Supplementary Figure Legends

sFig. 1. Immunoflorescence staining of Villin and Cre proteins in intestine villi

A. Sections of intestinal tissues were prepared from *Villin-Cre*$^{+}$*;Klf4*$^{fl/f}$ and *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice at age of 35-wks. Immunoflorescence staining was performed using specific first antibody against Villin (HPA006884, rabbit polyclonal; Sigma; 1:100 dilution). The respective secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Green; Invitrogen; 1:100 dilution). All sections were counterstained with DAPI. This was one representative experiment of many with similar results. B. The cre protein expression (predominantly in the nuclei) was further confirmed by immunohistochemical staining.

Note that strong positive staining of Villin in villi of intestine mucosal tissues of both strains, while Cre staining was only observed in *Villin-Cre*$^{+}$*;Klf4*$^{fl/f}$ mice.

sFig. 2. Immunoflorescence staining of Villin and Cre proteins in antrum mucosa

Sections of antral tissues were prepared from various strains of mice at age of 35-wks with or without MNU treatment. Double-immunoflorescence staining were performed using specific first antibodies against Cre (C7988, mouse monoclonal; Sigma; 1:100 dilution) and Villin (HPA006884, rabbit polyclonal; Sigma; 1:100 dilution). The respective secondary antibodies were Alexa Fluor 594 goat anti-mouse IgG (A-11005, Red; Invitrogen; 1:100 dilution) and Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Green; Invitrogen; 1:100 dilution). All sections were counterstained with DAPI. This was one representative experiment of many with similar results.

Note that positive staining of Cre/Villin in the antrum mucosa of *Villin-Cre*$^{+}$*;Klf4*$^{fl/f}$ mice; while the positive Cre/Villin staining was expanded in the antrum mucosa of *Villin-Cre*$^{+}$*;Klf4*$^{fl/f}$ mice with MNU treatment.

sFig. 3. Histopathological analysis of mucosal transformation in antrum

Tissue sections were prepared from corpus, antrum and colon of *Villin-Cre*$^{-}$*;Klf4*$^{fl/f}$ (*Klf4*$^{+/+}$) mice, and antrum of *Villin-Cre*$^{+}$*;Klf4*$^{fl/f}$ (*Klf4*$^{-/-}$) mice at age of 35, 50, or 80-wks with or with MNU treatment. Cell proliferation was determined by Ki67 staining was stained in antral mucosa (both normal and hyperplastic) and intramucosal neoplasia.

Note that hyperplasia in the antrum of mice at age of 50 and 80 wks of age, and MNU treatment accelerated the transformation of antrum at age of 35 wks. H&E, hematoxylin and eosin staining; P&A, periodic acid-Schiff and Alcian blue staining.

sFig. 4. Immunoflorescence staining of KLF4 and FoxM1 in an antral tumor

Sections of antral tissues were prepared from a *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mouse at age of 35-wks with MNU treatment. Immunoflorescence staining was performed using specific first antibodies against FoxM1 (AT2098a, mouse monoclonal, clone 5G10, Abgent; 1:100 dilution) and KLF4 (H-180, rabbit polyclonal; Santa Cruz Biotechnology; 1:100 dilution). The respective secondary antibodies were Alexa Fluor 594 goat anti-mouse IgG (A-11005, Red; Invitrogen; 1:100 dilution) and Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Green; Invitrogen; 1:100 dilution). All sections were counterstained with DAPI. This was one representative experiment of many with similar results.

Note that strong positive staining of FoxM1 in transformed mucosal and neoplastic cells (*left panels*), while KLF4 staining was only adjacent normal cells (*right panels*). N, normal; T, tumor; B, Brunners glands.

sFig. 5. *Foxa3-Cre*–mediated *Klf4* deletion in gastric corpus and antral mucosa in mice.

(*A*) Representative photographs of macroscopic views of the entire gastric mucosa in 35-week-old mice. (*B*) Representative microscopic views of massive hyperplasia in the gastric corpus (left panel) and an antral tumor (right panel) in a *Foxa3-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mouse. (*C*) Tumor incidence in the stomach (*C1*) and locations of tumors in the stomachs (*C2*). (*D*) The efficacy of *Klf4* deletion in the corpus in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ and *Foxa3-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice was measured using quantitative PCR analysis. Corpus DNA extracts from mice with wild-type and floxed *Klf4* alleles were used as controls.

sFig. 6. Histopathological analysis of gastric mucosal transformation of *Foxa3-Cre*$^{+}$*;Klf4*$^{fl/f}$ mice

Tissue sections were prepared from corpus and antrum of *Foxa-Cre*$^{+}$*;Klf4*$^{fl/f}$ mice at age of 5 and 35-wks with or without MNU treatment. Note that hyperplasia in both antrum and corpus (***A***) and hyperplasia and overexpression of FoxM1 (***B***).

Supplementary Materials and Methods

Cell Lines and Culture Conditions

The human gastric cancer cell line NCI-N87 was purchased from the American Type Culture Collection, and the gastric cancer cell line SK-GT5 was obtained from Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center). PANC02 murine pancreatic adenocarcinoma cell line was originally established by Corbett and Colleagues by implanting cotton thread-carrying 3-methyl-cholanthrene into the pancreas of C57BL/6 followed by serial *s.c.* transplantation (Corbett, T. H., Roberts, B. J., Leopold, W. R., Peckham, J. C., Wilkoff, L. J., Griswold, D. P. Jr, Schabel, F. M. Jr. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. Cancer Re *44*: 717-26, 1984) and was generously provided by Dr. James A. Nelson (The University of Texas M. D. Anderson Cancer Center). All cell lines were maintained in plastic flasks as adherent monolayers in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories). Cultures are free of *Mycoplasma*.

