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Activation of β -catenin Signaling by TFF1 Loss Promotes Cell Proliferation and Gastric Tumorigenesis

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Abstract

Objective—In this study, we investigated the role of TFF1 in regulating cell proliferation and tumor development through β -catenin signaling using in vivo and in vitro models of gastric tumorigenesis.

Design—TFF1-Knockout mice, Immunohistochemistry, luciferase reporter, qRT-PCR, immunoblot and phosphatase assays were used to examine the role of TFF1 on β -catenin signaling pathway.

Results—Nuclear localization of β -catenin with transcriptional upregulation of its target genes, *c-Myc* and *Ccnd1*, was detected in hyperplastic tissue at early age of 4-6 weeks and maintained during all stages of gastric tumorigenesis in the *Tff1*-knockout mice. The reconstitution of TFF1 or TFF1 conditioned media significantly inhibited the β -catenin/TCF transcription activity in MKN28 gastric cancer cells. In agreement with these results, we detected a reduction in the levels of nuclear β -catenin with downregulation of *c-MYC* and *CCND1* mRNA. Analysis of signaling

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DP: analysis of immunohistochemical data

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MBP: histopathology analysis of mouse and human tissues

MKW: histopathology analysis of mouse and human tissues

AB: analysis and interpretation of data; experimental troubleshooting; drafting of the manuscript; critical revision of the manuscript

PC: histopathology analysis of mouse and human tissues

WER: study concept and design; obtained funding; study supervision; experimental troubleshooting; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content

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molecules upstream of β -catenin revealed a decrease in p-GSK3 β (Ser9) and p-AKT (Ser473) protein levels following the reconstitution of TFF1 expression; this was consistent with the increase of p- β -catenin (Ser33/37/Thr41) and decrease of p- β -catenin (Ser552). This TFF1-induced reduction in phosphorylation of GSK3 β and AKT was dependent on PP2A activity. The treatment with okadaic acid or knockdown of PP2A abrogated these effects. Consistent with the mouse data, we observed loss of TFF1 and an increase in nuclear localization of β -catenin in stages of human gastric tumorigenesis.

Conclusion—Our data indicate that loss of TFF1 promotes β -catenin activation and gastric tumorigenesis through regulation of PP2A, a major regulator of AKT-GSK3 β signaling.

Keywords

TFF1; β -catenin; gastric cancer; PP2A

Introduction

Gastric cancer is one of the most common cancers worldwide and second in mortality after lung cancer [1]. Cancer development and progression are a result of an accumulation of contributing events such as infectious, environmental, and genetic risk factors. For instance, *Helicobacter pylori* infection and dietary factors are important risk factors for gastric carcinogenesis, while several genetic polymorphisms are found to be associated with a predisposition to cancer development [2]. Although the diagnostic capabilities and therapeutic options have improved, the prognosis for gastric cancer patients remains poor, especially in advanced stages [3].

Trefoil factor 1 (TFF1) is a member of the trefoil factor family peptides that are cysteine-rich proteins and form a characteristic trefoil domain through disulfide bonds, which make them resistant to hazardous conditions such as low pH and proteolysis [4]. TFF1 is expressed predominantly in the gastric epithelia and secreted by the mucus-secreting pit cells of the corpus and antropyloric regions of the stomach [5, 6]. *TFF1* has been reported as a gastric-specific tumor suppressor gene [7]. The TFF1 protein, which is secreted as a component of the protective mucus layer in the stomach, is highly expressed in response to mucosal injury [8]. Previous reports have shown loss of TFF1 protein expression in more than two-thirds of gastric adenocarcinomas because of mutation-independent mechanisms [9, 10]. The silencing of the *TFF1* gene expression in gastric cancer is predominantly induced by loss of heterozygosity (LOH) and hypermethylation of the *TFF1* promoter [9, 11, 12]. In addition, transcriptional regulation of TFF1 by COBRA1 have also been described in gastric cancers [10]. Using the *Tff1*-knockout mouse model, we and others have provided strong evidence supporting a tumor suppressor role for *Tff1* in gastric tumorigenesis, and demonstrated that *Tff1* is essential for normal differentiation of the antral and pyloric gastric mucosa [13, 14].

