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Disruption of Klf4 in Villin-Positive Gastric Progenitor Cells Promotes Formation and Progression of Tumors of the Antrum in Mice

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Abstract

BACKGROUND & AIMS—Krüppel-like factor 4 (*Klf4*) is a putative gastric tumor suppressor gene. Rare, villin-positive progenitor cells in the gastric antrum have multi-lineage potential. We investigated the function of Klf4 in these cells and in gastric carcinogenesis.

METHODS—We created mice with disruption of *Klf4* in villin-positive antral mucosa cells (*Villin-Cre+;Klf4fl/fl* mice). *Villin-Cre+;Klf4fl/fl* and control mice were given drinking water with or without 240 ppm *N*-methyl-*N*-nitrosourea (MNU) at 5 weeks of age and thereafter on alternating weeks for a total of 10 weeks. Gastric mucosa samples were collected at 35, 50, or 80

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weeks of age from mice that were and were not given MNU, and analyzed by histopathologic and molecular analyses. Findings were compared with those from human gastric tumor specimens.

RESULTS—Preneoplasia formed progressively in the antrum in 35- to 80-week-old *Villin-Cre+;Klf4fl/fl* mice. Gastric tumors developed in 29% of 80-week-old *Villin-Cre+;Klf4fl/fl* mice, which were located exclusively in the lesser curvature of the antrum. MNU accelerated tumor formation, and tumors developed significantly more frequently in *Villin-Cre+;Klf4fl/fl* mice than in control mice, at 35 and 50 weeks of age. Mouse and human gastric tumors had reduced expression of KLF4 and increased expression of FoxM1, compared with healthy gastric tissue. Expression of KLF4 suppressed transcription of FoxM1.

CONCLUSIONS—Inactivation of *Klf4* in villin-positive gastric progenitor cells induces transformation of the gastric mucosa and tumorigenesis in the antrum in mice. *Villin-Cre+;Klf4fl/fl* have greater susceptibility than control mice to chemical-induced gastric carcinogenesis and increased rates of gastric tumor progression.

Keywords

stomach cancer; mouse model; carcinogen; genetic

Gastric cancer is one of the most common cancers worldwide.[1] Its aggressive nature is related to a variety of intracellular events, including activation of oncogenes and inactivation of tumor suppressor genes.[2,3] However, the specific sequence of molecular changes leading to gastric cancer remains unclear.[1–3] Clinically relevant animal models would be particularly useful for further exploration of the molecular pathogenesis of gastric cancer and to serve as preclinical models for evaluation of therapeutic and chemoprevention strategies.[1–3]

The gastrointestinal tract epithelium is a continuously renewing tissue, which also is under continuous exposure to various kinds of carcinogens and injurious agents that can cause cellular stress and trigger epithelial transformation and tumorigenesis.[4] The gastric epithelium in particular contains functionally distinct pyloric and fundic mucosal lineages.[5] The geographically heterogeneous population of multiple cell types in each pyloric gland is generated by controlled division of gastric epithelial stem cells located in the glands.[5] Recent studies revealed that the majority of the pyloric glands are functionally monoclonal in the gastric antrum of adult mice and humans; specifically, all of the cellular progeny in the glandular gastric epithelium arise from a single stem cell.[6–9] Gastric progenitor cell (GPC) studies have identified a rare subpopulation of murine GPCs with robust Villin expression predominantly in the antrum.[6,10] These rare Villin-expressing GPCs are quiescent in the unstimulated stomach; however, they undergo both symmetric and asymmetric division and replace multiple entire pyloric glands during proinflammatory insults.[6]

Villin is an actin-bundling protein found in the apical brush border of absorptive tissue.[11] Villin is also one of the first structural genes to be transcriptionally activated in the embryonic intestinal endoderm.[10] The *Villin* gene is initially expressed in the intestinal hindgut endoderm 9 days post coitum during gut tube closure. Villin expression then rapidly extends throughout the small and large intestines and distal stomach.[12–14] At 16 days post coitum, intestinal cells have their highest levels of Villin expression, whereas neighboring stomach cells have low levels of Villin expression.[14] The promoter specificity in adult tissues has led to the use of Cre recombinase-expressing transgenic mouse models.[10] Researchers have identified several *cis*-regulatory sequences that drive the Villin promoter.[10,15,16]

Increasing evidence has established the relationship between chronic inflammation and gastric cancer.[2,3] Studies have suggested that Krüppel-like factor 4 (KLF4) plays an important role in mediating proinflammatory responses and that KLF4 expression is markedly induced by interferon-γ in macrophages and human colon cancer cell lines.[17] KLF4 is a zinc-finger transcription factor, and KLF4 mRNA expression is found primarily in postmitotic, terminally differentiated epithelial cells in organs such as the skin, lungs, and gastrointestinal tract.[18] Accumulating clinical, experimental, and mechanistic evidence shows that KLF4 is a potential tumor suppressor in patients with various cancers, including gastric cancer.[19–21]

Our recent study has shown that loss of KLF4 and overexpression of FoxM1 are evident in human gastric cancer and altered expression and function of FoxM1 contribute to gastric carcinogenesis.[22] FoxM1 is a member of the Forkhead box transcription factor family.[23] At the mRNA level, FoxM1 expression is ubiquitously expressed in mouse embryonic tissues and in proliferating mouse adult tissues but is extinguished in differentiated cells.[23] Recent studies have suggested that FoxM1 is required to couple the S and M phases of the cell cycle in part through regulating transcription of genes essential for cell cycle progression, including cyclin D1 and p27.[24,25] FoxM1 overexpression has been linked to oncogenesis.[25,26] However, the mechanistic role of FoxM1 in gastric carcinogenesis and its causal link to altered KLF4 function are unknown.

In the present study, we sought to determine the functional significance of *Klf4* inactivation in Villin-positive progenitor cells (GPCs) in gastric carcinogenesis. Our results clearly showed a protective role for *Klf4* in these cells from transformation and carcinogenesis.

Materials and Methods

Detailed materials and methods are described in the Supplementary Methods.

Mouse Strains, Derivations, and Maintenance

The derivation and use of *Klf4-LoxP*, *Villin-Cre*, and *Foxa3-Cre* mice were described previously.[10,21,27] All strains are on C57BL/6 genetic background. 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.

Genotyping and Identification of the Rearranged Alleles

Genomic DNA isolated from tail clippings from the study mice was assayed for the presence of *Klf4*-flox using polymerase chain reaction (PCR) with three primers in the *klf4* gene:[21] exon 1, 5′-ctgggccccacattaatgag-3′; exon 2, 5′-tcgctgacagccatgtcagac; and intron 3, 5′-ccagcagagccgttctggctg-3′. PCRs were carried out using the GoTaq PCR system (Promega, Madison, WI). In this report, the “floxed deletion” was referred as “deletion”, unless specifically stated or indicated otherwise.