Mouse Strains, Derivations, and Maintenance

The derivation and use of *Klf4-LoxP*, *Villin-Cre*, and *Foxa3-Cre* mice were described previously.[10,21,28] The *Klf4-LoxP* (*Klf4*$^{fl/fl}$) strain has loxP in the introns flanking exon 2 and exon 3. The mice were bred in the laboratory of Dr. Klaus Kaestner (University of Pennsylvania).[22] The *Villin-Cre* mice [004586, B6.SJL-Tg(*Vil-cre)*997Gum/J], which have Cre expressed under the control of a 12.4-kb regulatory region of the murine Villin promoter, were obtained from The Jackson Laboratory (Bar Harbor, ME).[10] *Foxa3-Cre* mice also were obtained from The Jackson Laboratory.[28] These three strains bred on a C57BL6 genetic background and maintained by crossing them with C57BL6J mice (The Jackson Laboratory). *Klf4*$^{fl/fl}$ mice were crossed with

Villin-Cre or *Foxa3-Cre* mice; pairs of mice heterozygous for the floxed *Klf4* allele and positive for *Villin-Cre* or *Foxa3-Cre* were intercrossed to obtain offspring that were homozygous for the floxed allele and positive for *Villin-Cre* or the *Foxa3-Cre* transgene. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the current regulations and standards of the US Department of Agriculture, US Department of Health and Human Services, and National Institutes of Health.

Isolation of Total RNA and Quantitative Real-Time PCR Analysis

The RNA(s) expression in murine gastric and intestinal mucosa specimens was measured using a two-step quantitative real-time PCR with TaqMan probe-based assays using an on-demand gene expression protocol (Applied Biosystems, Foster City, CA). Total RNA was isolated from the specimens using the PureLink RNA Mini Kit (Invitrogen). Fifty nanograms of total RNA was transcribed into cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative PCR analysis of gene expression was performed using TaqMan assays with a *KLF4* primer (Mm00516104_m1) according to the assay manufacturer's instructions. Each reaction was performed in triplicate with glyceraldehyde-3-phosphate dehydrogenase (Mm03302249_g1) used as an endogenous control. The $\Delta\Delta$Ct method was used for data analysis. Significant deviations from a fold difference of 1 (no change compared with the control samples) was tested using a *t*-test, for which *P* values less than .05 were considered statistically significant).

Small interfering RNA of KLF4

Human KLF4 small interfering RNA oligos (sense, 5'-uaacagcucaugccacccgtt-3'; and antisense, 5'-cggguggcaugagcuguuatt-3') were synthetically ordered from Ambion and used for knocking down of KLF4 expression in N87 or GT5 cells using the similar procedures described

previousely.[22] In some experiments, siRNA was cotransfected with KLF4 expression vector**.** Cell or protein samples were harvested at 48 h after transfection and processed for flow cytometric or Western blot analysis.

Western blot and immunoprecipitation analysis

Western blot and immunoprecipitation were performed using whole-cell lysates prepared from cultured cells and/or tumor tissue samples and relevant antibodies by following the procedures described previously.[22]

Cloning and Analysis of FoxM1 promoter

The proximal FoxM1 promoter construct p27-N-MB435 was used in cotransfection with pcDNA3.1-KLF4 or pcDNA3.1 for promoter activity assay as described previously.[22] In some experiments, p27-N-MB435 was cotransfected with pcDNA3.1-KLF4 vector, and pcDNA3.1 vector was used to adjust that equal amount of plasmid DNA was transfected to the cells in each well at this circumstance. The specific promoter activity was expressed as the fold changes of the experimental group versus the control group.[22]

Primary tumor cell culture

The second part of tumor was rinsed twice in cold (4°C) 70% alcohol and cold PBS and then cut into fine fragments using a sterile scalpel. The fragments were then subjected to sequential enzymatic digestion for 4 hours min at 37°C in a medium containing collagenase type I (500 μg/ml), trypsin inhibitor (0.25 mg/ml) and Elastase type (0.2 mg/ml). The cell suspensions were plated in 12-well cell culture dishes with medium containing 1mg/ml ampicillin and 500 μg/ml kanamycin. Two weeks later, DNA from the retrieved tumor cells, were extracted and subject to PCR-based assays for genotyping *Klf4* alleles, along with the DNA from gross tumor lesion and adjacent mucosa. Note that the retrieved gastric tumor cells exhibit complete *Klf4* gene deletion

compared to those of predominant deletion in gross tumor lesion, detectable deletion in antrum mucosa.

Generation of *Villin-Cre*; *Klf4-flox mice*

A. The *Klf4-flox* transgene contains LoxP sites (triangles) inserted in the first and third introns. In the tissues of Klf4-floxed mouse expressing Cre protein, the second and third exons are excised out, fusing the first exon out of frame with the fourth exon of the Klf4 gene. Deletion of exons 2 and 3 produces a frame shift in exon 4. Exon 1 encodes only the first amino acid of the Klf4 protein. Mice were genotyped by PCR using three primers as shown arrow 1, 2, and 3. The wild-type allele produced a band of 172 bp, the floxed allele a band of 296 bp, and the null allele a band of 425 bp. B. Klf4-floxed and Cre genotyping was done by PCR amplification. PCR screening for Klf4 revealed a band of 172 bp for wildtype allele, 296 bp for floxed allele, and 425bp for null allele. The presence of the Villin-Cre transgene was identified by PCR amplification with specific primers for the 12.4Kb VilCre transgene (1,100 bp) and Cre recombinase gene (233 bp). C. Detection of Villin-Cre-mediated rearrangement of Klf4 allele in different tissues. DNA was extracted from *different* tissues of previously verified conditional $Klf4^{-/-}$ mouse. PCR was performed according to Material and Methods. Representative tissues indicate varying amounts of rearrangement of Klf4 gene. D, Relative fold reduction of quantities of Klf4 transcripts in $Klf4^{-/-}$ mouse. Quantities of KLF4 transcripts in the intestine and stomach tissues of $Klf4^{fl/fl}$ and $Klf4^{-/-}$ was measured by Q-RT-PCR analysis. Klf4 transcripts are decreased about 95% in the intestine and 40% in the stomach of Klf4-null mouse compared to Klf4-flox mice.