Activation of β -catenin is a frequent molecular event associated with the malignant transformation in the colon and upper gastrointestinal adenocarcinomas [15, 16]. Previous reports have indicated that β -catenin promotes cell proliferation by the induction of transcription of several genes such as *CCND1* and *c-MYC* through the activation of T-cell

factor (TCF)/lymphoid enhancer factor (LEF) transcription factors [17, 18]. In normal epithelial cells, cytoplasmic β -catenin is tightly regulated by the adenomatous polyposis coli (APC) tumor-suppressor protein and glycogen synthase kinase-3 β (GSK3 β) leading to its phosphorylation at Ser33/34/Thr41 residues, ubiquitination, and proteasomal degradation [19, 20]. The accumulation of nuclear β -catenin is one of the hallmarks of activated β -catenin signaling in cancer; this activation was reported to be regulated by AKT-PP2A through phosphorylation of β -catenin at Ser552 and Ser675 [21]. In addition, AKT-dependent phosphorylation and inhibition of GSK3 β activity enhances β -catenin protein stability through decreased phosphorylation of β -catenin at Ser33/34/Thr41 residues [22, 23]. Protein phosphatases play an important role in regulating the phosphorylation and activity of several kinases in cancer [24]. Protein phosphatase 2A (PP2A) is a highly complex trimeric holoenzyme, which consists of catalytic, structural and regulatory subunits. The catalytic domain of PP2A (PP2Ac) has been implicated in the regulation of the AKT-GSK3 β - β -catenin signaling [25].

The role of TFF1 in regulating cell proliferation and the underlying mechanism in gastric tumorigenesis remain unclear. In this study, using the *Tff1*-knockout mouse model together with *in vivo*, *ex vivo*, and *in vitro* experiments; we demonstrated that TFF1 silencing enhances cell proliferation by activating β -catenin through regulation of the PP2A activity upstream of the AKT and GSK3 β signaling in gastric cancer.

Materials & Methods

Animals: histologic evaluation and immunohistochemical assessment

The *Tff1*-knockout mouse model of gastric tumorigenesis [13], *Tff1*-heterozygote and normal wild-type mice were maintained under barrier conditions in a pathogen-free state in accordance with Institutional Animal Care and Use Committee approved protocol at Vanderbilt University. Frozen and formalin fixed paraffin-embedded stomach tissue samples were collected from *Tff1*-knockout (n=49), *Tff1*-heterozygote (n=10), and wild-type mice (n=28). Histopathological classification and grading of the gastric tissues were performed by our pathologists on H&E stained sections.

Primary gastric epithelial cell extraction and short-term culture

For preparation of short-term cultures of primary gastric epithelial cells from the *Tff1*-knockout and wild-type mice, stomachs were removed from 8-week-old mice and opened as described above. After washing with HBSS, the gastric antrum was cut and incubated in 10 ml of 1 mmol/L dithiothreitol for 15 minutes at 37°C with shaking, washed in HBSS 3 times at 37°C, and incubated in 0.5 mg/ml collagenase for 30 minutes at 37°C. After the first collagenase digestion, tissues were washed again with HBSS 3 times and incubated for an additional 30 minutes in collagenase (0.37 mg/ml) at 37°C. Tissues were triturated using a wide-mouthed pipette, and larger fragments of tissue were allowed to settle under gravity for 45 seconds. The supernatant containing isolated gastric cells was removed and transferred to a clean 50-ml conical tube and left on ice to sediment for 45 minutes. The supernatant was then carefully removed and discarded, and isolated cell colonies were plated in chamber slides. Colonies of gastric epithelial cells were cultured in F-12 (Ham's medium)

supplemented with 10% FBS and 1% of antibiotic-antimycotic solution (Invitrogen Life Technologies). The cells were cultured for up to 72 hours in a humidified incubator at 37°C under an atmosphere of 5% CO₂, and the medium was changed every 24 hours.