Identification of the Villin-Cre Transgene

A mouse model with a 12.4-kb fragment of *Villin* promoter that drives *Cre* gene expression was used in this study.[10] The presence of the Villin-*Cre* transgene in specific tissues was identified using PCR amplification with specific primers for the 12.4-kb Vil*Cre* transgene (The Jackson Laboratory) (forward, 5′-gtgtgggacagagaacaaacc-3′; reverse, 5′-acatcttcaggttctgcggg-3′) and *Cre* gene (forward, 5′-gcggcatggtgcaagttgaat-3′; reverse, 5′-cgttcaccggcatcaacgttt-3′). Also, a 205-bp fragment of *Klf4* was amplified with the following

primers and used as an internal control: forward, 5′-caaatgttgcttgtctggtg-3′; reverse, 5′-tcagtcgagtgcacagttt-3′.

Treatment Protocol for N-Methyl-N-Nitrosourea

The alkylating agent *N*-methyl-*N*-nitrosourea (MNU; Sigma Chemical Co., St. Louis, MO), which is widely used to study gastric carcinogenesis,[28] was dissolved in distilled water at 240 ppm and freshly prepared three times a week for administration to the mice in drinking water in light-shielded bottles.[28] Five-week-old mice were given the MNU-containing drinking water on alternating weeks for a total of 10 weeks of exposure. The mice were killed using CO_2 asphyxiation and underwent a thorough postmortem examination at the age of 35, 50, or 80 weeks.

Statistical Analysis

The two-tailed Fisher exact test was used to determine the significance of the tumor incidence in each group. The two-tailed χ^2 or Fisher exact test was used to determine the significance of the difference between the covariates of KLF4 and FoxM1 expression. In all of the tests, *P* values less than .05 were considered statistically significant. The SPSS software program (version 12.0; SPSS Inc., Chicago, IL) was used for the statistical analyses.

Results

Villin-Cre–Mediated Deletion of the Klf4 Gene in Mice

The lesser curvature of the antrum is the most common site of gastric tumor formation in humans[3] and certain mouse models.[28] Strikingly, recently identified rare *Villin*-positive progenitor cells are localized in the antrum and exhibit multilineage potential as GPCs.[6] Although *Klf4* is regarded as a putative gastric cancer suppressor gene, the functional significance of *Klf4* inactivation in these *Villin*-positive cells is unknown. To determine the effects of *Klf4* ablation in Villin-positive cells on gastric mucosal transformation and tumorigenesis, we genetically generated and verified *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice (Figures 1*A* and 1*B*) using previously published procedures.[6,10,21,27] In *Villin-Cre* transgenic mice, we detected both Villin and Cre protein predominantly in the intestines (sFigure 1*A*), including the duodenum but rarely in the adjacent antrum (sFigure 2A). However, we observed an increase in Villin-positive cells in the antrum in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice (sFigure 2A) and a further rapid and patchy increase in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice upon treatment with MNU (sFigure 2B). This differing pattern of Villin expression in these tissues was consistent with the differing efficacy of *Villin-Cre*–mediated *Klf4* deletion in them (Figure 1*C*). *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice survived to at least 80 weeks of age. Our quantitative PCR analysis revealed that *Klf4* was deleted in more than 95% of the cells in the intestines and less than 20% cells in the antrum of a *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mouse (Figure 1*D*). Morphologically, we observed no discernible changes in the corpus mucosa, whereas loss of both Klf4 expression in small intestinal mucosa and goblet cells in the colonic mucosa further confirmed the expected function of the *Villin-Cre* transgene in *Klf4*$^{fl/fl}$ mice (Figure 1*E*).

Spontaneous Tumors in the Gastric Antrum in Villin-Cre^{+};Klf4$^{fl/fl}$ Mice

We then systematically examined gastric tumor formation in six different mouse strains: *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$, *Villin-Cre*$^{+}$*;Klf4*$^{+/fl}$, *Villin-Cre*$^{+}$*;Klf4*$^{+/+}$, *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$, *Villin-Cre*$^{-}$*;Klf4*$^{+/fl}$, and *Villin-Cre*$^{-}$*;Klf4*$^{+/+}$. No visible tumors formed in the stomachs of any of the mice by the age of 35 or 50 weeks (Figure 2*A*1). At the age of 80 weeks, 29% (5/17) of the *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice and 14% (1/7) of the *Villin-Cre*$^{+}$*;Klf4*$^{+/fl}$ mice had gastric tumors (Figure 2*A*1), and small percentages of these mouse strains also had tumors in other

organs (Figure 2*A*2) and their significance and underlying mechanisms are unclear. In contrast, no visible tumors formed in the stomachs or other organs in the control mouse strains (Figure 2*A*2), including *Villin-Cre*$^{+}$*;Klf4*$^{+/+}$, *Villin-Cre*$^{-}$*;Klf4*$^{+/fl}$, and *Villin-Cre*$^{-}$*;Klf4*$^{+/+}$ mice without MNU treatment (data not shown). More importantly, we found that all of the gastric tumors were located in the lesser curvature of the antrum (Figures 2*B* and 2*C*). Histological examination of gastric antral tumors showed that they were adenomas. The polypoid adenomatous lesions demonstrated elongated pits and enlarged glandular structures which led to additional branching and interglandular bridging in the thickened lamina propria and the intraepithelial compartments (Figure 2*B*2). Given the unique location (antrum) and time frame (advanced age) of gastric tumor formation, our model is highly relevant to human gastric cancer in the antrum.[3]

Susceptibility of Villin-Cre^{+};Klf4$^{fl/fl}$ Mice to Chemical Gastric Carcinogenesis

To further determine the functional significance of *Klf4* inactivation in gastric carcinogenesis, we treated the *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$, *Villin-Cre*$^{+}$*;Klf4*$^{+/fl}$, and *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice with MNU, which is widely used to induce gastric adenomas and adenocarcinomas in mice. The incidences of gastric tumors in the MNU-treated mice were significantly higher than those in the matched control mice that did not receive MNU treatment (Figures 3A1 & 3A2). However, the incidences were higher in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice than in *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice, which strongly suggested that *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice had increased susceptibility to chemical carcinogenesis in the stomach. Although we observed various locations of gastric tumors, the predominant location was the antrum (Figures 3A3, 3B & 3C).

Loss of Integrity and/or Expression of the Klf4 Gene in Gastric Tumors

To determine whether *Klf4* deletion was relevant to the development of gastric tumors in mice with MNU treatment, we performed PCR analysis using DNA from gastric tumors and matched corpus mucosal tissues obtained from *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ and *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. Whereas gastric tumors from *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice showed no *Klf4* deletions (Figure 4A1), all of the gastric antral tumors (n = 4) from *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice did exhibit *Klf4* deletions (Figure 4A2). As expected, we observed no discernible *Klf4* deletions in the matched corpus mucosal tissues in either mouse strain, i.e., *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice (Figure 4*A1*) and *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice (Figure 4*A2*).

To further characterize the specific cell populations with *Klf4* deletions in the *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ tumors, we extracted DNA from three sources (Figure 4*B*1): 1) matched corpus mucosal tissue, 2) an in vitro cell culture of half of an antral tumor to eliminate stromal cells (Figure 4*B*2), and 3) the other half of the antral tumor. Genotypic analysis showed complete deletion of the *Klf4* gene in the gastric tumor cells (Figure 4*B*3).