Human Tissue Specimens and Patient Information

Human gastric tumor specimens preserved in the Gastric Cancer Tissue Bank at The University of Texas MD Anderson Cancer Center were used for gene expression analysis using

immunohistochemistry, and information about the respective patients was obtained from the MD Anderson Upper Gastrointestinal Carcinoma Database. Primary gastric cancer in these patients was diagnosed and treated at MD Anderson from 1985 to 1998. The patients had well-documented medical histories and follow-up information. All of the patients underwent gastrectomy with lymph node dissection; none of them underwent preoperative chemotherapy or radiation therapy. Tumor specimens obtained from 86 patients with primary gastric cancer were selected for this study. The patients consisted of 56 men and 30 women, and their mean age was 62 years. Twenty patients had proximal cancer localization. Fifty-three patients had intestinal-type cancer, whereas 33 had diffuse-type disease. All of the patients underwent follow-up examinations at MD Anderson through the end of 1999. The median follow-up duration was 25.7 months. At the end of 1999, 30 of the patients were still alive. Patient characteristics are shown in sTable 1. Patients with gastric tumors centered at or above the gastroesophageal junction were not included in this study. However, those with proximal gastric tumors centered below the gastroesophageal junction were included. Furthermore, patients with tumors of at the gastroesophageal junction associated with Barrett esophagus were not included. Fifty-one lymph node metastasis specimens obtained from the 86 study patients and 57 normal gastric tissue specimens obtained from control patients without gastric cancer were also used in this study. The use of human specimens (Protocol # LAB03-0267) was approved by institutional review board of the University of Texas MD Anderson Cancer Center.

Western Blot Analysis

Whole cell lysates were prepared from normal gastric tissue, gastric tumors, and cultures of SK-GT5 or N87 gastric cancer cells with or without indicated treatment or manipulations. Standard Western blot analysis of the lysates was performed with specific anti-KLF4 (H-180; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-FoxM1 (AT2098a, clone 5G10, 1:100 dilution; Abgent, San Diego, CA) antibodies. Equal protein sample loading was monitored by incubating the

same membrane filter with an anti–glyceraldehyde-3-phosphate dehydrogenase antibody (FL-335; Santa Cruz Biotechnology). Probe proteins were detected using an enhanced chemiluminescence system (Amersham Life Sciences, Piscataway, NJ) according to the manufacturer's instructions.

Chromatin Immunoprecipitation

N87 and SK-GT5 cells were seeded to 80% confluence in 10-cm culture dishes and transfected with a recombinant adenovirus containing KLF4 (Ad-KLF4) or enhanced green fluorescent protein (EGFP) (Ad-EGFP) at a multiplicity of infection (MOI) of 10. Twenty-four hours later, chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. Briefly, DNA-crossbinding proteins were cross-linked with DNA and lysed in sodium dodecyl sulfate lysis buffer. The resulting lysate was sonicated to shear DNA to 200-500 bp. After preclearing with salmon sperm DNA/protein A agarose for 30 minutes at 4°C, chromatin samples were immunoprecipitated overnight with a control IgG antibody and H-180. The 258-bp region between -348 and -90 bp of the FoxM1 promoter was amplified using the indicated primers. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

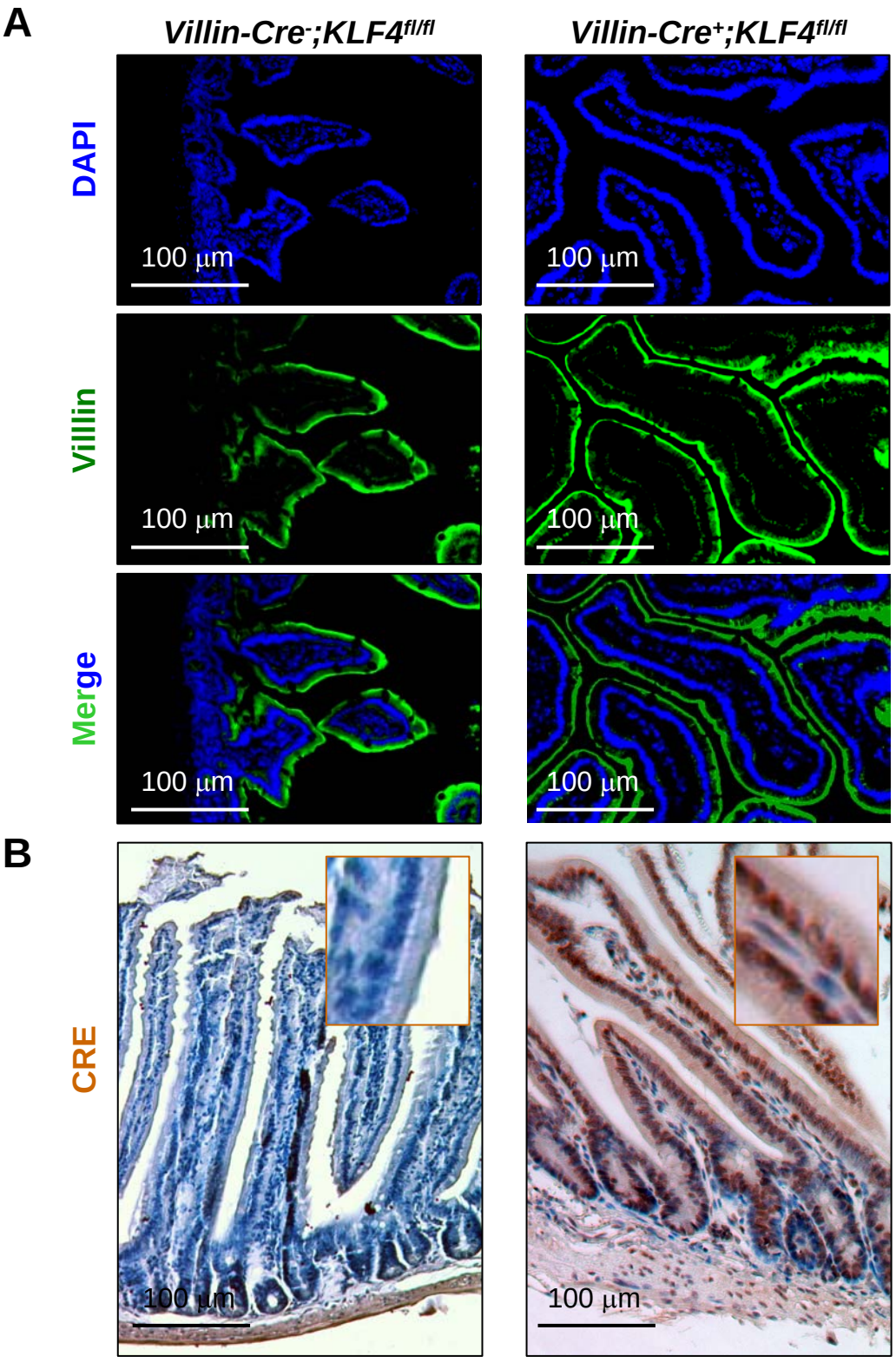
Preparation of Tissue Samples for Histopathologic Analysis

