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Abstract

BACKGROUND & AIMS—Trefoil factors (TFFs) regulate mucosal repair and suppress tumor formation in the stomach. *Tff1* deficiency results in gastric cancer, whereas *Tff2* deficiency increases gastric inflammation. *TFF2* expression is frequently lost in gastric neoplasms, but the nature of the silencing mechanism and associated impact on tumorigenesis have not been determined.

METHODS—We investigated the epigenetic silencing of *TFF2* in gastric biopsy specimens from individuals with *Helicobacter pylori*-positive gastritis, intestinal metaplasia, gastric cancer, and disease-free controls. *TFF2* function and methylation were manipulated in gastric cancer cell

lines. The effects of *Tff2* deficiency on tumor growth were investigated in the *gp130*[F/F] mouse model of gastric cancer.

RESULTS—In human tissue samples, DNA methylation at the *TFF2* promoter began at the time of *H pylori* infection and increased throughout gastric tumor progression. *TFF2* methylation levels were inversely correlated with *TFF2* messenger RNA levels and could be used to discriminate between disease-free controls, *H pylori*-infected, and tumor tissues. Genome demethylation restored *TFF2* expression in gastric cancer cell lines, so *TFF2* silencing requires methylation. In *Tff2*-deficient *gp130*[F/F]/*Tff2*^{−/−} mice, proliferation of mucosal cells and release of T helper cell type-1 (Th-1) 1 cytokines increased, whereas expression of gastric tumor suppressor genes and Th-2 cytokines were reduced, compared with *gp130*[F/F]controls. The fundus of *gp130*[F/F]/*Tff2*^{−/−} mice displayed glandular atrophy and metaplasia, indicating accelerated preneoplasia. Experimental *H pylori* infection in wild-type mice reduced antral expression of *Tff2* by increased promoter methylation.

CONCLUSIONS—TFF2 negatively regulates preneoplastic progression and subsequent tumor development in the stomach, a role that is subverted by promoter methylation during *H pylori* infection.

Keywords

Tumor Suppressor; Epigenetics; Stomach Cancer; Trefoil Factor 2

The trefoil factors (TFF) 1, TFF2, and TFF3 form a family of secreted proteins characterized by a triple loop structure, the trefoil domain.[1] Gastric TFF1 expression is restricted to surface mucus and pit epithelium of the fundus and antrum, whereas TFF2 is expressed in fundic mucus neck cells (MNCs), antrum, and duodenal Brunner's glands.[2,3] TFF3 expression is restricted to intestinal goblet cells.[4] TFF functions include mucus stabilization and barrier protection,[5] regulation of cell migration during wound healing,[6,7] gastric cell differentiation,[8] proliferation, and apoptosis.[9,10]

A body of published evidence shows that *TFF1* is a stomach-specific tumor suppressor gene (TSG). *Tff1*^{−/−} mice spontaneously develop antral/pyloric tumors,[10] and antral tumors in the *gp130*[Y757F/Y757F] (*gp130*[F/F]) mouse model of gastric cancer show reduced *Tff1* expression.[11] Approximately half of all human gastric cancers show loss of *TFF1* expression[12] through loss of heterozygosity and promoter methylation[13] as well as transcriptional inhibition by regulatory molecules.[14] *TFF1* inhibits gastric cancer cell line growth,[15] blocks G1/S phase cell cycle progression, increases *Retinoblastoma* gene expression,[9] and promotes gastric differentiation,[16] all consistent with a tumor suppressor role in the stomach.

By contrast, the role of *TFF2* in gastric cancer progression is less well understood. *TFF2* expression is rapidly induced in gastrointestinal ulcerative pathologies particularly in regenerating epithelium[17] or after nonsteroidal anti-inflammatory (NSAID) drug treatment.[18] Moreover, in vivo delivery of recombinant TFF2 ameliorates mucosal damage.[19]

TFF2 gives its former name to a mucus metaplasia, of mainly fundic origin known as *spasmolytic polypeptide expressing metaplasia* (SPEM).[20] Whereas SPEM is associated with *Helicobacter pylori*-dependent fundic gland atrophy[21] and may be a precursor to gastric cancer,[22] *TFF2* is dispensable for the development of SPEM-like metaplasia.[23] In addition, *Tff2*-deficient mice display only subtle alterations in mucosal proliferation, parietal cell activation, and reduced reparative function.[18]

An anti-inflammatory role for *Tff2* has recently been described. *Tff2*-deficient mice display immune deregulation and are more susceptible to dextran sodium sulphate-induced colitis and *Helicobacter felis*–induced gastritis, developing more severe inflammatory pathology than wild-type controls.[24,25] Furthermore, *H pylori*-infected *Tff2*-deficient mice develop more advanced premalignant lesions of atrophy, metaplasia, and dysplasia than wild-type littermates.[26] Therefore, in addition to immune modulation, *TFF2* may impose growth restraint and limit neoplastic progression in the context of chronic inflammation.

Here, we demonstrate that loss of *TFF2* expression occurs during the progression of human intestinal-type gastric cancer via the acquisition of aberrant promoter methylation. To determine the likely functional impact of *TFF2* loss (via promoter methylation) on gastric tumor growth, we have employed a genetic approach to disrupt *Tff2* function in murine gastric cancer. The $gp130^{Y757F/Y757F}$ ($gp130^{F/F}$) mouse is a model of gastric tumorigenesis that develops antral tumors with histopathologic similarities to human intestinal-type gastric cancer.[27] We show that genetic *Tff2* deficiency substantially accelerates tumor growth in $gp130^{F/F}$ mice, primarily via further loss of gastric TSGs and misexpression of hormones and cytokines that promote preneoplastic fundic atrophy and chronic gastritis. These data support the view that *TFF2* may act locally to suppress preneoplastic change and subsequent gastric tumor development, a role that is ultimately subverted by epigenetic silencing after *H pylori* infection.

Materials and Methods

Mice

$Tff2^{+/-}$ and $gp130^{F/+}$ heterozygous mice[11,18] were crossed to obtain wild-type, $Tff2^{-/-}$, $gp130^{F/F}$, and compound mutant $gp130^{F/F}/Tff2^{-/-}$ mice and maintained on a C57BL6/129-Sv mixed background. Age-matched littermate controls were used in all experiments. Wild-type (C57BL6) mice were infected with *H pylori* Sydney strain 1 (SS1) as described.[26] Experiments were performed in accordance with either the Royal Melbourne Hospital (approval No. 010/2005) or Murdoch Children's Research Institute Animal Ethics Committee (approval No. A583).

Human Tissue Sources

Gastric mucosal tissue was obtained from *H pylori*-positive individuals and disease-free controls as described.[28] Gastric cancers and matched preneoplastic adjacent to cancer tissues were obtained with informed consent (approval No. 174.2008; Kanazawa University Ethics Committee for Human Genome Research).

Morphometric and Histologic Analysis

Mouse stomachs were removed, pinned, and photographed for macroscopic morphometry. Paraffin sections (4 μm) stained with H&E or alcian blue periodic acid-Schiff (AB/PAS) were used for microscopic morphometry and histologic assessment. Morphometric analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij/index.html; National Institutes of Health, Bethesda, MD). The gastric mucosal area and length were outlined with the calibrated software drawing tool at the appropriate magnification. Inflammation was assessed by microscopy in a blinded fashion on AB/PAS and H&E-stained sections. Antral tumor tissues were analyzed with a minimum of 3 separate samples per animal cut and scored according to the density of inflammatory cells from minimum = 0 to maximum = 3[29] including infiltration around the muscularis mucosa, submucosal inflammation, and intramucosal infiltration of the tumors.

Mammalian Cell Culture

AGS and MKN28 gastric cancer cell lines were maintained in RPMI 1640 Glutamax I growth medium, 10% fetal bovine serum, 2 mmol/L nonessential amino acids, 50 IU/mL penicillin, 50 μg/mL streptomycin (all Invitrogen, Carlsbad, CA), at 37°C in a humidified incubator with 5% CO_2/air.