Additionally, we measured the KLF4 expression in gastric corpus tumors obtained from both *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ and *Villin-Cre*$^{-}$*;Klf4*$^{+/+}$ mice using quantitative real-time PCR analysis. Our results showed that the KLF4 expression was significantly lower in all of the tumors than in adjacent corpus mucosal tissues (Figure 4*C*1). Finally, we sought to determine the integrity of the *Klf4* gene in the corpus tumors obtained from *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice and found that some but not all of the tumors exhibited *Klf4* deletions (Figure 4*C*2).

Histopathology of the Stomach in Villin-Cre^{+};Klf4$^{+/+}$ Mice

We also systematically examined preneoplastic changes in the gastric mucosa. Without MNU-based treatment, the preneoplastic lesions were primarily located in the antrum and progressed as the mice aged. Treatment with MNU promoted the formation of preneoplastic

changes in these lesions, including hyperplasia (sFigure 3). As described above, neoplastic lesions formed in *Villin-Cre^{+};Klf4$^{+/+}$* mice that did not receive MNU (spontaneous tumors). However, we did not observe these lesions in control mouse strains, including *Villin-Cre^{+};Klf4$^{+/+}$*, *Villin-Cre^{-};Klf4$^{+/fl}$*, and *Villin-Cre^{-};Klf4$^{+/+}$* mice without MNU treatment, suggesting that gastric transformation was not impacted by the presence of floxed *Klf4* alleles or the Cre transgene.

Aberrant FoxM1 Overexpression in Gastric Tumors

To characterize the underlying mechanism by which deletion of the *Klf4* gene contributes to gastric transformation and tumorigenesis, we measured the KLF4 and FoxM1 expression in gastric antral tumors and normal gastric mucosal tissues obtained from *Villin-Cre^{+};Klf4$^{fl/fl}$*, *Villin-Cre^{+};Klf4$^{+/fl}$*, and *Villin-Cre^{+};Klf4$^{+/+}$* mice. In both Western blot and immunohistochemical analyses, lost KLF4 expression correlated with increased FoxM1 expression (Figure 5). Specifically, we found FoxM1 expression in the lower third and KLF4 expression in the upper third of the antral mucosa in *Villin-Cre^{-};Klf4$^{fl/fl}$* mice, whereas we observed KLF4 underexpression and FoxM1 overexpression in the antral mucosa in *Villin-Cre^{+};Klf4$^{fl/fl}$* mice (Figure 5*B*1). Concomitant underexpression of Klf4 and overexpression of FoxM1 were evident in intramucosal neoplastic lesions (Figure 5*B*2; sFigure 4) and tumors (Figure 5*C*) in *Villin-Cre^{+};Klf4$^{fl/fl}$* mice. These results suggested that FoxM1 is a prominent downstream gene whose expression is negatively regulated by KLF4.

Inverse Correlation between KLF4 and FoxM1 Expression in Human Gastric Tumors

To validate our observation in our mouse model, we examined KLF4 and FoxM1 expression in human gastric tumor specimens. Analysis of FoxM1 and KLF4 expression in all 86 gastric tumors revealed a significant inverse correlation between FoxM1 expression and KLF4 expression (sTable 1; Figure 6*A*). In most of the gastric tumor specimens, KLF4 expression was significantly decreased or lost, whereas FoxM1 overexpression was strikingly evident (Figure 6*B*). These findings also suggested that expression of FoxM1 is negatively regulated by KLF4.

Negative Regulation of FoxM1 Expression by KLF4

To determine whether KLF4 represses FoxM1 expression, we initially used a computer-bases software program to identify KLF4-binding sites on the FoxM1 promoter (Figure 6*C*). We found a putative KLF4-binding site between −230 and −210 bp in the FoxM1 proximal promoter (Figure 6*D*1). A ChIP assay indicated that KLF4 could bind to this region of the FoxM1 promoter in vivo (Figure 6*D*2). Consistently, increased KLF4 expression repressed FoxM1 expression in N87 and SK-GT5 cells (Figure 6*E*1), and increased KLF4 expression clearly bound to the target sequence of the FoxM1 promoter (Figure 6*E*2). Furthermore, transfection of a KLF4 expression vector repressed the activity of the pFXM1-360 proximal promoter, whereas knockdown of KLF4 expression by KLF4 small interfering RNA (siRNA) significantly increased the promoter activity (Figure 6*E*3). These results supported our hypothesis that KLF4 binds to the putative region of the FoxM1 promoter in vivo and represses FoxM1 transcription.

Gastric Antral Tumor Formation in Foxa3-Cre^{+};Klf4$^{fl/fl}$ Mice

A prior study has demonstrated that global deletion of *Klf4* in stomach caused preneoplastic changes.[27] However, whether those mice are prone to chemical carcinogenesis is unknown. In our final set of experiments, we bred and genetically verified *Foxa3-Cre^{+};Klf4$^{fl/fl}$* mice according to previously published procedures.[27] Consistent with that previous study, we observed extensive preneoplastic changes, including hyperplasia in gastric mucosa in both the corpus and antrum; furthermore, treatment with MNU promoted the formation of both

preneoplasia and neoplasia (sFigure 5A & sFigure 6). Interestingly, extensive hyperplasia formed in the corpus, and no tumors had formed in mice by the age of 35 weeks without MNU-based treatment. However, MNU treatment promoted gastric tumor formation in all of the mice and gastric tumors were located predominantly in the antrum (sFigures 5*B* and 5*C*).

Finally, we compared the effects of *Klf4* deletion in the corpus mucosa in *Foxa3-Cre*$^{+}$*;Klf4*$^{fl/fl}$ and *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. We observed extensive *Klf4* deletion in *Foxa3-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice, whereas we found no discernible deletions in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice (sFigure 5*D*). These findings suggested that the extent of *Klf4* deletion was directly correlated with that of gastric mucosal transformation.

Discussion

In this study, we observed selective inactivation of the *klf4* gene in a distinct putative progenitor cell population at the bases of the pyloric glands in the antrum and subsequent development of spontaneous gastric tumors in the lesser curvature of the antrum but not in the corpus in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. We also found that treatment with MNU accelerated gastric tumorigenesis in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. Moreover, loss of KLF4 expression resulted in upregulation of FoxM1 expression and contributed to gastric tumor formation in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. Consistently, KLF4 expression was inversely correlated with FoxM1 expression in human gastric tumors and downregulated FoxM1 expression in gastric cancer cells. Therefore, we provide clinical, experimental, and mechanistic evidence that loss of KLF4 expression in putative GPCs is an important step in gastric tumorigenesis and tumor progression. Because approximately 60–80% of intestinal-type gastric tumors form in the antrum, most often in the lesser curvature,[3,28,29] our present mouse model is a close recapitulation of human gastric cancer and a novel tool for further investigation into the underlying molecular basis for gastric carcinogenesis.

