

## Interleukin-1 $\beta$ induced by *Helicobacter pylori* infection enhances mouse gastric carcinogenesis



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### ABSTRACT

Interleukin-1 $\beta$  (*Il1b*) is considered to be involved in *Helicobacter pylori* (*HP*)-induced human gastric carcinogenesis, while the role of its polymorphisms in gastric cancer susceptibility remains controversial. Here, we aimed to clarify the role of *HP* infection-induced IL1B in gastric inflammation and carcinogenesis using *Il1b*<sup>-/-</sup> (*Il1b*-null) mice. In gastric mucosa of the *Il1b*<sup>+/+</sup> (WT) mice, *HP* infection induced *Il1b* expression and severe inflammation. In contrast, in *Il1b*-null mice, recruitment of neutrophils and macrophages by *HP* infection was markedly suppressed. In a carcinogenicity test, the multiplicity of gastric tumors was significantly suppressed in the *Il1b*-null mice (58% of WT;  $P < 0.005$ ). Mechanistically, *HP* infection induced NF- $\kappa$ B activation both in the inflammatory and epithelial cells in gastric mucosae, and the activation was attenuated in the *Il1b*-null mice. Accordingly, increased proliferation and decreased apoptosis of gastric epithelial cells induced by *HP* infection in the WT mice were attenuated in the *Il1b*-null mice. These results demonstrated that the IL1B physiologically induced by *HP* infection enhanced gastric carcinogenesis by affecting both inflammatory and epithelial cells.

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### 1. Introduction

Gastric cancer is the third leading cause of cancer death in the world [1], and chronic inflammation triggered by *Helicobacter pylori* (*HP*) infection is deeply involved in its development [2–4]. At the same time, only a minority (~3%) of *HP*-infected individuals develops gastric cancer after many years [5,6]. Whether a person with *HP* infection develops gastric cancer or not appears to be controlled by several factors, such as infection period, the type of *HP* [7], and host genetic factors regulating proinflammatory cytokines [8–12]. Among the host genetic factors, polymorphisms of interleukin-1B (*Il1b*) [8], interleukin-8 [13] and tumor necrosis factor- $\alpha$  [14], are reported to be associated with the risk of developing gastric cancers.

*Il1b* polymorphisms especially have attracted a considerable amount of attention [8,15,16]. Carriers of *Il1b*-511T and *Il1b*-

31C alleles, which have been shown to enhance IL1B production in the presence of *HP* infection [8,17,18], are considered to have a high risk of gastric cancer [8,19–21]. On the other hand, other epidemiological studies showed no association between *Il1b* polymorphisms and the risk of gastric cancer [22–25]. It is considered that the presence of confounders, such as differences in the infection period and genetic background, in human studies led to apparently discrepant epidemiological results [26]. Thus, the influence of *Il1b* polymorphisms that increase its expression in the presence of *HP* infection on gastric carcinogenesis is still unclear.

To minimize the effect of such confounders, an animal model has advantages because differences in environmental factors and genetic background can be controlled. Tu et al. constructed a transgenic mouse overexpressing human IL1B protein by the H/K-ATPase  $\beta$  subunit gene promoter, and IL1B overexpression in parietal cells clearly led to development of spontaneous gastric cancers [27]. However, whether IL1B physiologically induced by *HP* infection affects gastric carcinogenesis remains unclear. For this purpose, a mouse model, in which gastric cancers are induced by *N*-methyl-*N*-nitrosourea (MNU), a direct-acting alkylating agent, is useful [28]. Oral administration of MNU induces

Abbreviations: GC, gastric cancer; *HP*, *Helicobacter pylori*; IHC, immunohistochemistry; MNU, *N*-methyl-*N*-nitrosourea; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

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gastric cancers in mice without affecting the expression of IL1B [29–32], and the cancer induction is promoted by *HP* infection [29].

In this study, we aimed to clarify the role of IL1B physiologically induced by *HP* infection in gastric inflammation and carcinogenesis. To this end, we used *Il1b*-null mice [33], and analyzed the incidence of gastric cancer and the process of gastric carcinogenesis in the presence or absence of IL1B.

## 2. Materials and methods

### 2.1. Mice and sample preparation

*Il1b*<sup>+/+</sup> BALB/c (wild-type; WT) mice (Charles River Laboratories, Yokohama, Japan) and *Il1b*<sup>-/-</sup> BALB/c (*Il1b*-null) mice [33] were divided into three groups: *HP*-infected, *HP*-infected and *N*-methyl-*N*-nitrosourea (MNU)-treated, and a non-treated group (Figs. 1A, 2A, 3A and 4A). Mice were inoculated with the SS1 strain of *HP* ( $\sim 1.0 \times 10^8$  CFU/mouse) [34,35] at 5 weeks of age. In the MNU-treated group, 120 ppm of MNU (Sigma–Aldrich, St. Louis, MO) was given in drinking water in alternate weeks. The timing and duration of the treatment are shown in Figs. 1A, 2A, 3A, and 4A.

The stomach was resected, cut open along the greater curvature, and recorded by photography for tumor counting. Tumors whose area was larger than 2.0 mm<sup>2</sup> were counted by ImageJ software [36]. The antral region obtained from one half of the stomach was stored in RNAlater (Life Technologies, Tokyo, Japan)

for RNA and DNA extraction, and the other half was fixed in formalin or freshly embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) for histological analysis. RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) [37–39]. DNA was extracted by proteinase K digestion and the phenol/chloroform method. All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

### 2.2. Histological analysis

Formalin-fixed tissues were sliced along the longitudinal axis into strips of 5-mm width and embedded in paraffin. One side of each strip was histologically analyzed. For immunohistochemistry using formalin-fixed samples, sections of 3- $\mu$ m thickness were rehydrated and incubated in 10 mM citrate buffer (pH 6) at 120 °C for 5 min to unmask the antigen. After treatment with 1% H<sub>2</sub>O<sub>2</sub> followed by blocking with Blocking-One (Nacalai Tesque, Kyoto, Japan), sections were incubated with a primary antibody overnight. As the primary antibodies, we used anti-mouse IL1B (AF-401-NA, R&D Systems, Minneapolis, MN), anti-Ki67 (RM-9106-S0, Thermo Scientific Japan, Kanagawa, Japan), anti-single stranded DNA (A4506, Dako Cytomation, Kyoto, Japan), and anti-p65 (4764, Cell Signaling Technology Japan, Tokyo, Japan) antibodies. After washing the sections with PBS, they were incubated with a peroxidase-conjugated secondary antibody (Histofine Simple Stain Max PO, Nichirei, Tokyo, Japan) and visualized with diaminobenzidine (Wako Chemicals, Tokyo, Japan). Hematoxylin was used for counterstaining. Images covering all the areas of the sections were captured by TissueFAXS software (TissueGnostics, Vienna, Austria). By using HistoQuest software (TissueGnostics), the labeling index of IL1B was calculated as the ratio of positive cells to an area of the section, and that of Ki67, single-stranded DNA and p65 staining was calculated as the percentage of the positive cells relative to the total number of cells analyzed.

