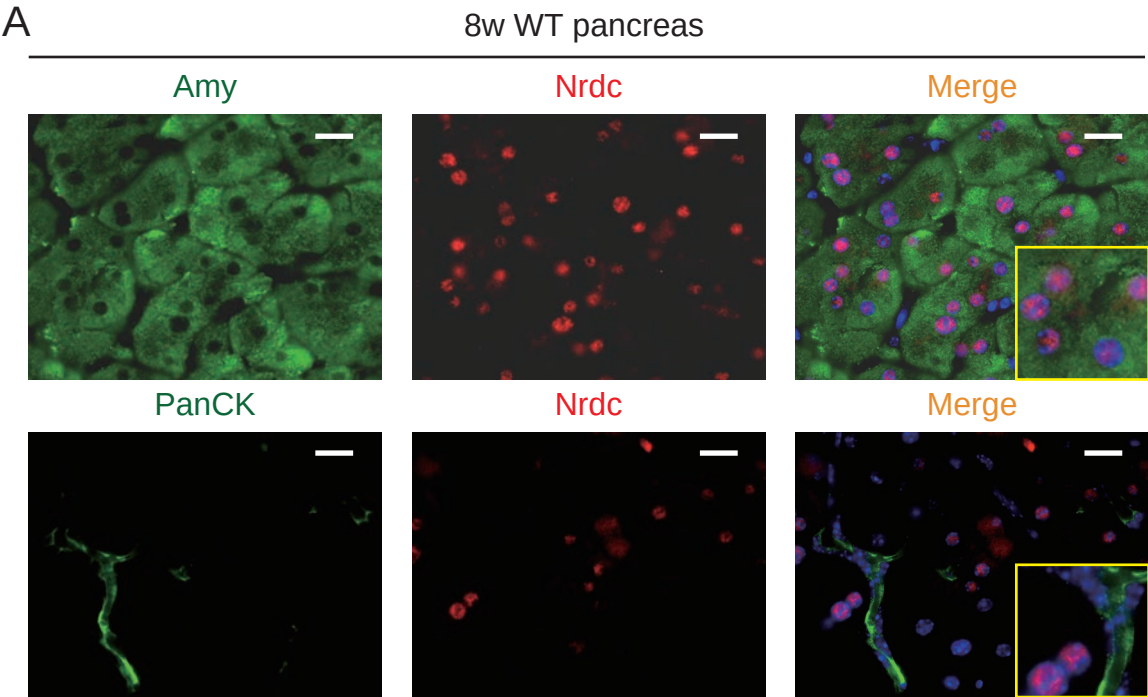
Supplementary figure S8

Subset of *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* mice developed invasive and metastatic pancreatic ductal adenocarcinoma (PDA) in mice.

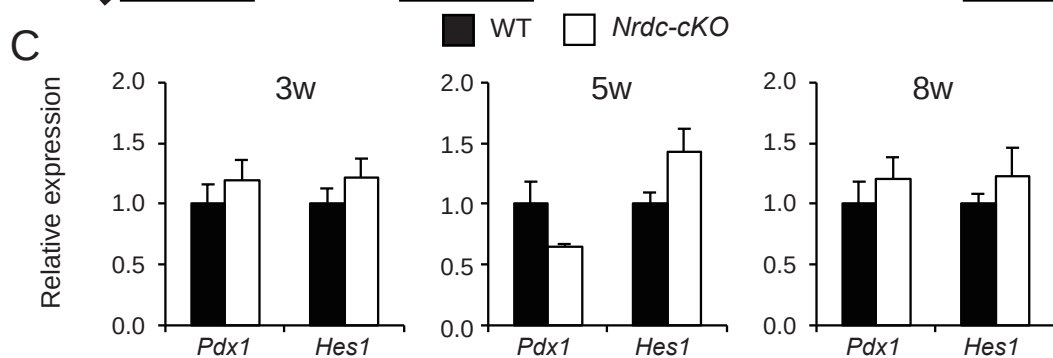
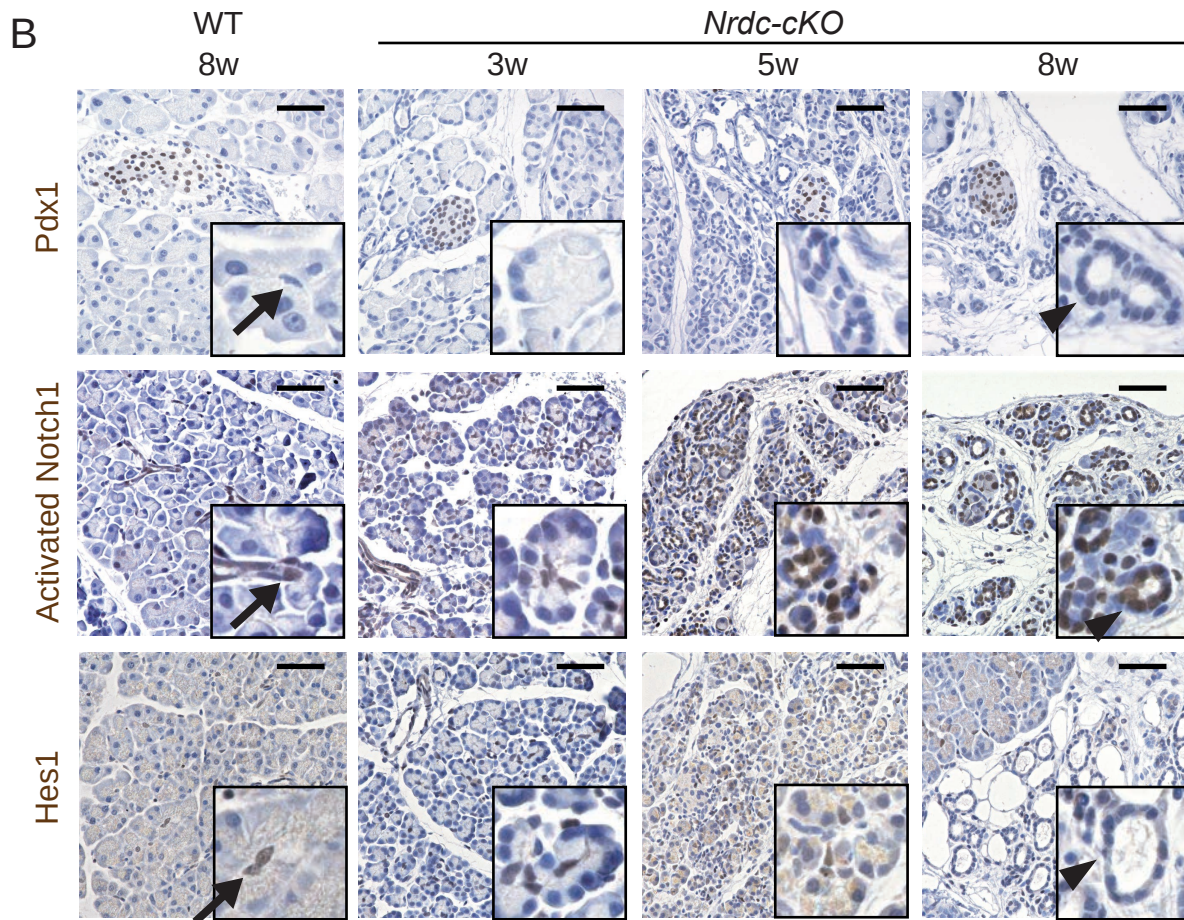
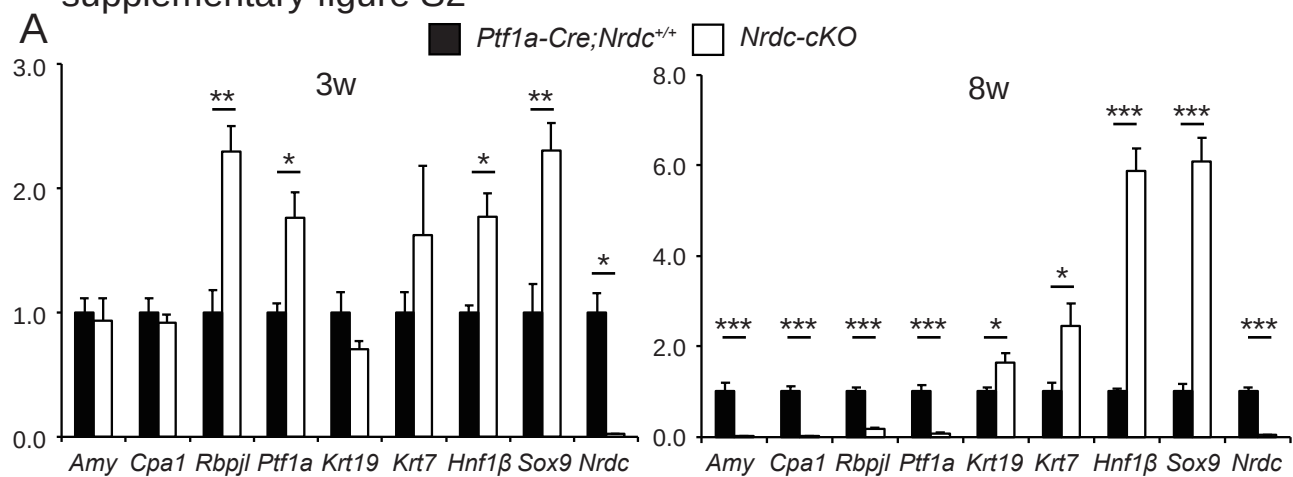
A. Macroscopic view of the *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* pancreas and liver at 19 weeks of age. Arrowhead indicates invasive PDA and liver metastasis.

B. H&E staining and immunostaining for Nrdc, F4/80, pan-cytokeratin (PanCK), and Pdx1 in *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flox/flox}* pancreata and livers from 19-week-old mice. Scale bars: 50 μ m.

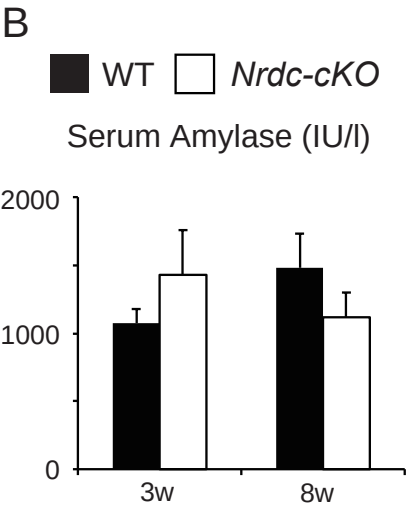
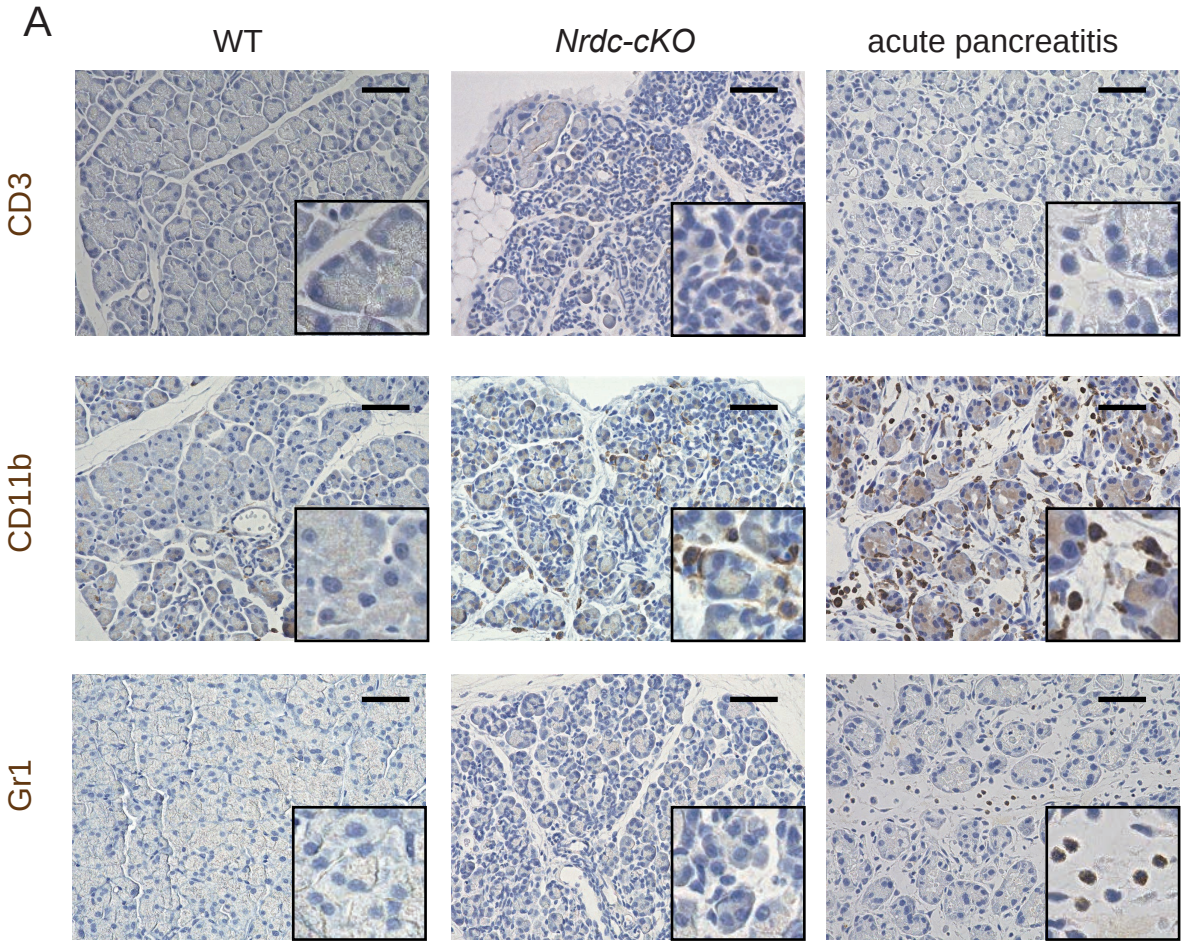
supplementary figure S1