The unique locations of both human intestinal-type gastric tumors and experimental gastric tumors in animals has spurred extensive investigations into their cellular origin.[29,30] Reported evidence supports the existence of gastric stem cells, which are considered prime candidates as cells of origin for cancer.[7,29,30] Studies tracing the in vivo lineage revealed that each gastric gland unit is functionally monoclonal in both the murine and human stomach, with all cell progeny arising from a single stem cell.[6–7] However, the identities of these putative gastric stem cells and their causal link to gastric carcinogenesis are not clear. At least three putative types of gastric stem cells exist, and they may all be found in the antrum. Also, gastric cancer can originate from bone marrow-derived cells in a mouse model of chronic *H. pylori* infection.[31] Interestingly, the biological characteristics of the pyloric glands in the antrum are developmentally similar to those in the small intestinal epithelium.[31] Researchers have identified Lgr5^{+} cells as stem cell markers in both the intestine and stomach in mice, and activation of a Wnt signal by conditional deletion of *APC* gene in Lgr5^{+} cells efficiently initiates tumor formation in the distal stomach.[7] Those researchers also recently observed that more active Lgr5^{+} stem cells affected the daily steady-state self-renewal of the pyloric epithelium in adults and both the corpus and pylorus in neonates.[7] Even more recently, investigators identified Villin-positive cells as prospective progenitor cells in the bottom third of the pyloric gland. These cells are normally quiescent and multiply with multilineage potential via both symmetric and asymmetric division in response to inflammatory insults.[6] All of these putative stem cell types appear to be capable of self-renewal and differentiation, with the cells' statuses ranging from pluripotent to multipotent to tissue-specific adult stem cells.[29] In the present study, selective inactivation of *Klf4* in Villin-positive progenitor cells led to the development of tumor phenotypes in the lesser curvature of the antrum in old mice. The fact that the tumors were located exclusively at the pyloric junction was also consistent with the notion that the lesser curvature of the

antrum is the predominant location of GPCs.[6–7] Paradoxically, the GPCs marked by the villin-Cre transgene do not express the endogenous villin locus.[6] Thus, to confirm efficient deletion of *KLF4* in those cells will require performing lineage tracing experiments.

Furthermore, we speculated that bone marrow-derived cells (BMDCs), $Lgr5^+$ cells, and Villin-positive cells all may contribute to gastric tumor formation, playing different roles linked with the differentiation status. BMDCs may be the most primitive uncommitted adult stem cells that can be recruited to tumor transformation by chronic inflammation. $Lgr5^+$ cells are located exclusively in the bases of pyloric glands and are active stem cells responsible for the daily self-renewal of the pyloric mucosa. Villin-positive cells are quiescent stem cells that require injurious insults such as inflammation (by interferon-γ) for their activation. Villin is not normally expressed in the stomach; however, its expression can be induced in atrophic human and murine stomachs in response to chronic *H. pylori* infection via extracellular signal-regulated kinase signaling.[32] Data from the present study also support this notion. Specifically, villin expression likely was induced by MNU-based treatment, which in turn drove *Klf4* gene deletion and contributed to increased gastric tumor incidence. Also, the extent of *Klf4* deletion varied in the antrum of mice at different ages or with exposure to chemical insults, further supporting that the size of Villin-positive cell subpopulation may change. Interestingly, these rare villin-positive cells are similar in many aspects (rarity, location, slow proliferation and morphology) to the controversial and recently identified rare DCAMKL1-positive intestinal cells[33] that are believed to be progenitor cells,[34] that were later identified as tuft cells not progenitor cells.[35] These cells are positive for villin, DCAMKL1, Cox1 and alpha tubulin. Further studies are clearly warranted to determine whether these rare villin-positive cells in antrum are also positive for either DCAMKL1, Cox1 and alpha tubulin or all of them; or whether these rare villin-positive gastric cells are in fact gastric tuft cells.[36]

Numerous genes are implicated in regulation of preneoplastic lesion and gastric tumor formation and progression in mouse models, including *K-Ras*,[37] cdx2,[38] gastrin,[39,40] Wnt,[7,41] and IKKβ/NF-κB.[42] A prior study showed that loss of KLF4 expression in *Foxa3-Cre$^+$;Klf4$^{fl/fl}$* mice led to gastric hypertrophy, mucus cell hyperplasia, glandular distortion, and polypoid lesion development,[27] and authors have reported reduced KLF4 expression in various types of human tumors,[19,20] supporting KLF4's tumor suppressor role. In the present study, we observed causal evidence of the critical role of loss of KLF4 expression in gastric carcinogenesis. Moreover, DNA analysis revealed *Klf4* gene deletion from a significant majority of the tumors that formed in MNU-treated *Villin-Cre$^+$;Klf4$^{fl/fl}$* mice, whereas all of the tumor cells derived from *Villin-Cre$^+$;Klf4$^{fl/fl}$* mice exhibited *Klf4* gene deletion. These results strongly suggest that these *Klf4*-deleted putative progenitor cells contribute to tumor formation in the gastric antrum in *Villin-Cre$^+$;Klf4$^{fl/fl}$* mice and give rise to hyperplastic lesions in the early stages of gastric carcinogenesis.

Finally, we observed that loss of KLF4 expression correlated directly with FoxM1 overexpression in a consistent manner in both human and murine gastric tumors. Several previous studies showed that FoxM1 expression is regulated by posttranslational modifications, degradation, and interaction with other transcription factors.[43] Our experiments established FoxM1 as a novel downstream target of KLF4 in that KLF4 negatively regulates FoxM1 transcription, providing a novel molecular basis that causally links frequent FoxM1 overexpression with loss of KLF4 expression in various types of cancer, including gastric cancer.[20,22] Given that FoxM1 positively regulates and KLF4 negatively regulates the progression of cell cycle, loss of expression of KLF4 and overexpression of FoxM1 should lead to dysregulated cell growth and cellular transformation as shown in the gastric antrum in *Villin-Cre$^+$;Klf4$^{fl/fl}$* mice and gastric

antrum and corpus in *Foxa3-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. Thus, targeting this aberrant pathway may be an effective strategy for cancer prevention and treatment.