For immunohistochemistry using frozen samples, sections of 5- $\mu$ m thickness were prepared from O.C.T.-embedded samples. The sections were fixed in acetone at -20 °C for 5 min and rehydrated with PBS. An Alexa Fluor 488-labeled anti-Ly6G (Gr-1) antibody (53-5931-80, eBioscience, San Diego, CA, USA), an Alexa Fluor 488-labeled anti-CD3 antibody (100321, BioLegend, San Diego, CA, USA), an anti-F4/80 antibody (14-4801-81, eBioscience, San Diego, CA, USA), a PE-labeled anti-EpCAM antibody (12-5791-82, eBioscience), a FITC-labeled anti-CD45 antibody (130-091-609, Milteny Biotec, Tokyo, Japan) and an anti-p65 antibody were applied to the sections and incubated overnight. To visualize the immunoreactivity of the primary antibodies without fluorescent dye, an anti-rat IgG-Alexa Fluor 594 antibody (A11007, Life Technology, Carlsbad, CA, USA), an anti-rabbit IgG (H + L)-Alexa Fluor 488 antibody (A11034, Life Technologies), and an anti-rabbit IgG Alexa 594 antibody (A11037, Life Technologies) were used as secondary antibodies. Six images per stomach were captured by the BZ-9000 microscope system (Keyence, Osaka, Japan) with a 20 $\times$  object lens. The labeling index of Ly6G and CD3 staining was calculated as the ratio of the positive cells relative to the total analyzed area, and that of F4/80 staining was calculated as the ratio of the positive area relative to the total analyzed area, as in previous studies [40,41].

### 2.3. Quantitative reverse-transcription PCR (qRT-PCR)

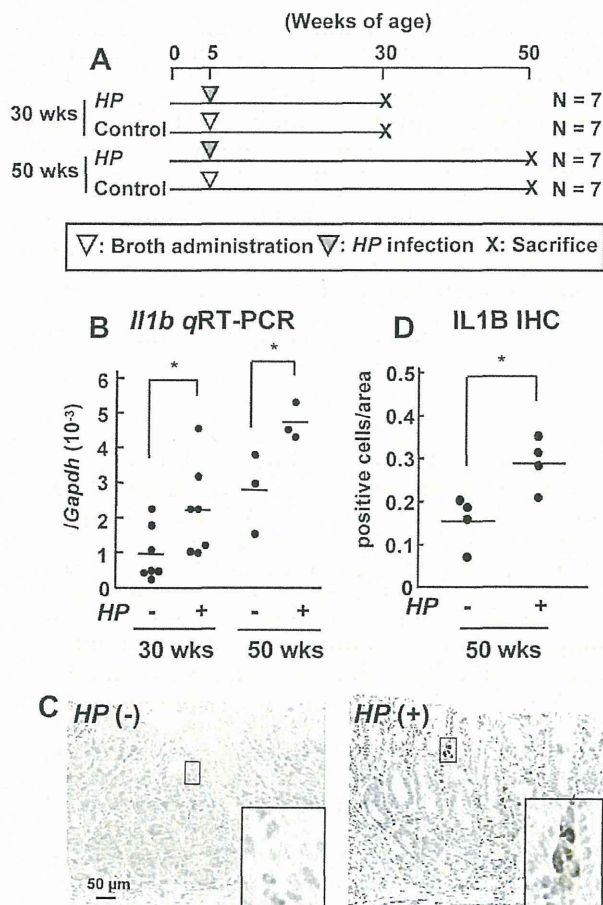
Complementary DNA (cDNA) was synthesized from 2  $\mu$ g of DNase-treated total RNA using a Superscript III kit (Life Technologies) with an oligo-d(T)<sub>12-18</sub> primer. Real-time PCR was performed using the IQ5 Real-time PCR Detection System (Bio-Rad, Tokyo, Japan) with an *Il1b*-specific primers (5'-TCACAGCAGCAC ATCAACAAG-3' and 5'-CATGTCTCTCATCTGGAAG-3') and *Gapdh*-specific primers (5'-CTGAACGGGAAGCTCACTGG-3' and 5'-ATGCTGCTTCAACCACTTC-3'). The number of cDNA molecules was obtained by comparing the amplification curves of a sample to those of standard samples with known copy numbers of template DNA. The copy number of *Il1b* was normalized to that of *Gapdh*.

