

Loss of *Klf4* in Mice Causes Altered Proliferation and Differentiation and Precancerous Changes in the Adult Stomach

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Background & Aims: The epithelial zinc-finger transcription factor *Klf4* (formerly GKLF) regulates cellular proliferation and differentiation in vitro. *Klf4* null mice die by postnatal day 1 and show changes in epithelial differentiation of skin and colon. **Methods:** We used tissue-specific gene ablation to generate mice lacking *Klf4* in their gastric epithelia. *Klf4* mutant mice and controls were killed for histology, immunohistochemistry, quantitative real-time polymerase chain reaction (qPCR), and serum gastrin levels. *Klf4* messenger RNA (mRNA) levels were analyzed in *Foxa3-Cdx2* transgenic mice and controls. Human gastric cancers and matched normal tissue were used for qPCR and immunohistochemistry for KLF4. **Results:** *Klf4* mutant mice survive to adulthood and show increased proliferation and altered differentiation of their gastric epithelia. *Klf4* mutants also display aberrant expression of acidic mucins and TFF2/SP-positive cells, findings characteristic of premalignant conditions, but no inflammation, intestinal metaplasia, dysplasia, or cancer up to 1 year of age. Expression of KLF4 is nearly absent in human gastric cancer, suggesting that failure to activate *KLF4* during normal cellular differentiation may be a common feature of gastric cancers. *p21^{WAF1/CIP1}* is an in vivo target of *Klf4*, but *Klf4* is not a mediator of *Cdx2*. **Conclusions:** Loss of a single genetic factor, *Klf4*, leads to dramatic changes in the gastric epithelia of mice, and *Klf4* is part of a regulatory pathway involving *p21^{WAF1/CIP1}* but not *Cdx2*. Thus, *Klf4* is critical for normal gastric epithelial homeostasis.

An understanding of gastric epithelial biology is essential for the identification of factors involved in the development and progression of gastric diseases, both benign and malignant. In the healthy adult, the rates of cell production and loss balance exactly and the gastric epithelial cell lineages differentiate in remarkably constant proportions.¹ Perturbation of this delicate balance leads to the development of peptic ulcers and gastric cancer. Thus, the ability of the gastric epithelium to maintain tight control of

proliferation and differentiation is critical for both normal homeostasis and tumor prevention.

The basic unit of proliferation and differentiation in the gastric epithelium is a tubular invagination of the mucosa called the pit-gland unit, which consists of the pit, isthmus, neck, and base.² Multipotent stem cells in the isthmus give rise to daughter cells, which proliferate and differentiate while migrating either to the surface or to the base of the gland. The adult gastric unit contains at least 5 different mature cell types: the pit or surface mucous cell, which produces mucins and other factors involved in mucosal protection; the parietal or oxyntic cell, which secretes acid; the zymogenic or chief cell, which secretes pepsin; the enteroendocrine cell, which elaborates a number of gastric hormones including gastrin; and the caveolated or brush cell.¹ The role of a sixth cell type, the mucus neck cell, remains controversial.^{3,4}

Animal models have provided valuable insight into the molecular mechanisms regulating gastric epithelial homeostasis. Of the 3 principal cell lineages (pit, zymogenic, parietal), only the parietal cell completes terminal differentiation in the stem cell zone, enabling parietal cells to influence the differentiation of other cell types.² Consistent with this, lineage ablation of parietal cells leads to increased proliferation in the stem cell compartment and altered differentiation of zymogenic and pit cells.⁵ Transgenic expression of SV40 T antigen in parietal cell progenitors leads to transdifferentiation to a neuroendocrine cell type and metastatic gastric cancer, showing the plasticity of these cells.⁶ Other mouse models highlight the ability of genetic alterations to change cellular differentiation pathways: expression of the *K-ras* oncogene in the gastric isthmus results in mucus neck

Abbreviations used in this paper: qPCR, quantitative real-time polymerase chain reaction; SPEM, SP-expressing metaplasia.

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cell hyperplasia and decreased parietal cell differentiation⁷; *transforming growth factor- α* overexpression produces expansion of the surface mucus cell population and depletion of parietal and chief cells, a condition resembling Menetrier's disease⁸; forced expression of *Cdx2* leads to the development of intestinal metaplasia⁹; and gastrin-deficient mice show altered differentiation of parietal, zymogenic, and mucus neck cells.¹⁰

Inflammation or disruption of the mucosal barrier also can alter gastric epithelial homeostasis profoundly. Mice lacking secretory phospholipase A2 infected with *Helicobacter felis* develop inflammation, gastric hypertrophy, and perturbed differentiation of parietal, chief, and mucus neck cells.¹¹ Combined with chronic hypergastrinemia, *H felis*-infected mice develop accelerated gastric cancer.¹² Targeted gene disruption of *TFF2/SP* leads to decreased cell proliferation and increased susceptibility to nonsteroidal anti-inflammatory drug injury.¹³ TFF2, which normally is expressed in mucus neck cells,³ is also an important marker for the development of gastric cancer.¹⁴ Aberrant expression of an SP-expressing metaplasia (SP-EM) cell lineage is seen in *H felis*-infected mice, in response to carcinogen administration in rats, and in human gastric adenocarcinoma.^{15–17} SP-EM is associated strongly with early gastric cancers and is observed in gastric biopsy specimens before the development of cancer.¹⁷ Despite these insights, the specific genetic factors that govern gastric epithelial homeostasis in vivo are not well understood.

The epithelial zinc-finger transcription factor Klf4 (*Krüppel*-like factor 4, previously known as GSKF) is an important regulator of cellular proliferation and differentiation in vitro.¹⁸ Klf4 is expressed highly in growth-arrested cells, is nearly undetectable in dividing cells, and controls a number of critical targets including the cdk-inhibitor *p21^{WAF1/CIP1}*.^{19,20} Klf4 also directly regulates a number of key gastrointestinal differentiation markers, including *keratin 4* and *intestinal alkaline phosphatase*.^{21,22} Decreased *Klf4* expression has been noted in a number of tumors, including adenomas of *APC^{Min}* mice and human colorectal cancer,²³ and *KLF4* has been identified as a potential tumor-suppressor gene in colorectal and bladder cancer.^{24,25} The functional analyses of Klf4 have been limited, however, owing to the early lethality of mice homozygous for a null mutation in *Klf4*.^{26,27} These mice show abnormal skin and colonic epithelial differentiation, no changes in proliferation, and die on postnatal day 1 from a defect in skin barrier function. As models such as the *p53* null mouse have shown,²⁸ the effect of genetic loss in vivo cannot always be predicted from in vitro data.

