Downregulation of Notch Signaling in Kras-Induced Gastric Metaplasia

Wen-Cheng Chung*, Yunyun Zhou†, Azeddine Atfi‡ and Keli Xu*,§

*Cancer Institute, University of Mississippi Medical Center, Jackson, MS, USA;†Department of Data Science, University of Mississippi Medical Center, Jackson, MS, USA;‡Cellular and Molecular Pathogenesis Division, Department of Pathology, Virginia Commonwealth University, Richmond, VA, USA;§Department of Neurobiology and Anatomical Sciences, University of Mississippi Medical Center, Jackson, MS, USA

Abstract
Activating mutations and amplification of Kras and, more frequently, signatures for Kras activation are noted in stomach cancer. Expression of mutant Kras\textsuperscript{G12D} in the mouse gastric mucosa has been shown to induce hyperplasia and metaplasia. However, the mechanisms by which Kras activation leads to gastric metaplasia are not fully understood. Here we report that Kras\textsuperscript{LSL-G12D/+};Pdx1-cre, a mouse model known for pancreatic cancer, also mediates Kras\textsuperscript{G12D} expression in the stomach, causing gastric hyperplasia and metaplasia prior to the pathologic changes in the pancreas. These mice exhibit ectopic cell proliferation at the base of gastric glands, whereas wild-type mice contain proliferating cells primarily at the isthmus/neck of the gastric glands. Notch signaling is decreased in the Kras\textsuperscript{LSL-G12D/+};Pdx1-cre gastric mucosa, as shown by lower levels of cleaved Notch intracellular domains and downregulation of Notch downstream target genes. Expression of a Notch ligand Jagged1 is downregulated at the base of the mutant gland, accompanied by loss of chief cell marker Mist1. We demonstrate that exogenous Jagged1 or overexpression of Notch intracellular domain stimulates Mist1 expression in gastric cancer cell lines, suggesting positive regulation of Mist1 by Notch signaling. Finally, deletion of Jagged1 or Notch3 in Kras\textsuperscript{LSL-G12D/+};Pdx1-cre mice promoted development of squamous cell carcinoma in the forestomach, albeit short of invasive adenocarcinoma in the glandular stomach. Taken together, these results reveal downregulation of Notch signaling and Mist1 expression during the initiation of Kras-driven gastric tumorigenesis and suggest a tumor-suppressive role for Notch in this context.

Neoplasia (2019) 21, 810–821

Introduction
Activation of the proto-oncogene KRAS occurs in many types of human malignancies. In gastric adenocarcinoma, activating mutations or amplification of KRAS are detected in approximately 15% of all cases (http://www.cbioportal.org), and signatures for activation of Kras are noted in at least 40% of gastric cancers [1,2]. Studies using mouse models have shown the consequences of Kras activation in the stomach. Systemic activation of Kras in adult mice resulted in rapid pathologic changes in the stomach, namely, hyperplasia of the forestomach squamous epithelium and hyperplasia/metaplasia in the glandular stomach, without obvious tumors in other organs shortly after Kras activation [3]. Mist1-CreERT2–mediated expression of constitutively activated Kras (Kras\textsuperscript{G12D}) in the gastric chief cells caused the full spectrum of metaplastic lineage transitions, including spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia (IM), two of the preneoplastic lesions associated with...
intestinal-type stomach cancer [4]. Expression of Kras^{G12D} in the corpus lineages including pit cells and their progenitors through Tff1-Cre caused foveolar hyperplasia, gastric atrophy, and pseudopyloric metaplasia with SPEM [5]. Furthermore, Kras activation has been shown to cooperate with loss of tumor suppressor genes in driving gastric cancers. Kras^{G12D} expression combined with inactivation of E-cadherin and p53 in the gastric parietal cells gave rise to both intestinal- and diffuse-type tumors [6], whereas Kras^{G12D} expression in rare Mist1-expressing gastric stem cells, in conjunction with Apc mutation or E-cadherin loss, culminated in intestinal- and diffuse-type carcinomas, respectively [7].

Notch signaling regulates cell fate decision, cell differentiation, and proliferation in a variety of tissues during development and homeostasis. Disruption of Notch signaling often leads to expansion of stem and progenitor cell population, impairs differentiation, and increased proliferation, ultimately contributing to tumor initiation and progression. Molecular alterations in NOTCH genes, including gain- and loss-of-function mutations and gene amplifications, have been found in approximately 22% of human stomach adenocarcinomas (http://www.cbioportal.org). Studies in murine models and human cancer cell lines as well as expression data from human patients suggest both oncogenic and tumor-suppressive roles for Notch receptors [8]. The paradoxically dual functions of Notch receptors suggest that cellular context may be critical for the outcome of Notch activation in the pathological process of gastric cancers.

The development of intestinal-type stomach cancer is preceded by the emergence of metaplastic cell lineages and dysplasia in the gastric mucosa. Expression of Kras^{G12D} in either isthmus stem cells or gastric chief cells induced metaplastic changes [4,9], suggesting multiple for histology and immunohistochemistry by standard procedures. Histology, Immunohistochemistry, and X-Gal Staining

strain was described previously [17]. All mouse experiments were institutional Animal Care and Use Committee of UMMC.