### 2.4. Detection and measurement of HP

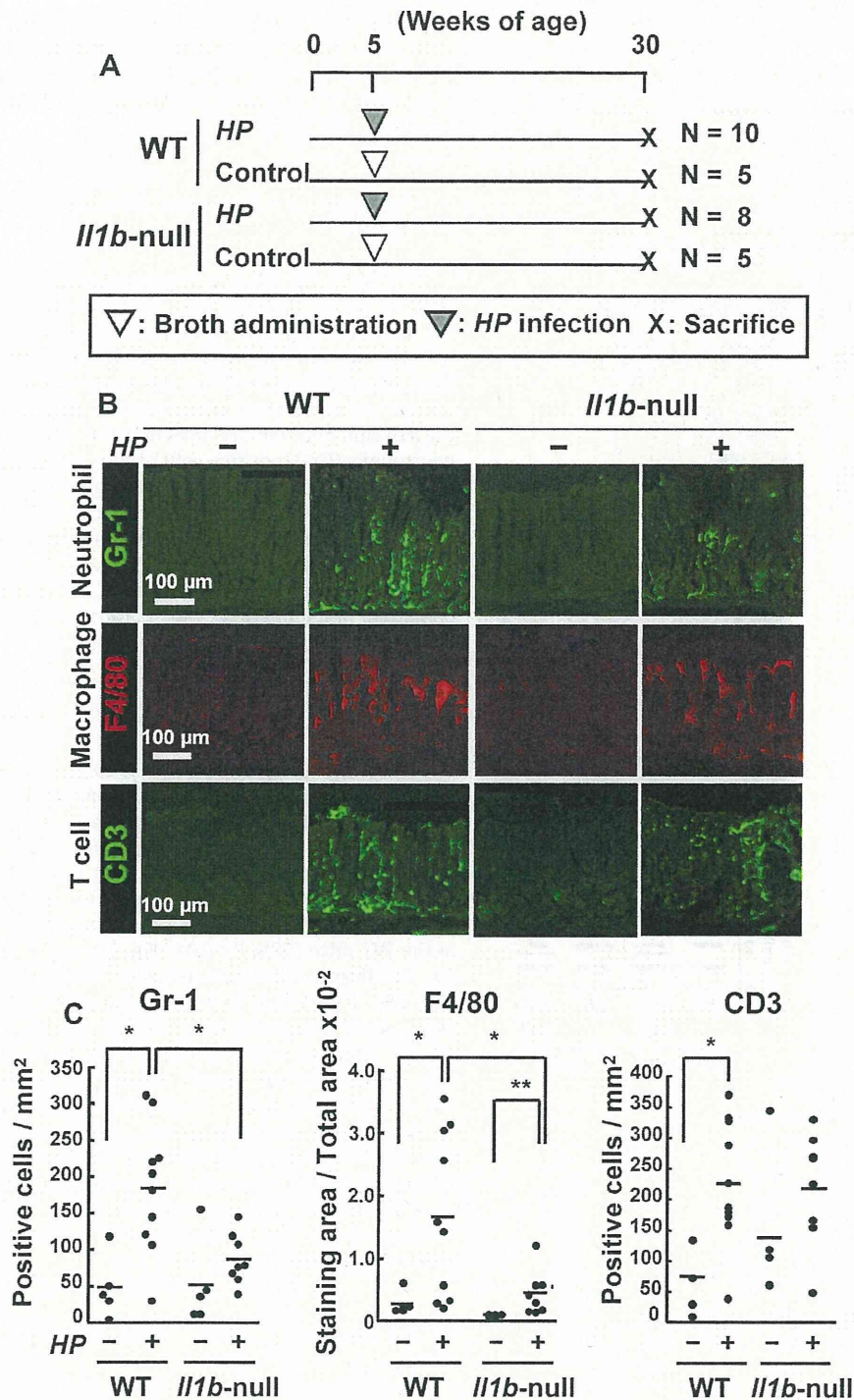
With 30 ng of genomic DNA extracted from the gastric antral region, real-time PCR was performed using primers specific to the *jhpr3* gene of *HP* (5'-AAGGATACTGCCTCCGTAAG-3' and 5'-ACAATCCGAAGTCACTGAGAGGTTG-3') and the *Il1a* gene of the mouse (5'-GATGCAAGCTATGGCTCACTTC-3' and 5'-GACGTGTGATACGTCCACCC-3'). The copy number of *jhpr3* was normalized to that of *Il1a*.

### 2.5. Statistical analysis

Statistical analyses were conducted with PASW statistics version 18.0.0 (IBM, Tokyo, Japan). Differences of the *Il1b* gene expression, the number of positive cells in immunohistochemical analyses, the amount of water with MNU, tumor area and tumor multiplicity, and body weight of the mice between two independent groups of sample data were evaluated by the Mann–Whitney *U* test. Tumor incidence was evaluated by Fisher's exact test. *p* values of 0.05 or less were considered statistically significant.



**Fig. 1.** Induction of IL1B by *HP* infection in the mouse stomach. (A) Experimental protocol of *HP* infection. (B) Expression level of *Il1b* mRNA analyzed by qRT-PCR in the stomach. (C) Representative IHC for IL1B in the stomach at 50 weeks of age. IL1B-positive cells localized mostly in the lamina propria, and no epithelial cell expressed IL1B. In the left panel, no positive cells were observed. (D) Quantification of the number of IL1B-positive cells in the stomach at 50 weeks of age. The number was larger with *HP* infection than without. Horizontal bar indicates the mean value. \*, *p* < 0.05.



**Fig. 2.** Suppression of infiltration of inflammatory cells in gastric mucosae by HP infection in *Il1b*-null mice. (A) Experimental protocol of HP infection in WT and *Il1b*-null mice. (B) Representative IHC for Gr-1 (green), F4/80 (red) and CD3 (green) in mouse gastric tissues. (C) Quantification of IHC. The numbers of infiltrating neutrophils and macrophages in *Il1b*-null mice were smaller than in WT mice. As for T cells, there was no significant difference between the two groups. Horizontal bar indicates the average. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