For proliferation analysis, AGS cells were serum starved overnight then seeded in 6-well plates at 2×10^5 cells/well in growth medium supplemented with 0.2% fetal bovine serum. Recombinant human (rh) TFF2 was used at 50 μg/mL. Viable cell counts were performed by 0.4% trypan blue dye exclusion on a hemocytometer. For apoptosis, cells were stained with annexin V-Alexa Fluor 488 conjugate (Invitrogen Molecular Probes, Carlsbad, CA) and propidium iodide according to the manufacturer's protocols. Apoptotic (annexin V positive, propidium iodide negative) cells (10,000 events) were resolved on an LSRII flow cytometer (BD Biosciences, San Diego, CA) and analyzed using FACS Diva software (BD Biosciences). For genome demethylation studies, AGS and MKN28 cells were plated in 25-cm flasks at 5×10^5 cells and allowed to attach overnight. Cells were treated with 10 μmol/L 5′-aza-2′-deoxycytidine (5′-Aza-dC) dissolved in dimethyl sulfoxide (Sigma) or dimethyl sulfoxide alone for 72 hours, with fresh 5′-Aza-dC added at 24-hour intervals.

Quantitative DNA Methylation Analysis

Genomic DNA was isolated from tissue using the DNeasy tissue kit (Qiagen, Hilden, Germany) or using Trizol (Invitrogen) and 200 – 500 ng of each sample was bisulphite converted using Zymo EZ DNA methylation reagents (Zymo Research, Orange, CA) according to the manufacturer's protocols. *TFF2* promoter fragments were recovered from bisulphite-converted DNA by polymerase chain reaction (PCR) (full details in Supplementary Materials and Methods). Quantitative methylation analysis of bisulphite PCR-amplified products was performed with the EPITYPER system (Sequenom Inc, San Diego, CA) using MassCLEAVE reagents (Sequenom) followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.[30] Data cleaning and 2-way hierarchical cluster analysis were performed using the R script gplots software (available: http://www.r-project.org/).

Results

TFF2 Epigenetic Silencing by Progressive Promoter Methylation During Human Intestinal-Type Gastric Cancer Development

To examine potential epigenetic mechanisms underlying loss of *TFF2* expression, we performed correlative *TFF2* messenger RNA (mRNA) expression and promoter methylation analysis on gastric mucosal specimens collected from 4 pathologic subgroups. These included normal stomach, *H pylori*-positive gastritis[31] (Supplementary Table 1), intestinal-type gastric cancer, and patient matched preneoplastic adjacent tissue showing intestinal metaplasia (IM) (Supplementary Table 2). Quantitative reverse transcription and polymerase chain reaction (QRT-PCR) (Supplementary Materials and Methods) revealed progressive loss of *TFF2* expression in *H pylori*-positive gastritis (fold change: -3.33 ± 0.77; $P = .035$), IM (fold change: -4.22 ± 0.679; $P = .009$), and gastric cancer (fold change: -5021.53 ± 2513.59; $P < .001$) compared with normal stomach (Figure 1*A*). Next, we quantified CpG methylation in the *TFF2* promoter region and first exon (Figure 1*B*) using the Sequenom EPI-TYPER assay. EPITYPER is equivalent to the bisulphite mutagenesis and sequencing technique in resolving methylation on individual CpG dinucleotides (Supplementary Figure 1) but superior in terms of quantitation, intrasample precision, and sample throughput. *TFF2* promoter methylation was significantly increased to a similar degree in *H pylori*-positive gastritis and IM tissues, respectively, but substantially increased in gastric cancer compared

with normal controls (Figure 1C) with the most pronounced differences observed at CpGs 8–12. Therefore, *TFF2* mRNA expression levels are inversely related to *TFF2* promoter methylation in gastric neoplasia. To examine the ability of *TFF2* methylation to discriminate between normal and gastric cancer tissues, we performed a 2-dimensional hierarchical cluster analysis using methylation ratio values from 8 of the 12 CpG units in the *TFF2* promoter (Figure 1D). The tissue specimens form 4 major clusters that separate normal antrum almost entirely from the gastric cancers. Of 28 cancers, only 3 are clustered with normal antrum, whereas none of the normal antrum tissues cluster with the cancers. Consistent with *TFF2* mRNA data, gastritis and IM are not distinguished by *TFF2* methylation levels but are collectively represented by 2 distinct clusters that cannot be classified as either normal or cancer. Our results indicate that *TFF2* promoter methylation is induced by *H pylori* infection, does not increase additionally in IM, but does increase significantly thereafter in established tumors in late disease. To confirm that *TFF2* silencing requires DNA methylation, we treated 2 human gastric cancer cell lines, AGS and MKN28, with the DNA methyltransferase inhibitor 5′-Aza-dC. Both cell lines cells show extremely low *TFF2* mRNA levels (Figure 2A) and very high *TFF2* promoter methylation levels compared with normal stomach (Figure 2B). *TFF2* expression was strongly up-regulated in AGS and MKN28 cells treated with 10 μmol/L 5′-Aza-dC for 72 hours compared to untreated controls (Figure 2C). These results argue strongly that the *TFF2* promoter is silenced by DNA methylation in gastric cancer cells.

Accelerated Tumor Growth in *Tff2*-Deficient Murine Gastric Cancer

To determine the role of *Tff2* in gastric tumor progression, in particular to recapitulate the likely functional impact of *TFF2* silencing by promoter methylation in human gastric cancer, we compared antral tumor growth between *gp130*$^{F/F}$ mice carrying wild-type or *Tff2*-null alleles (Figure 3A). Macroscopic morphometric analysis revealed that antral tumors in 6-week-old *gp130*$^{F/F}$/*Tff2*$^{-/-}$ compound mutant mice were more than double the size (105 ± 2.2 mm^2) of *gp130*$^{F/F}$ single mutant controls (48 ± 17 mm^2) (Figure 3B), and, by 12 weeks, *gp130*$^{F/F}$/*Tff2*$^{-/-}$ tumors were 35% larger than tumors of *gp130*$^{F/F}$ single mutant and *gp130*$^{F/F}$/*Tff2*$^{+/-}$ heterozygous null controls (154 ± 14 mm^2, 103 ± 68 mm^2, and 95 ± 92 mm^2, respectively) (Figure 3B). Microscopic analysis of antral tumor area at 12 weeks confirmed the increase in tumor size in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ mice (0.53 ± 0.03 mm^2) compared with *gp130*$^{F/F}$ (0.41 ± 0.01 mm^2) and *gp130*$^{F/F}$/*Tff2*$^{+/-}$ littermate controls (0.41 ± 0.04 mm^2) (Figure 3C). Consistent with these observations, mean antral gland height, cell number per antral gland, and Ki-67-positive cells (Figure 3D) were all increased in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ antral tumors compared with *gp130*$^{F/F}$ controls. No difference in apoptotic cell number was observed between *gp130*$^{F/F}$/*Tff2*$^{-/-}$ and *gp130*$^{F/F}$ antral tumors (Figure 3E), thus excluding decreased apoptosis as a mechanism for the accelerated tumor growth. In addition, expression of the gastric endocrine regulators *gastrin* and *somatostatin* were substantially reduced in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ mice compared with *gp130*$^{F/F}$ controls (Figure 3F) indicating that increased tumor growth occurs under hypogastrinemic conditions. We conclude that antral tumor growth is accelerated in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ mice, independently of apoptosis or gastrin, suggesting that *Tff2* negatively regulates gastric tumor progression.