By using tissue-specific gene ablation in mice, we show that *Klf4* controls gastric epithelial proliferation and differentiation of parietal, zymogenic, pit, and mucus neck cell lineages, showing a functional relationship between *Klf4* and proliferation and differentiation in the epithelia of adult animals. *Klf4* mutant mice have precancerous changes in the gastric epithelia, and we find a marked decrease in KLF4 expression in human gastric cancers. Finally, we identify *p21^{WAF1/CIP1}* as the first in vivo target of Klf4. Thus, *Klf4* is a critical regulator of both proliferation and differentiation in vivo and is required for normal gastric epithelial homeostasis.

Materials and Methods

Derivation of *Klf4* Mutant Mice

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. The derivation of embryonic stem cells heterozygous for the floxed *Klf4* allele was described previously.²⁷ A *Foxa3* YAC²⁹ was used to direct expression of Cre recombinase to the glandular gastric mucosa. Cre activity was confirmed by mating to a reporter line.³⁰ Mutant mice were homozygous for the *Klf4* floxed allele and hemizygous for the *Cre* transgene, and mice used for the experiments were of a mixed genetic background.

Histology

Stomachs were removed and tissues were processed as previously described.²⁷ Slides were stained with H&E, periodic acid-Schiff, or Alcian blue with nuclear fast red.²⁷ Stained sections were reviewed by a gastrointestinal pathologist who was blinded to genotype for evidence of inflammation, dysplasia, and neoplasia. The following numbers of matched littermate control and mutant mice were examined histologically: age 2 weeks, 2 pairs; age 6 months, 6 pairs; and age 1 year, 4 pairs. Images were captured on a Nikon Eclipse E600 microscope (Melville, NY) and Photometrics CoolSNAP CCD camera (Roper Scientific, Trenton, NJ).

Immunohistochemistry and Quantitation of Cells

We injected 6-month-old mice with bromodeoxyuridine Labeling Reagent (Zymed, South San Francisco, CA) 1 hour before death, removed the stomachs, and prepared them as described earlier. Human tissue from 2 gastric cancers and 2 control stomachs was obtained from the National Cancer Institute Cooperative Human Tissue Network. We also used tissue from 59 human gastric cancers and matched controls on the Histo-Array human stomach cancer tissue array (Imgenex, San Diego, CA). Rabbit polyclonal anti-Klf4/KLF4 was generated against amino acids 91–105 of mouse KLF4 (Biosource International/QCB, Hopkinton, MA).

We performed microwave antigen retrieval and processed the tissues,²⁷ followed by incubation with one of the following

primary antibodies: sheep antibromodeoxyuridine (1:500, US Biological, Swampscott, MA), rabbit anti-caspase 3 (1:750, R&D Systems, Minneapolis, MN), rabbit anti-chromogranin A (1:3000; Diasorin, Stillwater, MN), mouse anti-H⁺/K⁺ adenosine triphosphatase (1:2500, Medical & Biological Laboratories, Nagoya, Japan), rabbit anti-human intrinsic factor (1:5000, a gift from Dr. David Alpers, St. Louis, MO), rabbit anti-Klf4/KLF4 (1:10,000), rabbit anti-TFF1 (1:1000, a gift from Dr. Andrew Giraud, Melbourne, Australia), or rabbit anti-TFF2/SP (undiluted, a gift from Dr. Nicholas Wright, London, England). Species-specific secondary antibodies were added, and antibody binding was detected.²⁷ Images were captured on a Nikon Eclipse E600 microscope and Photometric Coolsnap CCD camera (Roper Scientific).

Gastric cell types, except enteroendocrine cells, were counted in 10 randomly selected gastric units from 2 mutant and 2 littermate control mice at 6 months of age.¹³ All cells were counted in the gastric body, except gastrin cells, which were measured in the antrum. Because of the low number of cells per gland, total endocrine, somatostatin, gastrin, and bromodeoxyuridine- or caspase 3-labeled cells were counted in 25 gastric units. Results were expressed as the mean number of cells per gastric unit \pm SEM.

Quantitative Real-Time Polymerase Chain Reaction

We isolated RNA from whole mouse stomach, from forestomach to pylorus, using the ToTALLY RNA kit (Ambion, Austin, TX). Human RNA from 3 gastric cancers and 2 control stomachs was obtained from the National Cancer Institute Cooperative Human Tissue Network. We reverse-transcribed RNA using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA). We designed primers and performed quantitative real-time polymerase chain reaction (qPCR) analysis in triplicate with complementary DNA from 3 *Klf4* mutant mice and 3 littermate controls at 6 months of age or 3 *Foxa3-Cdx2* transgenic mice⁹ and 3 littermate controls at 5 months of age. Analyses were performed on a Stratagene Mx4000 Multiplex Quantitative PCR System using Brilliant SYBR Green QPCR Reagents (Stratagene, La Jolla, CA). TATA-box binding protein was used as the internal control. Primer sequences are available on request.

