Downregulation of Notch Signaling in Kras-Induced Gastric Metaplasia

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Abstract

Activating mutations and amplification of Kras and, more frequently, signatures for Kras activation are noted in stomach cancer. Expression of mutant Kras^{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$^{LSL-G12D/+}$;Pdx1-cre, a mouse model known for pancreatic cancer, also mediates Kras^{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$^{LSL-G12D/+}$;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$^{LSL-G12D/+}$;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.

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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^{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. Dysregulation of Notch signaling often leads to expansion of stem and progenitor cell population, impaired 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 gastric cell types may serve as the cell of origin of premalignancy. However, oncogenic signaling involved in the initiation of gastric metaplasia is not fully understood. In this paper, we examined the effects of Kras^{G12D} expression in the stomach in *Kras$^{LSL-G12D/+}$; Pdx1-cre* mice. We previously reported drastic upregulation of Notch signaling during the initiation and progression of Kras-driven pancreatic ductal adenocarcinoma as well as Kras-induced gallbladder adenoma in the same mouse model [10,11]. To our surprise, Notch signaling is downregulated in Kras-induced gastric hyperplasia/metaplasia. Furthermore, expression of the chief cell marker Mist1 is lost in the *Kras$^{LSL-G12D/+}$;Pdx1-cre* corpus, and activation of Notch upregulates Mist1 in stomach cancer cells, providing insights into the Notch functions in stomach cancer pathogenesis.

Materials and Methods

Mice

Kras$^{LSL-G12D}$, *Pdx1-Cre*, *Rosa$^{LSL-lacZ}$*, and *Rosa$^{LSL-YFP}$* mouse strains were obtained from the Jackson Laboratory and have been previously described [12–15]. *Jag1^{flox}* strain was provided by Dr. Radtke and described previously [16]. Generation of *Notch3$^{β-Geo}$* 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 previously been described [19,20]. Other primer sequences are as follows: mouse *Mist1*: 5′ GTGGTGGCTAAAGCTACGTG 3′ (forward), 5′ GACTGGGGTCTGTCAGGTGT 3′ (reverse); human *MIST1*: 5′ CGGATGCACAAGCTAAATAACG 3′ (forward), 5′ CCGTCAGCGATTTGATGTAGTTC 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 transfected with plasmid DNA of 3XFlag-NICD2 [21] and hNICD3(3xFLAG)-pCDF1-MCS2-EF1-copGFP [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$^{G12D/+}$; 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$^{LSL-lacZ}$* reporter into *Pdx1-Cre* and performed X-gal staining for LacZ activity at 5 weeks of age. While the *Rosa$^{LSL-LacZ/+}$* control mice showed no positive staining in the stomach, *Rosa$^{LSL-LacZ/+}$;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%
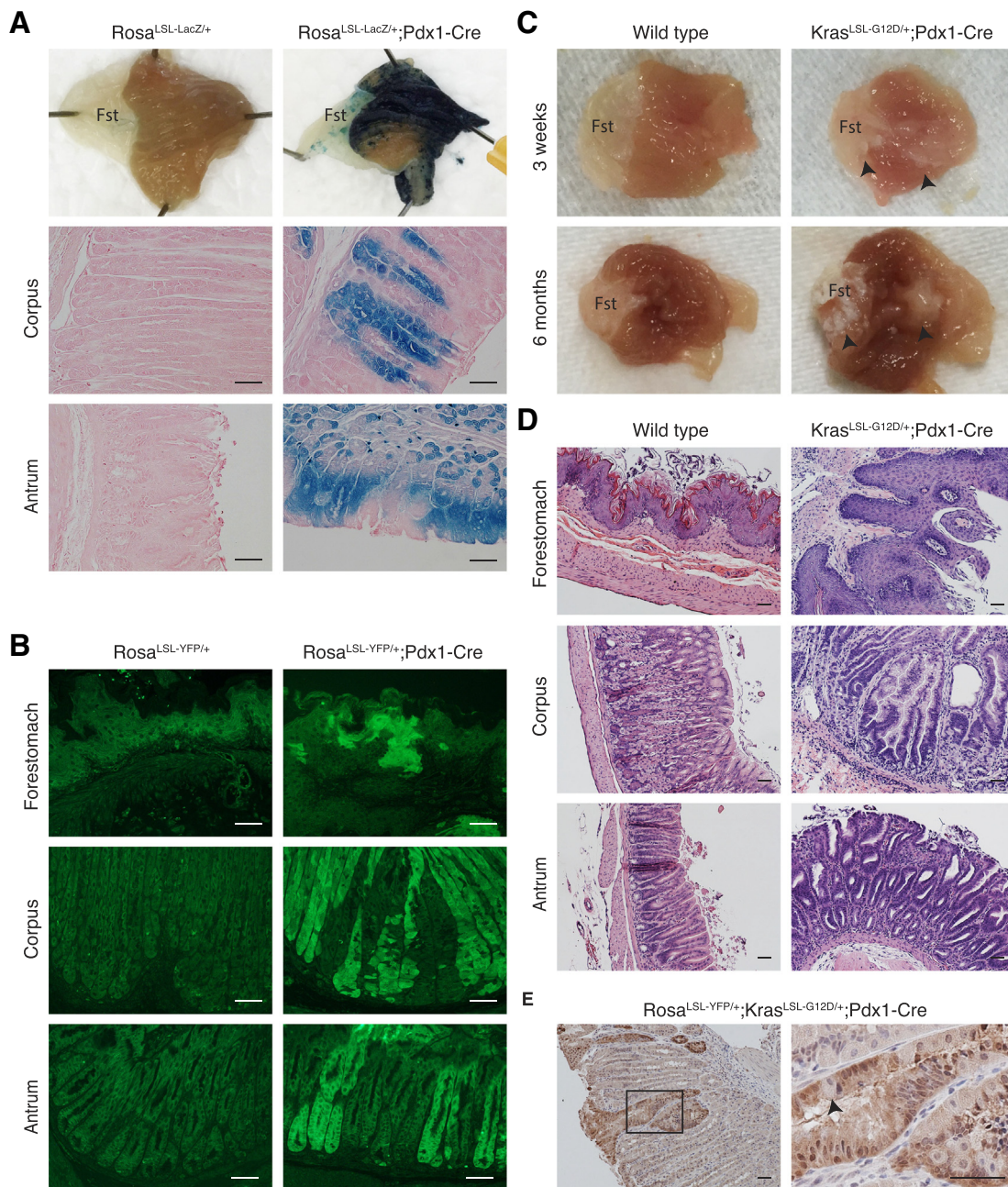