In summary, we derived mice with selective deletion of *Klf4* in GPCs and showed that loss of *Klf4* expression in these cells results in spontaneous gastric neoplasia and rapid development of gastric tumors in the setting of treatment with MNU, mostly in the lesser curvature of the antrum. The rapid progression to neoplasia observed in the *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice was associated with overexpression of FoxM1 protein. In addition, we showed that KLF4 inhibited FoxM1 expression in part through its binding site. Therefore, KLF4 plays an important role in the homeostasis of normal gastrointestinal tissue, and inactivation of *Klf4* in GPCs promotes carcinogenesis. Collectively, our findings strengthen the causative role gastric stem cells play in gastric carcinogenesis and the gastric tumor-suppressive role of Klf4 in response to carcinogen exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

GPC	Gastric progenitor cell
KLF4	Krüppel-like factor 4
MNU	*N*-methyl-*N*-nitrosourea
PCR	polymerase chain reaction
EGFP	enhanced green fluorescent protein
MOI	multiplicity of infection
DAPI	4′6-diamidino-2-phenylindole
ChIP	chromatin immunoprecipitation

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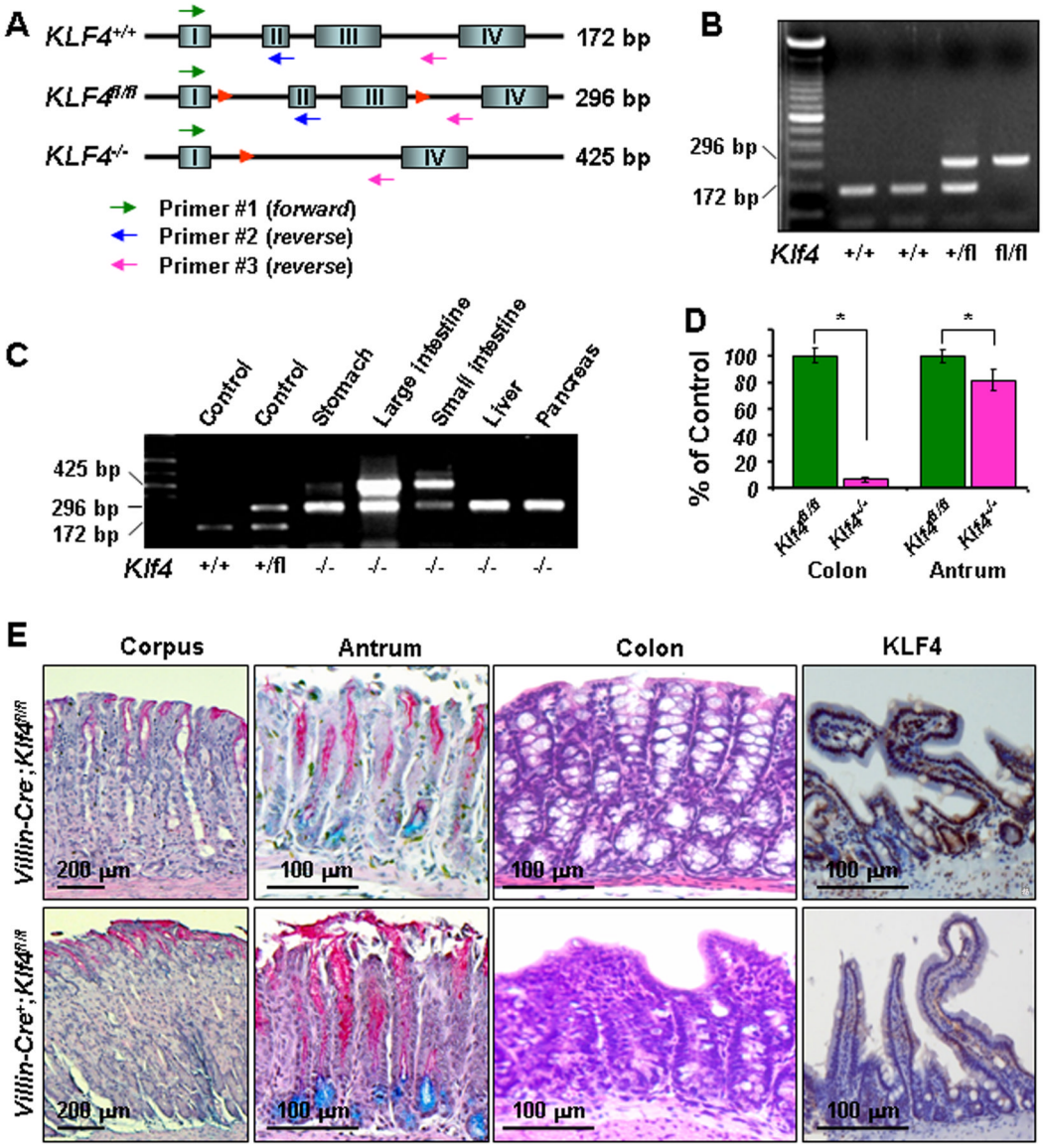

Figure 1. *Villin-Cre*–mediated *Klf4* deletion in the gastric antrum in mice

(*A*) Schematic diagram of the genomic structure of the *Klf4* gene with wild-type ($Klf4^{+/+}$), floxed ($Klf4^{fl/fl}$), and disrupted ($Klf4^{-/-}$) alleles. Shown are LoxP sites (red triangles), PCR primer positions (arrows), and expected PCR products using three primers: 172bp for the wild-type allele, 296bp for the floxed allele, and 425bp for the deleted allele. (*B*) Genotyping using mouse tail DNA at the age of 4 weeks. PCR screening for *Klf4* revealed a band of 172bp for the wild-type allele and 296bp for the floxed allele. (*C*) Detection of *Villin-Cre*–mediated rearrangement of the *Klf4* allele in different tissues in mice at the age of 20 weeks. DNA was extracted from different tissues from a previously genotyped $Klf4^{-/-}$ mouse. PCR analysis indicated various levels of rearrangement of the *Klf4* gene (425bp for the deleted allele). (*D*) The efficacy of *Klf4* deletion in the colon and gastric antrum in $Klf4^{fl/fl}$ and $Klf4^{-/-}$ mice was measured using quantitative PCR analysis of colon and antral DNA from those mice at the age of 35 weeks. (*E*) PAS/Alcian blue staining of gastric corpus and antral mucosa specimens obtained from $Villin\text{-}Cre^{-};Klf4^{fl/fl}$ and $Villin\text{-}Cre^{+};Klf4^{fl/fl}$ mice at the age of 50 weeks. $Villin\text{-}Cre^{+};Klf4^{fl/fl}$ antral mucosa exhibited increases in the

number of both PAS- and Alcian blue-positive cells. Hematoxylin and eosin staining of colons obtained from *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ and *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. Goblet cells were nearly absent from *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ colonic mucosa, whereas a normal contour and numerous goblet cells were observed along the crypts and surface epithelium in *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice. Positive Klf4 staining in the small intestinal mucosa cells of *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ mice but negative in that of *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice.