Mice

Kras^{LSL-G12D}, Pdx1-Cre, Rod^{LSL-lacZ}, and Rod^{LSL-YFP}

mouse strains were obtained from the Jackson Laboratory and have been previously described [12–15]. Jag1^floxed strain was provided by Dr. Radtke and described previously [16]. Generation of Notch3^{Geor} strain was described previously [17]. All mouse experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of UMMC.

Histology, Immunohistochemistry, and X-Gal Staining

Formalin-fixed paraffin-embedded stomach tissues were processed for histology and immunohistochemistry by standard procedures. Primary antibodies used for immunostaining were: GFP (Invitrogen, A11122, 1:200), Ki67 (Abcam, ab16667, 1:100), Mist1 (Santa Cruz, sc-80984, 1:100), Jagged1 (Santa Cruz, sc-6011, 1:100), Notch1 (Cell Signaling, No. 3608, 1:100), Notch2 (DSHB, University of Iowa, C651.6DbHN, 1:200), Notch3 (ProteinTech, 55114-1-AP, 1:100), Notch4 (Millipore, 09-089, 1:100), Cytokeratin 19 (Abcam, ab52625, 1:200), Muc5AC (Santa Cruz, sc-21701, 1:100), Clusterin (Santa Cruz, sc-6420, 1:100), and TFF3 (ProteinTech, 23277-1-AP, 1:100). X-Gal staining was performed as previously described [18].

Western Blot Analysis

Stomach tissues were lysed in RIPA buffer (Boston BioProducts) supplemented with protease inhibitor (Roche) and processed according to standard methodology. Protein concentrations were determined using BCA protein assay kit (Thermofisher). Antibodies for probing specified proteins are as follows: Notch1, Notch2, Notch3, and Notch4 are the same as above (all with 1:1000 dilution). β-Actin (Santa Cruz, sc-81178, 1:1000) was used as loading control.

Quantitative Reverse Transcription PCR

Total RNA was extracted from gastric tissues or cell lines using RNeasy Mini kit (Qiagen) and reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). PCR was performed using Quantitect SYBR Green PCR Kits (Qiagen) with a Bio-Rad CFX96 qPCR System. The relative abundance of mRNA for each gene was normalized with the expression level of Gapdh and presented as fold change of the control. The experiment was performed in triplicate and presented as mean ± standard error. Primer sequences for mouse Jag1, Hes1, Hes5, Hey1, and Hey2 and human HES1, HES5, HEY1, and HEY2 have been previously described [19,20]. Other primer sequences are as follows: mouse Mist1: 5′ GTGGTGCTAAAGCTACGTG 3′ (forward), 5′ GACTGGGTCTGTCAGGTGT 3′ (reverse); human MIST1: 5′ CGGATGCACAAGCTAAATAACG 3′ (forward), 5′ CCGTCAGCGATTGTAGTGTC 3′ (reverse).

Notch Overexpression and Jagged1 Treatment of Cell Lines

Human stomach cancer cell lines SNU-1 and Hs746T were purchased from ATCC. Cells were resuscitated from early passage liquid nitrogen stocks and cultured less than 1 month before reinitiating cultures. For the overexpression of Notch2IC and Notch3IC, cells were transected with plasmid DNA of 3XFlag-NICD2 [21] and hNIDCD3(3xFLAG)-pCDF1-MCS2-EF1-eopGFP [22], respectively, using TransIT-X2 transfection reagent (Mirus). Cells were harvested 48 hours posttransfection for RNA or cell lysate. Jagged1 treatment was performed by plating cells on six-well plates precoated with recombinant rat Jagged1 fused to human Fc (R&D Systems) or Fc protein as control. Briefly, 20 μg/ml Fc-specific human IgG (Sigma) in PBS was added to the wells and incubated for 2 hours at room temperature. The solution was then aspirated, and 2 μg of either Jagged1-Fc or Fc in PBS was added to each well and incubated at 4°C overnight. The solution was then aspirated before plating cells for 48 hours.

Statistical Analyses

All data are presented as mean ± standard error. Unpaired two-tailed t-test was performed for comparison between the control and experimental samples. P value of .05 or less was considered statistically significant.
Results

**Gastric Metaplasia and Forestomach Hyperplasia in Kras<sup>G12D</sup>/+; Pdx1-Cre Mice**

Pdx1-Cre is a transgenic mouse line commonly used for selective deletion or expression of genes in the pancreas through Cre-mediated recombination. Interestingly, endogenous Pdx1 expression was previously detected in gastric glands in the distal stomach (gastric antrum) [23], and Pdx1-Cre has been used to delete p53, Smad4, and E-cadherin in the stomach, causing diffuse-type gastric adenocarcinoma [24]. To determine the recombination activity of Pdx1-Cre in the stomach, we crossed a Rosa<sup>LSL-LacZ</sup> reporter into Pdx1-Cre and performed X-gal staining for LacZ activity at 5 weeks of age. While the Rosa<sup>LSL-LacZ</sup> control mice showed no positive staining in the stomach, Rosa<sup>LSL-LacZ</sup>+/Pdx1-Cre mice showed broad X-gal staining in both corpus and antrum of the glandular stomach, and punctuated staining in the forestomach near the border with corpus (Figure 1A). Sections of the stained tissue showed LacZ activity in approximately 40% of the stomach.