Figure 1. *Pdx1-Cre*–mediated gene recombination in the gastric epithelium and gastric phenotype of the *Kras$^{LSL-G12D/+}$;Pdx1-Cre* mice. (A) Whole-mount X-gal staining of the stomach from 5-week-old *Rosa$^{LSL-LacZ/+}$* and *Rosa$^{LSL-LacZ/+}$;Pdx1-Cre* mice and sections of the X-gal–stained corpus and antrum. (B) Anti-YFP immunofluorescence staining of the forestomach, gastric corpus, and antrum from *Rosa$^{LSL-YFP/+}$* and *Rosa$^{LSL-YFP/+}$;Pdx1-Cre* mice at 6 months of age. (C) Gross morphology of the stomach from wild-type and *Kras$^{LSL-G12D/+}$;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$^{LSL-YFP/+}$; Kras$^{LSL-G12D/+}$;Pdx1-Cre* mice at 6 weeks of age. Arrow points to a parietal cell that is YFP-negative. *Fst*, forestomach. Scale bars: 50 μm.
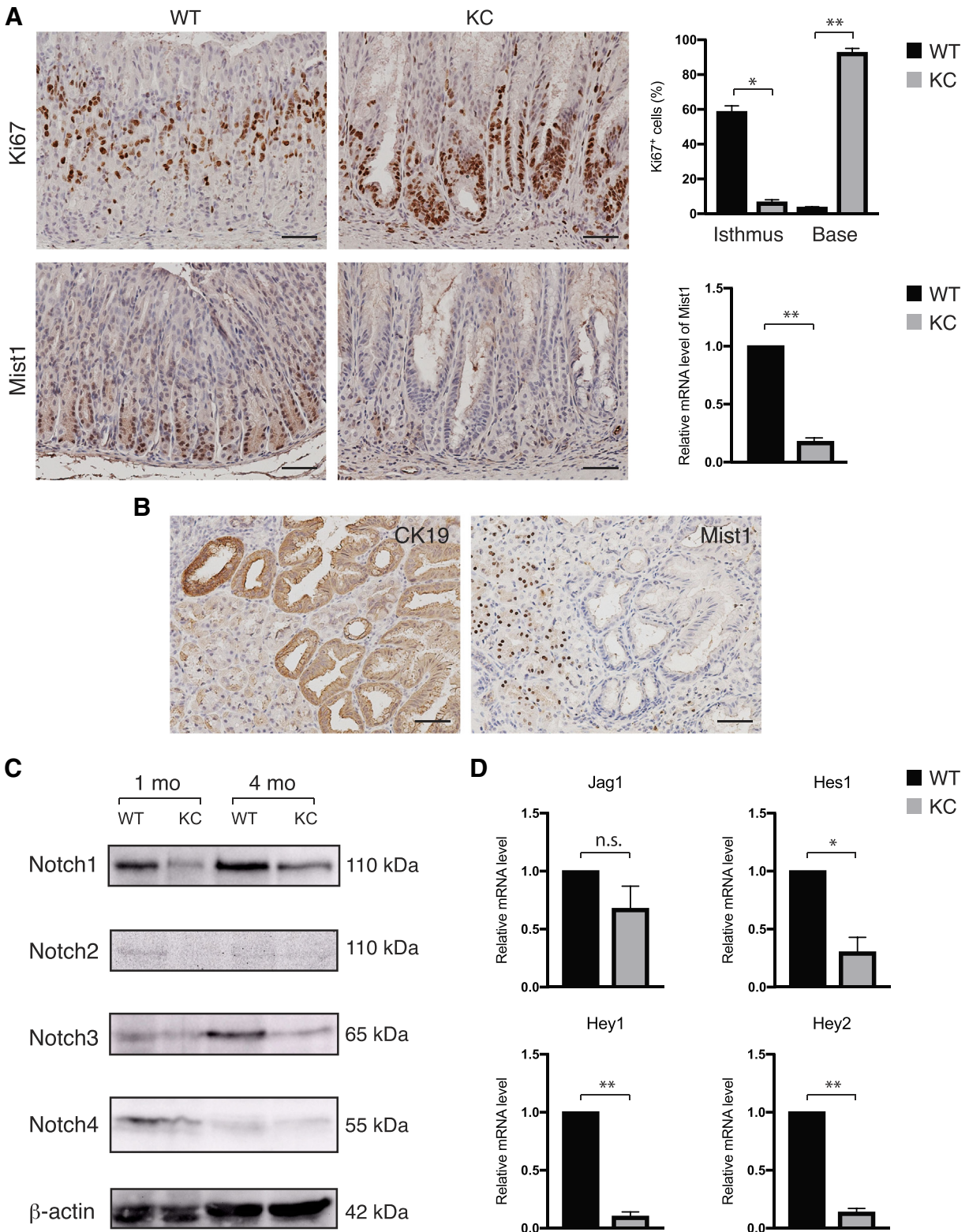
Figure 2. $Kras^{LSL-G12D/+}$;$Pdx1$-Cre mice exhibit increased proliferation at the base of gastric corpus associated with downregulation of Mist1 expression and decreased Notch signaling. (A) Immunostaining for Ki67 and Mist1 in the gastric corpus of wild-type (WT) and $Kras^{LSL-G12D/+}$;$Pdx1$-Cre (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. *$P \ll$ .05, **$P \ll$ .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 $Rosa^{LSL-YFP}$ 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 forestomach in $Rosa^{LSL-YFP/+}$;Pdx1-Cre mice but not in $Rosa^{LSL-YFP/+}$ control mice (Figure 1B). In agreement with the recombination activity of Pdx1-Cre in the stomach, $Kras^{LSL-G12D/+}$;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 $Rosa^{LSL-YFP}$ 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 $Kras^{G12D}$ (Figure 1E).