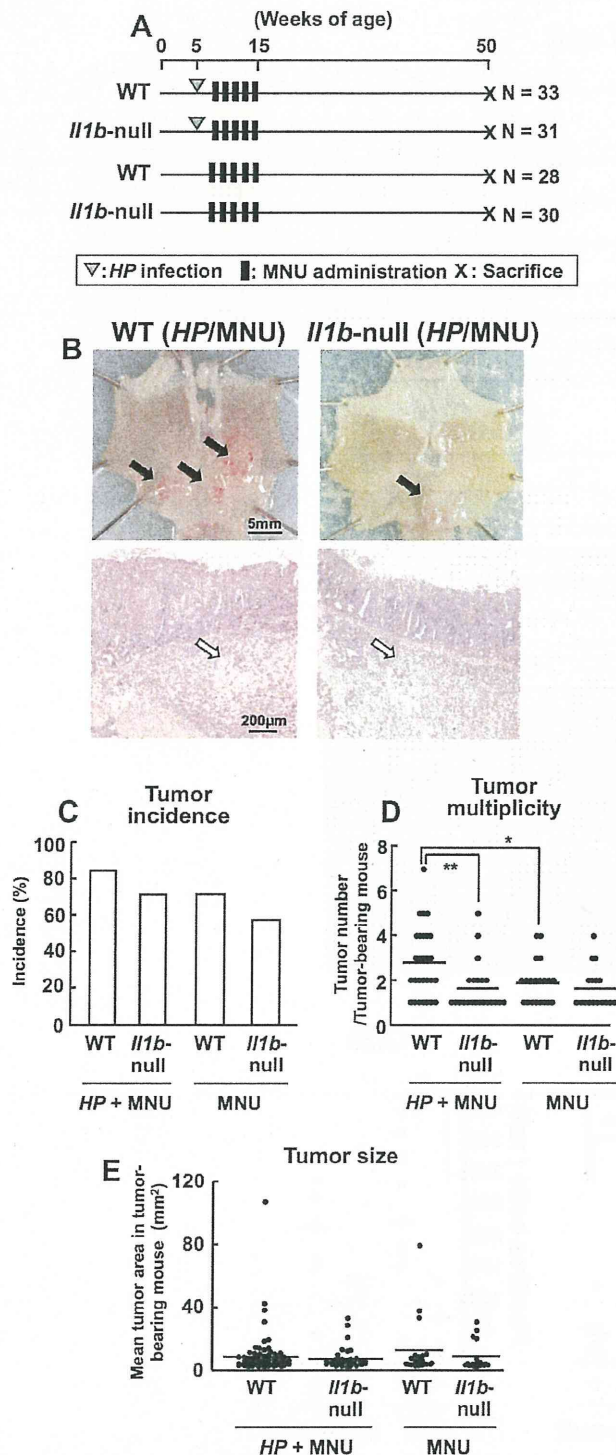
### 3. Results

#### 3.1. Induction of IL1B by HP infection in the mouse stomach

To confirm that IL1B is induced by HP infection in the mouse stomach, expression of *Il1b* mRNA was analyzed in the WT mice

(Fig. 1A). Expression levels of the *Il1b* mRNA were 2.3- and 1.7-fold higher at 30 and 50 weeks of age, respectively, in the HP-infected mice than in non-infected mice ( $p < 0.05$ ; Fig. 1B). Immunohistochemistry (IHC) of IL1B protein at 50 weeks revealed that inflammatory cells infiltrating the gastric mucosa expressed IL1B (Fig. 1C), and the number of IL1B-positive cells was 1.9-fold larger





**Fig. 3.** Suppression of MNU/HP-induced gastric tumors in *Il1b*-null mice. (A) Experimental protocol of gastric carcinogenicity test. *HP* was inoculated at 5 weeks of age. MNU (120 ppm) was administered in alternate weeks for a total of 5 weeks from 6 weeks of age. (B) Representative macroscopic view and histological sections of gastric tumors. Black arrows show the tumors. White arrows show invasion of the tumor cells in the submucosa and muscularis. (C) Tumor incidence. There was no significant difference between WT and *Il1b*-null mice. (D) The number of gastric tumors in tumor-bearing mice. Among *HP*-infected and MNU-treated groups, the number in the *Il1b*-null mice was smaller than that in WT mice. Among the WT mice, the number in *HP*-infected and MNU-treated mice was larger than that in only MNU-treated mice. (E) The size of gastric tumors. There was no significant difference between the WT and *Il1b*-null mice. A horizontal bar shows the mean value. \*\*,  $p < 0.01$ . \*,  $p < 0.05$ .

than that in the non-infected mice ( $p < 0.05$ ; Fig. 1D). No gastric tumors were observed at 30 or 50 weeks. These results confirmed that *HP* infection induced IL1B expression in the mouse stomach, and inflammatory cells were the source of the IL1B, as reported [42–44].

### 3.2. Suppression of inflammatory cell infiltration in *HP*-infected *Il1b*-null mice

To analyze the role of IL1B physiologically induced by *HP* infection on gastric inflammation and carcinogenesis, we employed *Il1b*-null mice (Fig. 2A). Before analysis of the influence of the lack of IL1B, we confirmed that *HP* infected all the WT and *Il1b*-null mice inoculated with *HP* to the same degree by measuring the number of *HP* in their antral regions (Supplementary Fig. 1). In the *Il1b*-null mice with *HP* infection, the numbers of the infiltrating neutrophils (Gr-1-positive cells) and macrophages (F4/80-positive cells) were markedly decreased, being 46% and 26%, respectively, of those in the WT mice with *HP* infection (Fig. 2B and C). On the other hand, the number of the infiltrating T cells (CD3-positive cells) was similar between the WT and *Il1b*-null mice (Fig. 2B and C). These results indicated that IL1B induced by *HP* infection enhanced inflammation via recruitment of neutrophils and macrophages.

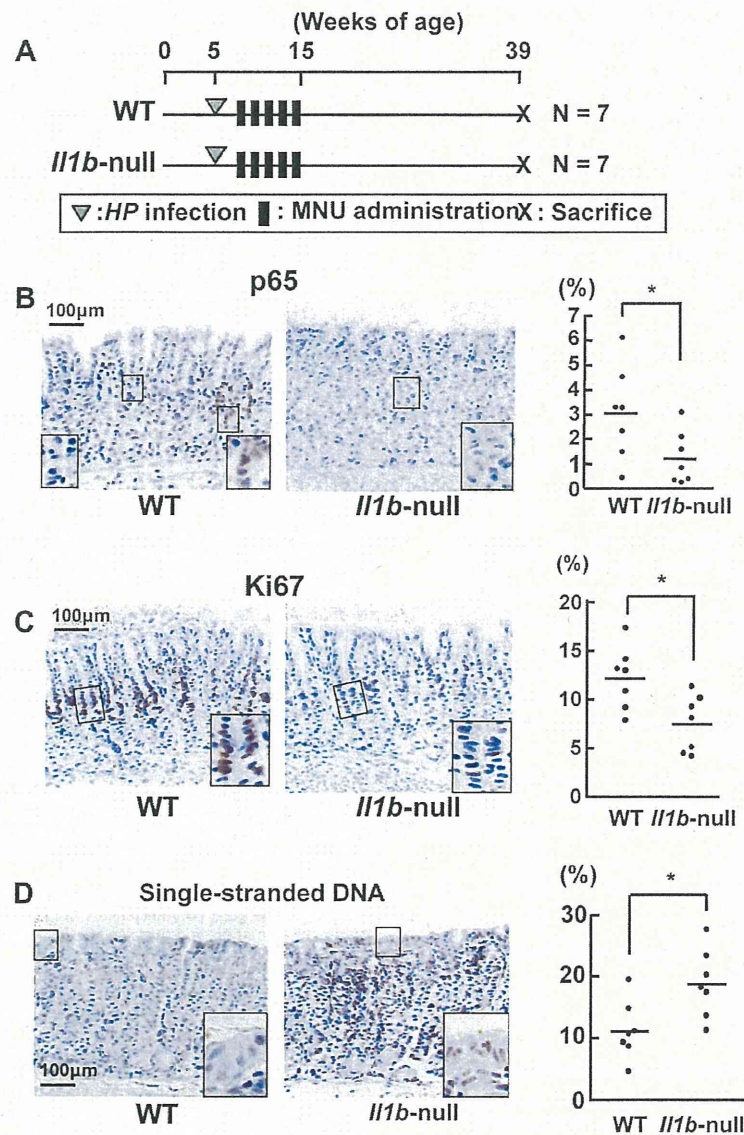
### 3.3. Suppression of gastric carcinogenesis in *Il1b*-null mice