Accelerated Tumorigenesis in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ Mice Is Independent of Signal Transducer and Activator of Transcription 3 but Correlated With Decreased Tumor Suppressor Gene Expression

Tumorigenesis in *gp130*$^{F/F}$ mice is due to hyperactivation of the signal transducer and activator of transcription (Stat)3 and reduced *Tff1* TSG expression downstream of disrupted Shp2-ras-Erk/Ap1 signalling.[11,32] We found no difference in phosphorylated Stat3 or extracellular signal-regulated kinase (Erk) protein levels between *gp130*$^{F/F}$/*Tff2*$^{-/-}$ and *gp130*$^{F/F}$ antrum (Supplementary Figure 2). Therefore, increased tumor growth in the

absence of functional *Tff2* is independent of gastric gp130 signalling. By contrast, *Tff1* protein (Figure 4*A*) and mRNA expression (Figure 4*B*) were significantly reduced in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ antral tumors compared with *gp130*$^{F/F}$. We also examined the recently described gastric TSGs *Gastrokine* (*Gkn*)*1*[33] and *Gkn2*[34] and, similar to *Tff1*, found that the mRNA expression of both was more substantially reduced in the antrum of *gp130*$^{F/F}$/*Tff2*$^{-/-}$ mice compared with *gp130*$^{F/F}$ controls (Figure 4*B*). Increased tumor growth in *gp130*$^{F/F}$/ *Tff2*$^{-/-}$ mice is therefore independent of gp130 signalling via Stat3, but correlates with reduced expression of gastric TSGs: *Tff1*, *Gkn1*, and *Gkn2*.

To substantiate further the impact of *TFF2* on tumor growth and regulation of gastric TSGs, we examined proliferation and apoptosis in AGS cells treated with rhTFF2. Treatment with rhTFF2 slowed the growth of AGS cells by 25% ± 4.3% (*P* < .05) after 48 hours compared with mock-treated cells (Figure 4*C*) and was dose dependent (Supplementary Figure 3). Apoptosis was unchanged between rhTFF2-treated cells and controls (Figure 4*D*), confirming our in vivo observation that *Tff2* inhibits proliferation of gastric epithelial cells without affecting programmed cell death. We hypothesized that altered TSG expression accounts for the observed rhTFF2-mediated growth inhibition of AGS cells. Consistent with this, *TFF1* expression was increased (fold change: 1.22 ± 0.06, *P* < .05) in rhTFF2 treated AGS cells (Figure 4*E*). Intriguingly, *TFF2* expression (fold change: 1.3 ± 0.1, *P* < .05) was also increased with rhTFF2 treatment, suggesting the existence of a positive feedback mechanism on *TFF2* transcription (Figure 4*E*).

Th1 Polarized Immune Response in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ Antral Tumors

Tumors in *gp130*$^{F/F}$ mice are associated with extensive inflammation. Therefore, we next examined inflammatory mediators associated with *gp130*$^{F/F}$/*Tff2*$^{-/-}$ compound mutant tumors. We observed increased leukocyte infiltration in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ compared with *gp130*$^{F/F}$ mice (Figure 5*A* and *B*), although numbers of activated macrophages (F4/80 positive) and neutrophils (myeloperoxidase [MPO] positive) were not significantly different (Figure 5*C*). Inflammatory leukocytes were mainly confined to desmin-positive lamina propria at the luminal margin of the epithelium (data not shown). In agreement with increased immunocyte infiltration, we observed a Th type 1 polarized immune response with significantly increased expression of the proinflammatory cytokines interleukin (*IL*)-*1*α, *IL-1*β, and interferon (*IFN*)-γ in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ antral tumors compared with *gp130*$^{F/F}$ controls (Figure 5*D*). We observed a concomitant decrease in the expression of the Th2 cytokine *IL-13*, with *IL-4* also trending to decrease (Figure 5*E*). Thus, ablation of *Tff2* in the context of gastric tumorigenesis results in a Th1 polarized response that may contribute directly to tumor progression by permitting increased leukocyte infiltration.

Increased Fundic Atrophy in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ Mice

Histologic analysis revealed significant glandular atrophy in the fundus of 12-week *gp130*$^{F/F}$/*Tff2*$^{-/-}$ compared with wild-type and *gp130*$^{F/F}$ mice (Figure 6*A* and *B*). To characterize the cellular composition of the fundus in *gp130*$^{F/F}$/*Tff2*$^{-/-}$ mice, sections were stained for neutral and acidic mucins (AB/PAS; surface mucus and MNCs), Tff2, Griffonia simplicifolia lectin II (*GS*II) (MNCs), and intrinsic factor (chief cells). H$^+$K$^+$ATPase mRNA (parietal cells) was quantified by QRT-PCR. In *gp130*$^{F/F}$/*Tff2*$^{-/-}$ and to a lesser extent in *gp130*$^{F/F}$/*Tff2*$^{+/-}$ mice, the fundic mucosa consisted predominantly of a *GS*II and AB/PAS-positive MNC-like population and was almost entirely devoid of parietal and chief cells (Figure 6*A*–*C*). Therefore, genetic ablation of *Tff2* in the context of pre-existing gastric pathology accelerates the loss of differentiated epithelial cell types leading to profound glandular atrophy.

H pylori Induces *Tff2* Promoter Methylation After Chronic Infection in Mice

To show definitively that *H pylori* infection can induce *Tff2* promoter methylation, we next analysed stomachs from wild-type (C57BL/6) mice experimentally infected with the mouse-adapted *H pylori* SS1 strain for 12 and 15 months. Tff2 expression was reduced in *H pylori* 12-month postinfection (MPI) stomach (Figure 7*A*), whereas *Tff2* promoter methylation was markedly increased at 12 MPI (and 15 MPI) compared with age-matched noninfected controls (Figure 7*B*). These observations were supported by hierarchical cluster analysis which effectively discriminated *H pylori*-infected mice from noninfected mice on the basis of *Tff2* methylation profiles (Figure 7*C*). Collectively, these results show that chronic *H pylori* infection directly induces *Tff2* promoter methylation in vivo. Because the progression of gastric pathology is clearly accelerated in *gp130*[F/F]/*Tff2*^{−/−} mice, and *H pylori* infection leads to gastric Tff2-deficiency via epigenetic silencing (Figure 7*A–C*), we also investigated whether short-term (3 months) *H pylori* infection in *gp130*[F/F] mice can phenocopy the effects of genetic *Tff2* deficiency by augmenting *Tff2* promoter methylation. We observed no effect of the *gp130*[F/F] mutation, *H pylori*, or their combined effect on *Tff2* expression or methylation (Supplementary Figure 4) in antral stomach after 3 months of infection.

Discussion

We have established *TFF2* epigenetic silencing, by promoter methylation, as a novel paradigm for loss of *TFF2* expression in human intestinal-type gastric cancer[35,36] and following experimental *H pylori* infection in the mouse stomach. We also assessed *Tff2* function in the context of mouse gastric tumor growth. Disruption of *Tff2* in *gp130*[F/F]/ *Tff2*^{−/−} mice produced a polarized immune response, with subdued Th2 and exacerbated Th1-type inflammation. *Tff2* deficiency in 6- and 12-week *gp130*[F/F]/*Tff2*^{−/−} mice caused larger tumors compared with *gp130*[F/F] mice because of increased proliferation and accumulation of mucus cells without affecting apoptosis. This demonstrates a novel function for *Tff2* in restraining tumorigenesis and acting as a TSG in the stomach (Supplementary Figure 5). Concordantly, development of gastric neoplasia is accelerated in *Tff2*-deficient mice compared with wild-type littermates after *H pylori* infection.[26]

Whereas tumorigenesis in *gp130*[F/F] mice is attributed to dysregulated gp130 signalling and repression of *Tff1*,[11] genetic ablation of *Tff2* conferred no additional dysregulation upon gp130 signalling via Stat3 or Shp2-ras-Erk/Ap1 pathways. However, *Tff2* deficiency led to reduced expression of *Tff1* and putative TSGs, *Gkn1*, and *Gkn2*. These 2 *gastrokines* are emerging as important endogenous regulators of gastric homeostasis and show profound loss of expression in *H pylori* infection, aspirin ingestion,[37,38] and neoplastic progression.[39] Thus, *TFF2* is a likely upstream regulator of *gastrokine* gene expression, and its inhibition may promote tumor growth because of loss of anti-proliferative gastrokine and *TFF1* genes. Additionally, human GKN2 protein exists predominantly as a heterodimer with TFF1,[40] thus attenuated expression of either protein may potentiate their combined loss of function.