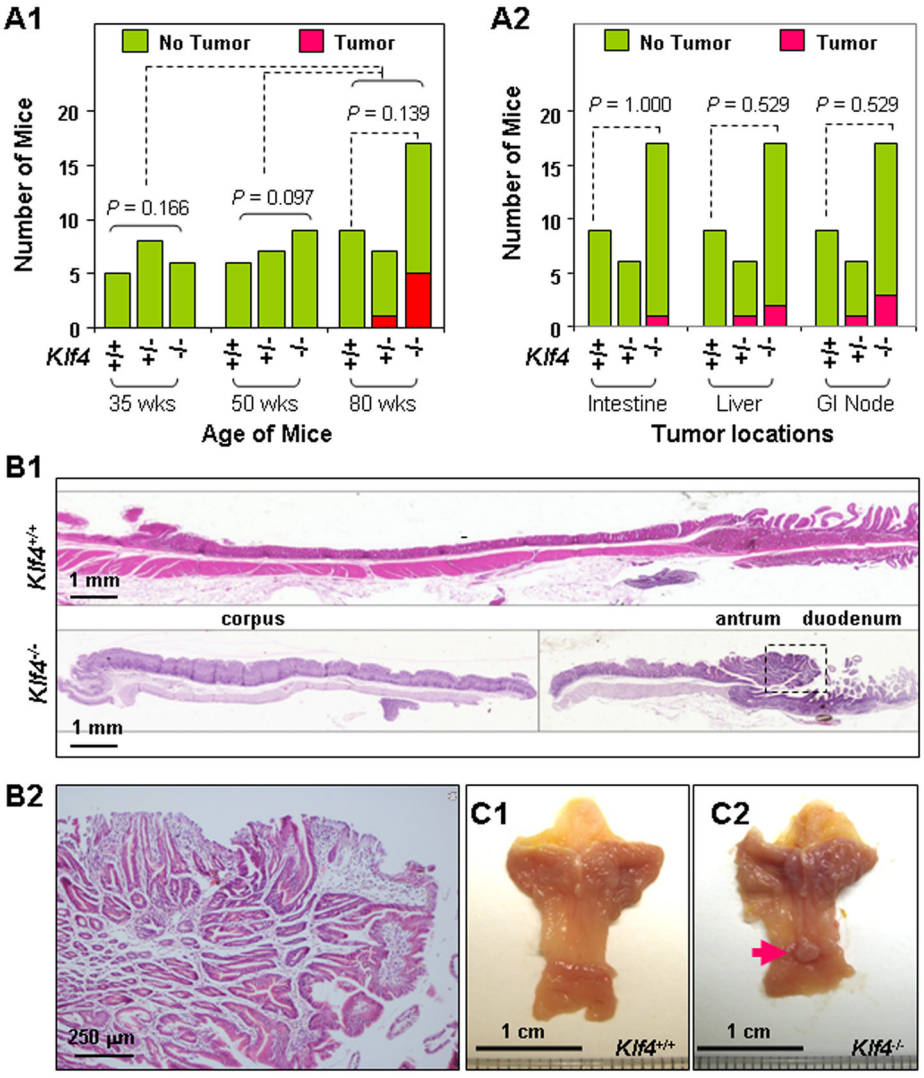

Figure 2.

Spontaneous gastric tumor development in the antrum in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. (*A*) Tumor incidence in the stomach (*A1*) and other locations (*A2*) in *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ (*Klf4*$^{+/+}$), *Villin-Cre*$^{+}$*;Klf4*$^{+/fl}$ (*Klf4*$^{+/-}$), and *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ (*Klf4*$^{-/-}$) mice at the ages of 35, 50, and 80 weeks. (*B*) Representative photographs of macroscopic views of the entire gastric mucosa in 80-week-old *Klf4*$^{+/+}$ and *Klf4*$^{-/-}$ mice (*B1*) and microscopic views of an antral tumor in the *Klf4*$^{-/-}$ mouse (*B2*). (*C*) Gross morphology of stomachs obtained from 80-week-old *Klf4*$^{+/+}$ (*C1*) and *Klf4*$^{-/-}$ (*C2*) mice. A visible antral tumor in a *Klf4*$^{-/-}$ mouse stomach is indicated by a red arrow.

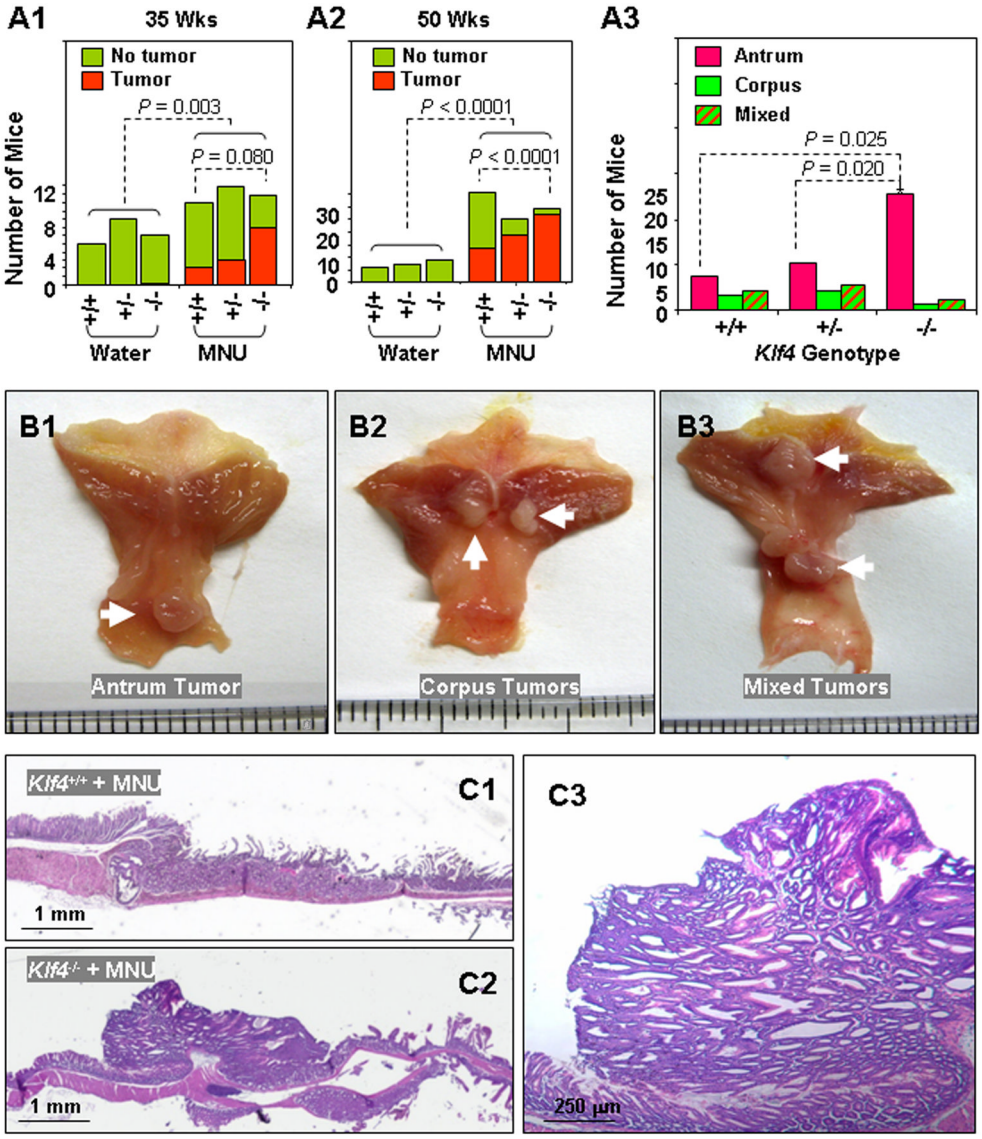

Figure 3.