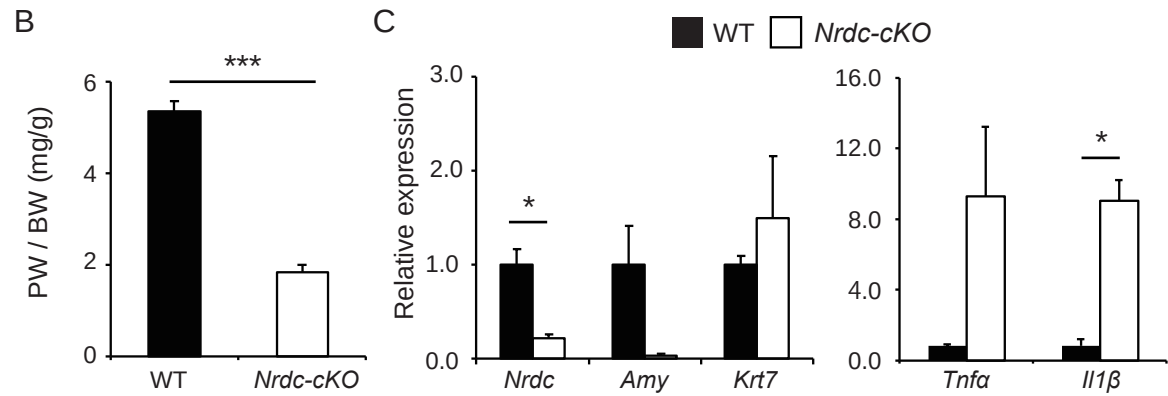
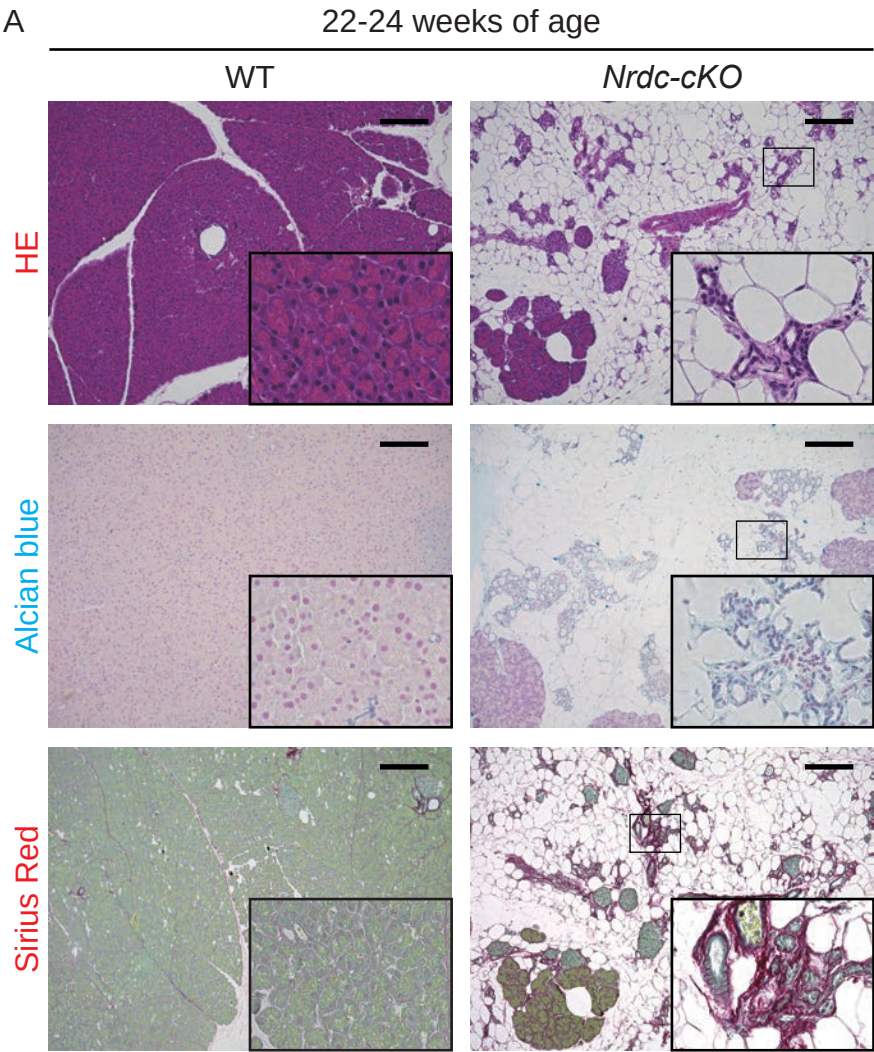
supplementary figure S2



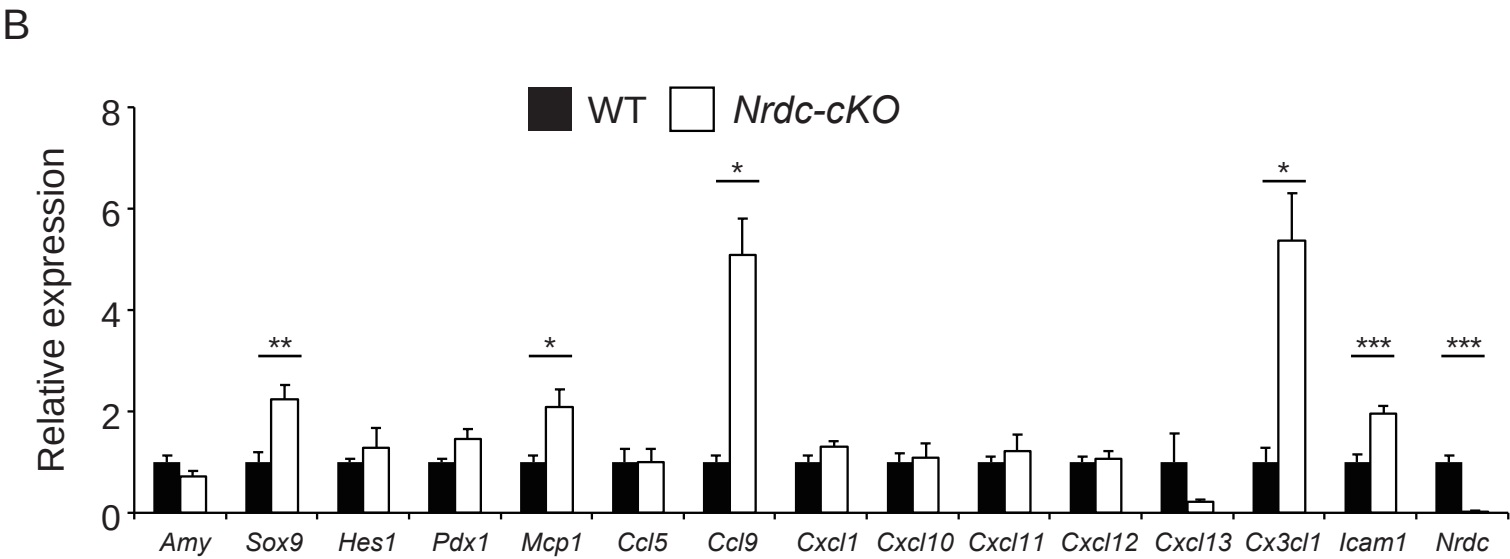
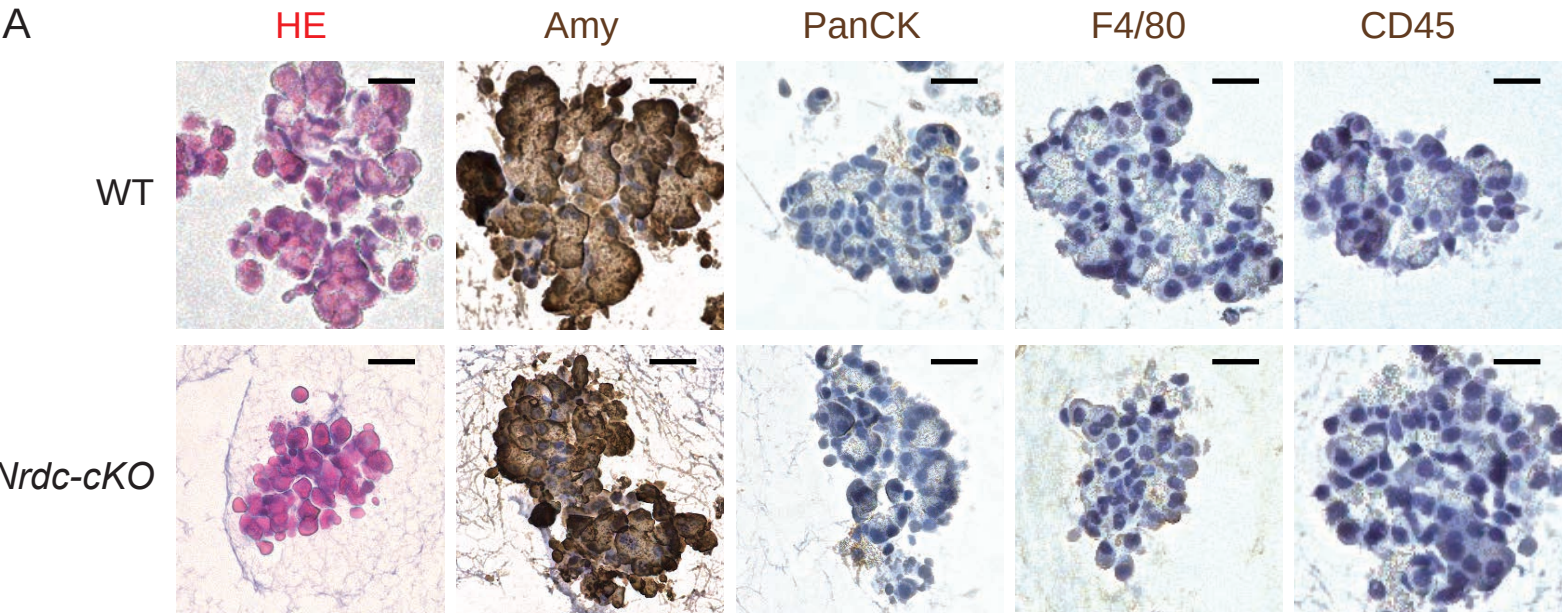
supplementary figure S3



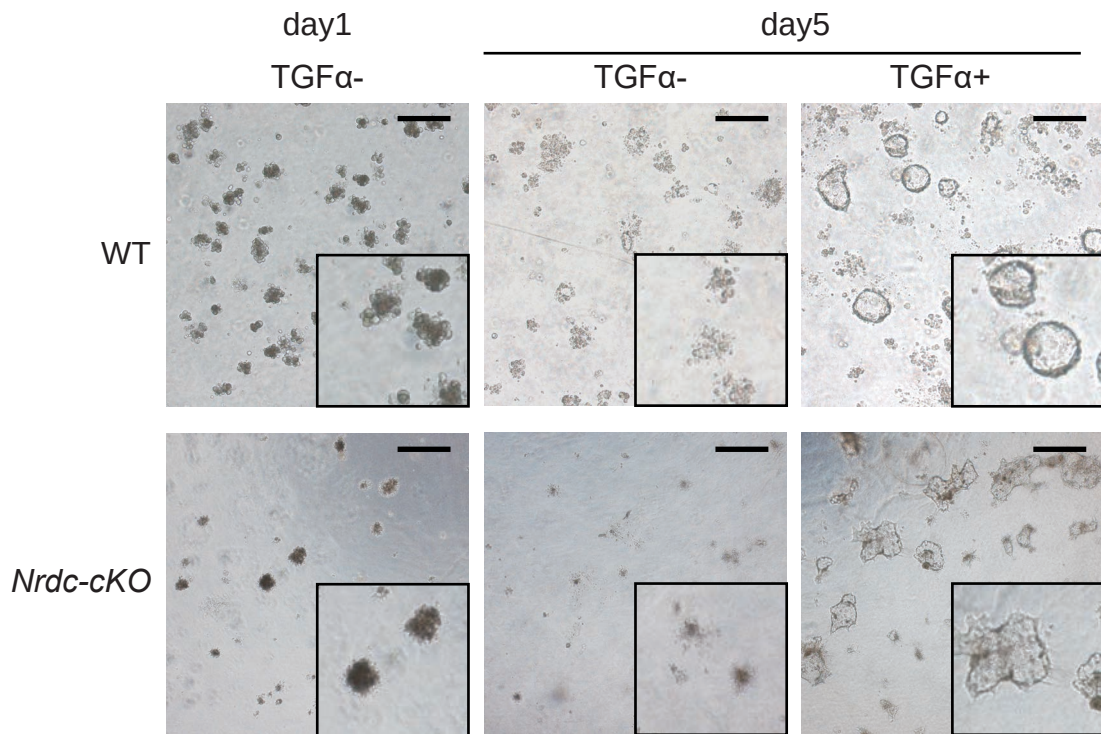
supplementary figure S4



supplementary figure S5



supplementary figure S6



A

Nrdc^{flox/+}

Nrdc^{flox/flox}

三

Alcian blue

Sirius Red

Claudin18

Nrdc^{flox/+}

Nrdc^{flox/flox}

Nrdc

F4/80

pErk1/2

B

C

PanIN Area (%)