Tff2 deficiency in *gp130*[F/F]/*Tff2*^{−/−} mice led to reduced *Tff1* expression. This finding provides a mechanism for increased proliferation because *TFF1* has previously been shown to inhibit the growth of the human gastric cancer AGS cell line.[15] Here we have shown that exogenous TFF2 peptide inhibits the growth of AGS cells without affecting apoptosis. Treatment with rhTFF2 increased *TFF1* and *TFF2* mRNA expression in cell lines, consistent with reported cross- and autoregulatory functions for TFF2.[41] Moreover, down-regulation of both *TFF1* and *TFF2* expression frequently occurs in gastric cancer,[35,36] consistent with a tumor suppressor function.

TFF1 and *TFF2* play critical roles in maintaining mucosal integrity,[42] and combined functional loss likely compromises the gastric mucosa, permitting increased inflammation

and damage while subduing repair processes. In this context, compound *gp130*[F/F]/*Tff2*^{−/−} mice displayed pervasive fundic atrophy by 12 weeks of age with significant loss of parietal and chief cell lineages and accumulation of undifferentiated *GS*II-positive MNC-like cells. Similarly, fundic atrophy is accelerated in *Tff2*-deficient mice chronically infected with *Helicobacter* species.[20,26]

Tumorigenesis in *gp130*[F/F]/*Tff2*^{−/−} mice was associated with augmented Th1 cytokine release and attenuated Th2 cytokine expression, particularly *IL-13*. Th1 cytokines, in particular IL-1β, promote mucosal inflammation, inhibit gastric acid secretion[43] and induce fundic atrophy leading to gastric adenocarcinoma.[44] Our data suggest that *Tff2* may modulate and prevent imbalanced levels of Th1 and Th2 cytokines leading to mucosal destruction and disrepair. It is unknown whether *TFF2* directly regulates inflammatory cytokines; however, these results highlight the important homeostatic role for *TFF2* in the normal gastric mucosa. TFF2 loss after *H pylori* infection provides a mechanism by which the bacterium may modulate the local immune response.

Expression of the hormones, gastrin and somatostatin, required for normal gastric mucosal development, and the former for parietal cell activation,[45] was almost completely ablated in *gp130*[F/F]/*Tff2*^{−/−} tumors. Gastrin deficiency results in chronic atrophic gastritis and predisposition to tumors via IFNγ/STAT3 activity,[46] thus decreased gastrin and somatostatin may contribute to the observed fundic atrophy. Additionally, because gastrin is a direct transcriptional regulator of *Tff1*,[47] its profound suppression in *gp130*[F/F]/*Tff2*^{−/−} mice may also contribute to loss of *Tff1* expression, independent but in concert with the effects of *Tff2*.

Quantitative analysis of normal human antral stomach, *H pylori*-dependent gastritis, intestinal metaplasia, and tumor cohorts revealed progressive loss of *TFF2* expression during gastric cancer development and concomitant gain of promoter methylation, particularly at CpG dinucleotides overlapping the transcription start site. This is the first description of *TFF2* promoter methylation in precancerous or cancerous stomach, supporting the notion that *TFF2* is a TSG targeted by epigenetic silencing. Whereas we believe that promoter methylation represents a principle means of TFF2 silencing, it is also possible that metaplasic induction leads to antral mucus cell loss and depletion of the source of TFF2 synthesis. Increased tumor growth in *gp130*[F/F]/*Tff2*^{−/−} mice clearly illustrates how perturbed *TFF2* expression might influence the rate of tumor progression in human gastric cancer. This is supported by our in vitro studies in which genome demethylation in AGS and MKN28 gastric cancer cell lines restores *TFF2* transcription, and recombinant TFF2 treatment in AGS cells activates *TFF1* transcription and inhibits cell proliferation.

Our human and mouse data show that *TFF2* methylation is likely initiated during *H pylori* infection, although the mechanism is unclear. This may not be a direct function of the bacterium itself but more likely arises from infection-dependent inflammation, in particular the activity of proinflammatory cytokines such as IL-1β, the expression of which is inversely correlated with that of *TFF2*. *IL-1*β expression is up-regulated during *H pylori* infection[48] and reportedly induces nitric oxide production, which stimulates DNA methyltransferases responsible for inappropriate hypermethylation and transcriptional gene silencing.[49] Thus, increased IL-1β release may indirectly mediate *TFF2* epigenetic silencing in human gastric cancer.

Inappropriate epigenetic silencing is one of the earliest detectable molecular defects in gastric tumorigenesis.[50] We demonstrate that *TFF2* promoter methylation effectively discriminates *H pylori*-positive gastritis/intestinal metaplasia, gastric cancer, and disease-free control tissue thereby warranting further evaluation of its diagnostic potential. This also raises the possibility of future clinical application of nontoxic DNA methylation inhibitors

such as 5′-Aza-C to reverse the inappropriate gene silencing of key gastric regulator genes. DNA methylation profiles are far more stable than mRNA markers and, as exemplified by our analysis of *TFF2*, provide a robust surrogate for transcriptional silencing during the formative stages of tumorigenesis. Determination of gastric *TFF2* methylation profiles may provide a novel approach for early intervention in gastric adenocarcinoma, a disease in which late diagnosis continues to present a major therapeutic challenge. In summary, we demonstrate that *TFF2* expression is epigenetically silenced during the progression to human intestinal-type gastric cancer by promoter hypermethylation. Taken together with our mouse genetic analysis, we conclude that *TFF2* functions as a novel stomach-specific and epigenetically regulated tumor suppressor gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this paper

|	
5′-Aza-C	5′-aza-2′-deoxycytidine
AB/PAS	alcian blue periodic acid-Schiff
Erk	extracellular signal-regulated kinase
IFN	interferon
IL	interleukin
IM	intestinal metaplasia
MNC	mucus neck cell
MPI	month postinfection
mRNA	messenger RNA
PCR	polymerase chain reaction
QRT-PCR	quantitative reverse-transcription polymerase chain reaction
rh	recombinant human
SPEM	spasmolytic polypeptide-expressing metaplasia
Stat	signal transducer and activator of transcription
TFF	Trefoil factor
TSG	tumor suppressor gene