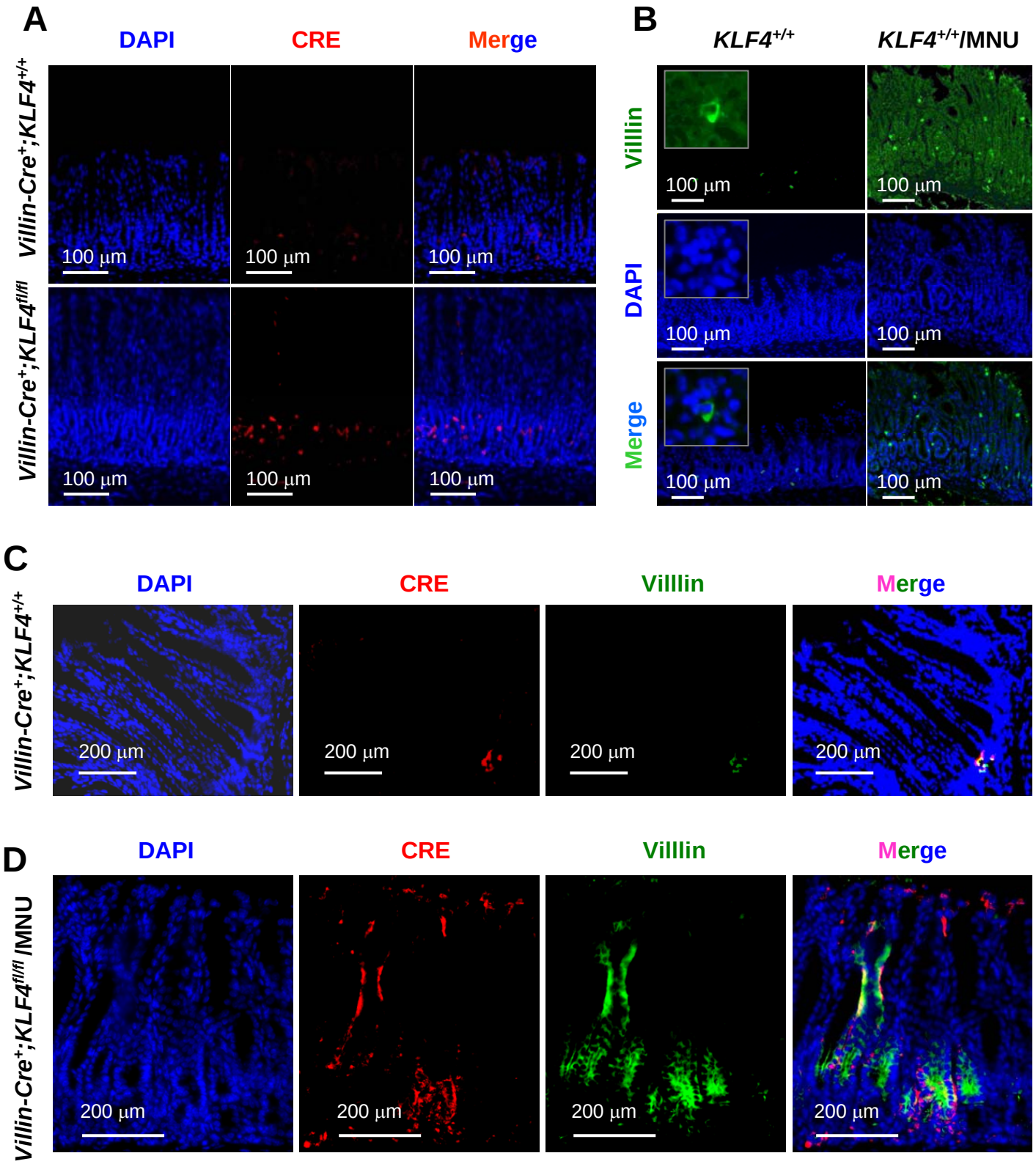
At the end of the experiment, all of the mice were killed using CO_2 asphyxiation, and their stomachs and other organs were carefully examined macroscopically. Their stomachs were then removed and opened along the greater curvature of the antrum. Tumors greater than 0.5 mm in diameter were mapped and counted by two observers blinded to the genotypes of the mice. The excised stomachs were fixed in 4% paraformaldehyde in phosphate-buffered saline, cut into strips, embedded in paraffin, and cut into 4-μm-thick sections. The sections were