Measurement of Serum Gastrin Levels

We obtained serum from 2 *Klf4* mutant mice and 2 littermate controls at 1 year of age by cardiac puncture at death. Before death, mice were fed ad libitum. Circulating gastrin concentrations were determined by radioimmunoassay using antibodies to the COOH terminus of gastrin as described previously.³¹

Results

Tissue-Specific Ablation of *Klf4*

To investigate the role of *Klf4* in epithelial proliferation and differentiation in the adult, we used tissue-

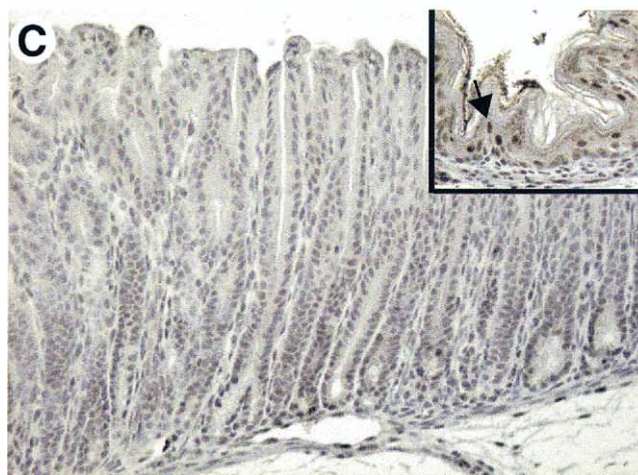
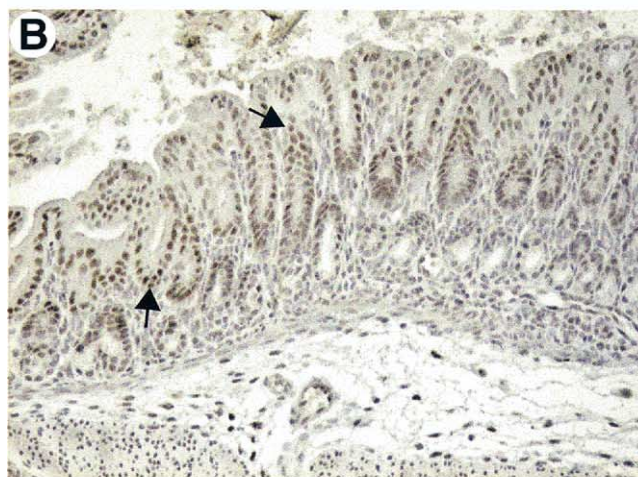
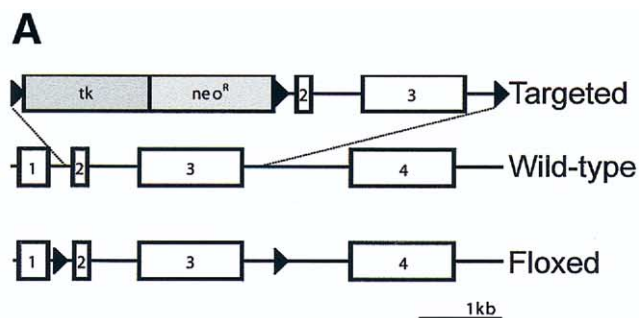
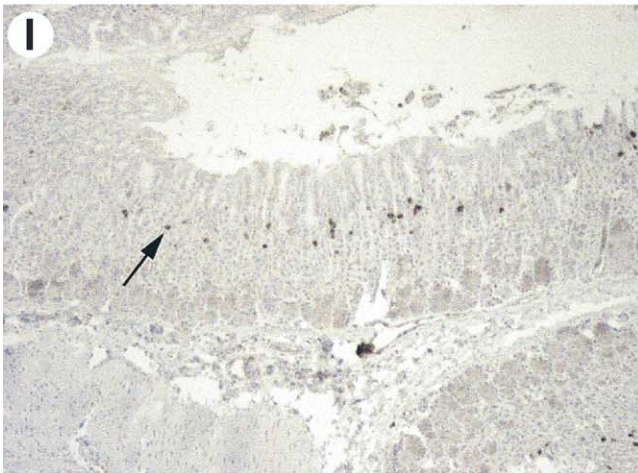
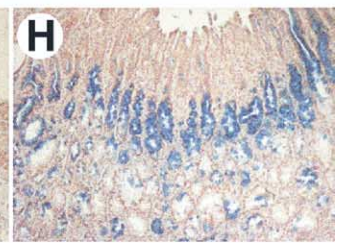
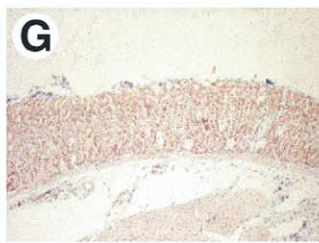
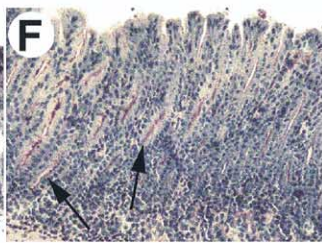
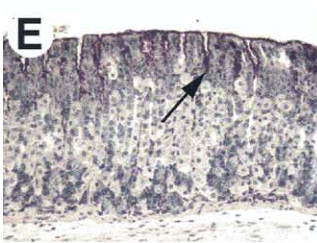
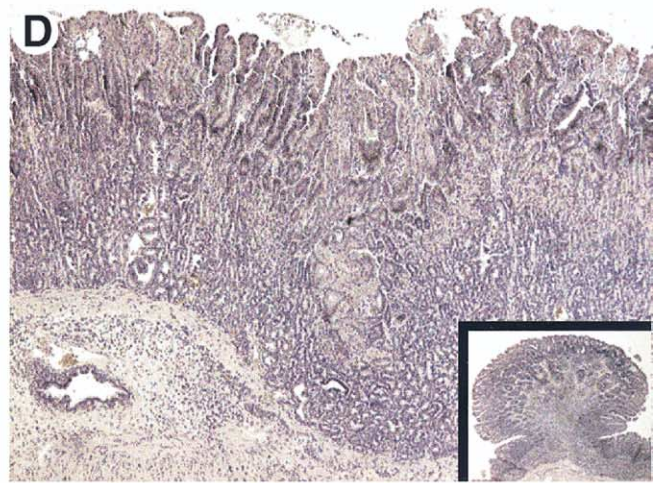
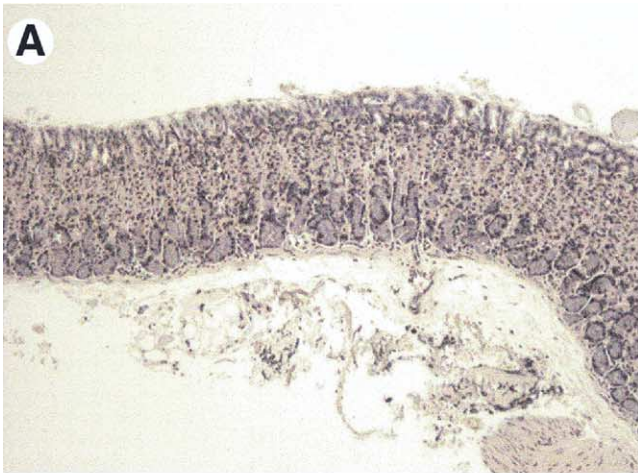