---

**Figure 1.** Pdx1-Cre–mediated gene recombination in the gastric epithelium and gastric phenotype of the Kras<sup>LSL-G12D</sup>/; Pdx1-Cre mice. (A) Whole-mount X-gal staining of the stomach from 5-week-old Rosa<sup>LSL-LacZ</sup> and Rosa<sup>LSL-LacZ</sup>/Pdx1-Cre mice and sections of the X-gal–stained corpus and antrum. (B) Anti-YFP immunofluorescence staining of the forestomach, corpus, and antrum from Rosa<sup>LSL-YFP</sup>/Pdx1-Cre mice at 6 months of age. (C) Gross morphology of the stomach from wild-type and Kras<sup>LSL-G12D</sup>/; Pdx1-Cre (KC) mice at 3 weeks and 6 months of age. Arrows point to lesions in KC mice. (D) Representative histology of the forestomach, gastric corpus, and antrum from wild-type and KC mice at 6 months of age. (E) Anti-YFP immunostaining of gastric corpus from Rosa<sup>LSL-YFP</sup>; Kras<sup>LSL-G12D</sup>/; Pdx1-Cre mice at 6 weeks of age. Arrow points to a parietal cell that is YFP-negative. Fst, forestomach. Scale bars: 50 μm.
Figure 2. KrasLSL-G12D+/+;Pdx1-Cre mice exhibit increased proliferation at the base of gastric corpus associated with downregulation of Mist1 expression and decreased Notch signaling. (A) Immunostaining for Ki67 and Mist1 in the gastric corpus of wild-type (WT) and KrasLSL-G12D+/+;Pdx1-Cre (KC) mice at 3 weeks of age. Quantification of Ki67-positive cells and quantitative RT-PCR for Mist1 are shown to the right. (B) Immunostaining for cytokeratin 19 (CK19) and Mist1 in KC mice at 5 months of age. (C) Western blot analysis for Notch receptors in the gastric corpus of WT and KC mice at 1 and 4 months of age. (D) Quantitative RT-PCR for Jag1, Hes1, Hey1, and Hey2 at 2 months of age. *P < .05, **P < .005. Scale bars: 50 μm.
of gastric glands in the corpus, most of them throughout the entire gland with some restricted to the base or the isthmus, and more than 50% of the antrum glands were LacZ-positive (Figure 1A). We also crossed a Rosa<sup>LSL-YFP</sup> reporter into Pdx1-Cre and performed anti-YFP immunofluorescence staining in the stomach at 6 months of age. Similarly, YFP-positive cells were identified in the majority of gastric glands as well as squamous epithelium of the Forrestomach in Rosa<sup>LSL-YFP</sup>;Pdx1-Cre mice but not in Rosa<sup>LSL-YFP</sup> control mice (Figure 1B). In agreement with the recombination activity of Pdx1-Cre in the stomach, Kra<sup>LSL-Gt(2E)Rosa</sup>;Pdx1-Cre (KC) mice exhibit hyperplasia in the gastric corpus and antrum, as well as in the forestomach, as early as 3 weeks after birth (Figure 1C). By 6 months of age, gastric lesions including hyperplasia in the forestomach and hyperplasia/metaplasia of the gastric glands in both corpus and antrum have occurred in the vast majority of these animals. In comparison, no lesions were found in age-matched wild-type mice (Figure 1D). Lineage tracing using the Rosa<sup>LSL-YFP</sup> reporter confirmed that metaplastic lineages have originated from cells expressing Cre (Figure 1E). Notably, parietal cells trapped in the lesion were YFP-negative, indicating that they were not arising from Cre-expressing cells and not expressing Kra<sup>G12D</sup> (Figure 1E).

**Increased Proliferation, Decreased Mist1, and Downregulation of Notch Signaling in the Gastric Mucosa of Kra<sup>LSL-G12D/+; Pdx1-Cre</sup> Mice**

We performed anti-Ki67 immunostaining to examine cell proliferation in the gastric corpus of KC mice in comparison with wild-type mice. Ki67-positive cells in wild-type mice are restricted to the isthmus/neck of the gastric glands, where gastric stem/progenitor cells are localized and proliferate normally (Figure 2A). To the contrary, gastric glands in KC mice contain fewer Ki67-positive cells at the isthmus/neck region; however, more than 90% of the cells at the base of these glands are Ki67-positive (Figure 2A). Thus, aberrant cell proliferation has occurred at the base of gastric glands in KC mice. Chief cells are normally localized at the base of gastric glands in the corpus [25]; therefore, we performed immunostaining of Mist1, a chief cell marker, in KC mice compared with wild-type mice. Nuclear and cytoplasmic staining of Mist1 was readily detected in chief cells of wild-type mice, whereas very few Mist1-positive cells were seen at the base of gastric corpus in KC mice, suggesting loss of chief cells and/or downregulation of Mist1 in KC mice (Figure 2A). Consistent with the immunostaining result, mRNA level of Mist1 in KC gastric corpus is significantly lower compared with wild-type mice (Figure 2A). In addition, metaplastic cells (staining positive for cytokeratin 19) lost expression of Mist1, whereas adjacent normal cells maintained strong Mist1 nuclear staining (Figure 2B).