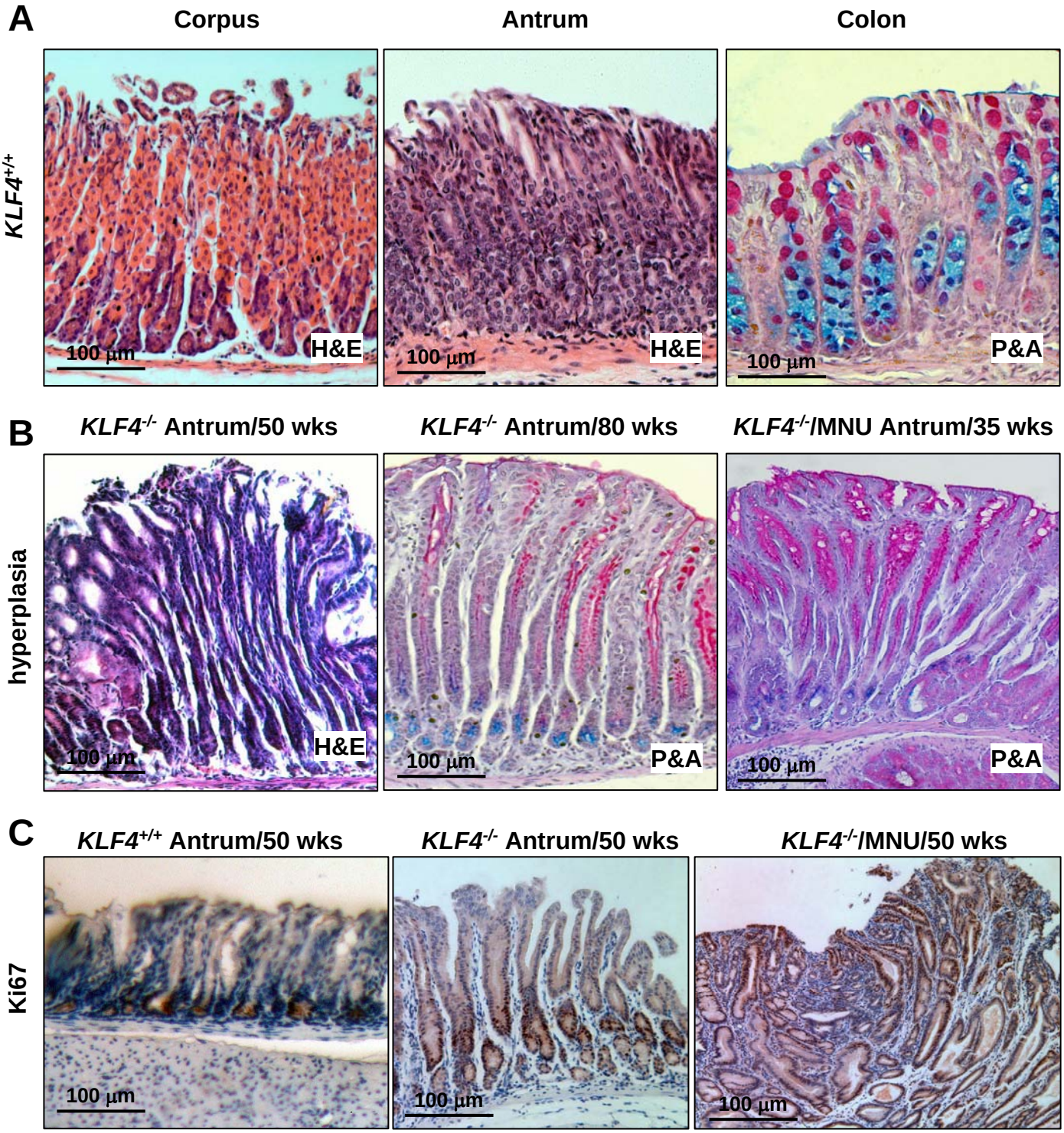
stained with hematoxylin and eosin (H&E) and with periodic acid-Schiff (PAS)/Alcian blue (P&A).