Figure 1. *Klf4* was ablated successfully in the gastric epithelia of *Foxa3-Cre/Klf4^{loxP/loxP}* mice. (A) Targeting strategy for generation of the *Klf4^{loxP}* allele. A targeting construct was designed to flank exons 2 and 3 of the *Klf4* gene with intronic loxP sites. The *Klf4* gene contains 4 exons, and deletion of exons 2 and 3 produced a functional null allele. (B and C) Immunohistochemistry for Klf4 in 2-week-old mice. (B) In control mice, Klf4 (arrows) was seen in nuclei of terminally differentiated cells in the mid- to upper part of the gastric unit. (C) *Klf4* mutant mice showed loss of Klf4 expression in the glandular gastric epithelia by 2 weeks of age. (C, inset) As expected, Klf4 staining (arrow) still was seen in the squamous gastric mucosa of *Klf4* mutant mice. (B and C) Magnification, 100 \times . (C) Inset shown at higher magnification.



specific gene ablation of *Klf4* using the Cre-loxP system. The *Klf4* gene contains 4 exons (Figure 1A), and deletion of exons 2 and 3 produces a functional null allele.^{26,27} *Klf4^{loxP/loxP}* mice had no abnormalities of development, histology, gene expression, or growth characteristics, indicating that the *Klf4^{loxP}* allele functionally is wild-type. To ablate the *Klf4* gene in the gastric mucosa, we used a *Foxa3-Cre* YAC transgene, which directs expression to all cells of the glandular stomach, as well as to cells of the liver, pancreas, small intestine, colon, ovary, testis, heart, and adipose tissue, but not the squamous stomach or skin.^{9,29} Developmentally, *Foxa3* messenger RNA (mRNA) first is detected in endodermal cells of the invaginating hindgut at E8.5, with no expression in embryonic ectoderm or mesoderm.³²

Mice that expressed Cre and were homozygous for the floxed *Klf4* allele (*Foxa3-Cre/Klf4^{loxP/loxP}* mice) survived to at least 1 year of age and appeared to grow normally. We successfully deleted *Klf4* from the gastric epithelium as determined by qPCR of whole gastric tissue, which showed a 95% decrease in *Klf4* expression (9.00 vs .46, normalized to TBP control; $P < .05$). *Klf4* immunostaining of control mice (Figure 1B) revealed expression in the mid- to upper portion of the gastric unit, a region populated by differentiating and terminally differentiated epithelial cells. In *Foxa3-Cre/Klf4^{loxP/loxP}* mice, this *Klf4* expression was lost (Figure 1C). Deletion of *Klf4* throughout the glandular stomach, including the antrum, was complete by at least 2 weeks of age, and the presence of any residual *Klf4* message likely was caused by the expected lack of *Klf4* deletion in the squamous stomach (Figure 1C, inset). In the small intestine and colon, *Klf4* deletion was mosaic (data not shown).

Gastric Epithelial Abnormalities in *Klf4* Mutant Mice

Foxa3-Cre/Klf4^{loxP/loxP} mice began to show changes in their gastric epithelia, with gastric hypertrophy and mucus cell hyperplasia, starting at 2 weeks of age (not shown). By 6 months of age, compared with control mice

Table 1. Effect of *Klf4* Deficiency on the Gastric Mucosa

	Control mice	<i>Klf4</i> -deficient mice
Total cells per gland	40.4 ± 1.1	72.4 ± 2.1 ^a
Proliferative index	1.3 ± .1	5.0 ± .3 ^a
Apoptotic rate	2.1 ± .2	2.0 ± .2
Surface mucus cells per gland	13.5 ± .7	29.1 ± 1.9 ^a
Mucous neck cells per gland	11.3 ± .7	42.1 ± .8 ^a
Parietal cells per gland	11.0 ± .9	5.4 ± .4 ^a
Zymogenic cells per gland	15.8 ± 1.0	6.9 ± .4 ^a
Endocrine cells per gland	1.4 ± .1	1.2 ± .1
Somatostatin-positive cells	.7 ± .1	.5 ± .0
Gastrin-positive cells ^b	2.7 ± .2	3.0 ± .6
Serum gastrin levels	30 ± 10 pmol/L	21 ± 10 pmol/L

NOTE. n = 2.

^a $P < .05$ vs control.

^bGastrin-positive cells measured from antrum.

(Figure 2A), *Klf4* mutant mice (Figure 2B) had severe distortion of gastric pit glands, with marked epithelial hypertrophy, further expansion of mucus cells, and decreased numbers of parietal cells (Table 1). At 1 year of age, compared with controls (Figure 2C), *Klf4* mutant mice (Figure 2D) showed greater hypertrophy and glandular distortion. Polypoid lesions of the mucosa (Figure 2D, inset) were present at 1 year of age as well, but no inflammation, dysplasia, or malignancies were seen in the gastric epithelia at any time point. Because *Klf4* mutant mice at stages from 6 months to 1 year of age had generally similar phenotypes, we chose to focus further studies on 6-month-old *Klf4* mutant mice.