A carcinogenicity test was then conducted using the WT and *Il1b*-null mice (Fig. 3A). *HP* infection and MNU treatment induced gastric tumors both in the *Il1b*-null and the WT mice (Fig. 3B). The tumor incidence in the *Il1b*-null mice (71%) tended to be lower compared with the WT mice (85%), but there was no statistically significant difference (Fig. 3C). However, the tumor multiplicity in the *Il1b*-null mice was significantly lower than that in the WT mice (58%,  $P < 0.01$ ; Fig. 3D) while tumor size showed no significant difference between the two groups of mice (Fig. 3E). To confirm that the difference in tumor multiplicity was dependent upon the *HP* infection, a carcinogenicity test without *HP* infection was also conducted. No significant difference was observed in the tumor multiplicity and size between the WT and *Il1b*-null mice. These data indicated that IL1B induced by *HP* infection enhanced gastric carcinogenesis. Increase of *Il1b* was confirmed in *HP*-infected and MNU-treated WT mice (Supplementary Fig. 2). Regarding the body weight change and intake of MNU-containing water, there was no significant difference between the WT and *Il1b*-null mice regardless of *HP* infection status (Supplementary Fig. 3).

### 3.4. Decrease of inflammatory and epithelial cells with NF- $\kappa$ B activation in the *Il1b*-null mice

The IL1B signal is mainly transduced by the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway [45]. To analyze the cell types receiving IL1B, immunohistochemical analysis of p65 (RelA), a component of NF- $\kappa$ B and the major transducer of IL1B signal [45], was conducted in the *HP*-infected mice with MNU treatment (Fig. 4A). p65 Protein was observed in the nucleus and cytoplasm of both infiltrating inflammatory and epithelial cells (Fig. 4B, left panel; Supplementary Fig. 4). The number of p65-positive cells in the *Il1b*-null mice was 40% of that in the WT mice (Fig. 4B, right panel). These results showed that the NF- $\kappa$ B signal was activated both in inflammatory cells and epithelial cells by *HP* infection, and the activation was attenuated in the *Il1b*-null mice.





**Fig. 4.** Activation of NF- $\kappa$ B signal (inflammatory and epithelial cells), increase of proliferation (Ki67; epithelial cells), and decrease of apoptosis (single-stranded DNA; epithelial cells) by *HP* infection, and their attenuation in the *Il1b*-null mice. (A) Experimental protocol. (B) Representative IHC of p65 (left), and its quantification (right). p65-positive cells were mostly localized in the lamina propria, and involved both in inflammatory and epithelial cells. The number of positive cells was smaller in *Il1b*-null mice than in WT mice. (C) Representative IHC for Ki67 (left), and its quantification (right). The number of positive cells was smaller in *Il1b*-null mice than in WT mice. (D) Representative IHC for single-stranded DNA (left), and its quantification (right). The number of positive cells was larger in *Il1b*-null mice than in WT mice. Horizontal bar shows the mean value. \*,  $p < 0.05$ .

### 3.5. Decreased proliferation and increased apoptosis in *Il1b*-null mice

The impact of the lack of IL1B on cell proliferation and apoptosis of epithelial cells was finally analyzed (Fig. 4A). Ki67 IHC showed that the number of proliferative cells in the *Il1b*-null mice was significantly smaller than that in the WT mice (58%,  $p < 0.05$ ; Fig. 4C). IHC of single-stranded DNA showed that the number of apoptotic cells in the *Il1b*-null mice was 1.7-fold larger than that in the WT mice (Fig. 4D). These results indicated that attenuated activation of the NF- $\kappa$ B pathway due to the lack of IL1B led to decreased proliferation and increased apoptosis of gastric epithelial cells.

## 4. Discussion

In this study, we demonstrated that the number of gastric tumors induced by *HP* was suppressed in *Il1b*-null mice. Our results

showed for the first time that the lack of IL1B decreased the number of gastric tumors, indicating that IL1B contributes to gastric carcinogenesis. This was in accordance with human epidemiological studies showing a positive association between increased IL1B production in gastric mucosa with *HP* infection and the high risk of gastric cancers [8,19–21,46]. The data here support that IL1B plays a crucial role in *HP*-induced gastric carcinogenesis.

In the carcinogenicity test, at 50 weeks of age, tumor multiplicity was smaller in the *Il1b*-null mice than in the WT mice, while tumor size was not different between the two groups. Based on the similar tumor size in the two genotypes, the timing of tumorigenesis was speculated to be similar between the genotypes. At the same time, based on the fewer number of tumors, the chance of initiation was speculated to be smaller in the *Il1b*-null mice. This suggested that the IL1B played an important role in the early stage of gastric carcinogenesis rather than in a late stage. In line with this



finding, at the age of 30 weeks (25 weeks after infection with *HP*), an increase of proliferation and a decrease of apoptosis of gastric epithelial cells were observed in the WT mice, and these reactions were attenuated in the *Il1b*-null mice. This indicated that IL1B induced by *HP* infection contributed to the increase of cell proliferation and the decrease of apoptosis of gastric epithelial cells. Mechanistically, we observed activation of NF- $\kappa$ B, the major downstream signal of IL1B [47,48], in gastric epithelial cells of *HP*-infected mice. Such activation of NF- $\kappa$ B is known to increase cell proliferation and suppress apoptosis in epithelial cells [49–52].