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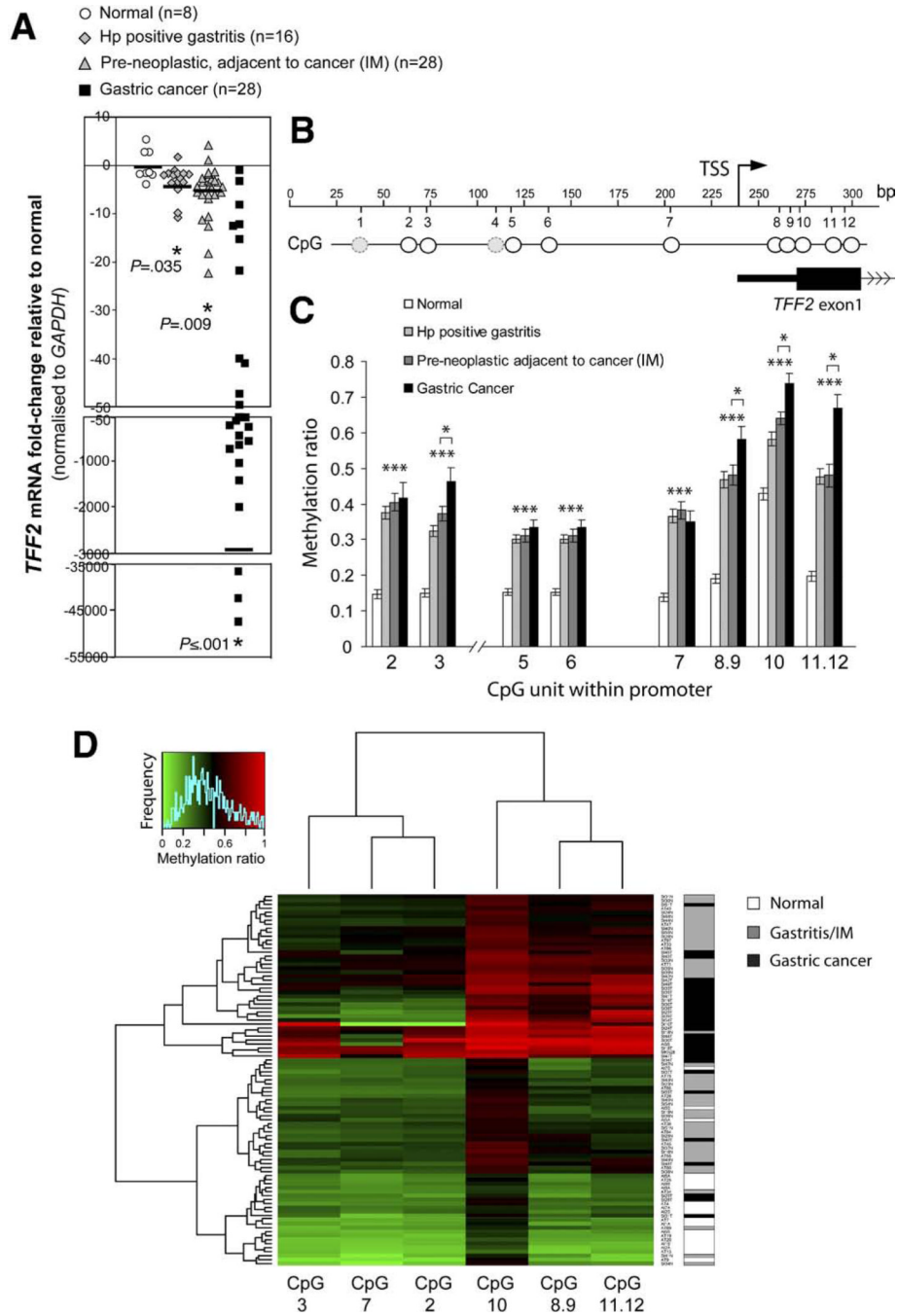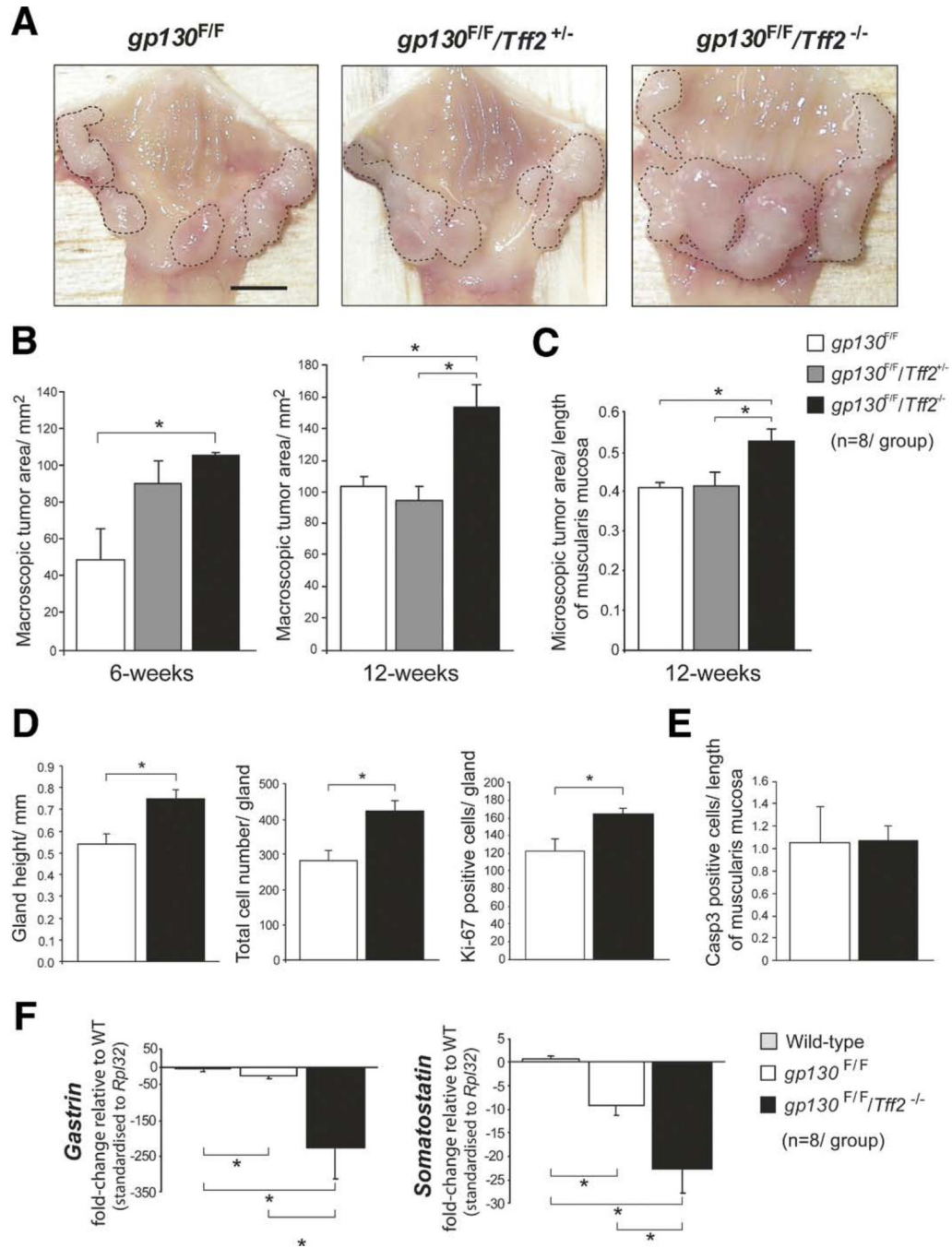
Figure 1.

Increased *TFF2* promoter methylation correlates with loss of expression in gastric cancer. (*A*) QRT-PCR analysis of *TFF2* messenger RNA (mRNA) levels in human gastric mucosal tissues (n = 91); *graph* shows mRNA fold-change relative to normal stomach; *black bars* show mean fold-change. *Asterisks* show statistical significance (*P* < .05). (*B*) *TFF2* promoter region scanned by EPITYPER methylation analysis. *Wide black box* shows coding regions; *narrow black box* shows 5′ UTR: *arrowheads* show transcriptional orientation; *TFF2* transcription start site (TSS); base pair (bp) positions are indicated; *solid-line circles* show CpGs covered by the analysis; *broken-line circles* show nonanalyzed CpGs. (*C*) EPITYPER