Nrhc^{flox/+} *Nrhc*^{flox/flox}

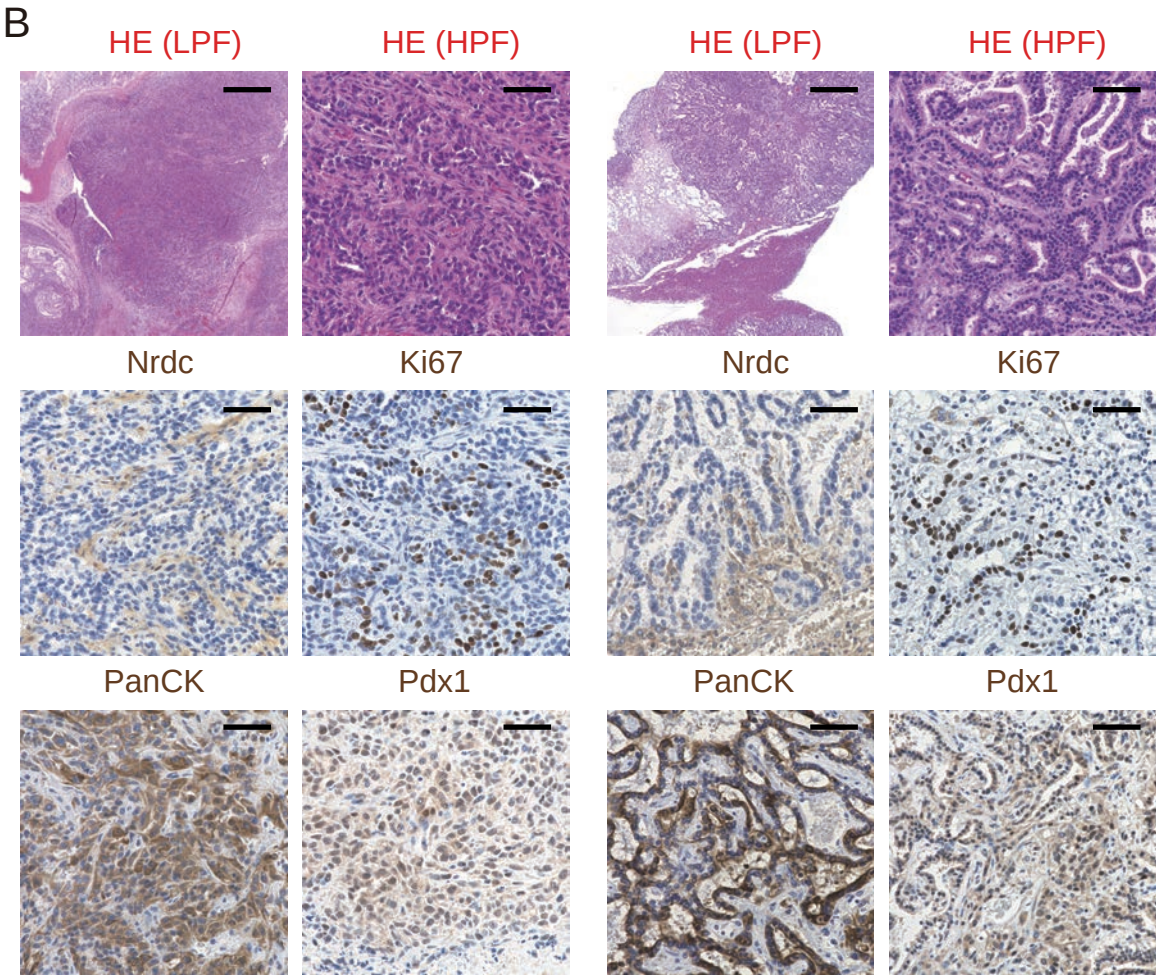
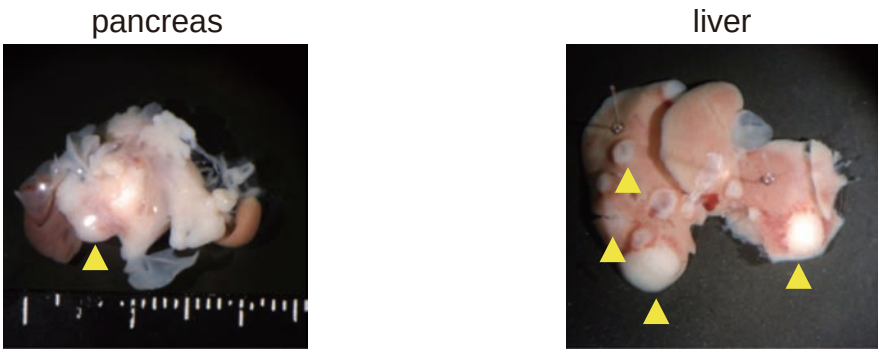
Ptf1a-Cre; Kras^{G12D}

Fibrotic area (%)

Nrhc^{lox/+} Nrhc^{lox/lox}
Ptf1a-Cre; Kras^{G12D}

supplementary figure S8

A *Ptf1a-Cre; Kras^{G12D}; Nrdc^{flx/flx}* (19weeks)



supplementary table S1. Primer sequences used for realtime PCR

Gene	primer	sequence	Gene	primer	sequence
<i>Gapdh</i>	forward	AGGTCGGTGTGAACGGATTTG	<i>Ccl9</i>	forward	CCCTCTCCTTCCTCATTCTTACA
	reverse	TGTAGACCATGTAGTTGAGGTCA		reverse	AGTCTTGAAAGCCCATGTGAAA
<i>Nrdc</i>	forward	GACTTGCTGGTGAATGCTGA	<i>Cxcl1</i>	forward	CTGGGATTACCTCAAGAACATC
	reverse	TTCGATGATTCTTCGCACAG		reverse	CAGGGTCAAGGCAAGCCTC
<i>Amy</i>	forward	TTGCCAAGGAATGTGAGCGAT	<i>Cxcl10</i>	forward	CCAAGTGCTGCCGTCAATTTTC
	reverse	CCAAGGTCTTGATGGGTTATGAA		reverse	GGCTCGCAGGGATGATTTCAA
<i>Rbpjl</i>	forward	CAGCAAAGATGTGAGCAGACT	<i>Cxcl11</i>	forward	TGTAATTTACCCGAGTAACGGC
	reverse	GGTAGACACAGGGTGGAGGA		reverse	CACCTTTGTCGTTTATGAGCCTT
<i>Cpa1</i>	forward	CAGTCTTCGGCAATGAGAACT	<i>Cxcl12</i>	forward	TGCATCAGTGACGGTAAACCA
	reverse	GGGAAGGGCACTCGAACATC		reverse	TTCTTCAGCCGTGCAACAATC
<i>Ptf1a</i>	forward	TCCCATCCCCTTACTTTGATGA	<i>Cxcl13</i>	forward	GGCCACGGTATTCTGGAAGC
	reverse	GTAGCAGTATTCGTGTAGCTGG		reverse	GGGCGTAACCTGAATCCGATCTA
<i>Krt19</i>	forward	GGGGGTTCAGTACGCATTGG	<i>Cx3cl1</i>	forward	ACGAAATGCGAAATCATGTGC
	reverse	GAGGACGAGGTCACGAAGC		reverse	CTGTGTCGTCTCCAGGACAA
<i>Krt7</i>	forward	CACGAACAAGGTGGAGTTGGA	<i>Icam1</i>	forward	GTGATGCTCAGGTATCCATCCA
	reverse	TGTCTGAGATCTGCGACTGCA		reverse	CACAGTTCTCAAAGCACAGCG
<i>Hnf1β</i>	forward	GTTACTGCCGTCCCCGAATTT	<i>Ccr2</i>	forward	ATCCACGGCATACTATCAACATC
	reverse	CGGCTTGGTGTGCGAGATCC		reverse	GCATTAGCTTCAGATTACGGGT
<i>Sox9</i>	forward	AGTACCCGCATCTGCACAAC	<i>Cxcr3</i>	forward	TACCTTGAGGTAGTGAACGTCA
	reverse	ACGAAGGGTCTCTTCTCGCT		reverse	CGCTCTCGTTTTCCCAATATC
<i>Pdx1</i>	forward	TTCCCGAATGGAACCGAGC	<i>Tlr3</i>	forward	GTGAGATACAACGTAGCTGACTG
	reverse	GCGTGAGCTTTGGTGGATT		reverse	TCCTGCATCCAAGATAGCAAGT
<i>Hes1</i>	forward	ATAGCTCCCGCATTCCAAG	<i>Tnfrsf11a</i>	forward	CCAGGAGAGGCATTATGAGCA
	reverse	GCGCGGTATTTCCCAACA		reverse	ACTGTGCGAGGTAGGAGTGC
<i>Tnfa</i>	forward	CCCTCACACTCAGATCATCTTCT	<i>Traf1</i>	forward	AGGGTGGTGAATTACAGCAA
	reverse	GCTACGACGTGGGCTACAG		reverse	GCAGTGTAGAAAGCTGGAGAG
<i>Il1β</i>	forward	GCAACTGTTCTGAACTCAACT	<i>Trem2</i>	forward	CTGGAACCGTCACCATCACTC
	reverse	ATCTTTGGGGTCCGTCAACT		reverse	CGAAACTCGATGACTCCTCGG
<i>Mcp1</i>	forward	TTAAAAACCTGGATCGGAACCAA	<i>Pla2g5</i>	forward	CCAGGGGGCTTGCTAGAAC
	reverse	GCATTAGCTTCAGATTACGGGT		reverse	AGCACCAATCAGTGCCATCC
<i>Ccl5</i>	forward	GCTGCTTTGCCTACCTCTCC	<i>Bmper</i>	forward	GCCTGGGATTACCTGCTGC
	reverse	TCGAGTGACAAACGACTGC		reverse	ACACATTATGCAAGGGTTGTCTG

All primer pairs were selected from the Primer Bank database.



Deletion of Nardilysin Prevents the Development of Steatohepatitis and Liver Fibrotic Changes

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Abstract

Nonalcoholic steatohepatitis (NASH) is an inflammatory form of nonalcoholic fatty liver disease that progresses to liver cirrhosis. It is still unknown how only limited patients with fatty liver develop NASH. Tumor necrosis factor (TNF)- α is one of the key molecules in initiating the vicious circle of inflammations. Nardilysin (N-arginine dibasic convertase; *Nrd1*), a zinc metalloendopeptidase of the M16 family, enhances ectodomain shedding of TNF- α , resulting in the activation of inflammatory responses. In this study, we aimed to examine the role of *Nrd1* in the development of NASH. *Nrd1*^{+/+} and *Nrd1*^{-/-} mice were fed a control choline-supplemented amino acid-defined (CSAA) diet or a choline-deficient amino acid-defined (CDAA) diet. Fatty deposits were accumulated in the livers of both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice by the administration of the CSAA or CDAA diets, although the amount of liver triglyceride in *Nrd1*^{-/-} mice was lower than that in *Nrd1*^{+/+} mice. Serum alanine aminotransferase levels were increased in *Nrd1*^{+/+} mice but not in *Nrd1*^{-/-} mice fed the CDAA diet. mRNA expression of inflammatory cytokines were decreased in *Nrd1*^{-/-} mice than in *Nrd1*^{+/+} mice fed the CDAA diet. While TNF- α protein was detected in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mouse livers fed the CDAA diet, secretion of TNF- α in *Nrd1*^{-/-} mice was significantly less than that in *Nrd1*^{+/+} mice, indicating the decreased TNF- α shedding in *Nrd1*^{-/-} mouse liver. Notably, fibrotic changes of the liver, accompanied by the increase of fibrogenic markers, were observed in *Nrd1*^{+/+} mice but not in *Nrd1*^{-/-} mice fed the CDAA diet. Similar to the CDAA diet, fibrotic changes were not observed in *Nrd1*^{-/-} mice fed a high-fat diet. Thus, deletion of nardilysin prevents the development of diet-induced steatohepatitis and liver fibrogenesis. Nardilysin could be an attractive target for anti-inflammatory therapy against NASH.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition in which excess fat accumulates in the hepatocytes of patients without a history of alcohol abuse [1]. NAFLD is a hepatic manifestation of metabolic syndromes, such as obesity, type-II diabetes mellitus, and hyperlipidemia. Its prevalence is increasing particularly in the developed countries [1,2]. Nonalcoholic steatohepatitis (NASH) is a severe form of NAFLD, in which liver inflammation is observed and which progresses to liver fibrosis. A part of NAFLD patients develops NASH that leads to liver fibrosis. However, the exact causes and mechanisms of the development of NASH remain unknown.

Recent investigations have suggested a “multi-hit process” model for the development of NASH [3]. Liver inflammation including NASH can be initiated or enhanced by multiple cytokines secreted mainly by Kupffer cells or macrophages [4].

During liver fibrogenesis, myofibroblasts, that are not present in normal liver, also contribute to liver fibrogenesis through the remodeling of extracellular matrix [5]. In pro-inflammatory cascades, there are several key factors that play a crucial role in initiating or halting inflammation. Tumor necrosis factor (TNF)- α is one of such key molecules, and anti-TNF- α therapies are used widely to treat human inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel diseases [6,7]. To activate TNF- α , a membrane-bound pro-TNF- α must be appropriately and sufficiently cleaved by the prototypical sheddase, TNF- α -converting enzyme (TACE) [8]. Previously, we showed that nardilysin (N-arginine dibasic convertase; *Nrd1*), a zinc metalloendopeptidase of the M16 family that ubiquitously localizes in various organs, enhances the shedding of TNF- α through TACE activation [9–13]. Nardilysin binds to TACE and directly enhances its catalytic activity [10,11]. It also promotes the ectodomain shedding of