To characterize the expansion of mucus cells in the *Klf4* mutant mice, we stained gastric mucosa with periodic acid–Schiff and Alcian blue. PAS staining detects both neutral and acidic mucins, whereas Alcian blue detects only acidic mucins, not seen normally in the stomach. Compared with controls (Figure 2E), elaboration of periodic acid–Schiff–positive mucins was more extensive in the *Klf4* mutant mice (Figure 2F), especially in the mucus cells of the neck. Although no Alcian blue staining was seen in control mice (Figure 2G), the gastric

Figure 2. *Klf4* mutant mice had marked abnormalities of their gastric epithelia. (A–D) H&E-stained gastric mucosa from (A and C) control and (B and D) *Klf4* mutant mice. (A) Compared with controls, (B) *Klf4* mutant mice at 6 months of age had dramatic distortions of the gastric units, marked epithelial hypertrophy, striking increases in the number of mucus cells, and decreased numbers of parietal cells. (C) At 1 year of age, control mice showed normal gastric architecture (D) whereas mutant mice showed continued epithelial hypertrophy and glandular distortion. (D, inset) Polypoid lesions also were seen in 1-year-old mutant mice, but no gastric malignancies and no inflammation were seen at any time point. (E and F) Periodic acid–Schiff staining of (E) control and (F) *Klf4* mutant mice indicated aberrant expression of gastric mucins in the neck regions of mutant mice. (G and H) Staining of (G) control and (H) *Klf4* mutant mice with Alcian blue detected acidic mucins not normally found in the stomach. (I and J) Immunohistochemistry for bromodeoxyuridine indicated dividing cells in the gastric epithelia. (I) In control mice, each gastric unit had, in general, 1 to 2 proliferating cells (arrow) located in the isthmus or neck region of the gland. (J) *Klf4* mutant mice showed a 4-fold increase ($P < 1 \times 10^{-17}$) in the number of proliferating cells (arrows) and a shift in the location of labeled cells toward the surface, with scattered proliferating cells throughout the remainder of the gastric unit. Magnification was as follows: (E and F) 200×, (A, B, G–J) 100×, (C and D) 40×. (D) Inset shown at decreased magnification.

Table 2. Expression of Intestine-Specific Markers in the Gastric Mucosa

Gene name	Control	Mutant
<i>muc2</i>	.11 ± .05	.11 ± .05
<i>TFF3</i>	.19 ± .08	.20 ± .12
<i>villin</i>	5.0 ± 1.5	4.1 ± .8

NOTE. Expression relative to *TBP* control.
n = 3.

epithelia of *Klf4* mutant mice (Figure 2H) contained Alcian blue–positive mucins in the neck region. Ectopic expression of acidic mucins is found in intestinal metaplasia,⁹ but we saw no goblet cells in the *Klf4* mutant mice and no gastric expression of the intestine-specific markers *muc2*, *TFF3*, and *villin* by qPCR (Table 2). Gastric expression of acidic mucins also is seen in gastric adenocarcinomas, both mucinous and nonmucinous, including those of the diffuse and intestinal types.³³ Because we found no evidence of gastric cancer in the mice analyzed, the presence of acidic mucins in the gastric mucosa of *Klf4* mutant mice represented a premalignant condition.

Given the marked hypertrophy of gastric epithelia in *Foxa3-Cre/Klf4^{loxP/loxP}* mice, we investigated whether proliferative and/or apoptotic pathways were altered in *Klf4* mutant mice. In control mice (Figure 2I), proliferation occurred in a narrow band localized to the isthmus of the gastric gland. *Klf4* mutant mice (Figure 2J) had a shift of proliferating cells toward the lumen, with scattered proliferation throughout the gastric unit, and a 4-fold increase in the number of proliferating cells (Table 1). Staining with an antibody to caspase-3 revealed no difference in the number of apoptotic cells in the gastric epithelia of control and *Klf4* mutant mice (Table 1). Thus, *Klf4* impacted on cell proliferation but not apoptosis in the gastric epithelium.

We next focused on the differentiation pathways in the gastric epithelium. In control mice (Figure 3A), parietal cells were seen throughout the gastric unit. *Klf4* mutant mice (Figure 3B) had a more than 50% decrease in the number of parietal cells (Table 1). Mature zymogenic cells also were decreased by more than 50% in *Klf4* mutant mice (Table 1). Control mice (Figure 3C) showed prominent staining for mature zymogenic cells in the base of the gastric gland. In *Klf4* mutant mice (Figure 3D), staining for these cells was decreased and confined to the most basal segment of the gland. Mucus neck cells may represent a distinct, functional cell lineage, secreting a number of peptides with luminal protective features or merely a transit cell population, intermediate between stem cells and differentiated zymogenic cells.^{3,4} Compared with control mice (Figure 3E), *Klf4* mutant

mice (Figure 3F) showed a 4-fold increase in the number of TFF2/SP-positive mucus cells (Table 1). Such SPEM lineages are seen in premalignant and malignant lesions of the stomach.^{7,16,17}

Surface mucus (pit) cells were confined to the gastric pits in both control (Figure 3G) and *Klf4* mutant mice (Figure 3H), but the number of pit cells was increased approximately 2-fold in *Klf4* mutants (Table 1). The number of total enteroendocrine, somatostatin, and gastrin cells were unchanged in *Klf4* mutant mice (Table 1). Serum gastrin levels also were similar in control and mutant mice (Table 1), indicating that alterations in gastrin levels were not responsible for the gastric phenotype in *Klf4* mutant mice. Although a 2-fold increase in pit cells and loss of zymogenic cells were noted with lineage ablation of parietal cells,⁵ a decrease and not an increase in mucus neck cells was seen in this model. Thus, loss of parietal cells alone was not responsible for the phenotype in *Klf4* mutant mice.

KLF4 Expression Is Decreased Dramatically in Human Gastric Cancers

Because *Klf4*-deficient mice develop premalignant changes in their gastric epithelia, we hypothesized that loss of KLF4 expression might be seen in human gastric cancers. In fact, KLF4 expression was decreased dramatically in both intestinal and diffuse-type human gastric cancer. As in the mouse (Figure 1B), nuclear KLF4 staining was seen in cells in the mid- to upper portion of the gastric unit in adjacent normal tissues from gastric cancer patients (Figure 4A, B). Interestingly, KLF4 expression was not seen in all terminally differentiated cells, including some pit and parietal cells. In contrast, KLF4 expression was absent in nearly all cells of intestinal (Figure 4C) and diffuse-type (Figure 4D) gastric cancers. By qPCR, human gastric cancers had a 96% decrease in *KLF4* mRNA expression compared with normal controls (39.6 vs 1.6, normalized to TBP control; $P < .05$). Recently, *KLF4* was identified as a potential tumor suppressor in colorectal cancer.²⁵ Thus, the failure to activate *KLF4* during normal cellular differentiation may be a common feature of gastrointestinal carcinogenesis.