DNA methylation analysis in human gastric mucosal tissues described in *A*. *Bar graph* shows mean CpG methylation ratios (levels). CpG units in pairs 8–9 and 11–12, respectively, are shown as combined averages (the constituent CpG units were not resolved by the EPITYPER assay). *Asterisks* show statistical significance ($P < .05$). (*D*) Two-way hierarchical cluster analysis of *TFF2* promoter methylation levels (*columns*) against human tissue samples (*rows*). Classification of the human gastric mucosal tissues is shown on the right: *open boxes* (normal stomach), *shaded boxes* (*H pylori*-positive gastritis/preneoplastic adjacent to cancer with IM), *solid boxes* (gastric cancer). CpG methylation ratios 0 (*green*) to 1.0 (*red*; see color key). *Histogram* shows CpG/methylation ratio frequency.
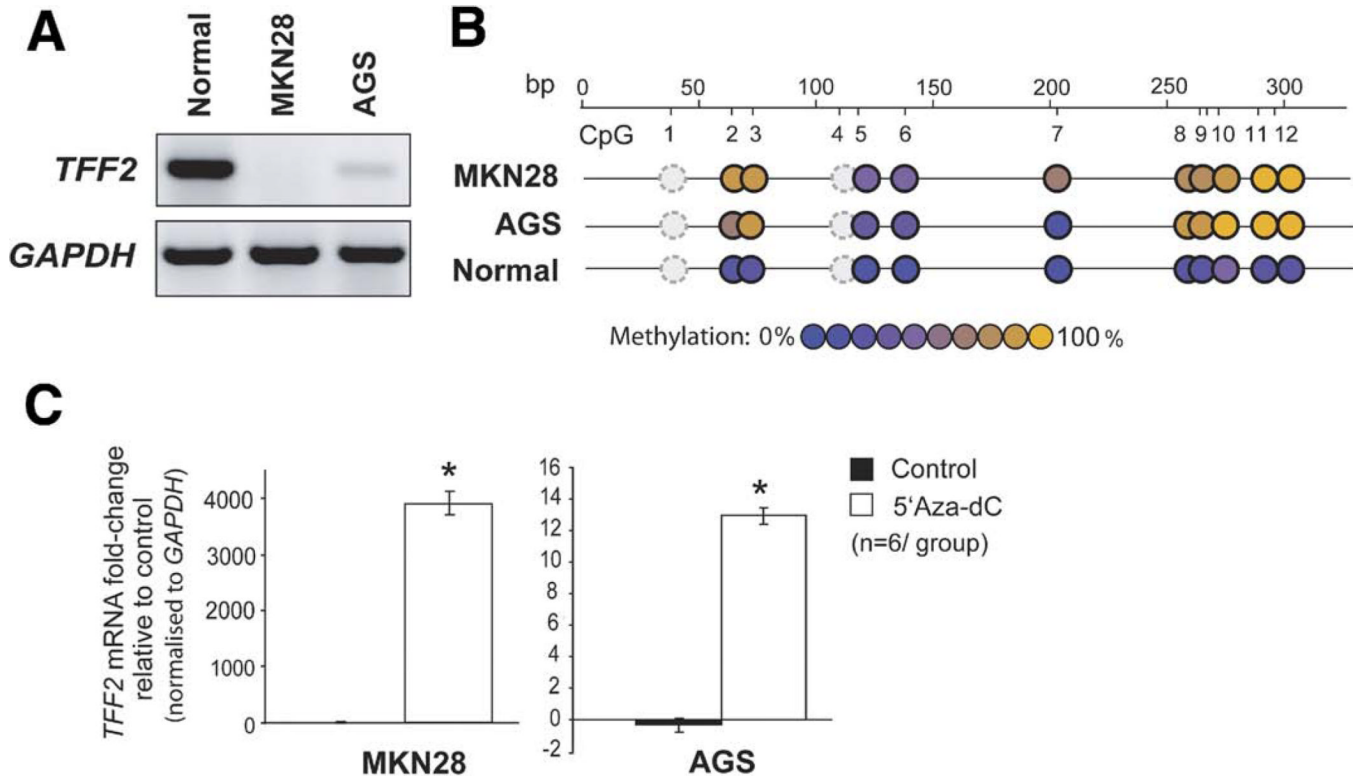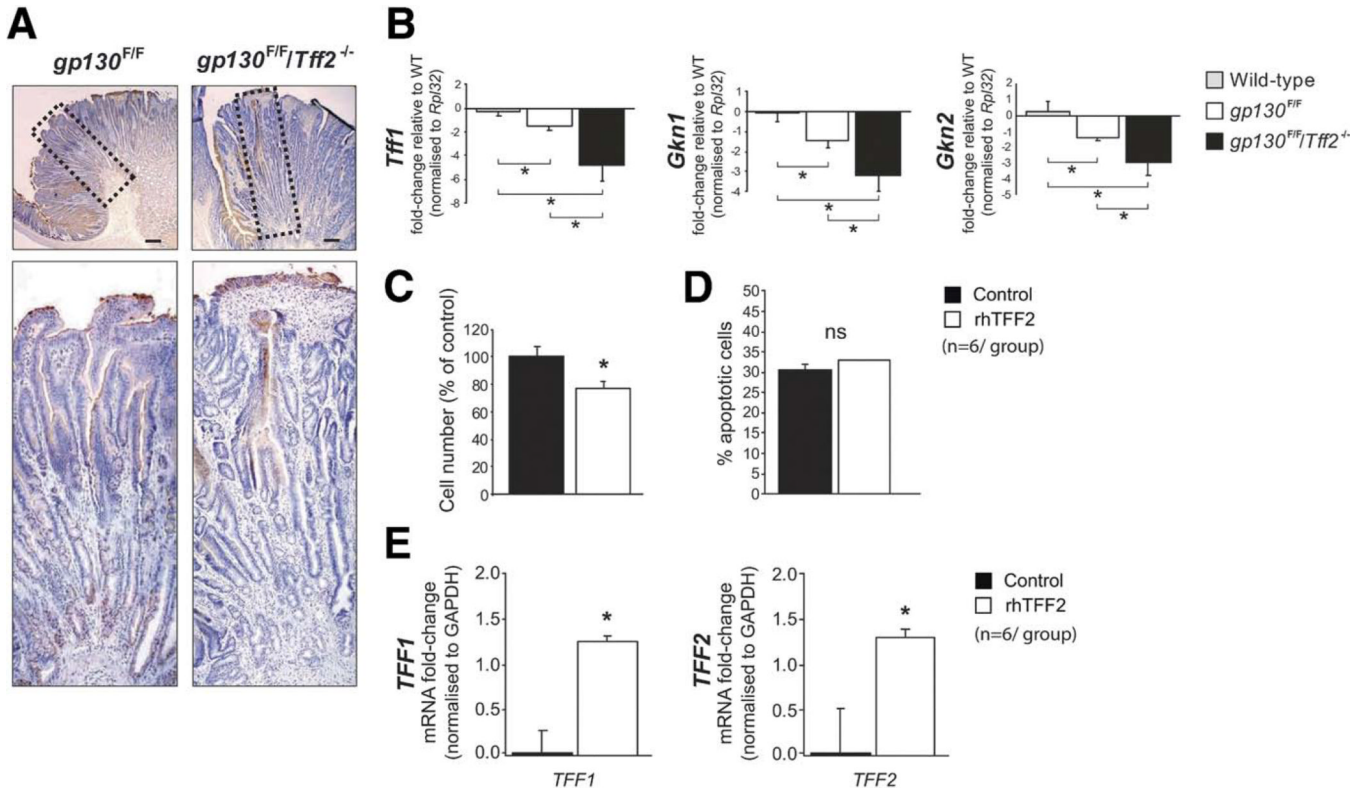
Figure 2.
TFF2 is re-expressed following genome demethylation. (*A*) Semiquantitative RT-PCR analysis of *TFF2* messenger RNA (mRNA) expression in normal gastric antrum, MKN28 cells, and AGS cells. *GAPDH* demonstrates complementary DNA (cDNA) integrity. (*B*) EPITYPER analysis of *TFF2* promoter methylation of samples shown in *A*. *Epigrams* show CpG methylation ratios: graded from *blue* (*0*; hypomethylated) to *yellow* (*1*; hypermethylated). Base pair (*bp*) positions are indicated; *solid circles* show CpGs covered by the analysis; *faded circles* show nonanalyzed CpGs. (*C*) QRT-PCR analysis of *TFF2* expression in MKN28 cells and AGS cells treated with 10 μmol/L 5′Aza-dC for 72 hours compared with mock-treated controls (n = 6). *Histograms* show mean mRNA fold-change relative to untreated control. *Asterisks* show statistical significance (*P* < .05).
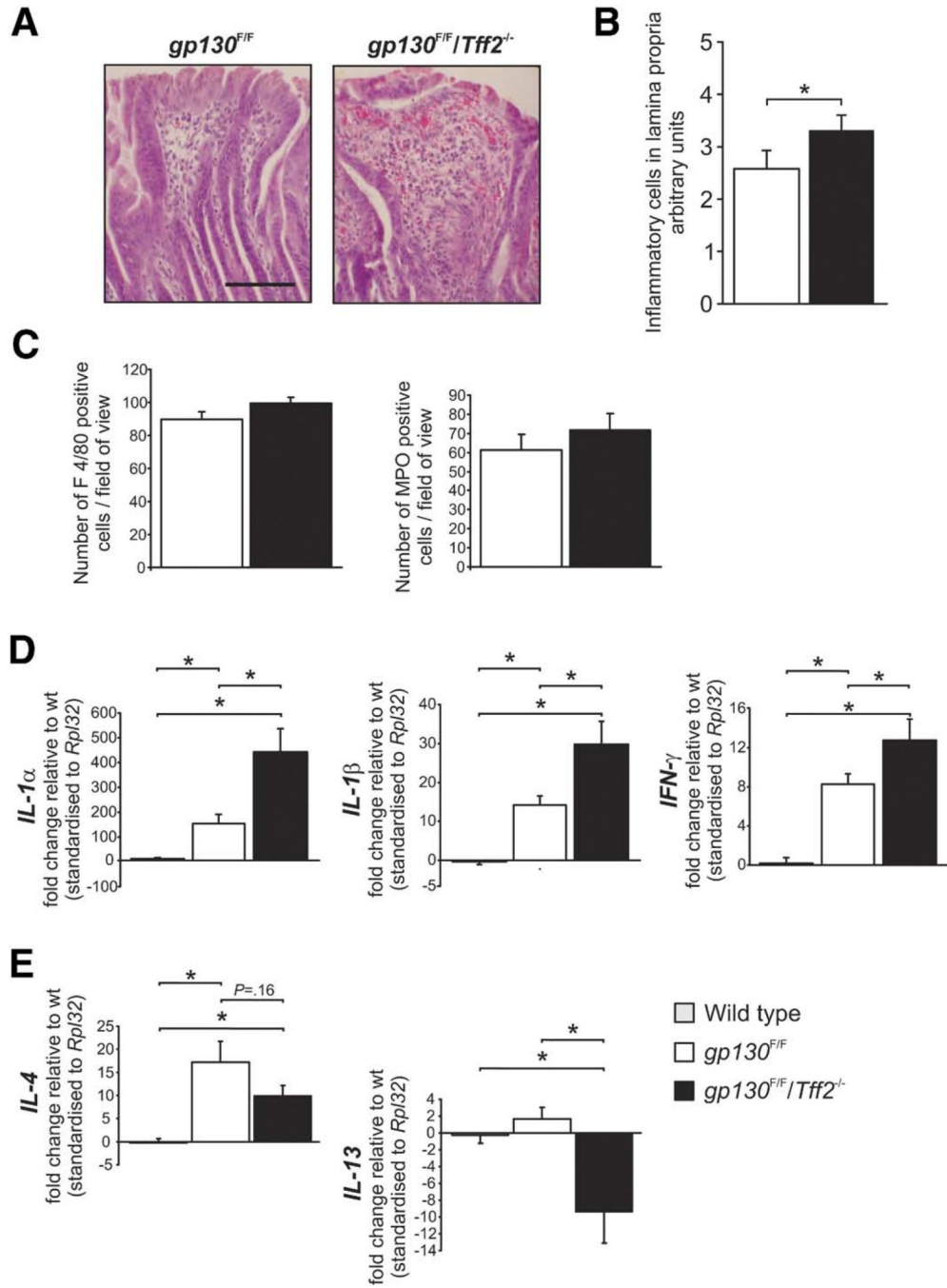
Figure 3.
Morphometric analysis of antral tumors in *gp130*^F/F^/*Tff2*^−/−^ mice. (*A*) Images of antral tumors in 12-week-old mice; tumor areas are defined with *dotted outlines*; *scale bar* shows 5 mm. (*B*) Macroscopic morphometric analysis of antral tumor area in 6- and 12-week-old mice. (*C*) Microscopic morphometric analysis of tumor area (standardized to length of muscularis mucosa) in 12-week-old mice. (*D*) Antral gland height; cell number per antral gland; proliferating cells by quantitative Ki-67 immunohistochemistry. (*E*) Localization of apoptotic cells by quantitative activated caspase (casp) 3 immunohistochemistry. (*F*) QRT-PCR analysis of *Gastrin* and *Somatostatin* messenger RNA (mRNA) expression.