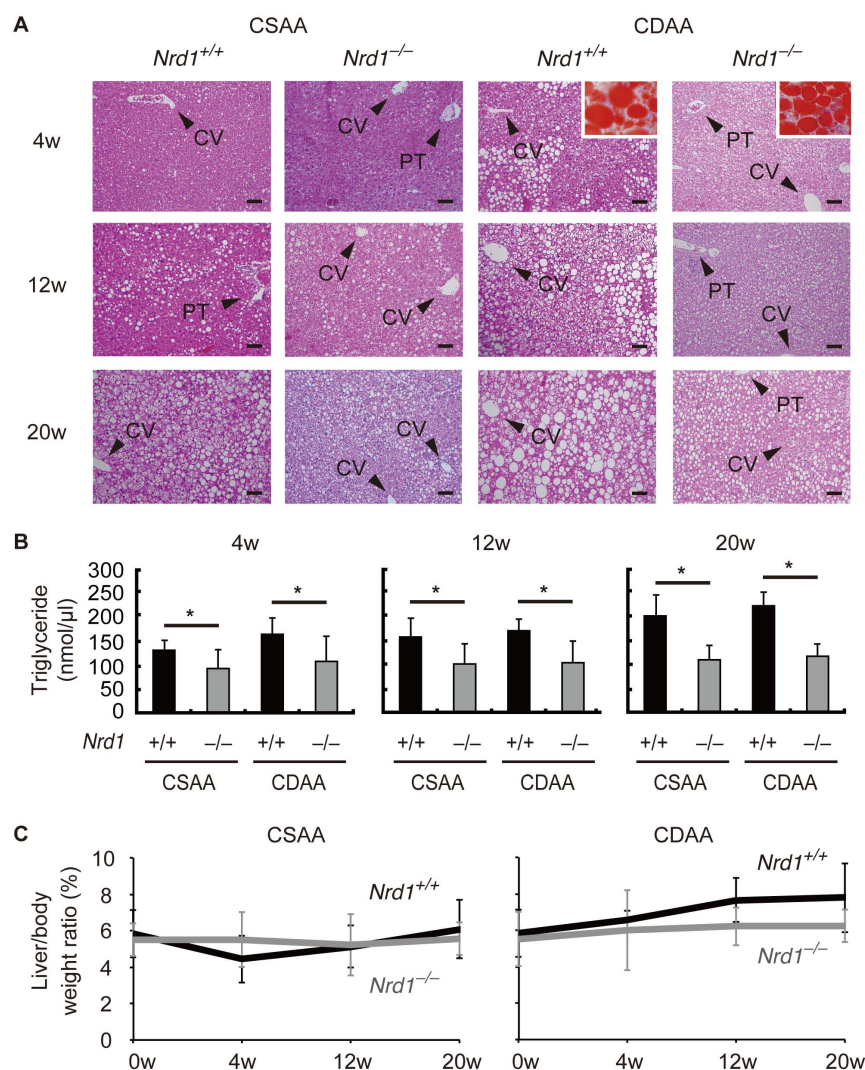


Figure 1. CDAA diet caused hepatic steatosis in *Nrd1*^{+/+} and *Nrd1*^{-/-} mice. A. Histology of the livers of *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CSAA (left) or CDAA (right) diets. Representative changes of the liver with regard to fat deposition at 4 (upper), 12 (middle), and 20 (lower) weeks during the experiments are depicted. Fat deposits were confirmed by oil red O staining shows (orange, inset). CV indicates central vein, and PT marks portal triad. Bars indicate 100 μm. B. Quantification of triglyceride in the liver. Triglyceride in the liver was increased in the livers of both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice during administration of the CDAA or CSAA diets, although it was significantly more prominent in *Nrd1*^{+/+} mice. n = 4–5, each. **P* < 0.05. C. There was no significant difference in the liver/body weight ratio between *Nrd1*^{+/+} and *Nrd1*^{-/-} mice during the experiments. doi:10.1371/journal.pone.0098017.g001

TNF-α, resulting in activation of the TNF-α/nuclear factor-κB pro-inflammatory signaling cascade [12].

In this study, we aimed to elucidate the mechanisms that distinguish NASH from simple liver steatosis. We examined the role of nardilysin, that is known to enhance TNF-α shedding, in the development of steatohepatitis using *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed a choline-deficient and amino acid-defined (CDAA) diet and a high-fat diet (HFD), that are used widely to reproduce the natural course of NASH and liver fibrosis in mice as well as in humans.

Materials and Methods

Ethics statement

All animal experiments were undertaken in accordance with institutional guidelines. The Review Board of Kyoto University granted ethical approval for this study.

Animal models

Nardilysin-deficient (*Nrd1*^{-/-}) mice (CDB0466K: <http://www.cdb.riken.jp/arg/mutant%20mice%20list.html>) were previously described [13]. Male *Nrd1*^{+/+} and *Nrd1*^{-/-} mice with the BL6/CBA background were bred and housed in a temperature- and light-controlled facility with unlimited access to food and water. To induce steatohepatitis and liver fibrotic changes, 10–12-week old male mice were fed a control choline-supplemented amino acid-defined (CSAA) diet or a choline-deficient amino acid-defined (CDAA) diet (Research Diets, New Brunswick, NJ, USA) for 4, 12, or 20 weeks according to the previous reports [14,15]. As another diet-induced model of steatohepatitis and liver fibrosis, mice were fed a HFD (Oriental Bio Service, Kyoto, Japan) for 20 weeks on the basis of previous studies [16,17]. Triglyceride levels in the livers were determined with Triglyceride Quantification kit (Abcam, Cambridge, MA, USA) according to the manufacture's protocol. Serum levels of alanine aminotransferase (ALT) were

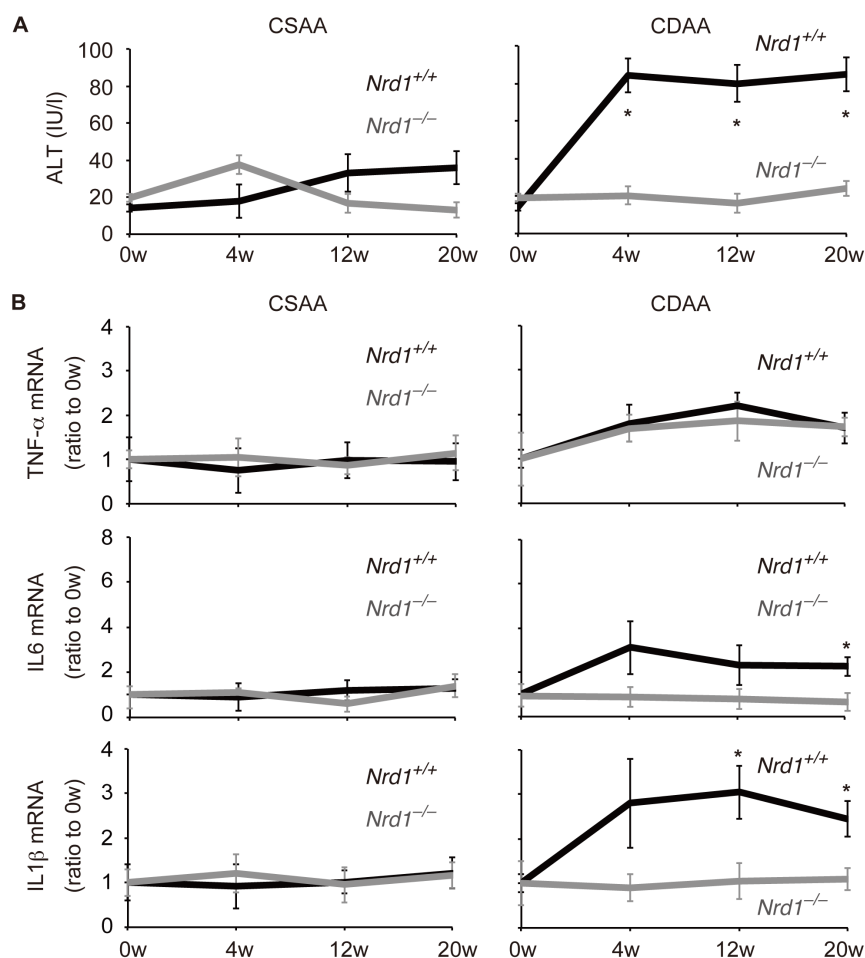


Figure 2. CDAA diet did not cause steatohepatitis in *Nrd1*^{-/-} mice. A. Serum ALT was not elevated in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CSAA diet (left). Serum ALT levels were significantly elevated in *Nrd1*^{+/+} mice, but were not elevated in *Nrd1*^{-/-} mice upon administration of the CDAA diet (right). Each value depicts the mean $\pm$ standard errors ($n=5-15$, each). * $P<0.05$. B. Relative expression of mRNA are shown as relative values compared to those at 0 w. mRNA of TNF- α was increased in both *Nrd1*^{+/+} (significantly) and *Nrd1*^{-/-} mice fed the CDAA diet compared with those fed the CSAA diet, with no significant difference between the two groups. In contrast, the mRNA expression levels of IL6 and IL1- β were increased only in *Nrd1*^{+/+} mice, and these levels were significantly higher than respective values in *Nrd1*^{-/-} mice fed the CDAA diet. ($n=5-16$, each) * $P<0.05$.

doi:10.1371/journal.pone.0098017.g002

measured using a Transaminase CII-Test Wako kit (Wako Pure Chemical Industries, Osaka, Japan).

Histological analyses and immunostainings

The liver was resected at various time points, fixed with 4% buffered paraformaldehyde solution, embedded in paraffin, and sectioned into 5- μ m thickness. Oil red O (Wako Pure Chemical Industries) staining was performed to confirm fatty deposition. Sirius red (saturated picric acid containing 0.1% Direct Red 80 and 0.1% Fast Green FCF; Sigma-Aldrich, St. Louis, MO, USA) staining was done to visualize collagen deposition. Stained fibrotic areas were measured as percentage area in a representative $\times 100$ high-power field in each mouse using Image J software. For the immunostainings the sections were incubated overnight with the primary antibodies at 4°C, after which the secondary antibodies were added. Kupffer cells or macrophages were stained with rat anti-F4/80 monoclonal antibody (Abcam). TNF- α staining was performed with anti-TNF- α goat polyclonal antibodies (R&D systems, Minneapolis, MN, USA). Activated myofibroblasts were stained with anti- α -smooth muscle actin (SMA) rabbit polyclonal

antibody (Abcam). Negative controls were prepared with isotype IgG.

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 (cDNA) was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Basel, Switzerland). qRT-PCR was performed using SYBR Green I Master (Roche Applied Science) and Light Cycler 480 (Roche Applied Science). Values are expressed as arbitrary units relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sets used were: TNF- α -Forward, CCCTCACACTCAGATCATCTTCT, TNF- α -Reverse, GCTACGACGTGGGCTACAG; interleukin (IL) 6-Forward, TAGTCCTTCCCTACCCCAATTTCC, IL6-Reverse, TTGGTCCTTAGCCACTCCTTC; IL1- β -Forward, GCAACTGTTCTGTA-CTCAACT, IL1- β -Reverse, ATCTTTTGGGGTCCGTCACCT; CCR2-Forward, ATCCACGGCATACTATCAACATC, CCR2-Reverse, CAAGGCTCACCATCATCGTAG; collagen I-For-

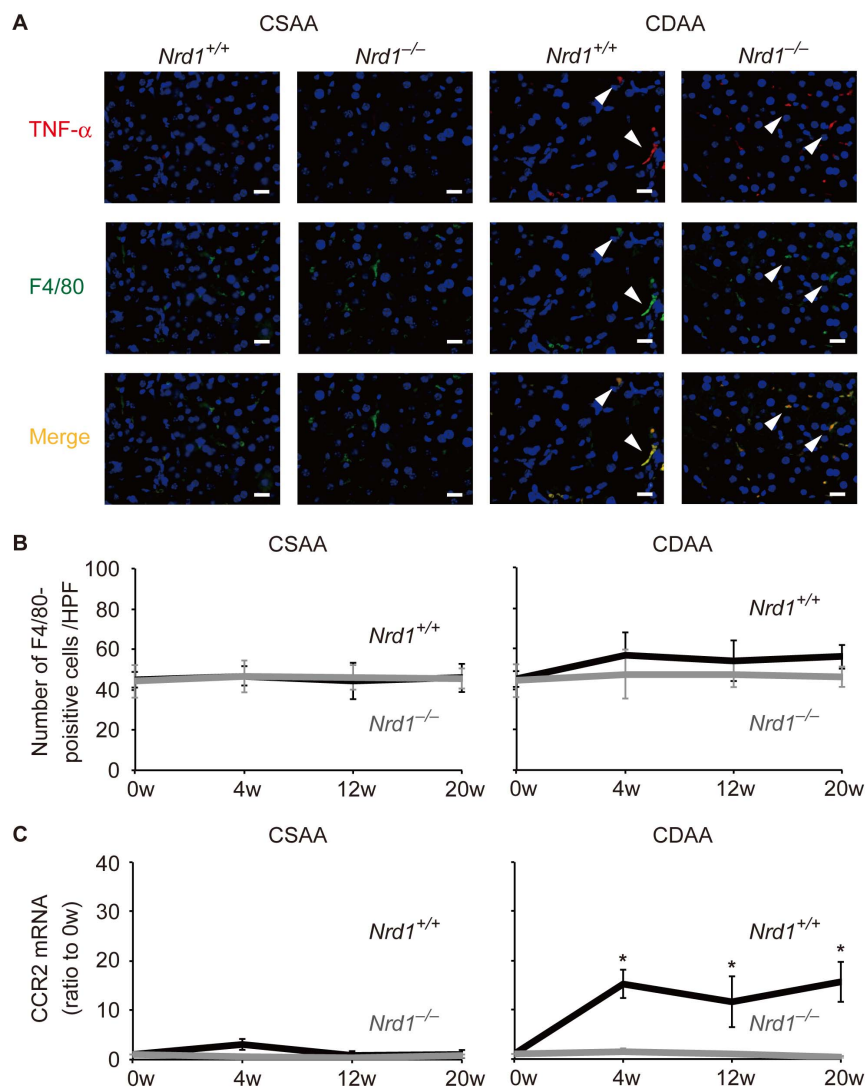


Figure 3. TNF- α was expressed in *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet. A. Immunohistochemistry showed that TNF- α protein (red, arrowheads) was expressed in F4/80-positive Kupffer cells or macrophages (green, arrowheads) in the livers of both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet for 20 weeks (right), but not in mice fed the CSAA diet for 20 weeks (left). A blue color indicates DAPI-positive nuclei. Bars indicate 50 μ m. B. The number of F4/80-positive cells/ $\times 100$ high-power field (HPF) in livers slightly increased (approximately 1.2 times) only in *Nrd1*^{+/+} mice fed the CDAA diet (right). C. Relative expression of mRNA are shown as relative values compared to those at 0 w. The mRNA expression level of CCR2 was increased in *Nrd1*^{+/+} mice fed the CDAA diet, and the levels were significantly higher than respective values in *Nrd1*^{-/-} mice fed the CDAA diet. * $P < 0.05$.

doi:10.1371/journal.pone.0098017.g003

ward, GCTCCTCTTAGGGGCCACT, collagen I-Reverse, ATTGGGGACCTTAGGCCAT; collagen IV-Forward, TCCGGGAGAGATTGGTTTCC, collagen IV-Reverse, CTGGCCTATAGCCCTGGT; tissue inhibitor of metalloproteinase (Timp) 1-Forward, CTTGGTTCCCTGGCGTACTC, Timp1-Reverse, ACCGTATCCGTCCACAAACAG; transforming growth factor (TGF)- β 1-Forward, CTCCCGTGGCTTCTAGTGC, TGF- β 1-Reverse, GCCTTAGTTTGGACAGGATCTG; α -SMA-Forward, GTCCAGACATCAGGGAGTAA, α -SMA-Reverse, TCGGATACTTCAGCGTCAGGA.