Increased Proliferation, Decreased Mist1, and Downregulation of Notch Signaling in the Gastric Mucosa of $Kras^{LSL-G12D/+}$; Pdx1-Cre 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 $Kras^{G12D}$ 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 in 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 $Kras^{LSL-G12D/+}$;Pdx1-cre (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. *$P \ll$ .05, **$P \ll$ .005.
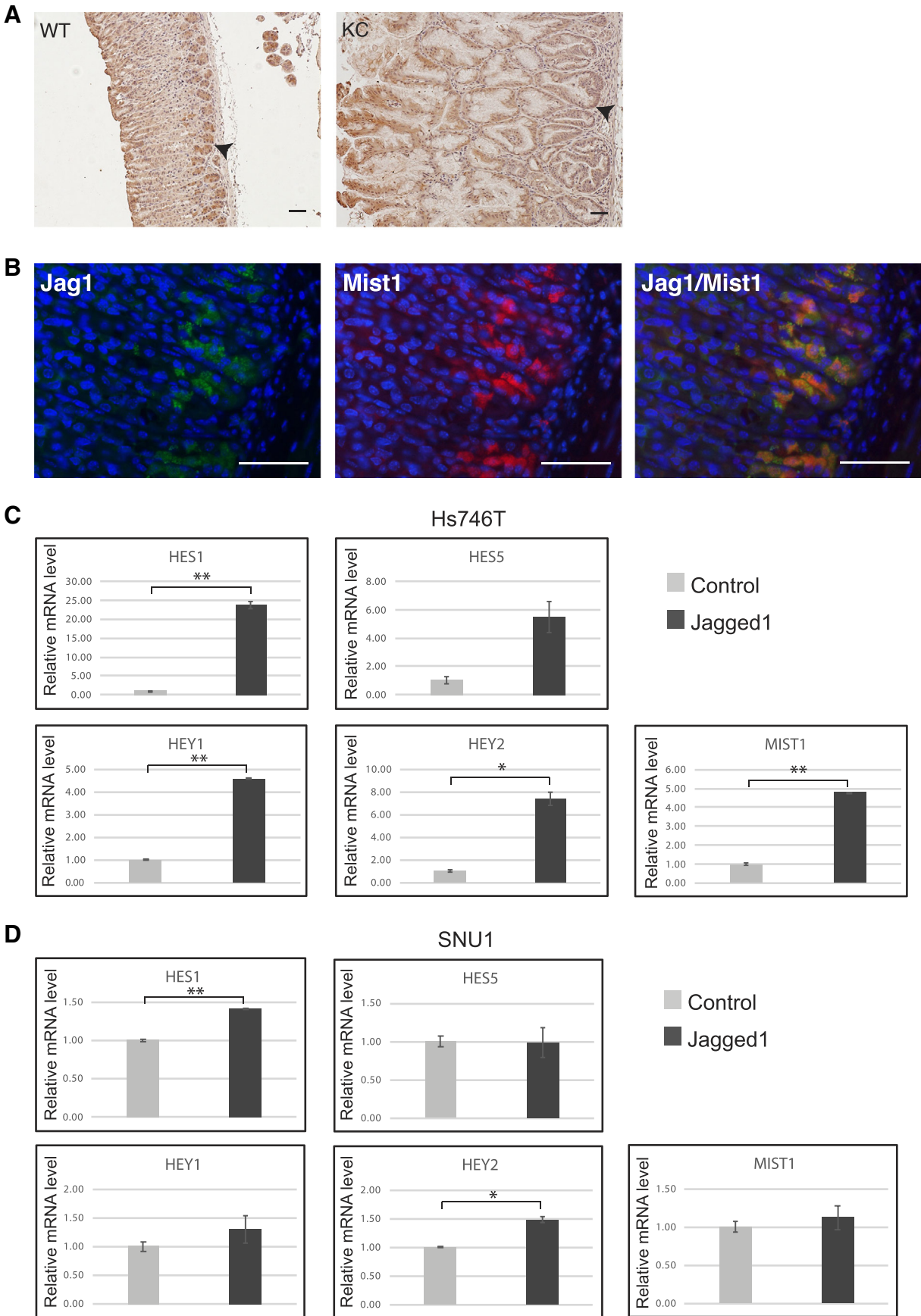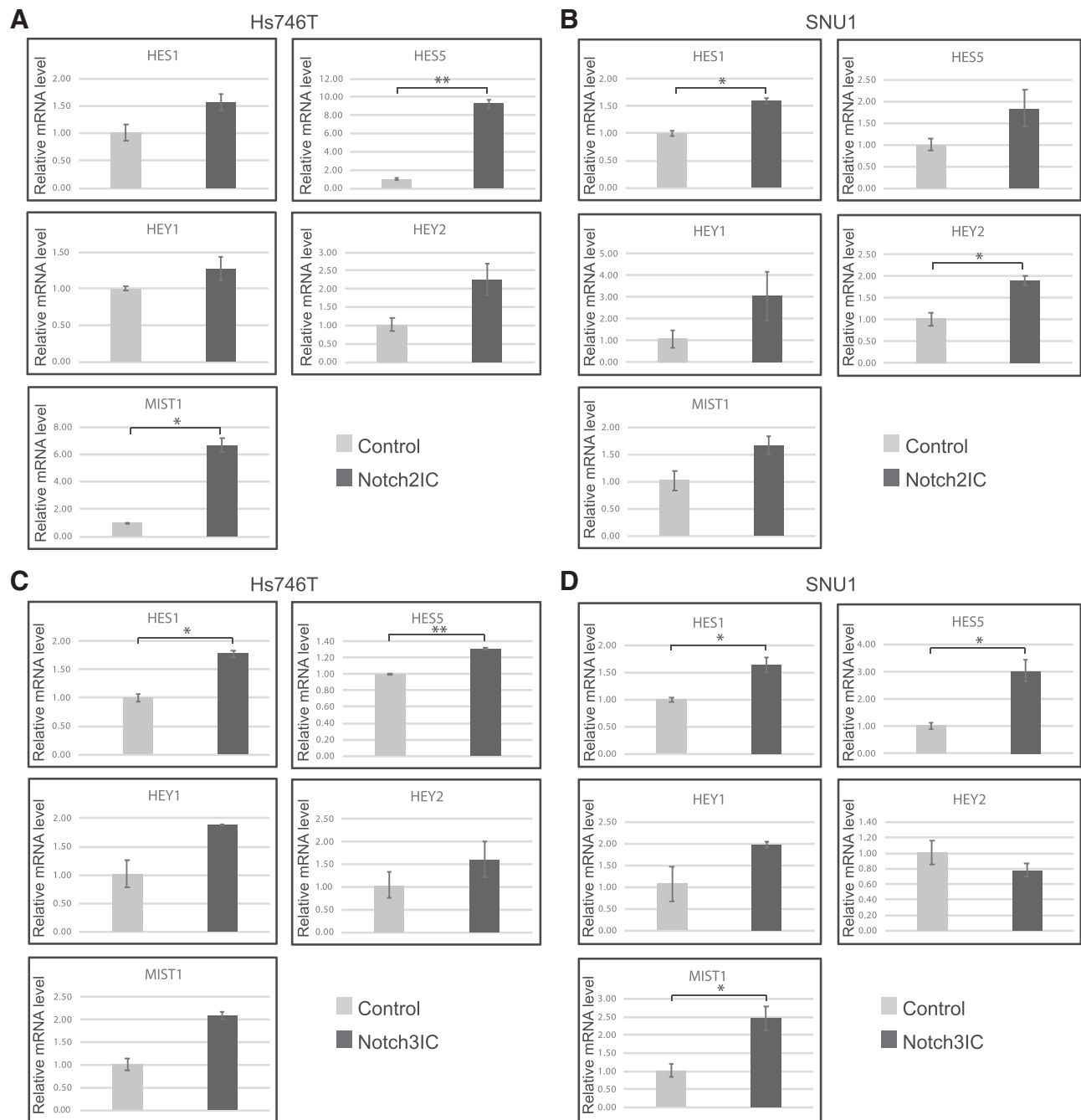
Figure 4. Activation of Notch2 or Notch3 upregulates Mist1 expression in stomach cancer cell lines. (A and B) Quantitative RT-PCR for HES1, HES5, HEY1, HEY2, and MIST1 in Hs746T (A) and SNU1 (B) cells transfected with a control plasmid or plasmid overexpressing Notch2 intracellular domain (Notch2IC). (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). *$P \ll .05$, **$P \ll .005$.