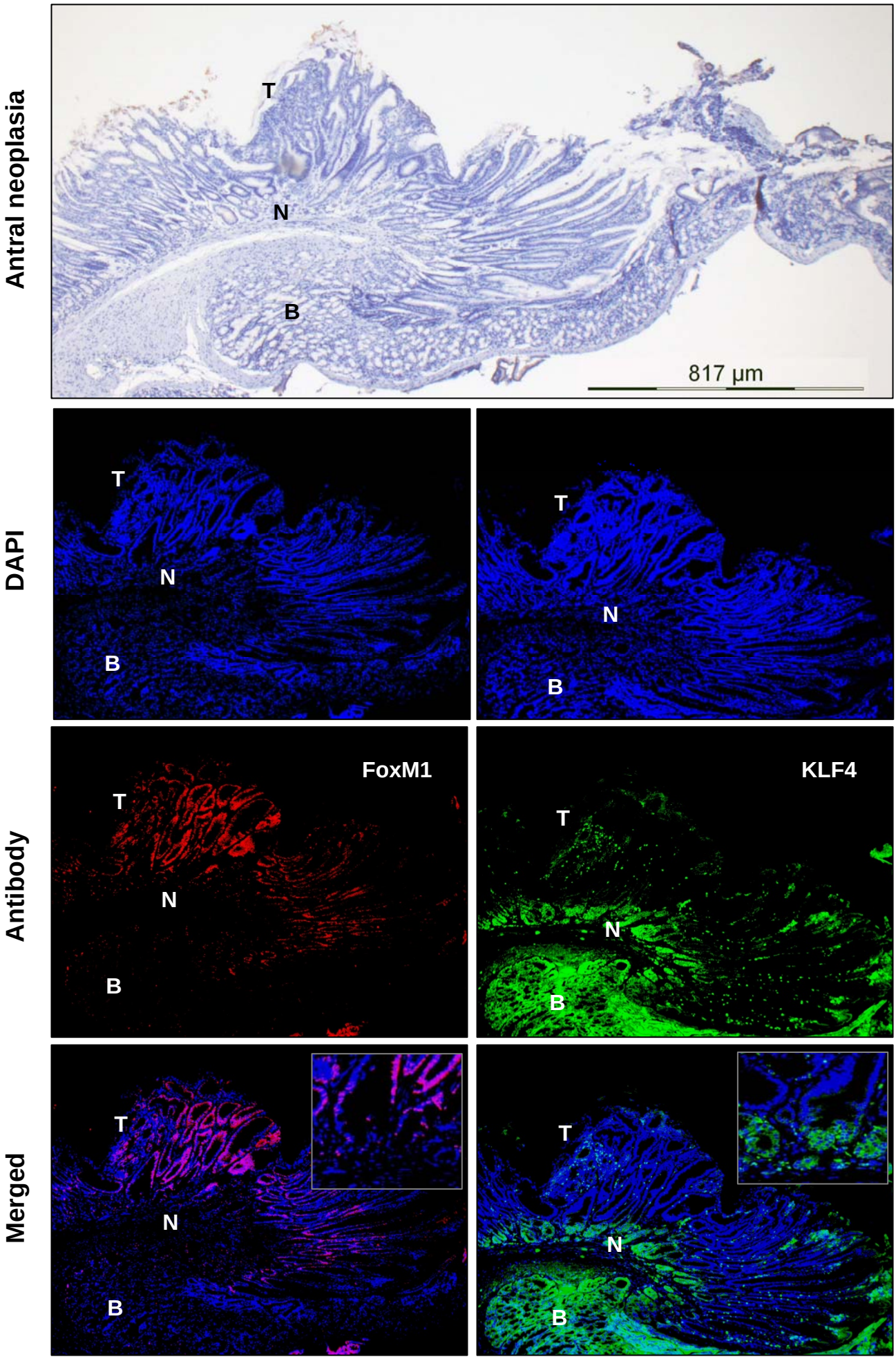
Immunohistochemistry and Immunofluorescent Analyses

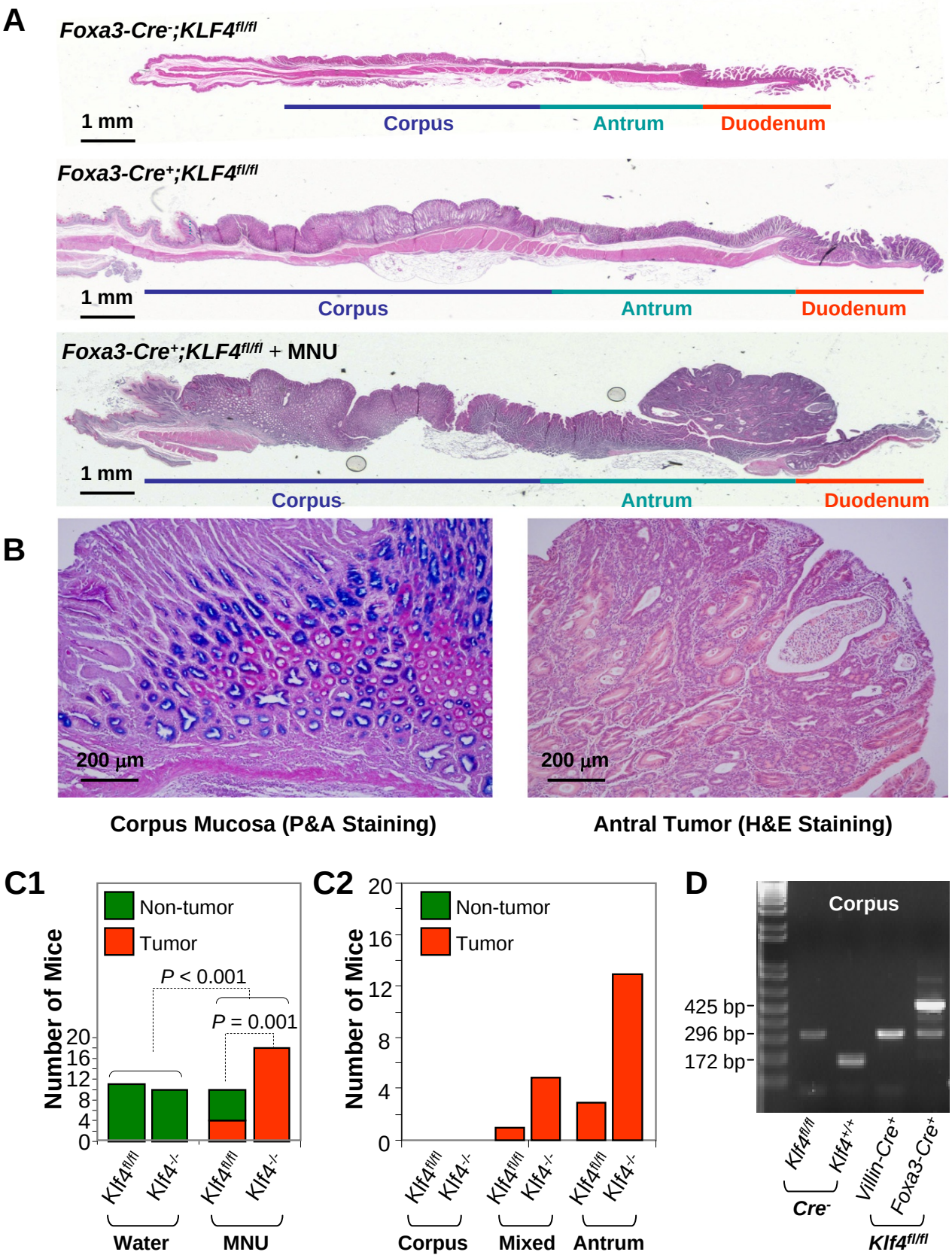
Sections (4 μm thick) of 4% paraformaldehyde-fixed, paraffin-embedded normal gastric mucosa and gastric tumor specimens were prepared and processed for immunostaining with a murine monoclonal antibody against KLF4 (NBP1-21047, clone AT4E6, 1:100 dilution; Novus Biologicals, Littleton, CO), murine monoclonal antibody against FoxM1 (AT2098a, clone 5G10, 1:100 dilution; Abgent), murine monoclonal antibody against CRE (C7988, 1:100 dilution; Sigma Chemical Co.), rabbit polyclonal antibody against human proliferating cell nuclear antigen (sc-7907, 1:200 dilution; Santa Cruz Biotechnology), and rabbit polyclonal antibody against Villin (HPA006884, 1:100 dilution; Sigma Chemical Co.). The HistoMouse-MAX kit (87-9551; Invitrogen, Carlsbad, CA) was used to detect a horseradish peroxidase-conjugated secondary antibody and the chromogen 3,3'-diaminobenzidine in the tissue samples. The molecular markers were identified in different sections of the same tissue blocks. Staining without a primary antibody was used as a negative control. For immunofluorescent analysis of CRE and Villin, the secondary antibodies Alexa Fluor 594 goat anti-mouse IgG (A-11005, 1:100 dilution; Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (A-11008, 1:100 dilution; Invitrogen), respectively, were used and then counterstained with 4',6-diamidino-2-phenylindole. No specific staining was observed in the negative control slides prepared without using a primary antibody.