We previously reported upregulation of Notch signaling in the pancreas and gallbladder in KC mice, in which expression of Kra<sup>G12D</sup> induced development of pancreatic ductal adenocarcinoma and gallbladder adenoma, respectively [10,11]. To determine whether Notch signaling is altered in the stomach in KC mice, we performed Western blot analysis for Notch receptors in the gastric mucosa. Surprisingly, levels of cleaved intracellular domains of Notch1, 2, and 3 (activated Notch) were lower in KC mice compared with wild-type mice (Figure 2C). Consistent with the Western blot result, mRNA levels of Notch downstream target genes including Hes1, Hey1, and Hey2 were decreased significantly in KC gastric mucosa compared to the wild-type (Figure 2D). Taken together, our findings showed that Notch signaling is downregulated in the gastric mucosa with constitutively activated Kras.

**Regulation of Mist1 by Jagged1-Mediated Notch Signaling in Gastric Cells**

Jagged1 is the major ligand mediating Notch signaling in the gallbladder and pancreas and is upregulated in these tissues in KC mice [10,11]. We wondered whether Jagged1 was downregulated in the gastric mucosa in KC mice. Although no significant decrease in jag1 mRNA level was observed (Figure 2D), immunostaining showed almost complete absence of Jagged1 expression at the base of gastric glands in KC mice, in contrast to the wild-type mice (Figure 3A). Of note, strong Jagged1 immunoreactivity was detected in surface mucous cells in KC glands, comparable to wild-type glands (Figure 3A). Interestingly, immunofluorescence staining showed that Jagged1-expressing cells are either juxtaposed to Mist1-expressing cells or co-expressing Mist1 at the base of gastric glands in wild-type mice (Figure 3B). Given that KC gastric glands showed decreased expression of both Jagged1 and Mist1 at the base, we sought to determine whether Jagged1-mediated Notch signaling regulates Mist1 expression in gastric cells. Hs746T and SNU-1 are two of the human stomach cancer cell lines with relatively low JAG1 expression, according to expression data from Cancer Cell Line Encyclopedia. We treated these two cell lines with exogenous Jagged1 ligand and examined the effect. Hs746T cells plated on Jagged1-precoated plates showed five-fold increase in MIST1 mRNA expression, associated with a drastic upregulation of the Notch downstream target genes HES1, HES5, HEY1, and HEY2 (Figure 3C). Expression of MIST1 in SNU1 cells cultured in the Jagged1-precoated plate did not change significantly compared with the control, nor did the expression of Notch target genes (Figure 3D). Apparent ineffectiveness of precoated-Jagged1 on SNU-1 is likely due to the suspension (nonadherent) culture of this cell line. Next, we performed overexpression of Notch2 or Notch3 intracellular domain in these two cell lines. As expected, overexpression of either Notch2 or Notch3 caused upregulation of the Notch downstream target genes (i.e., HES1, HES5, HEY1, and HEY2) as well as MIST1 in both cell lines (Figure 4, A-D). Thus, Jagged1-mediated Notch signaling positively regulates Mist1 expression in gastric cells.

**Differential Expression of Notch Receptors in the Forestomach and Glandular Stomach**

We performed immunohistochemistry for Notch receptors in the stomach of adult KC mice. In the forestomach, Notch1 expression is restricted to the basal layer of normal squamous epithelium and squamous cell carcinoma (Figure 5, A and B). Notch2 immunostaining is negative in the squamous epithelium, with very few positive cells in the

**Figure 3.** Jagged1 and Mist1 are localized at the base of gastric corpus, and Jagged1 stimulates Mist1 expression in stomach cancer cells. (A) Anti-Jagged1 immunostaining in the gastric corpus of WT and Kra<sup>LSL-G12D/+;Pdx1-cre</sup> (KC) mice at 1 month of age. Arrows: base of gastric corpus. (B) Double immunofluorescence staining for Jagged1 and Mist1 in the gastric corpus of WT mice at 3 weeks of age. (C) Quantitative RT-PCR for HES1, HES5, HEY1, HEY2, and Mist1 in Hs746T cells cultured in plates precoated with control Fc or Jagged1-Fc protein. (D) Quantitative RT-PCR for HES1, HES5, HEY1, HEY2, and Mist1 in SNU1 cells cultured in plates precoated with control Fc or Jagged1-Fc protein. Scale bars: 50 μm. *P < .05, **P < .005.
stroma (Figure 5, D and E). Notch3 immunostaining is weak in the squamous epithelium but strong in smooth muscle layer underlying normal squamous epithelium and in fibroblasts surrounding the carcinoma (Figure 3, G and H). Notch4 was detected mostly in the stroma, including the endothelial cells of blood vessels and fibroblasts (Figure 3, J and K). In the glandular stomach, Notch1, Notch2, and Notch3 were all undetectable in the metaplastic lineages in gastric corpus, while weak Notch4 immunostaining was noted in these cells (Figure 3, C, F, I, and L). Collectively, immunohistochemical studies indicate that Notch receptors are differentially expressed in the forestomach and glandular stomach in KC mice.