Klf4 Is Part of a Regulatory Pathway Involving *p21^{WAF1/CIP1}* But Not *Cdx2*

To understand the mechanisms by which *Klf4* regulates gastric epithelial proliferation and differentiation, we examined putative upstream regulators and downstream targets of *Klf4*. Previously, ectopic expression of the caudal-related homeobox gene *Cdx2* induced intestinal metaplasia in the gastric mucosa of transgenic

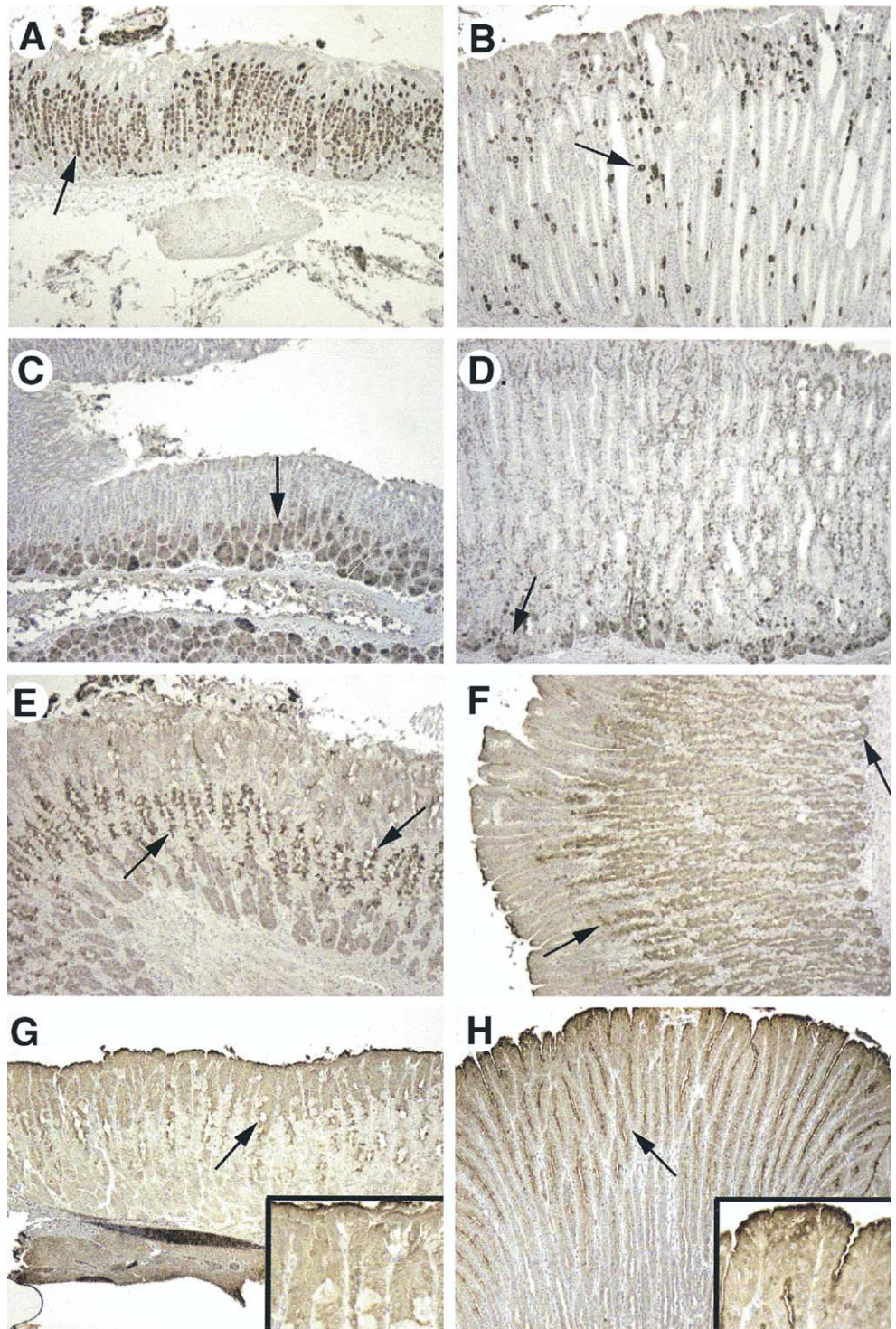


Figure 3. *Klf4* mutant mice had altered differentiation of parietal, zymogenic, pit, and mucus neck cells. (A and B) Staining for the H⁺/K⁺ adenosine triphosphatase indicated the numbers of parietal cells (arrows). (A) In control mice, parietal cells (arrows) were located throughout the gastric gland. (B) *Klf4* mutant mice had dramatically decreased numbers of parietal cells throughout the gastric epithelia. (C and D) Staining for intrinsic factor showed (C) mature zymogenic cells (arrows) at the base of the gastric glands in control mice, (D) with decreased numbers and a more basilar location of zymogenic cells in *Klf4* mutant mice. (E and F) The trefoil protein TFF2/SP was found in (E) mucus neck cells (arrows) of control mice, (F) but the number of TFF2/SP-positive cells was increased markedly in *Klf4* mutant mice, encompassing most of the lower two thirds of the gastric unit. (G and H) Staining for the trefoil protein TFF1 showed increased numbers of surface mucus cells (arrows) in (G) control and (H) *Klf4* mutant mice. (A–H) Magnification, 100×. (G and H) Insets shown at higher magnification.

mice.⁹ Because expression of *Klf4* is dependent on *Cdx2* in vitro,³⁴ we considered the possibility that *Klf4* was a target of *Cdx2* in vivo. If this were the case, we would expect to see decreased expression of *Klf4* in *Foxa3-Cdx2* transgenic mice,⁹ contributing to the abnormal gastric epithelial differentiation and metaplasia in these mice. However, expression of *Klf4* was not altered significantly

in the gastric epithelia of 5-month-old *Cdx2* transgenic mice (1.2-fold increase, *P* = .63; data not shown). By 5 months of age, the alterations in the gastric mucosa of the *Foxa3-Cdx2* transgenic mice already are well-established.⁹ Thus, the dramatic changes in gastric epithelial differentiation in both *Cdx2* transgenic mice and *Klf4*-deficient mice must occur by independent mechanisms.