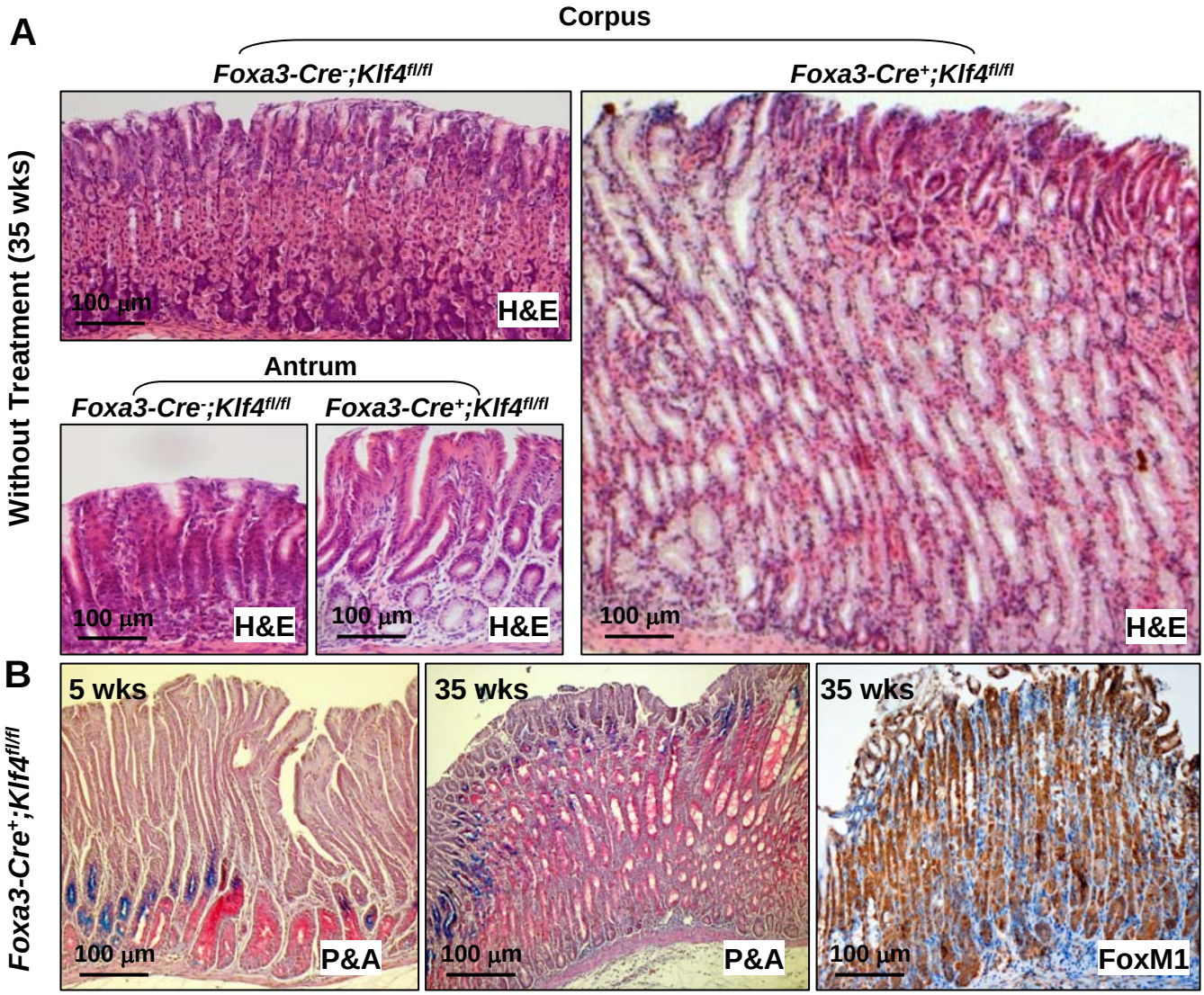

A
Villin-Cre-;KLF4fl/fl
Villin-Cre+;KLF4fl/fl
DAPI
Villlin
Merge
100 μm
B
CRE
100 μm