Induction of gastric tumor development in the antrum in *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ mice. (*A*) Incidence (*A1* & *A2*) and locations (*A3*) of gastric tumor formation in *Villin-Cre*$^{-}$*;Klf4*$^{fl/fl}$ (*Klf4*$^{+/+}$), *Villin-Cre*$^{+}$*;Klf4*$^{+/fl}$ (*Klf4*$^{+/-}$), and *Villin-Cre*$^{+}$*;Klf4*$^{fl/fl}$ (*Klf4*$^{-/-}$) mice at the ages of 35 and 50 weeks with or without MNU-based treatment. (*B*) Gross morphology of visible tumors (white arrows) in the gastric antrum (*B1*), corpus (*B2*), or both ("mixed," *B3*) in 50-week-old *Klf4*$^{-/-}$ mice. (*C*) Representative photographs of macroscopic views of the entire gastric mucosa in 50-week-old *Klf4*$^{+/+}$ (*C1*) and *Klf4*$^{-/-}$ (*C2*) mice and a microscopic view of an antral tumor in the *Klf4*$^{-/-}$ mouse (*C3*).

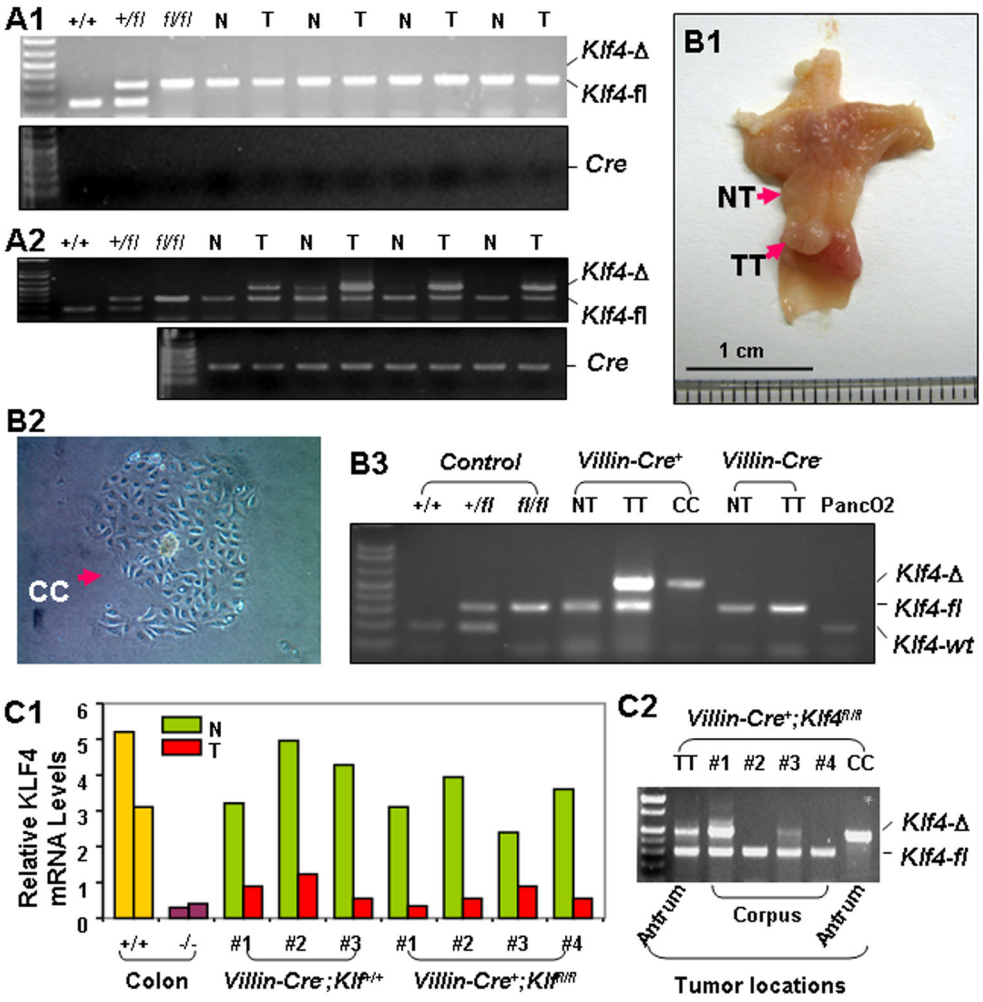

Figure 4.

Analysis of the integrity and expression of the *Klf4* gene in gastric tumors. (*A*) DNA was isolated from matched gastric tumor specimens (T) and nontumorous corpus mucosa specimens (N) obtained from *Villin-Cre*$^{-}$;*Klf4*$^{fl/fl}$ (*A1*) and *Villin-Cre*$^{+}$;*Klf4*$^{fl/fl}$ (*A2*) mice. PCR analysis was performed for genotyping of *Klf4* alleles. (*B*) An individual antral tumor was obtained immediately after surgery (*B1*) and divided into two parts: one for primary culture (CC, *B2*) and one for DNA extraction (TT). DNA was also extracted from a nontumorous corpus mucosa specimen (NT). PCR analysis was performed for genotyping of *Klf4* alleles (*B3*). (*C*) *Klf4* mRNA expression and genetic integrity in corpus tumors. Real-time PCR analysis was performed using total RNA extracted from corpus tumors and adjacent nontumorous tissue specimens from *Klf4*$^{+/+}$ and *Klf4*$^{-/-}$ mice. Total RNA obtained from colonic mucosa in *Klf4*$^{+/+}$ and *Klf4*$^{-/-}$ mice was used as a control (*C1*). DNA was extracted from *Klf4*$^{-/-}$ corpus tumors, and PCR analysis was performed for genotyping of *Klf4* alleles using DNA from TT and CC as a control (*C2*).

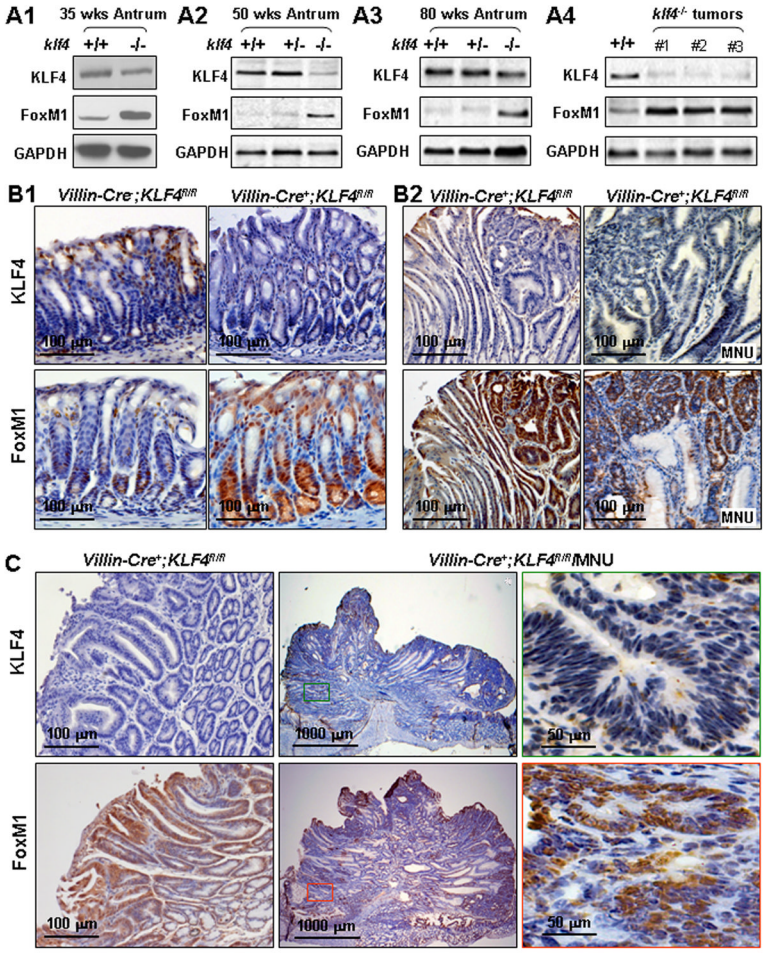

Figure 5.