*Il1b*-null mice also showed attenuated infiltration of inflammatory cells in the stomach with *HP* infection compared with WT mice. Among the infiltrating inflammatory cells in *HP*-infected gastric mucosa, the *Il1b*-null mice showed a significant decrease in the number of neutrophils and macrophages compared with the WT mice. On the other hand, both groups showed a similar level of lymphocyte infiltration. Considering that IL1B does not directly affect lymphocyte functions, such as differentiation and expansion [53], the small effect of IL1B deficiency on the recruitment of lymphocytes into inflammatory lesions was reasonable.

IL1B, which is known to be secreted mainly by macrophages [53–55], enhanced the *HP*-triggered inflammation by activating NF- $\kappa$ B in the macrophages and the neutrophils. This suggested that the macrophages stimulate themselves through IL1B secretion in *HP*-infected gastric mucosa. The same phenomenon, that macrophages stimulate themselves through IL1B, has also been reported for some tumors [56–58]. Thus, it might in part explain the mechanisms by which IL1B intensifies the *HP*-triggered inflammation in gastric mucosae.

In conclusion, our study demonstrated that IL1B physiologically induced by *HP* infection enhanced the number of gastric tumors possibly by affecting an early stage of gastric carcinogenesis.

#### Conflict of interest

The authors have declared that no competing interests exist.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2013.07.034>.

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# Dependence receptor UNC5D mediates nerve growth factor depletion–induced neuroblastoma regression

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**Spontaneous regression of neuroblastoma (NB) resembles the developmentally regulated programmed cell death (PCD) of sympathetic neurons. Regressing tumor cells express high levels of the nerve growth factor (NGF) receptors TRKA and p75<sup>NTR</sup> and are dependent on NGF for survival; however, the underlying molecular mechanism remains elusive. Here, we show that UNC5D, a dependence receptor that is directly targeted by p53 family members, is highly expressed in favorable NBs. NGF withdrawal strongly upregulated UNC5D, E2F1, and p53 in human primary favorable NBs. The induced UNC5D was cleaved by caspases 2/3, and the released intracellular fragment translocated into the nucleus and interacted with E2F1 to selectively transactivate the proapoptotic target gene. The cleavage of UNC5D and its induction of apoptosis were strongly inhibited by addition of netrin-1. *Unc5d*<sup>-/-</sup> mice consistently exhibited a significant increase in dorsal root ganglia neurons and resistance to NGF depletion–induced apoptosis in sympathetic neurons compared with wild-type cells. Our data suggest that UNC5D forms a positive feedback loop with p53 and E2F1 to promote NGF dependence–mediated PCD during NB regression.**

## Introduction

Neuroblastoma (NB) is one of the most common solid tumors in children and arises from the sympathoadrenal lineage of the neural crest. The enigma of NB is that many tumors found in infants less than 1 year of age frequently regress spontaneously, even though the tumor metastasizes to the liver, skin, and/or bone marrow, designated as stage 4s (1). Accumulating evidence suggests that both genetic and epigenetic changes may affect the clinical behavior of NBs. However, the molecular and biological bases of NB spontaneous regression and aggressiveness remain elusive.

One of the breakthroughs for understanding how NB regresses was the discovery that both TRKA and p75<sup>NTR</sup>, high- and low-affinity receptors, respectively, for nerve growth factor (NGF), are expressed at significantly high levels in favorable NBs (2, 3), whereas TRKB and its ligands, brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT4/5), are highly expressed in aggressive NBs in an autocrine and/or paracrine manner (4). It has been hypothesized that the quantitative relationship between NGF and its receptor complexes within tumor tissue, as well as the acquisition of NGF dependence, may be crucial to inducing NB regression (5).

Recent progress in developmental neurobiology has shown that some important molecules, such as p53 (6), p63 (7), and E2F1 (8, 9), as well as the c-JUN/EGLN3/KIF1B $\beta$  pathway (10–12), are involved in the regulation or induction of NGF depletion–

induced programmed cell death (PCD) of sympathetic neurons. Recently, Bredesen and colleagues proposed a new “dependence receptor” concept, which was originally initiated from the idea of the NGF dependence of developing neuronal cells: some receptors display 2 completely opposing actions depending on the availability of their ligands (13). In the presence of their ligands, the receptors transduce a “positive” signal for differentiation, migration, or survival; conversely, those receptors conduct a “negative” signal to trigger apoptosis in the absence of any ligand (13). To date, a growing number of dependence receptors have been identified, including deleted in colorectal cancer (DCC; ref. 14), Ret (15), UNC5 (16, 17), Patched (18), neogenin (19), ALK (20), and integrins such as  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  (21).

To identify the genes that play a key role in both developmentally regulated neuronal PCD and spontaneous regression of NB, we previously generated cDNA libraries from primary NB tissues and identified UNC5D as one of the genes highly expressed in the favorable subset (22). UNC5D is the fourth member of the human dependence receptor UNC5 family (17) and shares the same ligand, netrin-1 (encoded by *NTN1*), with other UNC5 family members and the tumor suppressor DCC (23). The UNC5 family possesses the intracellular Zo-1–like (ZUS) domain and death domain (DD) as well as putative caspase cleavage sites (13). Previous *in vitro* and *in vivo* studies of the UNC5 mouse homologs UNC5A, UNC5B, and UNC5C suggest that they all function as proapoptotic receptors by receiving cleavage of the intracellular region by caspases, which is strongly inhibited by binding of netrin-1 (13, 16). Tanikawa et al. have shown that *UNC5B* is a direct transcriptional target