**Accelerated Gastric Metaplasia and Forestomach Squamous Cell Carcinoma When Jag1 or Notch3 Is Deleted**

To determine the role of Jagged1-mediated Notch signaling in KrasG12D-induced pathologic changes in the stomach, we crossed a conditional deletion allele of Jag1 into KC mice. Gross examination of stomach found increased incidence and size of lesions in Jag1flox/flox;KrasLSL-G12D/+;Pdx1-Cre (JKC) mice compared to KC mice (Figure 6, A and C). Histological examination indicated many of the JKC mice developed squamous cell carcinoma of forestomach and extensive lesions in the gastric corpus (Figure 6B). For comparison, forestomach in KC mice showed hyperplasia, and only a few of them

---

**Figure 4.** Activation of Notch2 or Notch3 upregulates Mist1 expression in stomach cancer cell lines. (A and B) Quantitative RT-PCR for HES1, HES5, HEY1, HEY2, and MIST1 in Hs746T (A) and SNU1 (B) cells transfected with a control plasmid or plasmid overexpressing Notch2 intracellular domain (Notch2IC). (C and D) Quantitative RT-PCR for HES1, HES5, HEY1, HEY2, and MIST1 in Hs746T (C) and SNU1 (D) cells transfected with a control plasmid or plasmid overexpressing Notch3 intracellular domain (Notch3IC). *P < .05, **P < .005.
progressed to squamous cell carcinoma. Although the percentage of animals showing gastric metaplasia was similar in age-matched KC and JKC mice, the average area of metaplastic lesions in JKC mice was increased compared with KC mice (49% versus 36% of the examined areas, \( P < .05 \)). We also crossed a null allele of \( \text{Notch3} \) (\( \text{Notch3}^{\beta-Geo/\beta-Geo} \)) into KC mice. The resulting \( \text{Notch3}^{\beta-Geo/\beta-Geo};Kras^{LSL-G12D/+};Pdx1-cre \) (NKC) mice showed complete penetrance of gastric metaplasia as well as squamous cell carcinoma of forestomach at young ages as early as 1-2 months (Figure 6, \( A \) and \( C \)). Glandular epithelium was highly disorganized in NKC mice and was partially replaced by squamous epithelium expanded out of the forestomach, especially in old animals. There was also inflammatory cell infiltration in the gastric mucosa in these mice (Figure 6B). However, neither JKC nor NKC mice developed invasive carcinoma in the glandular stomach up to 7 months of age. We performed Western blot analysis for Notch intracellular domains in the forestomach squamous cell carcinoma of NKC mice. As expected, NKC forestomach showed no Notch3 intracellular domain; however, increased levels of Notch1 and Notch4 intracellular domains compared with wild-type were observed (Figure 6D). Despite the increase in Notch1/Notch4 intracellular domains, mRNA levels of \( \text{Hes1}, \text{Hes5}, \text{Hey1} \), and \( \text{Hey2} \) all decreased in NKC forestomach (Figure 6E), suggesting that Notch3 is the major receptor mediating Notch signaling in the forestomach. Taken together, deletion of Jagged1 or Notch3

**Figure 5.** Differential expression of Notch receptors in the forestomach and glandular stomach lesions in \( \text{Kras}^{LSL-G12D/+};Pdx1-cre \) mice. (A-C) Immunostaining of Notch1 in normal squamous epithelium (A) and squamous cell carcinoma (B) of the forestomach, and metaplastic lineages of the corpus (C). (D-F) Immunostaining of Notch2 in normal squamous epithelium (D), squamous cell carcinoma (E), and metaplastic lineages of the corpus (F). (G-I) Immunostaining of Notch3 in normal squamous epithelium (G), squamous cell carcinoma (H), and metaplastic lineages of the corpus (I). (J-L) Immunostaining of Notch4 in normal squamous epithelium (J), squamous cell carcinoma (K), and corpus metaplastic lineages (L). Scale bars: 50 \( \mu \text{m} \).
Notch Signaling in Gastric Metaplasia

Chung et al.

Neoplasia Vol. 21, No. 8, 2019

A

Fst

JKC

KC

NKC

B

Foregut stomach

Corpus

C

|        | 1-2 months of age                                      | 3-5 months of age                                      |
|--------|--------------------------------------------------------|--------------------------------------------------------|
| KC     | Forestomach squamous cell carcinoma (0/10)              | Forestomach squamous cell carcinoma (2/7)               |
|        | Metaplasia in the glandular stomach (9/10)             | Metaplasia in the glandular stomach (6/7)              |
| JKC    | Forestomach squamous cell carcinoma (4/11)              | Forestomach squamous cell carcinoma (9/11)             |
|        | Metaplasia in the glandular stomach (10/11)            | Metaplasia in the glandular stomach (11/11)            |
| NKC    | Forestomach squamous cell carcinoma (4/4)               | Forestomach squamous cell carcinoma (8/8)               |
|        | Metaplasia in the glandular stomach (4/4)              | Metaplasia in the glandular stomach (8/8)              |