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 $Kras^{G12D}$-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 $Jag1^{flox/flox};Kras^{LSL-G12D/+};Pdx1-Cre$ (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
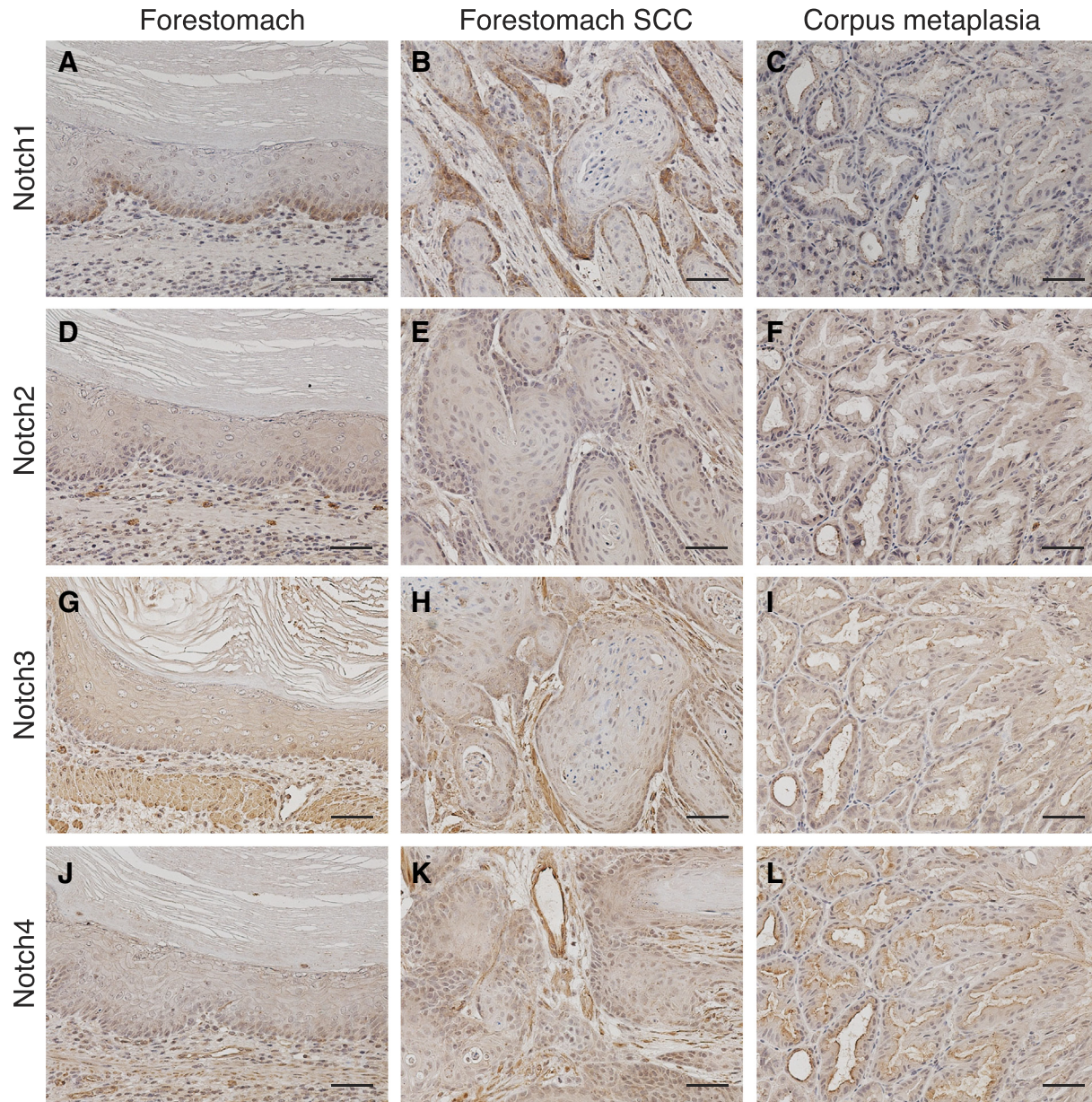
Figure 5. Differential expression of Notch receptors in the forestomach and glandular stomach lesions in $Kras^{LSL-G12D/+}$;$Pdx1$-cre mice. (A-C) Immunostaining of Notch1 in normal squamous epithelium (A) and squamous cell carcinoma (B) of the forestomach, and metaplastic lineages of the corpus (C). (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 μm.