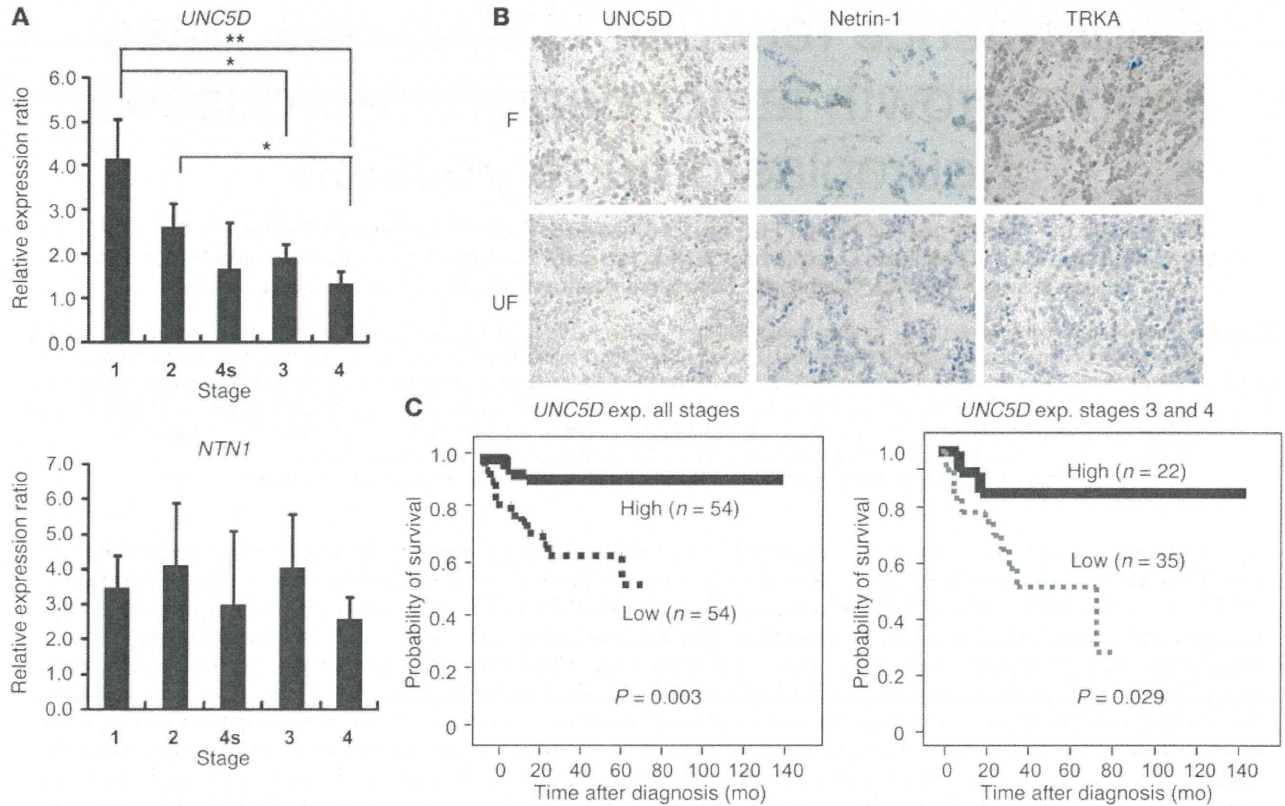
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research article



**Figure 1**

UNC5D is a novel favorable prognostic indicator for NB. (A) Expression of *UNC5D* and *NTN1* mRNA in 108 primary NBs detected by quantitative RT-PCR. Data represent mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ . (B) Representative images of *UNC5D*, netrin-1, and *TRKA* immunohistochemical analysis in favorable (F) and unfavorable (UF) NBs. Original magnification,  $\times 400$ . (C) Kaplan-Meier survival curves based on higher or lower expression levels of *UNC5D* mRNA in 108 patients and the unfavorable subset.

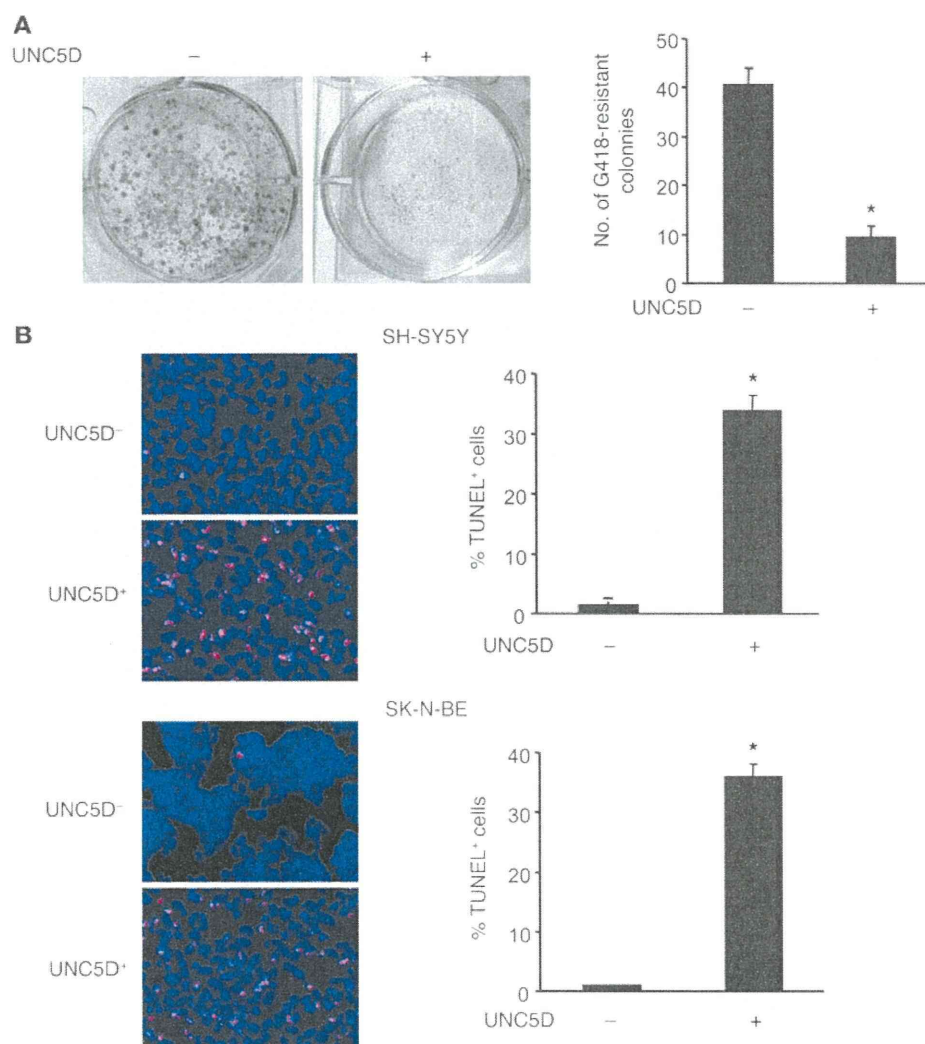
of the tumor suppressor p53 and mediates p53-induced cell death (24). We also previously found that *UNC5D* is directly targeted by p53 for its expression (17). In addition, knockout mice of *Unc5a*, *Unc5b*, and *Unc5c* have shown different phenotypes (25–27). However, the functional role of *UNC5D* remains unclear.