Lost KLF4 expression and FoxM1 overexpression in gastric tumors. (*A*) Western blot analyses were performed using total protein lysates extracted from antral mucosa in mice at the ages of 35 (*A1*), 50 (*A2*), and 80 (*A3*) weeks and from antral tumors (*A4*). *KLF4*$^{+/+}$, *Villin-Cre*$^{-}$;*Klf4*$^{fl/fl}$; *KLF4*$^{+/-}$, *Villin-Cre*$^{+}$;*Klf4*$^{+/fl}$; *KLF4*$^{-/-}$, *Villin-Cre*$^{+}$;*Klf4*$^{fl/fl}$. (*B*) Immunohistochemical staining for KLF4 (upper panels) and FoxM1 (lower panels) protein. From left to right were: normal, hyperplastic, intramucosal neoplastic, and neoplastic tissue from mice at age of 80 weeks. (*C*) Immunohistochemical staining of two gastric tumors for KLF4 and FoxM1 protein from mice at age of 80 weeks. The right panels are views of the middle panels at higher magnification.

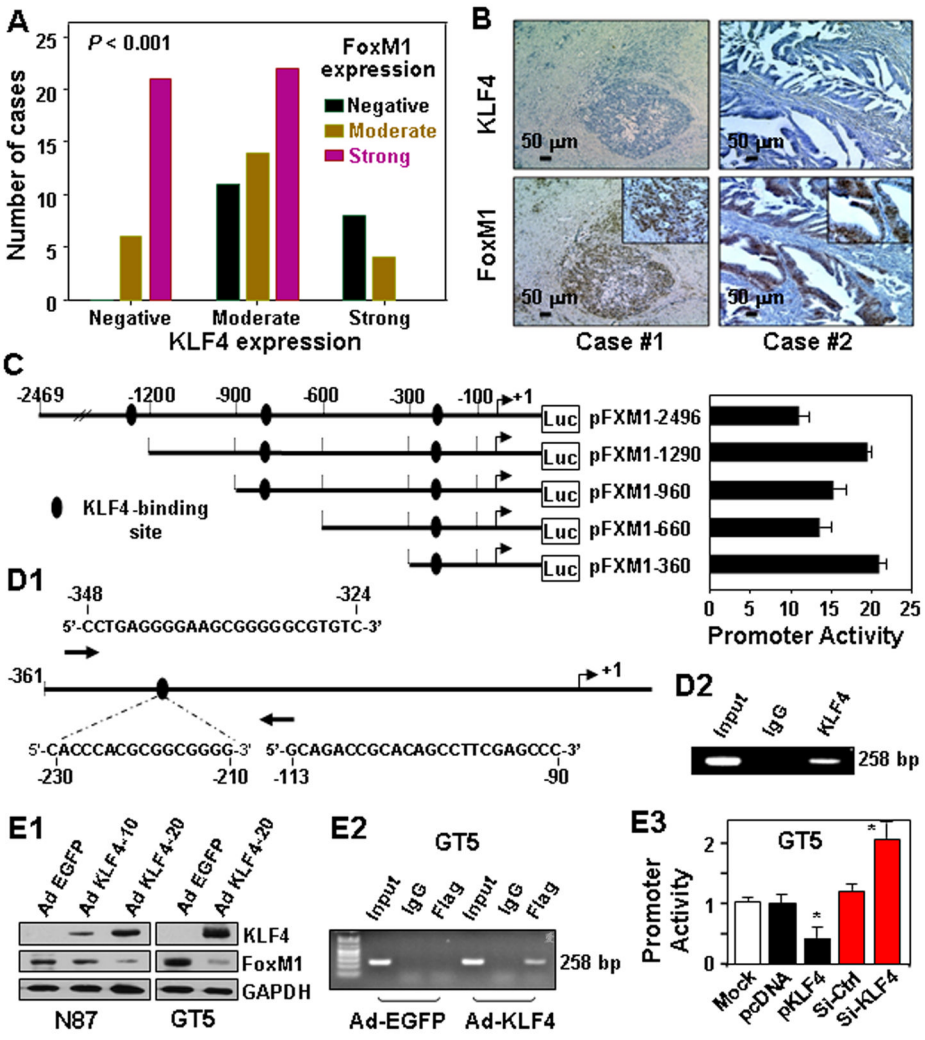

Figure 6.

KLF4 regulation of FoxM1 expression in human gastric tumors. (*A*) Tissue sections were prepared from 86 formalin-fixed, paraffin-embedded human gastric tumor specimens. Immunostaining of the sections was performed using specific anti-KLF4 and -FoxM1 antibodies. FoxM1 expression levels were inversely correlated with KLF4 expression levels (P<001; χ^2 test). (*B*) Representative photographs of two cases showing KLF4 underexpression and FoxM1 overexpression. (*C*) Deletion mutants of FoxM1 promoter reporters were transfected into SK-GT5 cells in triplicate, and the relative promoter activities were measured 24 hours after transfection. (*D*) Schematic diagram of the FoxM1 proximal promoter. The nucleotide positions and sequences of the putative KLF4 binding site and PCR forward and reverse primers for ChIP analysis are shown (*D1*). Chromatin was extracted from SK-GT5 cells, and the ChIP assay was performed using a specific anti-KLF4 antibody and oligonucleotides flanking the FoxM1 promoter region containing the KLF4-binding site (*D2*). (*E*) N87 and SK-GT5 cells were transduced with a control Ad-EGFP (*Neo*) or Ad-KLF4 at an MOI of 10 or 20 for 24 hours. Total protein lysates were harvested from the cell cultures, and the levels of KLF4 (exogenous, as determined using an anti-FLAG antibody) and FoxM1 expression were determined using Western blot analysis (*E1*). SK-GT5 cells were transduced with Ad-KLF4 or Ad-EGFP at an MOI of 10 for 24 hours. Chromatin fragments were prepared for ChIP analysis using control IgG and an anti-Flag

antibody (*E2*). The pFXM1-360 proximal promoter was transfected into SK-GT5 cells in triplicate with pcDNA3.1, a KLF4 expression vector, nontargeting control siRNA, or FoxM1-siRNA. The relative promoter activities were assessed 24 hours after transfection (*E3*).