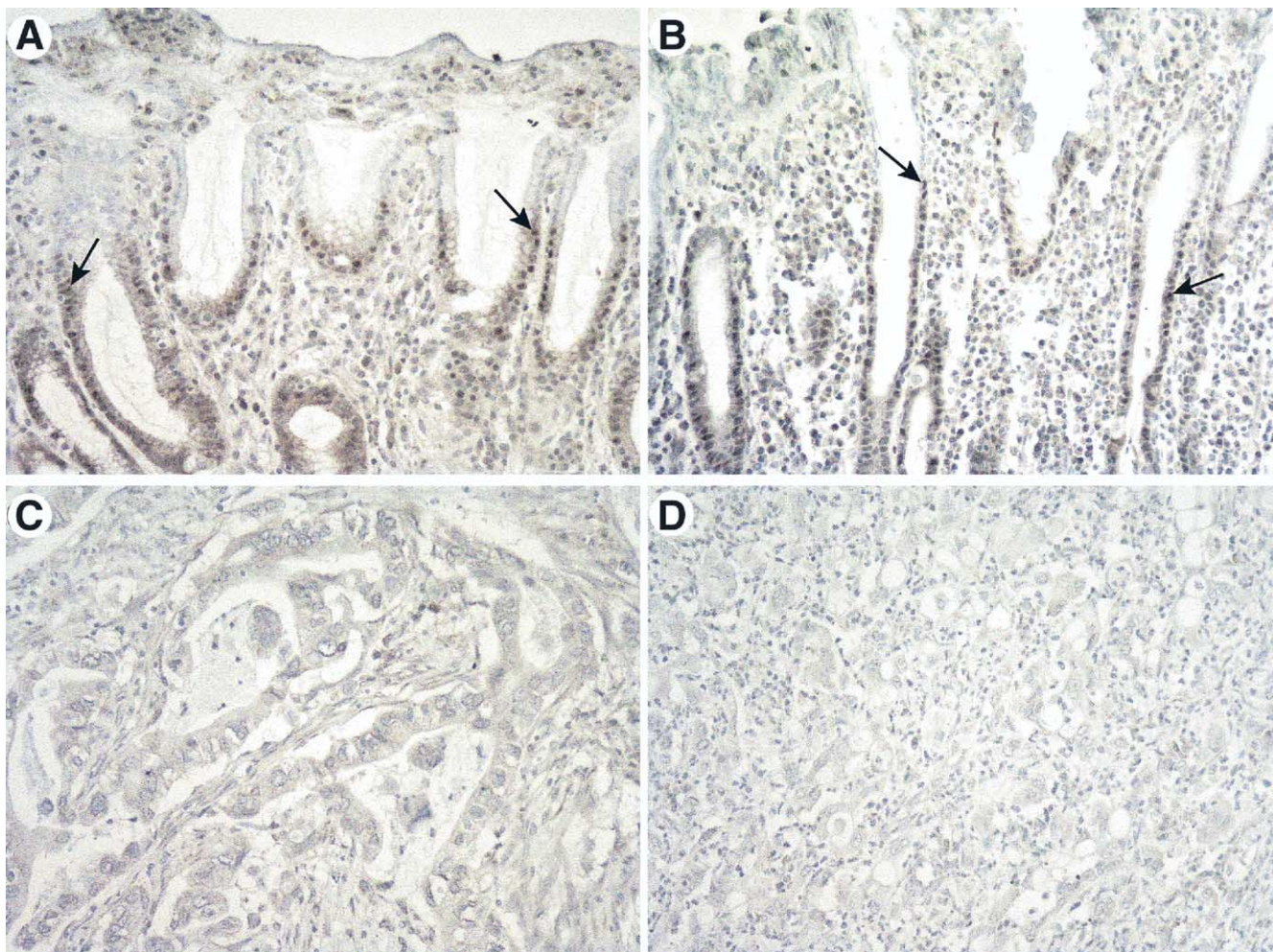


Figure 4. *Klf4* expression was decreased dramatically in human gastric cancers compared with adjacent normal tissue. In normal stomach from patients with (A) intestinal and (B) diffuse-type gastric cancer, nuclear KLF4 expression (arrows) was seen in the mid- to upper portion of the gastric unit. Staining for KLF4 was lost in (C) intestinal and (D) diffuse-type gastric adenocarcinoma. Sections are representative of 6 human gastric cancers analyzed. (A–D) Magnification, 200 \times .

This notion is reinforced by the lack of intestinal metaplasia in the gastric mucosa of *Klf4* mutant mice (Table 2).

Klf4 has been shown to regulate transcriptionally a number of genes in vitro, including the cdk inhibitor *p21*^{WAF1/CIP1}, *cyclinD1*, and *ornitine decarboxylase*.^{19,35,36} By qPCR, we found a 45% decrease in *p21*^{WAF1/CIP1} mRNA expression in *Klf4*-deficient mice ($P < .05$), whereas mRNA levels of *cyclinD1* and *ornitine decarboxylase* were unchanged (data not shown). In addition, mRNA levels of the tumor-suppressor genes *p53* and *p63* were not altered in *Klf4* mutant mice (data not shown). Notably, *p53* has been shown to coordinately regulate the *p21*^{WAF1/CIP1} promoter with *Klf4*.¹⁹ Thus, the decreased *p21*^{WAF1/CIP1} expression was likely a direct consequence of *Klf4* deletion in the stomach.