In the present study, we explored the functional role of *UNC5D* in NGF depletion-induced PCD using primary NB cells as well as *Unc5d*<sup>-/-</sup> mouse-derived neuronal cells. Our results showed that NGF depletion-inducible *UNC5D* formed a positive feedback loop with p53, E2F1, and caspases to accelerate NGF deficiency-mediated cell death.

**Results**

*High-level expression of UNC5D, but not netrin-1, is associated with favorable prognosis of NB.* *UNC5D* was selected from our NB cDNA project as a differentially expressed gene between favorable and unfavorable subsets (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI65988DS1). Quantitative RT-PCR analysis using 108 primary NBs revealed that expression levels of *UNC5D* mRNA were significantly higher in favorable NBs than unfavorable ones (Figure 1A). Immunohistochemical analysis also showed that, similar to *TRKA*, *UNC5D* was positive in tumor cells from favorable NBs with a tendency to regress spontaneously (Figure 1B, Supplemental Figure 2,

and Supplemental Table 1). Furthermore, high levels of *UNC5D* mRNA expression were significantly associated with a favorable outcome ( $P = 0.003$ ; Figure 1C and Supplemental Table 2). More interestingly, expression levels of *UNC5D* significantly segregated the patients with NB in advanced stages 3 and 4 into those with good and poor prognosis ( $P = 0.029$ ; Figure 1C). However, expression of *UNC5A* as well as *UNC5B* and *UNC5C* showed no prognostic significance in primary NBs (Supplemental Figure 1 and Supplemental Table 2). Multivariate analysis indicated that expression of *UNC5D* was a significant prognostic factor independent of age, stage, and *TrkA* expression and marginally of DNA ploidy, but not of *MYCN* amplification (Supplemental Table 3). Notably, expression of *NTN1*, a ligand of *DCC* and the *UNC5* family, was similar at the mRNA level among all stages of NB (Figure 1A). At protein levels, netrin-1 was barely positive in both favorable and unfavorable NB cells, except in some stromal cells (Figure 1B and Supplemental Figure 2). Thus, netrin-1 appeared to be relatively deficient within the NB tissues, which suggested that the expression pattern of *UNC5D*/netrin-1 was very similar to that of *TRKA*/NGF in primary NBs (2). Furthermore, enforced expression of *UNC5D* led to a decrease in the number of G418-resistant colonies and induced apoptotic cell death in several NB cell lines (Figure 2), which suggests that *UNC5D* may play a proapoptotic role in NB.



**Figure 2**

UNC5D inhibits cell growth and induces apoptosis in NB cell lines. (A) Colony formation assay for SK-N-BE cells transfected with the indicated plasmids. 48 hours after transfection, cells were transferred to fresh medium containing G418 (400  $\mu$ g/ml) for 2 weeks. G418-resistant colonies were stained with Giemsa solution and photographed. Data represent mean  $\pm$  SD of 2 independent experiments. \* $P < 0.01$ . (B) Apoptosis was evaluated by TUNEL assay in SH-SY5Y and SK-N-BE cells transfected with the indicated plasmids for 48 hours. Data represent mean  $\pm$  SD of 3 independent experiments. \* $P < 0.01$ . Original magnification,  $\times 100$ .

*UNC5D* is induced during NGF withdrawal-triggered apoptosis. We previously hypothesized that, similar to the physiological PCD that occurs in developing sympathetic neurons, the NGF dependence of NB cells could be one of the main causes of NB spontaneous regression (2, 5). We therefore examined the expression of the UNC5 family before and after depletion of NGF in favorable NB cells in primary culture. NGF treatment enhanced cell survival and induced neurite outgrowth in NB cells prepared from 2 tumors with favorable biology (case 1, 9 months old, stage 1, single copy of *MYCN*, high TRKA expression, and aneuploidy; case 2, 18 months old, stage 3, single copy of *MYCN*, high TRKA expression, and aneuploidy; Figure 3, B and E). Subsequent withdrawal of NGF triggered apoptotic cell death (Figure 3, A and C), which was significantly inhibited by addition of netrin-1 (Figure 3, D and E). Of interest, only *UNC5D* among the *UNC5* family members was strongly induced – along with *p53*, *E2F1*, and *BAX*, which are already known to be induced in response to NGF depletion (6, 9, 28) in NGF-deprived cells – at both the mRNA and the protein level (Figure 3, B and C), whereas expression of *NTN1* was consistently almost undetectable. It was notable that the induced *UNC5D* protein appeared to be mainly localized in the nuclei of

NB cells, as observed using the antibody recognizing the C terminus of the protein (Figure 3C). Another netrin-1 receptor, *DCC*, was weakly induced in case 1 tumor cells on day 6 after NGF treatment, but was not further induced after NGF deprivation (Figure 3B), which suggests that *DCC* is more likely to be involved in NGF-induced NB differentiation.

To test the *in vivo* function of *UNC5D*, we generated mice with knockout of *Unc5d*, a mouse homolog of human *UNC5D* (S. Haraguchi and A. Nakagawara, unpublished observations). The primary sympathetic neurons were isolated from superior cervical ganglia (SCG) of P0 *Unc5d*<sup>-/-</sup> or wild-type mice. NGF treatment induced neurite outgrowth of primary sympathetic neurons obtained from both wild-type and *Unc5d*<sup>-/-</sup> mice to a similar extent. However, after NGF depletion, neuronal death was attenuated in *Unc5d*<sup>-/-</sup> versus wild-type sympathetic neurons (Figure 3F). Consistently, the number of neuronal cells isolated from the dorsal root ganglia (DRG) of *Unc5d*<sup>-/-</sup> mice was significantly higher than that from wild-type mice (Table 1).

Because most NB-derived cell lines cannot respond to NGF treatment owing to defects in their NGF/TRKA signaling pathway, we used rat PC12 pheochromocytoma cells, a model sys-