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, $P \ll .05$). We also crossed a null allele of $Notch3$ ($Notch3^{\beta-Geo/\beta-Geo}$) into KC mice. The resulting $Notch3^{\beta-Geo/\beta-Geo}$;$Kras^{LSL-G12D/+}$; $Pdx1$-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 $Hes1, Hes5, Hey1$, and $Hey2$ 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
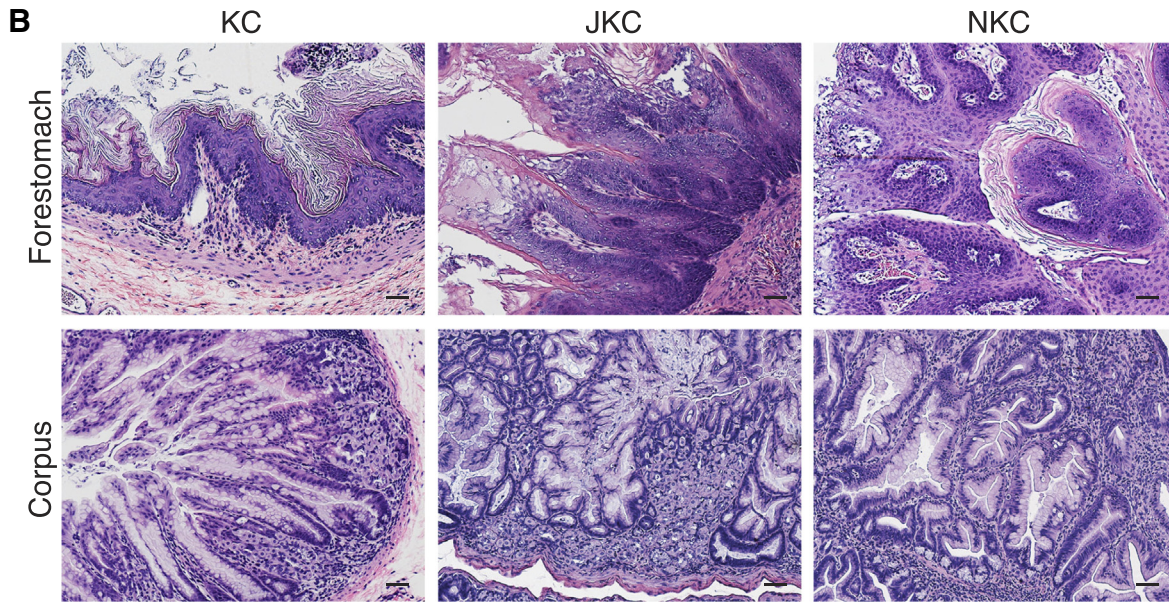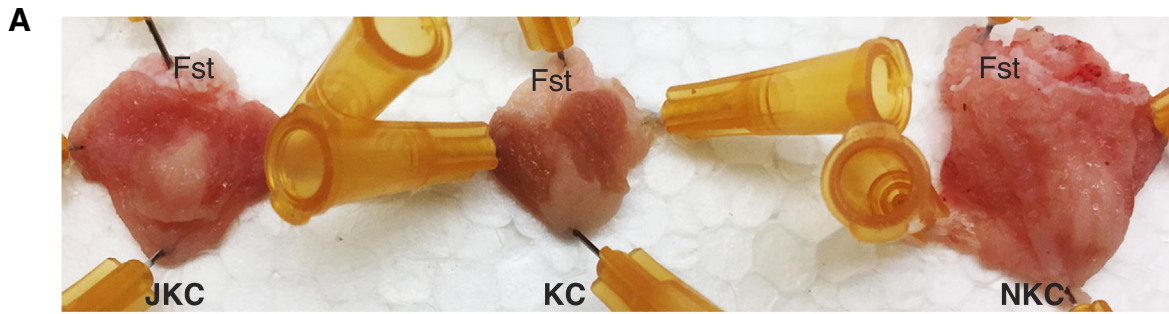
|	1-2 months of age	3-5 months of age
KC	Forestomach squamous cell carcinoma (0/10)	Forestomach squamous cell carcinoma (2/7)
	Metaplasia in the glandular stomach (9/10)	Metaplasia in the glandular stomach (6/7)
JKC	Forestomach squamous cell carcinoma (4/11)	Forestomach squamous cell carcinoma (9/11)
	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)