Measurement of cytokine levels by enzyme-linked immunosorbent assay (ELISA)

To determine the production and secretion of TNF- α protein in CDAA-treated mouse liver, a modified protocol that described in previous reports was used [18,19]. In brief, a liver fragment was

divided into two specimens (100 μ g each). One specimen was subjected directly to protein extraction, and the amount of protein extracted was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The other was cultured in a 24-well flat-bottomed culture plate in serum-free Dulbecco's modified Eagle's medium (D-MEM; Life Technologies) supplemented with penicillin and streptomycin (Life Technologies). After 12 hours, the supernatant was collected and the protein level measured. The amounts of TNF- α , IL6, and IL1- β proteins were measured using a Mouse ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

Mouse peritoneal macrophage experiments

Mouse peritoneal macrophages were isolated from 8-week-old female C57BL/6J mice. Peritoneal cells were harvested by peritoneal lavage with 10 ml PBS. Cells were re-suspended and

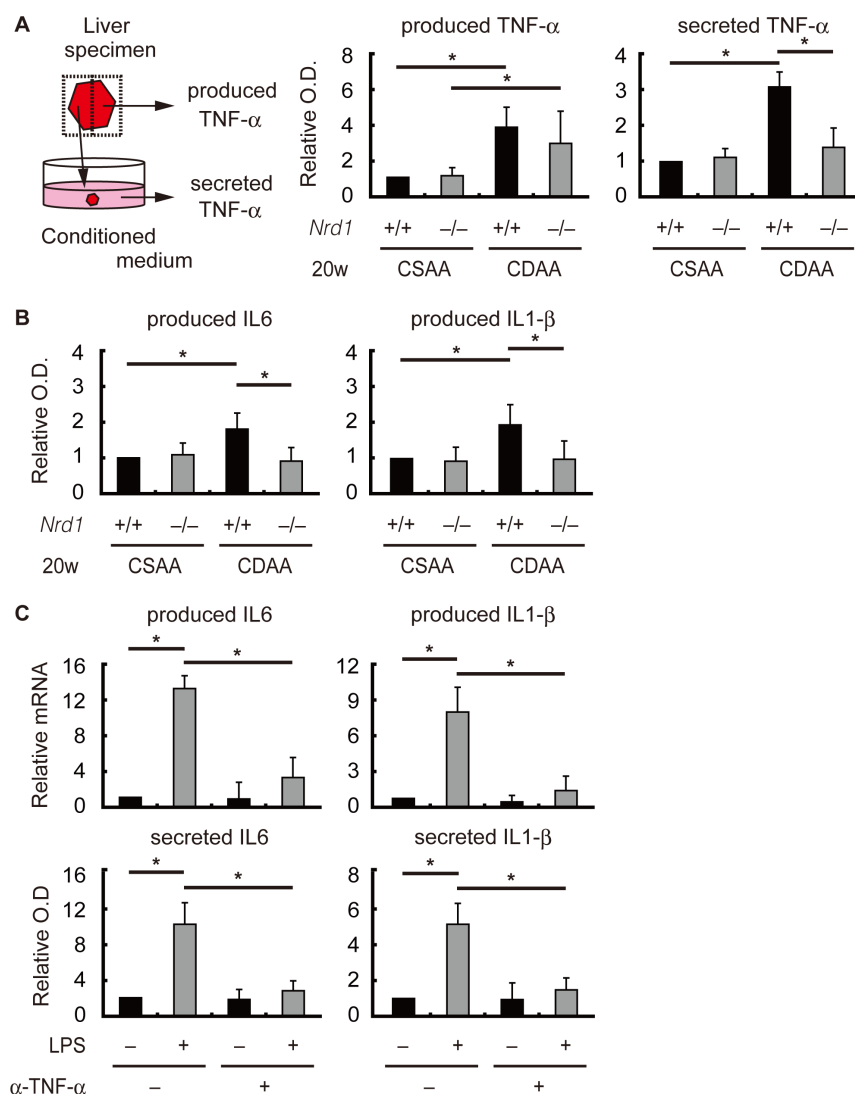


Figure 4. TNF- α was not sufficiently secreted from in *Nrd1*^{-/-} mice fed the CDAA diet. A. A liver fragment was divided into two pieces. One was directly subjected to protein extraction directly and measurement of TNF- α (produced TNF- α). The other piece was cultured in serum-free medium for 12 hours, and the supernatant was subjected to measurement of TNF- α (secreted TNF- α). The relative optical density (O.D.) to that of *Nrd1*^{+/+} fed the CSAA diet was determined by ELISA for TNF- α . Production of TNF- α from liver specimens were not significantly different between *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet for 20 weeks ("produced TNF- α "). In contrast, TNF- α secreted from liver specimens was significantly increased in *Nrd1*^{+/+} mice fed the CDAA diet for 20 weeks compared with those fed the CSAA diet; however, the elevation was not observed in *Nrd1*^{-/-} mice under the same condition ("secreted TNF- α "). * $P < 0.05$. B. Production of IL6 and IL1- β proteins were significantly increased only in *Nrd1*^{+/+} mice fed the CDAA diet for 20 weeks compared with those in *Nrd1*^{-/-} mice fed the CSAA diet for 20 weeks. * $P < 0.05$. C. mRNA (upper, 'produced') and protein (lower, 'secreted') production of IL6 and IL1- β were significantly increased after LPS treatment in *Nrd1*^{+/+} mouse peritoneal macrophages. However, administration of anti-TNF- α neutralizing antibodies significantly suppressed the production of IL6 and IL1- β in the presence of LPS. * $P < 0.05$. doi:10.1371/journal.pone.0098017.g004

cultured in D-MEM supplemented with 10% FCS, 100 mg/ml of penicillin, 100 mg/ml of streptomycin, and 1.25 μ g/ml of amphotericin B. 1.0×10^6 peritoneal cells were seeded into a 48-well dish, and incubated for 2 hours. Then, cells were washed in PBS, and re-cultured in the serum-free medium. To inhibit TNF- α activity, either control serum or 0.4 μ g/ml of anti-TNF- α neutralizing polyclonal antibodies (R&D systems) was administered into the culture medium. After 30 minutes later, 1 μ g/ml of lipopolysaccharide (LPS) were added. Medium and cells were collected 2 hours after the stimulation, and subjected to the analyses according to the methods described above.

Statistical analyses

Results are the mean $\pm$ standard deviation unless stated otherwise. Differences between treatments, groups, and strains were analyzed using the two-tailed Student's t-test.

Results

Nrd1^{-/-} mice did not develop steatohepatitis with CDAA diet

The CDAA diet is deficient in choline only, but contains methionine, allowing observation of the sequential development of steatohepatitis and liver fibrotic changes in a longer experimental

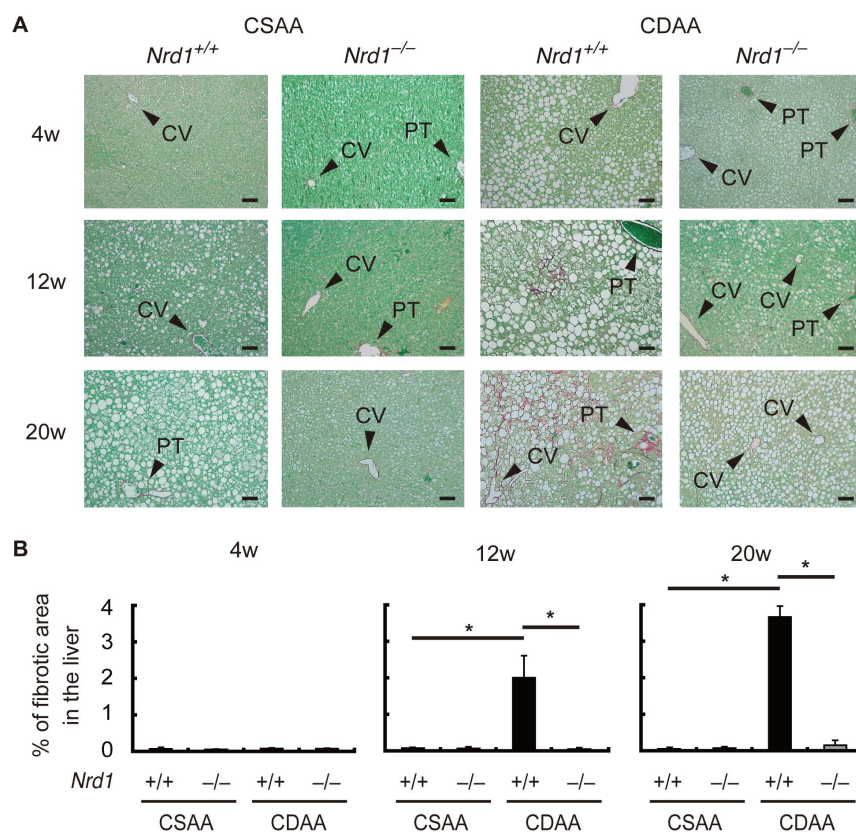


Figure 5. Liver fibrotic area was not observed in *Nrd1*^{-/-} mice fed the CDAA diet. A. Liver fibrosis was determined by Sirius red staining (red) in *Nrd1*^{+/+} and *Nrd1*^{-/-} mice at 4 (upper), 12 (middle), and 20 (lower) weeks in the livers of *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CSAA or CDAA diet. Fibrotic changes were not observed in *Nrd1*^{+/+} or *Nrd1*^{-/-} mice fed the CSAA diet (left). Fibrotic changes were prominent in *Nrd1*^{+/+} mice, but not in *Nrd1*^{-/-} mice fed the CDAA diet (right). Bars indicate 100 μ m. B. Quantification of fibrotic areas. Fibrotic areas was observed and increased in a time-dependent manner only in the livers of *Nrd1*^{+/+} mice fed the CDAA diet. n = 5–8, each. **P* < 0.05. doi:10.1371/journal.pone.0098017.g005

period in mice [4,14]. The control CSAA diet also causes mild steatosis, but does not result in steatohepatitis and liver fibrotic changes in mice [4,14]. To study the role of nardilysin during the development of steatohepatitis followed by liver fibrosis, *Nrd1*^{+/+} and *Nrd1*^{-/-} mice were fed the CSAA or CDAA diets. Histology and oil red O staining showed that fat accumulation in the livers of both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice occurred during administration of the CDAA or CSAA diets and increased in a time-dependent manner, although fat accumulation in *Nrd1*^{+/+} mice was more prominent than that in *Nrd1*^{-/-} mice (Figure 1A). Size of fat deposition was greater in *Nrd1*^{+/+} mice than in *Nrd1*^{-/-} mice in both diet groups at each time point (Figure 1A), and triglyceride levels in the liver were significantly higher in *Nrd1*^{+/+} mice (Figure 1B). There was no significant difference in the liver/body weight ratio between *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed CSAA or CDAA diets (Figure 1C). Thus, administration of CSAA or CDAA diets induced hepatic steatosis in mice to a varying degree. However, serum ALT levels were significantly increased in *Nrd1*^{+/+} mice upon administration of the CDAA diet, whereas they were not increased in *Nrd1*^{-/-} mice fed the CDAA diet (Figure 2A). Serum ALT level was elevated in neither *Nrd1*^{+/+} nor *Nrd1*^{-/-} mice fed the CSAA diet (Figure 2A). Consistent with these findings, qRT-PCR showed that mRNA expression of inflammatory cytokines, such as IL6 and IL1- β , was significantly increased only in *Nrd1*^{+/+} mice fed the CDAA diet when fat accumulation and elevation of ALT were prominent, whereas they were not increased in *Nrd1*^{-/-} mice fed the CDAA diet, and in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the

CSAA diet (Figure 2B). These data indicated that nardilysin played an important role in the development of steatohepatitis and accompanied the production of inflammatory cytokines in mice fed the CDAA diet.

Nrd1 was required for sufficient secretion of TNF- α

TNF- α is one of the key molecules that are involved in the development of NASH [4–7,20]. Because secretion of activated TNF- α is the initial step in nardilysin-mediated production of inflammatory cytokines [12], we hypothesized that sufficient secretion of TNF- α by nardilysin is required for the development of steatohepatitis. Thus, we aimed to ascertain whether TNF- α was produced and secreted sufficiently in the livers of *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet. qRT-PCR showed that the mRNA of TNF- α was increased in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet, and that in contrast to the results looking at IL6 and IL1- β mRNA levels, there was no significant difference between *Nrd1*^{+/+} and *Nrd1*^{-/-} mice (Figure 2B). Immunohistochemistry showed that TNF- α protein was detected in F4/80-positive Kupffer cells or macrophages in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet for 20 weeks (Figure 3A, right, arrowheads). Conversely, TNF- α protein was barely detected in F4/80-positive Kupffer cells or macrophages in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the control CSAA diet for 20 weeks (Figure 3A, left). The number of F4/80-positive cells/ $\times$ 100 high power field (HPF) in the liver was slightly increased only in *Nrd1*^{+/+} mice fed the CDAA diet but not in *Nrd1*^{-/-} mice fed the CDAA diet and those