D

|        | WT | WT | NKC | NKC |
|--------|----|----|-----|-----|
| Notch1 |    |    |     |     |
|        |    |    |     |     |
|        | 110 kDa |   |   |   |
| Notch2 |    |    |     |     |
|        | 110 kDa |   |   |   |
| Notch3 |    |    |     |     |
|        | 65 kDa   |   |   |   |
| Notch4 |    |    |     |     |
|        | 55 kDa   |   |   |   |
| β-actin|    |    |     |     |
|        | 42 kDa   |   |   |   |

E

Hes1

Hes5

WT        NKC

Hey1

Hey2

Relative mRNA level

### Table C

|        | 1-2 months of age                                      | 3-5 months of age                                      |
|--------|--------------------------------------------------------|--------------------------------------------------------|
| KC     | Forestomach squamous cell carcinoma (0/10)              | Forestomach squamous cell carcinoma (2/7)               |
|        | Metaplasia in the glandular stomach (9/10)             | Metaplasia in the glandular stomach (6/7)              |
| JKC    | Forestomach squamous cell carcinoma (4/11)              | Forestomach squamous cell carcinoma (9/11)             |
|        | Metaplasia in the glandular stomach (10/11)            | Metaplasia in the glandular stomach (11/11)            |
| NKC    | Forestomach squamous cell carcinoma (4/4)               | Forestomach squamous cell carcinoma (8/8)               |
|        | Metaplasia in the glandular stomach (4/4)              | Metaplasia in the glandular stomach (8/8)              |

### Diagrams D and E

- **D**: Western blot analysis of Notch proteins (Notch1, Notch2, Notch3, Notch4, β-actin) with corresponding molecular weights (110 kDa, 65 kDa, 55 kDa, 42 kDa).
- **E**: Relative mRNA levels of Hes1, Hes5, Hey1, and Hey2 for WT and NKC groups. Significant differences are indicated (*** for p < 0.001, ** for p < 0.01, **** for p < 0.0001).
accelerated KrasG12D-initiated metaplasia in the glandular stomach and promoted development of forestomach squamous cell carcinoma, suggesting a tumor-suppressive role for Notch signaling in the initial stages of gastric tumorigenesis.

We characterized gastric lesions in these mice by Alcian blue staining and immunohistochemistry. While the wild-type mice were completely negative, KC, JKC, and NKC mice all showed ectopic Alcian blue–positive mucins in the corpus gland, suggesting metaplastic changes caused by KrasG12D (Figure 7, A-D). These lesions are negative for Muc5AC, a marker for foveolar hyperplasia (Figure 7, E-H). We also performed immunostaining of Clusterin and TFF3, markers for SPEM and IM lesions, respectively. KC, JKC, and NKC mice showed robust expression of Clusterin in affected areas of the gastric corpus, whereas wild-type mice contained very few cells with weak Clusterin immunoreactivity (Figure 7, I-L). TFF3 was highly expressed in KC, JKC, and NKC corpus and completely negative in wild-type mice (Figure 7, M-P). Collectively, gastric lesions induced by Pdx1-Cre–mediated KrasG12D expression represent metaplastic lineages including both SPEM and IM.

**Discussion**

Previous studies in mice suggested an oncogenic role for Notch signaling in the stomach, as forced expression of Notch1 intracellular domain in dedifferentiated parietal cells, Sox2+ corpus stem cells, or Lgr5+ antral stem cells resulted in the development of adenoma [26–28]. Although no lesion was found in the stomach of Jag1fl/fl mice.
Notch Signaling in Gastric Metaplasia Chung et al.

Pdx1-Cre and Notch3BEGo/β-Geo mice (data not shown), deletion of Jagged1 or Notch3 in KC mice promoted development of gastric metaplasia and forestomach squamous cell carcinoma, suggesting that Jagged1 and Notch3 are tumor suppressive in Kras-driven stomach tumorigenesis. The apparent discrepancy may reflect versatile roles of Notch signaling in various cell types of the gastric gland. Notch may exert a tumor-promoting or tumor-suppressive function, dependent on the cell of origin and/or stage of gastric cancers. In the Pdx1-Cre-mediated KC model, gastric metaplasia may have arisen from chief cells in the corpus (see discussion below), where Notch signaling appears to maintain Mist1 expression. Interestingly, a recent study found organoids derived from KrasG12D, Tji1-Cre gastric corpus exhibited accelerated growth and abnormal differentiation with a loss of chief cell marker [5]. In contrast, chronic Notch activation in parietal cells confers stem cell properties, ultimately leading to adenoma formation [28], and Notch activation in antral stem cells induces undifferentiated polyps [26].

Studies in human gastric adenocarcinoma also suggest dual roles for individual Notch receptors (reviewed by Huang et al.) [8]. Interestingly, an immunohistochemical study of primarily resected gastric cancer and pretherapeutic biopsies found that NOTCH1-negative tumors demonstrated worse survival, whereas high NOTCH1 expression was associated with early-stage tumors and significantly increased survival in this subgroup [29]. Meanwhile, higher NOTCH2 expression was associated with early-stage and intestinal-type tumors and better survival in this type [29]. The expression of NOTCH3 has been associated with the intestinal/glandular differentiation of gastric carcinoma cells, hinting a possible role as a favorable prognostic indicator [30]. NOTCH4 promotes gastric cancer growth [31], and no opposite role has been reported. JAGGED1 expression in gastric cancer was decreased compared with that in nontumor tissue, and low expression of JAGGED1 predicted a role as a favorable prognostic indicator [30]. NOTCH4 promotes glandular differentiation of gastric carcinoma cells, hinting a possible role as a favorable prognostic indicator [30]. NOTCH4 promotes gastric cancer growth [31], and no opposite role has been reported. JAGGED1 expression in gastric cancer was decreased compared with that in nontumor tissue, and low expression of JAGGED1 predicted a dismal outcome [32]. Consistent with these studies in human patients, we observed reduced Jagged1 expression at the base of the gastric gland, accompanied by decreased Notch activation in a model of KrasG12D-driven gastric metaplasia.