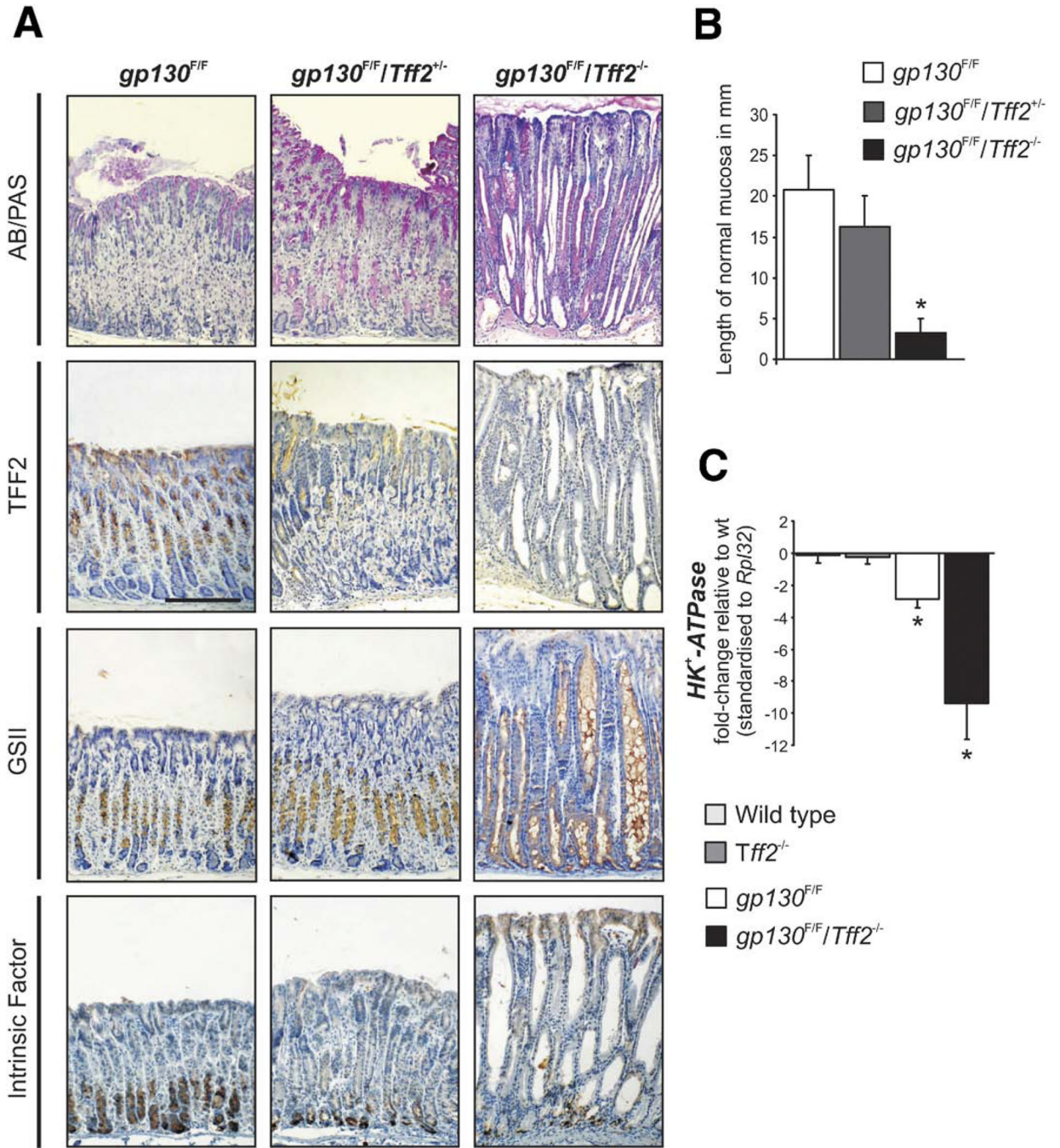*Histograms* show mean mRNA fold change relative to wild-type. *Asterisks* show statistical significance ($P < .05$).