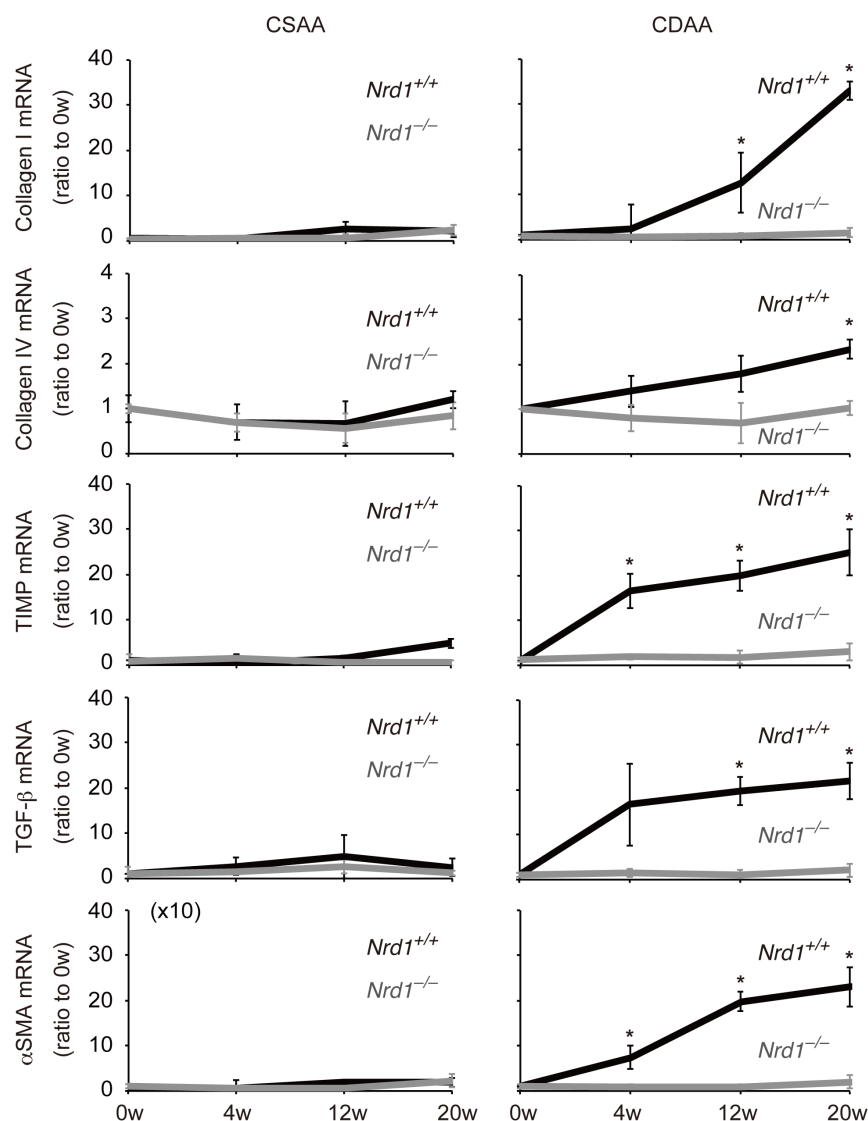


Figure 6. Fibrogenic factors were not elevated in *Nrd1*^{-/-} mice fed the CDAA diet. During CDAA diet administration, mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA were significantly increased in the livers of *Nrd1*^{+/+} mice but not in those of *Nrd1*^{-/-} mice. Those factors were not altered by administration of the CSAA diet in *Nrd1*^{+/+} or *Nrd1*^{-/-} mice. **P*<0.05. doi:10.1371/journal.pone.0098017.g006

in *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CSAA diet (Figure 3B). qRT-PCR showed that mRNA expression of CCR2, a recruited macrophage marker, was significantly increased in *Nrd1*^{+/+} mice, but not in *Nrd1*^{-/-} mice (Figure 3C). This suggested that macrophages are not sufficiently recruited in *Nrd1*^{-/-} mice. At 20 weeks of a CDAA feeding, production of TNF-α protein was significantly upregulated in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mouse livers (Figure 4A, produced TNF-α), but the increase in TNF-α protein secretion from liver specimens into the conditioned medium was decreased significantly (0.46-fold) by *Nrd1* knockout (Figure 4A, secreted TNF-α). In contrast, production of IL6 and IL1-β proteins were not increased in *Nrd1*^{-/-} mice fed a CDAA diet (Figure 4B). These data suggested that nardilysin was required for the shedding of TNF-α in mice fed the CDAA diet and possibly the induction of inflammation. To further investigate that possibility, we examined whether blocking TNF-α suppresses the production of IL6 and IL1-β. We used *Nrd1*^{+/+} mouse peritoneal macrophages as substitutes for Kupffer cells and recruited macrophages in the liver, and examined

the effect of pre-incubation with anti-TNF-α neutralizing antibodies on the production of IL6 and IL1-β. Following LPS stimulation mRNAs and secreted proteins of both IL6 and IL1-β from macrophages were significantly increased, and administration of anti-TNF-α neutralizing antibodies significantly suppressed the production of IL6 and IL1-β (Figure 4C). This also suggested that TNF-α secretion played an important role to induce IL6 and IL1-β production in mice.

Nrd1^{-/-} mice were resistant to CDAA diet-induced liver fibrotic changes

Persistent steatohepatitis results in hepatic fibrosis [1–4]. Using Sirius red staining we investigated whether secretion/production of inflammatory cytokines enhanced by nardilysin was associated with the development of liver fibrotic changes. Four weeks after CDAA feeding, fibrotic changes were not prominent in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice (Figure 5A and B). Twelve weeks after CDAA feeding, fibrotic changes were observed in *Nrd1*^{+/+} mice,

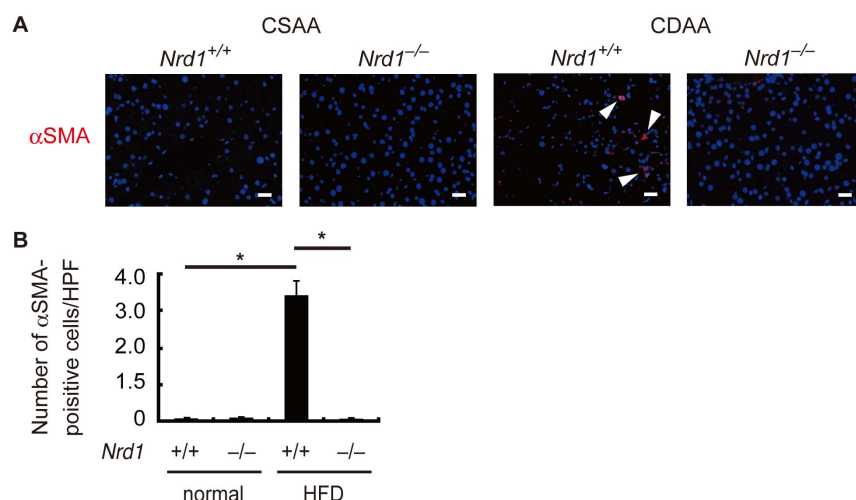


Figure 7. Activated myofibroblasts were not observed in *Nrd1*^{-/-} mice fed the CDAA diet. A. Immunostainings for αSMA demonstrated that activated myofibroblasts were detected in *Nrd1*^{+/+} mice fed a CDAA diet for 20 weeks, but not in *Nrd1*^{-/-} mice. Activated myofibroblasts were hardly detected by administration of the CSAA diet in *Nrd1*^{+/+} or *Nrd1*^{-/-} mice. Bars indicate 100 μm. B. The number of αSMA-positive cells/×400 high-power field (HPF) in livers increased only in *Nrd1*^{+/+} mice fed the CDAA diet for 20 weeks. **P*<0.05. doi:10.1371/journal.pone.0098017.g007

whereas such changes were not prominent in *Nrd1*^{-/-} mice (Figure 5A and B). At 20 weeks of CDAA diet administration, fibrotic changes in *Nrd1*^{+/+} mice became more prominent, while they were not observed in *Nrd1*^{-/-} mice (Figure 5A and B). Fibrotic changes were not observed throughout the experiments in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed a CSAA diet (Figure 5A and B). Consistently, the increased mRNA expression of fibrogenic markers such as collagen I, collagen IV, TIMP1, TGF-β, and αSMA in *Nrd1*^{+/+} mouse livers were not observed in *Nrd1*^{-/-} mice fed the CDAA diet (Figure 6). Immunostainings for αSMA demonstrated that activated myofibroblasts were detectable only in *Nrd1*^{+/+} mice fed a CDAA diet (Figure 7A and B). Thus, nardilysin played a pivotal role in the development of liver fibrosis caused by the CDAA diet.

Nrd1^{-/-} mice were resistant to high fat diet-induced liver fibrogenesis

To further confirm the role of nardilysin in the development of steatohepatitis followed by liver fibrotic changes, *Nrd1*^{+/+} and *Nrd1*^{-/-} mice were also fed HFD. Similar to the CDAA diet, HFD administration for 20 weeks induces hepatic steatosis and liver fibrogenesis [16]. In the present study, steatosis was observed more prominently in *Nrd1*^{+/+} mice compared to *Nrd1*^{-/-} mice at 20 weeks of HFD administration, but not in mice fed a normal control diet (Figure 8A). Consistently, triglyceride in the liver were elevated in *Nrd1*^{+/+} and *Nrd1*^{-/-} mice (Figure 8B). However, serum ALT levels were significantly increased in *Nrd1*^{+/+} mice upon 20-week administration of the HFD, whereas they were not increased in *Nrd1*^{-/-} mice fed the HFD (Figure 8C). Furthermore, fibrotic changes were detected only in *Nrd1*^{+/+} mice fed a HFD (Figure 8D and E). Consistent with this finding, qRT-PCR showed that the mRNA expression of IL1-β was significantly increased only in *Nrd1*^{+/+} mice at 20 weeks of HFD feeding, but not in that of *Nrd1*^{-/-} mice (Figure 9A). mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA were significantly increased in the livers of *Nrd1*^{+/+} mice fed a HFD for 20 weeks, but not in those of *Nrd1*^{-/-} mice (Figure 9B). Therefore, nardilysin also played an important role in the development of steatohepatitis and liver fibrogenesis induced by HFD in mice.

Discussion

In the present study, we demonstrated that steatosis was induced by the CDAA diet in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice, although fatty changes were less prominent in *Nrd1*^{-/-} mice. Importantly, steatohepatitis followed by liver fibrotic changes was observed only in *Nrd1*^{+/+} mice and not in *Nrd1*^{-/-} mice. Secretion of TNF-α, and the production of inflammatory cytokines and fibrogenic factors were not upregulated in *Nrd1*^{-/-} mice as compared with *Nrd1*^{+/+} mice. In the HFD model, steatohepatitis and liver fibrogenesis were hardly observed in *Nrd1*^{-/-} mice. These data suggested that nardilysin plays an important role in the development of steatohepatitis followed by liver fibrosis.

In mice fed with the CDAA diet, the levels of hepatic triglyceride content were lower in *Nrd1*^{-/-} mice compared with those in *Nrd1*^{+/+} mice, suggesting the possibility that nardilysin is involved in the regulation of hepatic lipid synthesis. A decreased steatosis in *Nrd1*^{-/-} mice may partly affect hepatic inflammation. However, steatosis did occur in the liver of *Nrd1*^{-/-} mice; on the other hand, hepatic inflammation was not observed despite the presence of steatosis in *Nrd1*^{-/-} mice. This indicated that nardilysin has an important role in the initiation and/or promotion of inflammatory responses induced by the CDAA diet. Persistent inflammation distinguishes steatohepatitis from simple hepatic steatosis [1–3]. Among pro-inflammatory factors, TNF-α is one of the key molecules that initiate inflammatory cascades, and its role in the progression of NASH has been discussed [4–7]. For example, apoptotic change in the liver, which contributes to the progression of NASH, is inhibited by an anti-TNF receptor neutralizing antibody or pentoxifylline in a mouse model of NASH [20]. The absence of TNFR1, a receptor for TNF-α, reduces IL6 mRNA production in the liver fed with the HFD even in the presence of elevated serum TNF-α [21]. The absence of TNFR1 also reduces liver lipid accumulation and macrophage accumulation in livers of HFD-fed mice [21]. Thus, inhibition of TNF-α signaling appears to play a pivotal role to suppress inflammatory reactions in NASH as well as other inflammatory disorders [22]. Although clinical application of anti-TNF-α therapy has not been established in the treatment of human NASH, anti-TNF-α neutralizing antibodies are effectively used to treat various human

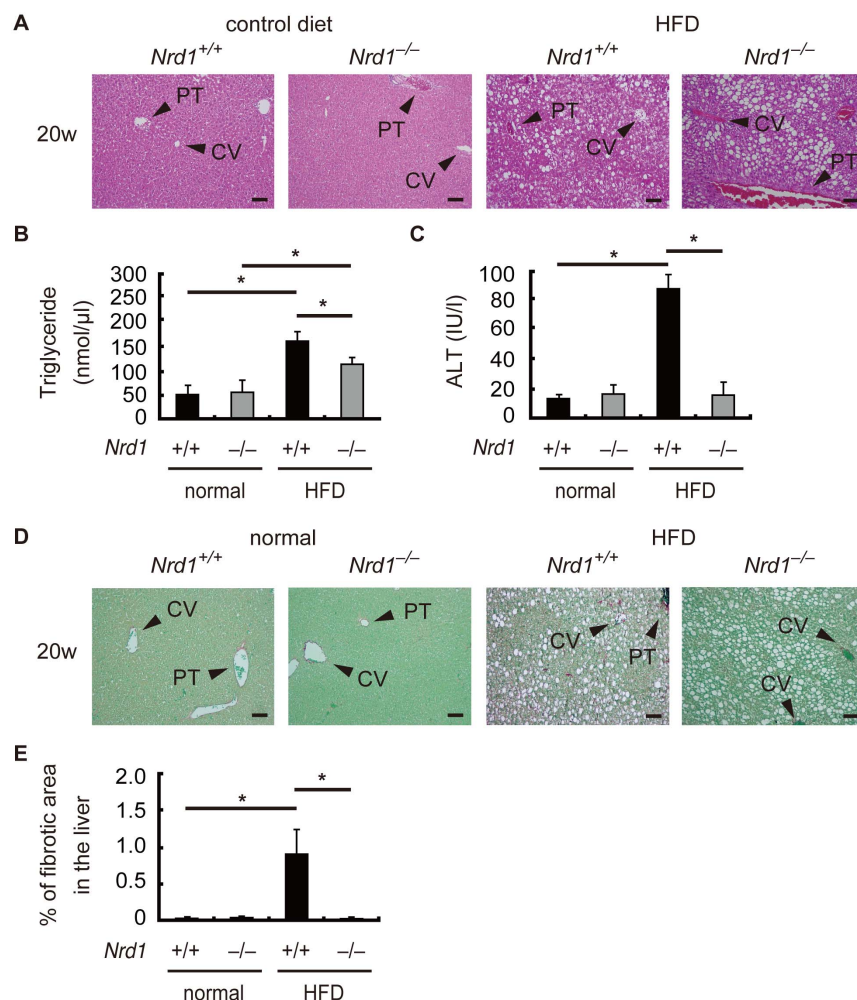


Figure 8. Liver fibrogenesis was not observed in *Nrd1*^{-/-} mice fed the HFD. A. Steatosis was observed in both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice after 20-week HFD administration (right), but not in those fed a normal control diet (left). Bars indicate 100 μm. B. Quantification of triglyceride in the liver. Triglyceride was elevated in the livers of both *Nrd1*^{+/+} and *Nrd1*^{-/-} mice after 20-week HFD administration, although it was significantly higher in *Nrd1*^{+/+} mice. n=4, each. **P*<0.05. C. Serum ALT levels were significantly elevated in *Nrd1*^{+/+} mice upon administration of the HFD, but were not elevated in other mouse groups. **P*<0.05. D. Fibrotic area was less prominent in *Nrd1*^{-/-} mice than in *Nrd1*^{+/+} mice (right). Bars indicate 100 μm. E. Fibrotic area was observed only in the livers of *Nrd1*^{+/+} mice fed the HFD (right). n=5, each. **P*<0.05. doi:10.1371/journal.pone.0098017.g008

inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel diseases [6,7]. We previously reported that nardilysin is essential for the sufficient activation of TNF-α in cooperation with TACE [10–12]. By the knockdown of *Nrd1*, TNF-α secretion is decreased concomitantly with decreased TACE activity, and the production of inflammatory cytokines such as IL6 and IL1-β is significantly suppressed [10–12]. In the present study, it is worth noting that TNF-α secretion from liver specimens was decreased significantly in *Nrd1*^{-/-} mice fed the CDAA diet, while TNF-α production was not different between *Nrd1*^{+/+} and *Nrd1*^{-/-} mice fed the CDAA diet. Consistently, the production of various inflammatory cytokines were not increased in the livers of *Nrd1*^{-/-} mice. Although the precise mechanism of the decreased inflammatory responses in *Nrd1*^{-/-} mice was not clear, it appeared likely that the impaired release of TNF-α in *Nrd1*^{-/-} mouse livers was one of the reasons for the reduced inflammatory reactions in *Nrd1*^{-/-} mice. As well, impaired recruitment of macrophages into the liver may also contribute to the reduced inflammatory reactions in *Nrd1*^{-/-} mice. It would be also possible that different activation status of TNF-α and

inflammatory responses conversely affect difference of fatty contents between *Nrd1*^{+/+} and *Nrd1*^{-/-} mice. Whatever the case, nardilysin seemed to play an important role in the development of steatohepatitis and liver fibrosis presumably through TNF-α activation.

Previous studies have shown that Kupffer cells and recruited macrophages interact with hepatic stellate cells, accelerate their activation, and promote the fibrogenic responses [4,17]. Activated myofibroblasts also promote the remodeling of the extracellular matrix and contribute to liver fibrosis [5]. Indeed, our immunohistochemical analyses showed that Kupffer cells and macrophages were major producers of TNF-α in the livers of mice fed the CDAA diet, and that αSMA-positive myofibroblasts were not prominent in *Nrd1*^{-/-} mice. Decreased release of TNF-α from Kupffer cells and recruited macrophages could be one of the mechanisms for the suppression of diet-induced steatohepatitis in *Nrd1*^{-/-} mice, and thus nardilysin in Kupffer cells and recruited macrophages may be required for the progression of NASH and liver fibrosis, concomitantly with the recruitment of myofibroblasts. However, we could not completely exclude the possible

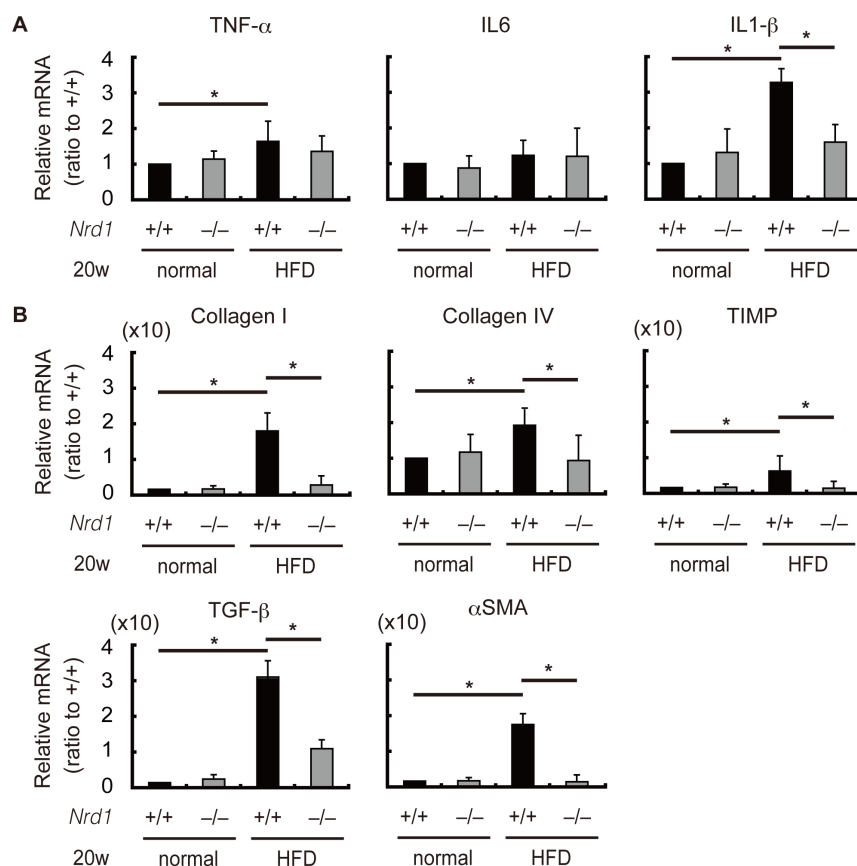


Figure 9. Inflammatory and fibrogenic factors were not increased in *Nrd1*^{-/-} mice fed the HFD. A. mRNA of TNF-α was slightly increased in both *Nrd1*^{+/+} (significantly) and *Nrd1*^{-/-} mice. In contrast to *Nrd1*^{+/+} mice, the mRNA expression level of IL1-β was not increased in *Nrd1*^{-/-} mice. **P*<0.05. B. mRNA expression levels of collagen I, collagen IV, TIMP, TGF-β, and αSMA in the livers of *Nrd1*^{+/+} mice fed a HFD for 20 weeks were significantly higher than the respective values of *Nrd1*^{+/+} mice fed the control diet. However, they were not altered by HFD in *Nrd1*^{-/-} mice. **P*<0.05. doi:10.1371/journal.pone.0098017.g009

contribution of nardilysin in other cells such as hepatocytes or endothelial cells for the development of NASH and liver fibrosis. Therefore, genetically-engineered mice lacking or strongly expressing nardilysin in Kupffer cells and macrophages may be required to confirm our hypothesis in future studies.

In summary, the present study indicates that nardilysin contributes to the development of diet-induced NASH and liver fibrotic changes by regulating chronic liver inflammation.

Nardilysin could be an attractive target for anti-inflammatory therapy against NASH and liver fibrosis.

Author Contributions

Conceived and designed the experiments: SIH HS. Performed the experiments: SIH YM KI MT Yoshito Kimura YT Yuto Kimura YN KK. Analyzed the data: SIH HS KK. Contributed reagents/materials/analysis tools: EN HK. Wrote the paper: SIH HS TC.

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SCIENTIFIC REPORTS

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Nardilysin regulates inflammation, metaplasia, and tumors in murine stomach

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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.

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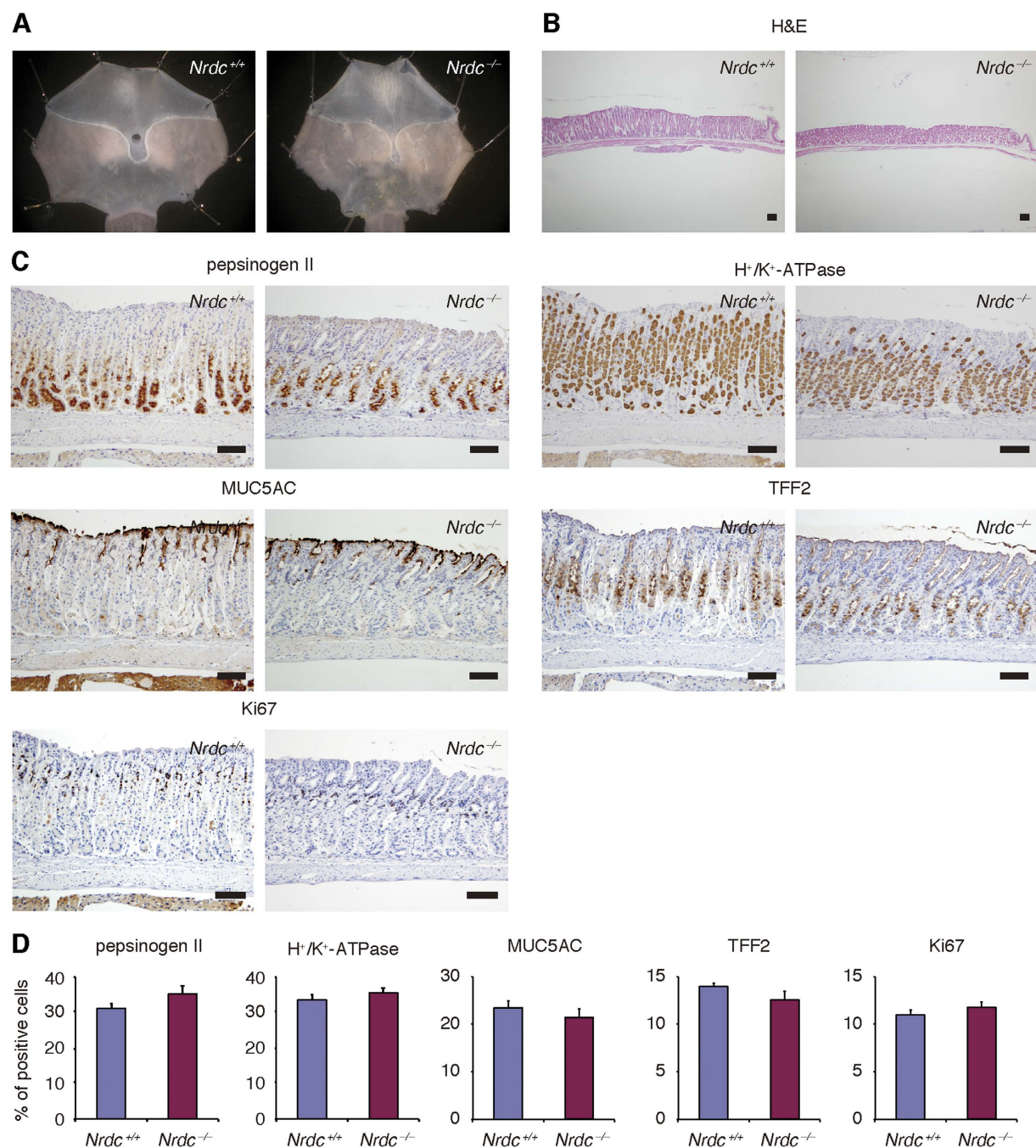


Figure 1. Stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice under physiological conditions. (A) Representative macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice. (B) H&E staining of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs. Bars = 100 μ m. (C) Immunohistochemistry for pepsinogen 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

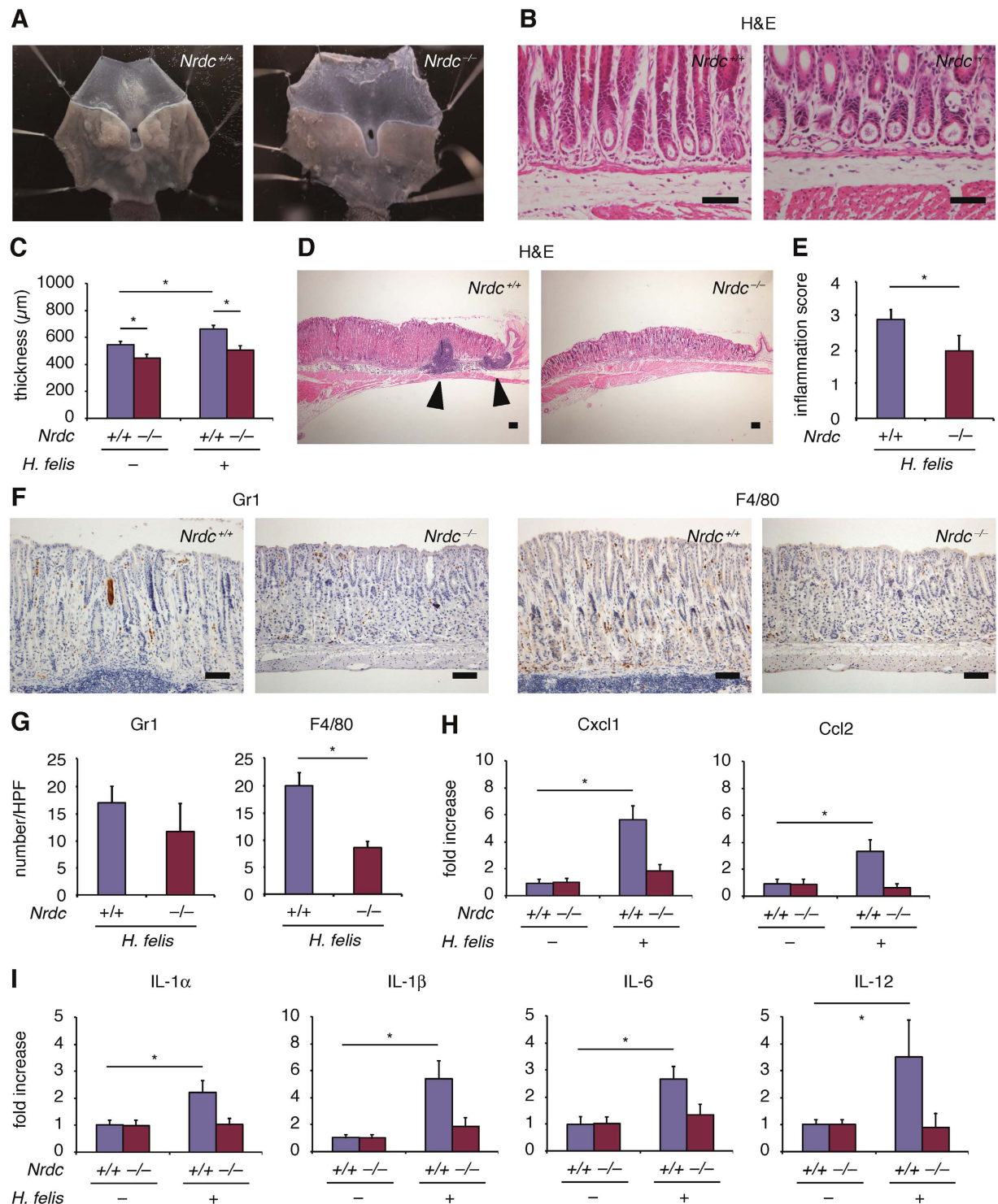


Figure 2. Stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice infected with *Helicobacter felis*. (A) Representative macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice infected with *Helicobacter felis*. (B) Detection of *Helicobacter felis* in the mucosae of *Nrdc*^{+/+} and *Nrdc*^{-/-} 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.