We next examined the expression of other *Krippel*-like factor (KLF) family members. The tissue-restricted *Klf5* and the ubiquitously expressed *Klf6* (*Zf9*) have been

implicated in cellular proliferation and differentiation and have been shown to interact with *Klf4* in vitro.^{37–39} All of these factors bind similar CACCC DNA elements. Although *Klf4* has not been shown to regulate *Klf5* or *Klf6* directly, we speculated that up-regulation of these genes, especially the pro-proliferative *Klf5*, could contribute to the gastric phenotype in the *Klf4* mutant mice. However, we found no changes in the mRNA expression of *Klf5* and *Klf6* in *Klf4*-deficient mice (data not shown).

Finally, we investigated levels of the putative gastric tumor-suppressor *Runx3* and *transforming growth factor- α* , which produces gastric hyperplasia and changes in cellular differentiation when overexpressed.^{8,40} We hypothesized that alterations in these factors could play a role in the phenotype of *Klf4* mutant mice. However, we found no changes in the mRNA expression of *transforming growth factor- α* or *Runx3* in *Klf4*-deficient mice (data not

shown). Thus, we have identified $p21^{WAF1/CIP1}$ as a specific target of *Klf4* in vivo and a possible contributor to the gastric phenotype in the *Klf4*-deficient mice.

Discussion

Perturbations of the pathways that govern normal gastric epithelial homeostasis, whether through genetic modifications or more general processes such as inflammation or defective mucosal barrier function, lead to the development of gastritis, ulceration, metaplasia, and cancer.⁴¹ In this study, we provide insight into the genetic factors regulating gastric homeostasis by showing that the epithelial zinc-finger protein *Klf4* plays a critical role in maintaining normal gastric epithelial homeostasis. Loss of *Klf4* in the stomach leads to striking changes in the gastric epithelia, in the absence of inflammation, ulceration, intestinal metaplasia, dysplasia, or neoplasia. The lack of inflammation in *Klf4* mutant mice indicates that these alterations are not the result of bacterial overgrowth. In addition, serum gastrin levels are normal in *Klf4* mutant mice, excluding the possibility that these changes are secondary effects of hypergastrinemia or hypogastrinemia.

Klf4 mutant mice have a 4-fold increase in proliferation, a 2-fold increase in the number of pit cells, a 4-fold increase in the number of mucus neck cells, and half the number of parietal and zymogenic cells. How does loss of *Klf4* produce such a dramatic gastric phenotype? Our data suggest that *Klf4* is required to direct the cell-fate decisions of the gastric epithelial precursor cells. Normally, the multipotential gastric stem cells divide asymmetrically to produce 1 stem cell and 1 partially committed precursor cell, which undergoes further division and eventual differentiation into the mature cell lineages.² The differentiation of these precursor cells can be perturbed by a number of processes, including ablation of a single cell type, deletion of a critical trophic factor, oncogenic expression, and inflammation.^{5,7,10,16} Recent experiments have shown the plasticity of the gastric epithelial lineage progenitors.⁶ In the stomach, *Klf4* is expressed in the nuclei of cells in the mid- to upper portion of the gastric unit, including the neck region, placing this transcriptional regulator in a critical position to affect the differentiation of precursor cells. Moreover, *Klf4* functions as both a transcriptional activator and a transcriptional repressor in gastrointestinal epithelia,^{21,22,42} consistent with a role in cell-fate decisions in multiple lineages.

Because *Klf4* mediates p53-dependent G1/S cell-cycle arrest via synergistic induction of $p21^{WAF1/CIP1}$,^{19,20} the decrease in $p21^{WAF1/CIP1}$ expression in *Klf4* mutant mice may contribute to the phenotype in the gastric epithelium. Nonetheless, the lineage-specific *Klf4* targets that

mediate the differentiation of the gastric epithelial precursor cells are not yet clear. Interestingly, homozygous deletion of SP/TFF2 does not lead to the ablation of mucus neck cells,¹³ indicating that this factor marks mucus neck cells but does not control their differentiation. Although *Klf4* regulates goblet cell differentiation in the colon,²⁷ the role of *Klf4* in the regulation of the trefoil proteins TFF1 and TFF2 and the gastric mucins, *Muc5AC* and *Muc6*,⁴³ is not known. Overall, the multitude of *Klf4* targets in the gastric epithelia may best be identified by future functional genomic analyses.

The phenotype of *Klf4* mutant mice resembles that of *H felis*-infected C57BL/6 mice, which have increased proliferation, SPEM, and decreased numbers of parietal and zymogenic cells.^{11,16} In contrast to the *H felis*-infected mice, however, the changes in the *Klf4* mutant mice occur in the absence of an inflammatory response. Particularly intriguing is the presence of SPEM in the *Klf4* mutant mice. Although SPEM in premalignant and malignant lesions of the stomach is well-established,^{7,16,17} its role in malignant transformation is not known. Moreover, expression of *TFF2* has been implicated in the maintenance of epithelial integrity and mucosal healing in vivo.¹³ Given these ambiguities, *Klf4* mutant mice offer a unique opportunity to dissect the complex pathways regulating gastric homeostasis and to investigate the changes in gastric carcinogenesis. Future studies will involve additional perturbations to the gastric epithelia of the *Klf4* mutant mice, including infecting these animals with *Helicobacter* and mating these animals with *p53* null mice, as well as investigation of the molecular mechanisms governing the loss of *KLF4* expression in human gastric cancer.

Here, we have used tissue-specific gene ablation to show that *Klf4* controls both proliferation and differentiation in the gastric epithelium. We have identified *Klf4* as part of a regulatory pathway in the gastric epithelium involving $p21^{WAF1/CIP1}$ but not *Cdx2*. Because human gastric cancers lack *KLF4* expression, failure to activate *KLF4* during normal cellular differentiation may be a common feature of these cancers. In summary, we show that loss of a single genetic factor, *Klf4*, leads to dramatic changes in the gastric epithelia of mice, indicating that *Klf4* is required for normal gastric epithelial homeostasis in vivo.

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