Although not the focus of this study, forestomach neoplasia was noted in KC mice. Interestingly, deletion of either Jagged1 or Notch3 drastically accelerated the development of squamous cell carcinoma of forestomach. Notch has been shown to function as a tumor suppressor in the squamous epithelium of skin, lung, esophagus, and bladder [33–35]. Our results support a tumor-suppressive role for Notch in the squamous epithelium of forestomach.

Notch signaling appears to suppress differentiation in the antrum [26]; however, the role of Notch in the gastric corpus is less clear. Here we show that Notch signaling regulates Mist1, which is predominantly expressed in the corpus chief cells [25]. Notch signaling is known to regulate secretory cell differentiation in a number of tissues including the mammary gland, intestine, and airway epithelium, where Jagged1 serves as a major ligand [36–39]. Given that Mist1 is necessary and sufficient to induce and maintain secretory cell architecture and function [40], perhaps it is not surprising that Notch signaling regulates Mist1 in gastric lineages. Interestingly, expression of MIST1 was lost in gastric metaplasia, dysplasia, and carcinoma in human patients [41]. Our study linked downregulation of Mist1 to the decrease of Jagged1-mediated Notch signaling in Kras-induced gastric metaplasia.

Expression of KrasG12D in gastric chief cells using Mist1-Cre results in full range of metaplastic lineage transitions [4]. Notewor-
Hayakawa Y, Ariyama H, Stancikova J, Sakitani K, Asaha S, Renz BW, Dubeykovskaya ZA, Shibata W, Wang H, and Westphalen CB, et al (2015). Mirt1 expressing gastric stem cells maintain the normal and neoplastic gastric epithelium and are supported by a perivascular stem cell niche. Cancer Cell 28, 800–814.

Huang T, Zhou Y, Cheng AS, Yu J, To KFTang W (2016). NOTCH receptors in gastric and other gastrointestinal cancers: oncogenes or tumor suppressors? Mol Cancer 15, 80.

Hayakawa Y, Fox JG, and Wang TC (2017). Isthmus stem cells are the origins of metaplasia in the gastric corpus. Cell Mol Gastroenterol Hepatol 4, 89–94.

Zhang S, Chung WC, and Xu K (2016). Lunatic Fringe is a potent tumor suppressor in Kras-initiated pancreatic cancer. Oncogene 35, 2485–2495.

Chung WC, Wang J, Zhou Y, and Xu K (2017). Kras(G12D) upregulates Notch signaling to induce gallbladder tumorigenesis in mice. Oncoscience 4, 131–138.

Hirogori S, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenastra TD, and Hirt BA, et al (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4, 437–450.

Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, Jacks T, Soriano P (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21, 70–71.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, and Goff SP (2001). The Parahox gene Pdx1 is required to maintain positional identity in the adult pancreas. Genes Dev 15, 3243–3248.

Soriano P (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21, 70–71.

Parahox gene Pdx1 is required to maintain positional identity in the adult pancreas. Genes Dev 15, 3243–3248.

Mancini SJ, Mantei N, Dumortier A, Suter U, MacDonald HR, and Radtke F (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Cell Biochem Funct 23, 317–323.

Xu K, Nieuwenhuis E, Cohen BL, Wang W, Cany AJ, Danska JS, Coulas L, Rossant J, Wu MY, and Piccione TD, et al (2010). Lunatic Fringe-mediated Notch signaling is required for lung alveogenesis. Am J Physiol Lung Cell Mol Physiol 298, L45–L56.

Xu K, Usry J, Kousis PC, Prat A, Wang DY, Adams JR, Wang W, Loc AJ, Deng T, and Zhao W, et al (2012). Lunatic fringe deficiency cooperates with the Met/Caveolin gene amplicon to induce basal-like breast cancer. Am J Physiol Lung Cell Mol Physiol 298, L234–L242.

Zhang S, Loc AJ, Radtke F, Egan SE, and Xu K (2013). Jagged1 is the major regulator of notch-dependent cell fate in proximal airways Dev Dyn 2013 .

Zhang S, Chung WC, Wu G, Egan SE, Miele L, and Xu K (2015). Manic fringe promotes a claudin-low breast cancer phenotype through notch-mediated PI3KCG induction. Cancer Res 75, 1936–1943.

Ong CT, Cheng HT, Chang LW, Ohtsuka T, Kageyama R, Stormo GD, and Kopan R (2006). Target selectivity of vertebrate notch proteins. Collaboration between discrete domains and CSL-binding site architecture determines activation specificity. J Biol Chem 281, 5106–5119.