A
DAPI
CRE
Merge
Villin-Cre+;KLF4+/+
Villin-Cre+;KLF4fl/fl
100 μm
B
KLF4+/+
KLF4+/+/MNU
Villlin
DAPI
Merge
100 μm
C
DAPI
CRE
Villlin
Merge
Villin-Cre+;KLF4+/+
200 μm
D
DAPI
CRE
Villlin
Merge
Villin-Cre+;KLF4fl/fl /MNU
200 μm


A
Corpus
Antrum
Colon
KLF4+/+
100 μm
H&E
100 μm
H&E
100 μm
P&A
B
KLF4-/- Antrum/50 wks
KLF4-/- Antrum/80 wks
KLF4-/-/MNU Antrum/35 wks
hyperplasia
100 μm
H&E
100 μm
P&A
100 μm
P&A
C
KLF4+/+ Antrum/50 wks
KLF4-/- Antrum/50 wks
KLF4-/-/MNU/50 wks
Ki67
100 μm
100 μm
100 μm


Antral neoplasia
T
N
B
817 μm
DAPI
T
N
B
T
N
B
Antibody
FoxM1
T
N
B
KLF4
T
N
B
Merged
T
N
B
T
N
B


A
Foxa3-Cre-;KLF4fl/fl
1 mm
Corpus
Antrum
Duodenum
Foxa3-Cre+;KLF4fl/fl
1 mm
Corpus
Antrum
Duodenum
Foxa3-Cre+;KLF4fl/fl + MNU
1 mm
Corpus
Antrum
Duodenum
B
200 μm
200 μm
Corpus Mucosa (P&A Staining)
Antral Tumor (H&E Staining)
C1
Number of Mice
Non-tumor
Tumor
P < 0.001
P = 0.001
Klf4fl/fl
Klf4-/-
Klf4fl/fl
Klf4-/-
Water
MNU
C2
Number of Mice
Non-tumor
Tumor
Klf4fl/fl
Klf4-/-
Klf4fl/fl
Klf4-/-
Klf4fl/fl
Klf4-/-
Corpus
Mixed
Antrum
D
Corpus
425 bp
296 bp
172 bp
Klf4fl/fl
Klf4+/+
Villin-Cre+
Foxa3-Cre+
Cre-
Klf4fl/fl


A
Corpus
Foxa3-Cre-;Klf4fl/fl
Foxa3-Cre+;Klf4fl/fl
Without Treatment (35 wks)
100 μm
H&E
Antrum
Foxa3-Cre-;Klf4fl/fl
Foxa3-Cre+;Klf4fl/fl
100 μm
H&E
100 μm
H&E
100 μm
H&E
B
Foxa3-Cre+;Klf4fl/fl
5 wks
100 μm
P&A
35 wks
100 μm
P&A
35 wks
100 μm
FoxM1

Patient Characteristics and FoxM1 Expression

Patient Characteristic		FoxM1 Expression				KLF4 Expression			
		Negative	*Moderate*	*Strong*		*Negative*	*Moderate*	*Strong*	
	Total n=86	n=19	n=24	n=43	*p* value	n=27	n=47	n=12	*p* value
Gender									
Male	56	10	15	31	0.317	20	29	7	0.487
Female	30	9	9	12		7	18	5	
Completeness of resection									
R0	69	17	19	33	0.504	16	41	12	0.003
R1, R2	17	2	5	10		11	6	0	
Disease Stage									
I	14	7	4	3	0.135	0	12	2	0.005
II	28	6	8	14		5	17	6	
III	30	4	9	17		15	11	4	
IV	14	2	3	9		7	7	0	
Pathology type									
Papillary	12	2	2	8	0.141	1	9	2	0.358
Tubular	28	8	10	10		7	15	6	
Diffuse	8	2	3	3		2	4	2	
Mucinous	5	2	1	2		2	2	1	
Signet ring	21	4	8	9		9	11	1	
Mixed	12	1	0	11		6	6	0	
Lauren's classification									
Intestinal	53	15	15	23	0.163	14	30	9	0.351
Diffuse	33	4	9	20		13	17	3	

Sections (5 μm thick) of formalin-fixed, paraffin-embedded gastric tumor specimens were prepared and processed for immunostaining using rabbit polyclonal antibodies against human FoxM1 and KLF4 (Santa Cruz Biotechnology). A positive reaction was indicated by a reddish-brown precipitate in the nuclei and/or cytoplasm. FoxM1- and KLF4-positive staining was classified as negative positive, moderate, or strongly positive according to the percentage of positive cells and staining intensity. Two independent investigators examined five random fields (one field = 0.159 mm^2 at a magnification of ×100) in each specimen and scored each specimen without knowledge of patient outcome (double-blinded). Mean values of the two investigators' scores were presented. Note that Pearson's χ^2 test was performed to determine the statistical significance of the relationships between the expression levels of FoxM1 and KLF4 and various parameters.