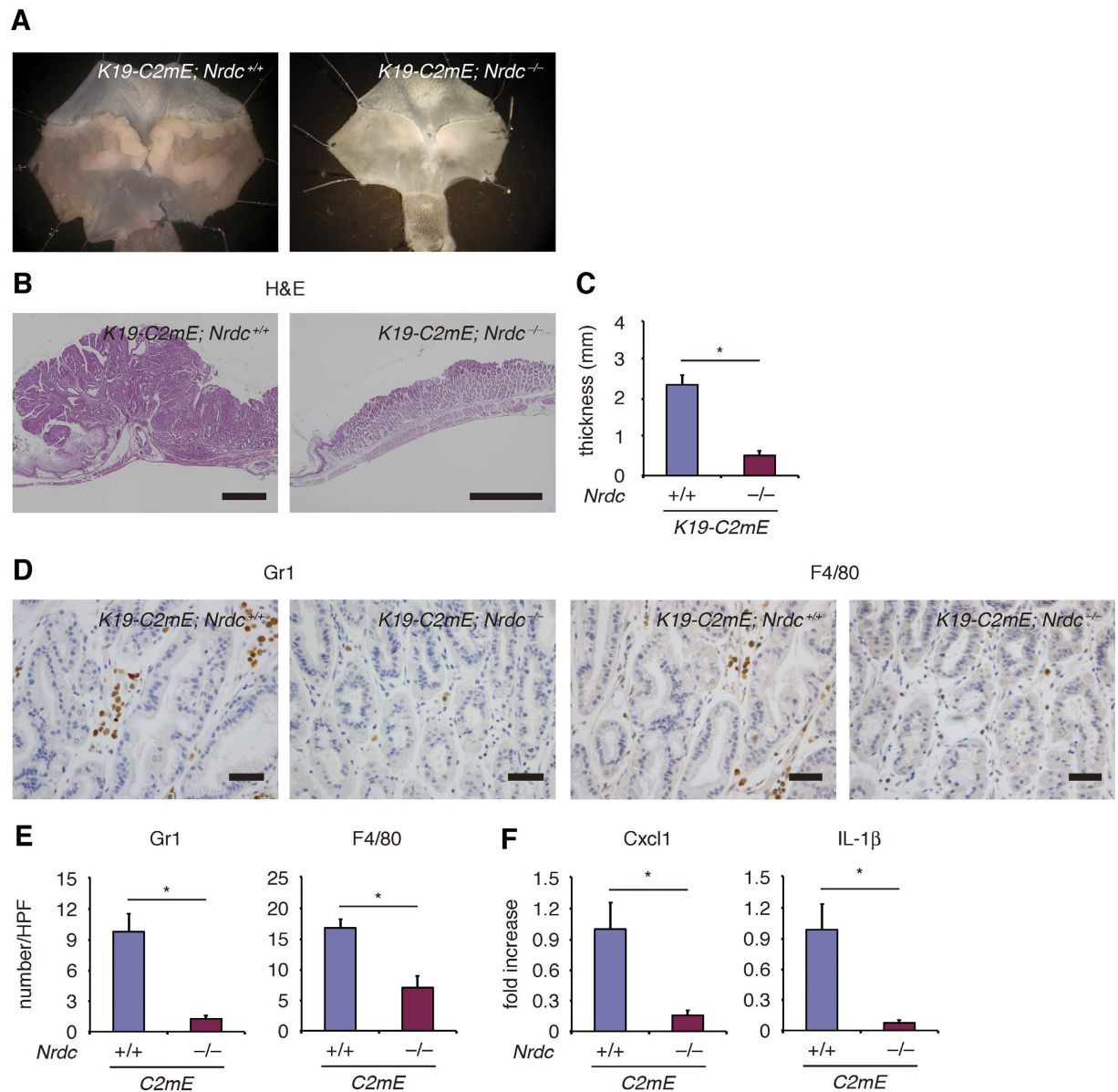


Figure 3. Gastritis caused by forced expression of prostaglandin E₂. (A) Representative macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice with forced expression of PGE₂ by the insertion of *K19-C2mE* alleles. (B) H&E staining of *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs with 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.

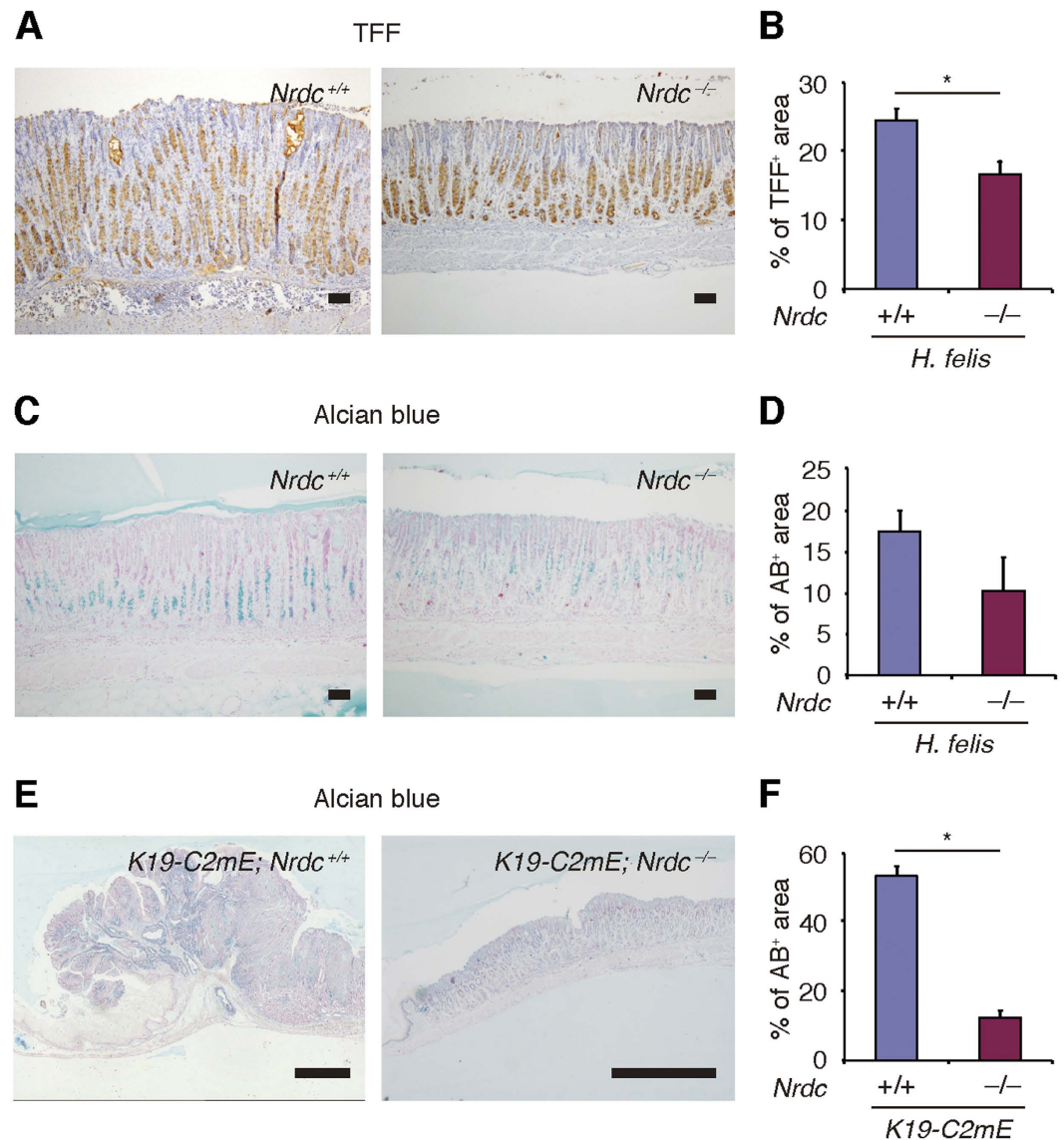


Figure 4. Metaplastic changes in *Nr4c*^{+/+} and *Nr4c*^{-/-} mouse stomachs. (A) Immunohistochemistry for TFF2 in *Nr4c*^{+/+} and *Nr4c*^{-/-} mouse stomachs with *Helicobacter felis* infection. Bars = 100 μ m. (B) Areas stained for TFF2 in *Nr4c*^{+/+} and *Nr4c*^{-/-} mouse stomachs with *Helicobacter felis* infection. **P* < 0.05. (C) Alcian blue staining of *Nr4c*^{+/+} and *Nr4c*^{-/-} mouse stomachs with *Helicobacter felis* infection. Bars = 100 μ m. (D) Areas stained with Alcian blue in *Nr4c*^{+/+} and *Nr4c*^{-/-} mouse stomachs with *Helicobacter felis* infection. **P* < 0.05. (E) Alcian blue staining of *Nr4c*^{+/+} and *Nr4c*^{-/-} mouse stomachs with PGE₂ expression. Bars = 1000 μ m. (F) Areas stained with Alcian blue in *Nr4c*^{+/+} and *Nr4c*^{-/-} 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 *Nr4c*^{-/-} than in *Nr4c*^{+/+} mice, and the inflammation score was less severe in *Nr4c*^{-/-} mice. Infiltration of neutrophils and macrophages was decreased in *Nr4c*^{-/-} 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 *Nr4c*^{-/-} mice. Thus, the deletion of *Nr4c* attenuates gastric inflammation, indicating that *Nr4c* also plays a role in the intravital stomach. It is also important that the deletion of *Nr4c* attenuated inflammation caused by the forced expression of PGE₂. This indicated that the deletion of *Nr4c* attenuates gastric inflammation regardless of the cause of inflammation, and a global therapeutic approach may be possible by targeting *Nr4c*.

We next questioned whether *Nr4c* 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 *Nr4c*. More importantly, gastric

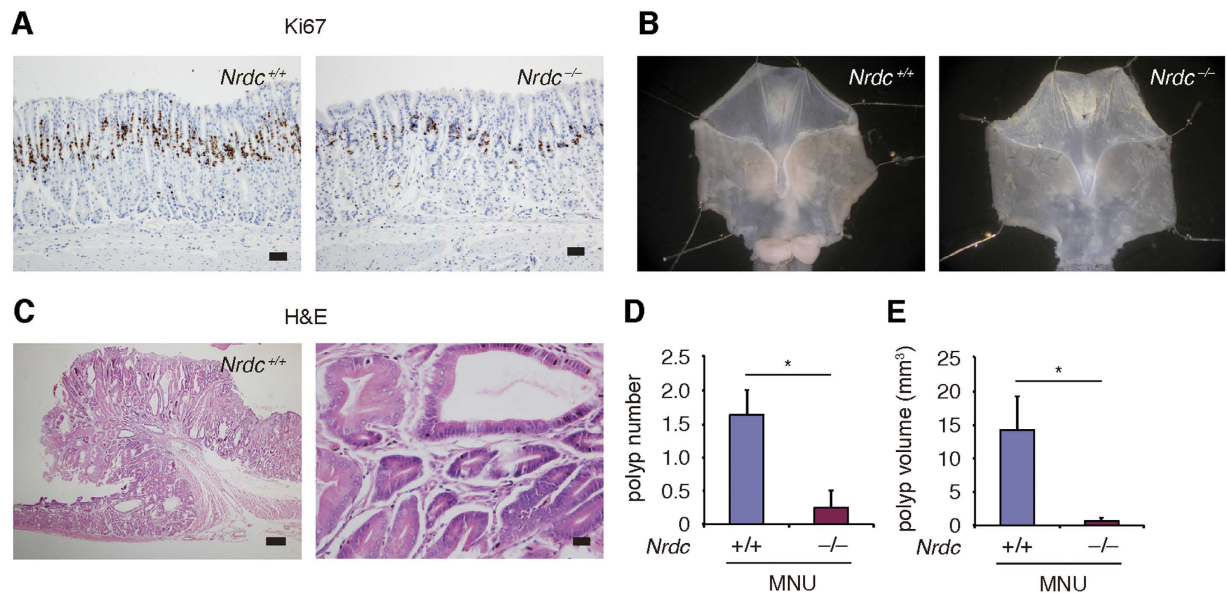


Figure 5. Formation of gastric tumors in *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs. (A) Ki67 immunostaining in *Nrdc*^{+/+} and *Nrdc*^{-/-} mouse stomachs with the forced PGE₂ expression. Bars = 50 μ m. (B) Macroscopic views of the stomachs of *Nrdc*^{+/+} and *Nrdc*^{-/-} mice treated with N-methyl-N-nitrosourea (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, TGGTGCTGAGATAGTGTGTC; 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, CTGGGATTACCTCAAGAACATC; Cxcl1-reverse, CAGGGTCAAGGCAAGCCTC; Ccl2-forward, ATCCACGGGCATACTATCAACATC; Ccl2-reverse, CAAGGCTCACCATCATCGTAG; Gapdh-forward, AGGTCGGTGTGAACGGATTTC; 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.

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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

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