Liu H, Kennard S, and Lilly B (2009). NOTCH3 expression is induced in mural stem cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. Circ Res 104, 466–475.

Holland AM, Garcia S, Naselli G, Macdonald RJ, and Harrison LC (2013). The Parathox gene Pdx1 is required to maintain positional identity in the adult foregut. Int J Dev Biol 57, 391–398.

Park JW, Jiang SH, Park DM, Lim NJ, Deng C, Kim DY, Green JE, and Kim HK (2014). Cooperativity of E-cadherin and Smad4 loss to promote diffuse-type gastric adenocarcinoma and metastasis. Mol Cancer Res 12, 1088–1099.

Choi E, Roland JT, Barlow BJ, O’Neal R, Rich AE, Nam KT, Shi C, and Goldenring JR (2014). Cell lineage distribution atlas of the human stomach reveals heterogeneous gland populations in the gastric antrum. Gut 63, 1711–1720.

Demitrack ES, Gifford GB, Keeley TM, Carulli AJ, VanDussen KL, Thomas D, Giordano TJ, Liu Z, Kopan R, and Samuelson LC (2015). Notch signaling regulates gastric antral LGR5 stem cell function. EMBO J 34, 2522–2536.

Demitrack ES, Gifford GB, Keeley TM, Horita N, Todison A, Turgon DK, Siebel CW, and Samuelson LC (2017). NOTCH1 and NOTCH2 regulate epithelial cell proliferation in mouse and human gastric corpus. Am J Physiol Gastrointest Liver Physiol 312, G133–G144.

Kim TH and Shvidasani RA (2011). Notch signaling in stomach epithelial stem cell homeostasis. J Exp Med 208, 677–688.

Bauer L, Takacs A, Slotta-Huspenina J, Langer R, Becker K, Novotny A, Ott K, Walch A, Hapfelmeier A, and Keller G (2015). Clinical significance of NOTCH1 and NOTCH2 expression in gastric carcinomas: an immunohistochemical study. Front Oncol 5, 94.

Kang H, An HJ, Song JY, Kim TH, Heo JH, Ahn DH, and Kim G (2012). Notch3 and Jagged2 contribute to gastric cancer development and to glandular differentiation associated with MUC2 and MUC5AC expression. Histopathology 61, 576–586.

Qian C, Liu F, Ye B, Zhang X, Liang Y, and Yao J (2015). Notch4 promotes gastric cancer growth through activation of Wnt/beta-catenin signaling. Mol Cell Biochem 401, 165–174.

Liu H, Zhang H, Shen Z, Wang X, Wang Z, Xu J, and Sun Y (2015). Expression of Jagged1 predicts postoperative clinical outcome of patients with gastric cancer. Int J Clin Exp Med 8, 14782–14792.

Maraver A, Fernandez-Marcos PJ, Cash TP, Mendez-Pertuz M, Duenas M, Maitia P, Martinelli P, Munoz-Martín M, Martinez-Fernandez M, and Camanero M, et al (2015). NOTCH pathway inactivation promotes bladder cancer progression. J Clin Invest 125, 824–830.

Agrawal N, Jiao Y, Bettegowda C, Hutless SM, Wang Y, David S, Cheng Y, Twaddell WS, Latt NL, and Shin EJ, et al (2012). Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. Cancer Discov 2, 899–905.

Wang NJ, Sanborn Z, Arnett KL, Bayston LJ, Liao W, Proby CM, Leigh IM, Collisson EA, Gordon PB, and Jakkula L, et al (2011). Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. Proc Natl Acad Sci U S A 108, 17761–17766.

Gorni K, Staudt MR, Salir J, Kaner RJ, Heddrich J, Rogalski AM, Arbelaez V, Crystal RG, and Walters MS (2016). JAG1-mediated notch signaling regulates secretory cell differentiation of the human airway epithelium. Stem Cell Res 12, 454–463.

Zhang S, Loc AJ, Radtke F, Egan SE, and Xu K (2013). Jagged1 is the major regulator of Notch-dependent cell fate in proximal airways Dev Dyn 2013 .

Zhang S, Chung WC, Wu G, Egan SE, Miele L, and Xu K (2015). Manic fringe promotes a claudin-low breast cancer phenotype through notch-mediated PI3KCG induction. Cancer Res 75, 1936–1943.

Fre S, Bardin A, Robine S, and Louvard D (2011). Notch signaling in intestinal homeostasis across species: the cases of Drosophila, Zebrafish and the mouse. Exp Cell Res 317, 2740–2747.

Lo HGJ, Jin RY, Siibbel G, Liu D, Karki A, Joens MS, Madison BB, Zhang B, Blanc V, and Fitzpatrick JA, et al (2017). A single transcription factor is sufficient to induce and maintain secretory cell architecture. Genes Dev 31, 154–171.

Lennnerz JK, Kim SH, Oates EL, Huh WJ, Doherty JM, Tian X, Bredemeyer AJ, Goldenring JR, Lauwers GY, and Shin YK, et al (2010). The transcription factor MIST1 is a novel human gastric chief cell marker whose expression is lost in metaplasia, dysplasia, and carcinoma. Am J Pathol 177, 1514–1533.