Figure 4.
Loss of gastric tumor suppressor gene expression in $gp130^{F/F}/Tff2^{-/-}$ antral tumors. (*A*) Immunohistochemical localization of Tff1 in antral tumors. (*B*) QRT-PCR analysis of *Tff1, Gkn1*, and *Gkn2*. *Histograms* show mean messenger RNA (mRNA) fold change relative to wild type. (*C*) Proliferation determined by viable cell counts in AGS cells following treatment with 50 μg/mL recombinant human (rh) TFF2 for 48 hours (n = 20). (*D*) Apoptosis measured by Annexin V flow cytometry in AGS cells treated with 50 μg/mL rhTFF2 for 48 hours (n = 5). (*E*) QRT-PCR analysis of *TFF1* and *TFF2* mRNA expression in AGS cells treated with 50 μg/mL rhTFF2 for 48 hours (n = 6). *Asterisks* show statistical significance (*P* < .05).
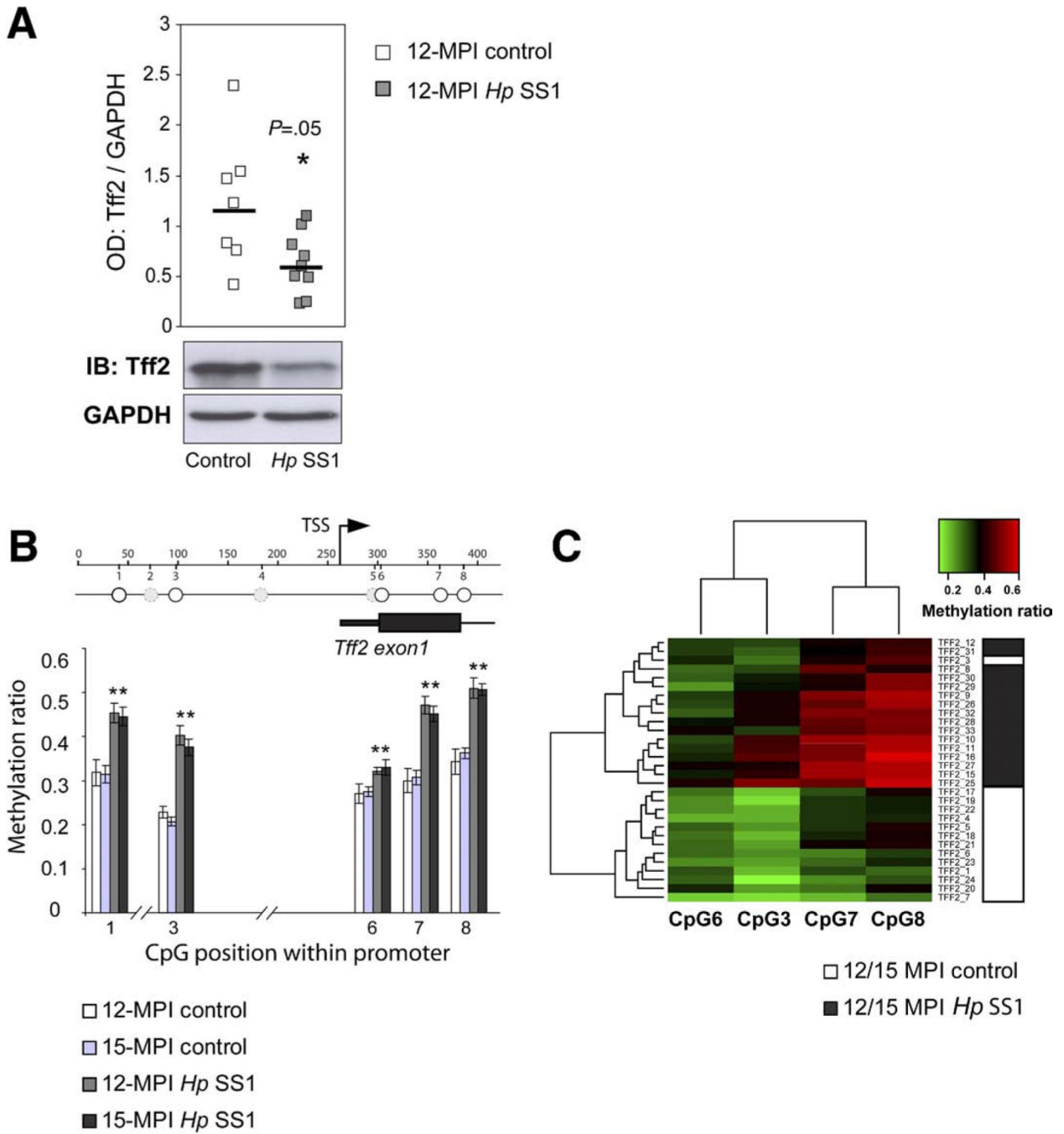
Figure 5.
Analysis of inflammatory mediators in *gp130*^F/F^/*Tff2*^−/−^ antral tumors. (*A*) Representative H&E sections showing intramucosal inflammation in the lamina propria within tumors; *scale bar* shows 50 μm. (*B*) Semiquantitative gradation of tumor-associated inflammatory leukocyte infiltrate. (*C*) Quantitative immunohistochemistry of tumor-associated, activated macrophages (*F4/80*) and neutrophils (*MPO*). QRT-PCR expression analysis in antral tumors of (*D*) Th1 cytokines: *IL1*α, *IL1*β, *IFN*γ. (*E*) Th2 cytokines: *IL-13, IL-4. Histograms* show mean messenger RNA fold change relative to wild type. *Asterisks* show statistical significance (*P* < .05).

Figure 6.
Analysis of fundic atrophy in *gp130*F/F/*Tff2*−/− mice. (*A*) Immunohistochemical localization of Tff2 (MNCs), intrinsic factor (chief cells), *GS*II (MNCs); neutral/acidic mucins (*AB/PAS*). *Scale bar* shows 50 μm. (*B*) Quantitative morphometry of normal vs atrophic fundic mucosa. (*C*) QRT-PCR analysis of H+K+ATPase expression; *histogram* shows mean messenger RNA fold change relative to wild type. *Asterisks* show statistical significance (*P* < .05).

Figure 7.

*Tff*2 promoter methylation is induced by experimental *H pylori* infection in mice. (*A*)
Immunoblot analysis of Tff2 protein expression in stomachs of wild-type (C57BL6) mice at
12-month postinfection (*12-MPI*) with *H pylori* SS1 (*Hp* SS1) compared with noninfected
controls. *Horizontal black bars* show mean optical density (OD) of Tff2 protein bands
normalized to GAPDH. Representative protein bands from each group are shown below the
scatterplots. (*B*) Quantitative *Tff*2 promoter methylation analysis (EPITYPER) in 12 and 15
MPI *Hp* SS1 and control stomach. *Histograms* show mean CpG methylation ratios. *Asterisks*
show statistical significance (*P* < .05). *Wide black box* shows exon1 coding region; *narrow*

black box shows exon1 5′ UTR; transcription start site (TSS); base pair (bp) positions are indicated; *solid-line circles* show CpGs covered by the analysis; *broken-line circles* show nonanalyzed CpGs. (*C*) Two-way hierarchical cluster analysis of *Tff2* promoter methylation data (*columns*) against mouse samples (*rows*). *Hp* SS1 infection status classification is shown on the right: *white boxes* (*12/15 MPI* noninfected controls), *black boxes* (*12/15 MPI Hp* SS1). CpG methylation ratios: 0.2 (*green*; low) to 0.6 (*red*; high; see color key).