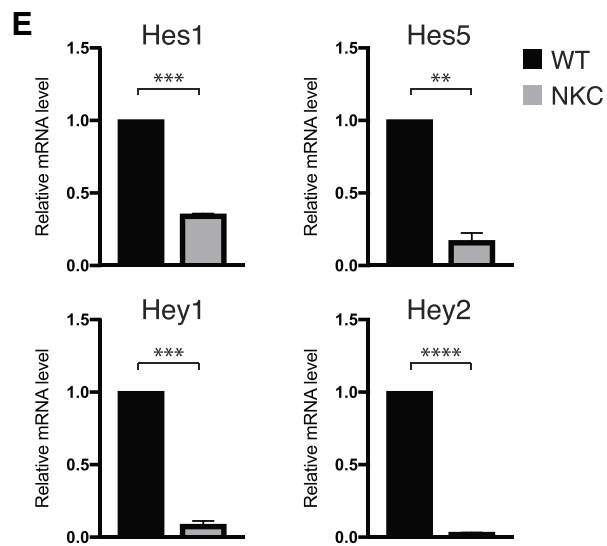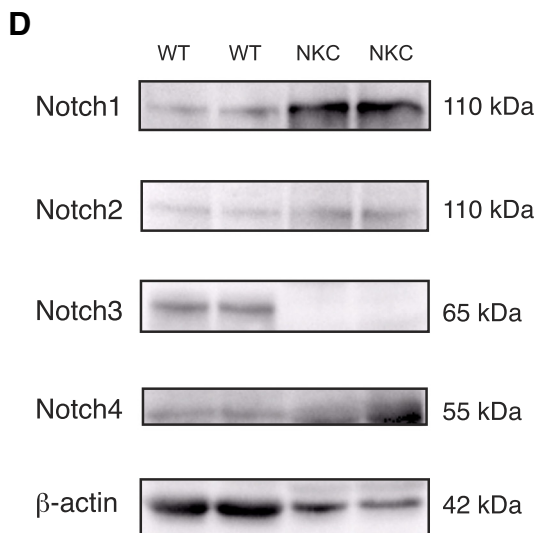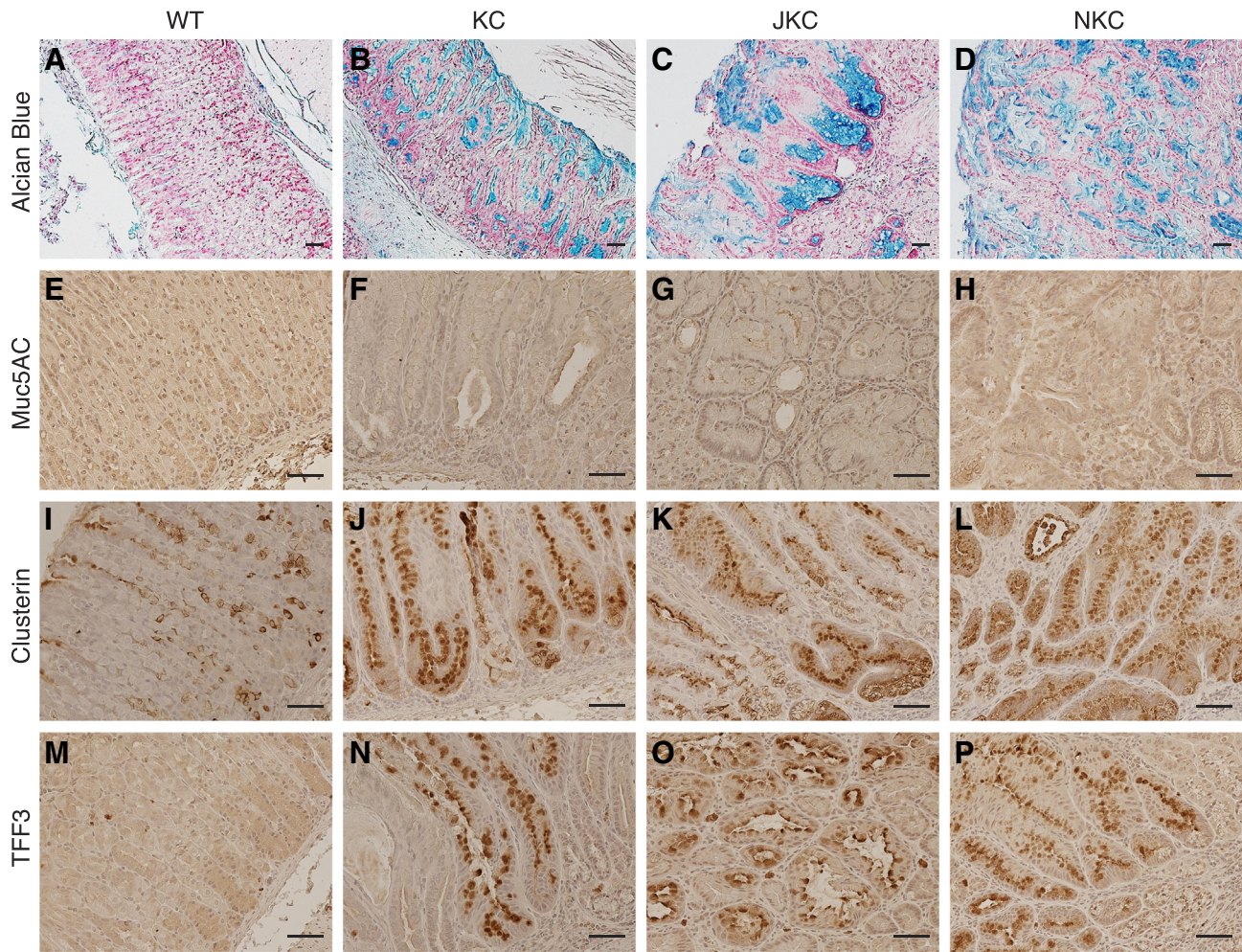
Figure 7. Histological and immunohistochemical analysis of metaplastic lesions in the gastric corpus of $Kras^{LSL-G12D/+}$;$Pdx1$-cre (KC), $Jag1^{flox/flox}$;$Kras^{LSL-G12D/+}$;$Pdx1$-cre (JKC), and $Notch3^{\beta-Geo/\beta-Geo}$;$Kras^{LSL-G12D/+}$;$Pdx1$-$cre$ (NKC) mice. (A-D) Alcian Blue staining of the gastric corpus in WT, KC, JKC, and NKC mice. (E-H) Immunostaining for Muc5AC in the gastric corpus of WT, KC, JKC, and NKC mice. (I-L) Immunostaining for Clusterin in the gastric corpus of WT, KC, JKC, and NKC mice. (M-P) Immunostaining for TFF3 in the gastric corpus of WT, KC, JKC, and NKC mice. These animals were examined at 3-5 months of age. Scale bars: 50 μm.

accelerated $Kras^{G12D}$-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 $Kras^{G12D}$ (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 $Kras^{G12D}$ 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 $Jag1^{flox/flox}$;

Figure 6. Deletion of Jagged1 or Notch3 promoted Kras-driven squamous cell carcinoma in the forestomach. (A) Gross morphology of the stomach from $Kras^{LSL-G12D/+}$;$Pdx1$-cre (KC), $Jag1^{flox/flox}$;$Kras^{LSL-G12D/+}$;$Pdx1$-cre (JKC), and $Notch3^{\beta-Geo/\beta-Geo}$;$Kras^{LSL-G12D/+}$;$Pdx1$-$cre$ (NKC) mice at 3 months of age. (B) Representative histology of the forestomach and gastric corpus in KC, JKC, and NKC mice at 4 months of age. Scale bars: 50 μm. (C) Quantification of stomach lesions in KC, JKC, and NKC mice. (D) Western blot analysis for Notch receptors in the forestomach of WT and NKC mice at 3 months of age. (E) Quantitative RT-PCR for Hes1, Hes5, Hey1, and Hey2 in the WT and NKC forestomach at 3 months of age. **$P \ll .005$, ***$P \ll .001$, ****$P \ll .0001$,

Pdx1-Cre and *Notch3*$^{\beta-Geo/\beta-Geo}$ 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 *Kras*$^{LSL-G12D/+}$;*Tff1*-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 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 Kras^{G12D}-driven gastric metaplasia.

Albeit 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 Kras^{G12D} in gastric chief cells using Mist1-Cre results in full range of metaplastic lineage transitions [4]. Notewor-

thily, Mist1 expression also marks rare quiescent stem cells in the gastric corpus isthmus. These cells can serve as a cell of origin for intestinal-type cancer with the combination of Kras and Apc mutations as well as for diffuse-type cancer with the loss of E-cadherin [7]. Thus, distinct types of Mist1-expressing cells in the stomach can serve as cell of origin of metaplasia and carcinoma. Notch regulation of Mist1 in distinct gastric cell types may underlie the differential, sometimes opposite, roles of Notch signaling in gastric cancers with different cellular origins, histological types, or pathological stages.

Conclusions

In this paper, we showed by lineage tracing that *Pdx1-Cre*–mediated recombination occurred in the stomach in addition to the previously known pancreas and duodenum. We reported for the first time that Kras-induced gastric metaplasia involved downregulation of Notch signaling and loss of Mist1 expression in the corpus chief cells. Immunohistochemistry demonstrated colocalized/juxtaposed Jagged1 and Mist1 expressions at the base of the corpus gland, and activation of Notch signaling upregulated Mist1 expression in gastric cancer cell lines. Ablation of Jagged1 or Notch3 accelerated Kras-induced gastric metaplasia as well as squamous cell carcinoma of the forestomach. These results shed light on the roles of Notch signaling in gastric cancer pathogenesis.

Conflict of Interests

All authors declare no conflict of interest.

Author Contributions

W. C. performed most of the experiments described in this study. Y. Z. and A. A. participated in data acquisition, analysis, and interpretation. K. X. conceived and designed the study, participated in data analysis and interpretation, and wrote the manuscript. All authors contributed to manuscript writing, review, and/or revision and approved the submitted version.

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