Reconstitution of TFF1 expression in cell lines

MKN28 cells were obtained from ATCC and were cultured in Ham's F-12 supplemented with 10% FBS (Invitrogen Life Technologies) at 37°C in an atmosphere containing 5% CO₂. In order to reconstitute the expression of TFF1 in MKN28 cells using adenovirus system, The TFF1 coding sequence from pcDNA3.1/TFF1 plasmid was subcloned into the adenoviral shuttle vector (pACCMV). The recombinant adenovirus-expressing TFF1 was generated by co-transfecting HEK-293 cells with the shuttle and backbone adenoviral (pJM17) plasmids using the Calcium Phosphate Transfection kit (Applied Biological Materials). After infection, the MKN28 cells were analyzed for the expression of *TFF1* by qRT-PCR.

Statistical analysis

Using GraphPad Prism software, a 1-way ANOVA Newman-Keuls Multiple Comparisons Test was used to compare the differences between 3 groups or more, and a 2-tailed Student's test was used to compare the statistical difference between 2 groups. The correlation between 2 parameters was determined by Spearman correlation. The differences were considered statistically significant when the P value was < 0.05.

Supplementary Methods—The methods that describe conditioned media, immunofluorescence, quantitative PCR, luciferase reporter, Western blotting, proliferation, phosphatase assay immunoprecipitation, shRNA knockdown, and immunohistochemistry on tissue microarrays assays are included in the Supplementary Materials and Methods.

Results

Loss of *Tff1* activates β -catenin in gastric hyperplasia and subsequent tumorigenesis cascade in mouse

Immunohistochemistry analysis of β -catenin staining of the pyloric antrum gastric tissues of the *Tff1*-knockout (*Tff1*-KO) mice revealed a moderate-strong cytosolic and nuclear immunostaining of β -catenin as early as 4-6 weeks of age. In contrast, the expression of β -catenin was limited to the cell membrane in *Tff1*-wild-type (*Tff1*-WT) and *Tff1*-heterozygote (*Tff1*-HET) mice of similar age (Figure 1A). To confirm the activation of β -catenin at an early age, the mRNA expression of β -catenin target genes, *c-Myc* and *Ccnd1*, was evaluated by qRT-PCR. The results showed a significant increase of mRNA expression of *c-Myc* ($P < 0.01$) and *Ccnd1* ($P < 0.05$) in *Tff1*-KO as compared to *Tff1*-WT and *Tff1*-HET (Figure 1B). Notably, the mRNA expression of *c-Myc* and *Ccnd1* genes was not significantly different between *Tff1*-WT and *Tff1*-HET. The mRNA expression of the *Tff1* gene was confirmed in all three groups of mice using qRT-PCR (Supplemental Figure S1). Indeed, the results demonstrated no significant correlation between the mRNA expression of *c-Myc* or *Ccnd1* and different age groups in *Tff1*-KO mice (Supplemental Figure S2). Next, the immunohistochemistry analysis of gastric tissues revealed an increase of nuclear β -catenin

expression in hyperplasia, low-grade dysplasia (LGD), high-grade dysplasia (HGD), and adenocarcinoma (AC) in the pyloric antrum of *Tff1*-KO mice as compared to normal *Tff1*-WT mice (Figure 1C). To confirm the activation of β -catenin in the tissue samples, the mRNA expression of β -catenin target genes, *c-Myc* and *Ccnd1*, was evaluated by qRT-PCR. The results showed a significant increase of mRNA expression of *c-Myc* ($P < 0.001$) and *Ccnd1* ($P < 0.05$) in hyperplasia and remained elevated in advanced lesions (LGD, HGD, and AC) in *Tff1*-KO mice as compared to normal wild-type (Figure 1D). These results are concordant with previous reports indicating that β -catenin nuclear translocation is an early event in gastric adenocarcinoma and several other cancers [26]. Taken together, the data suggest that loss of Tff1 promotes β -catenin activation at an early age (4-6 weeks) and stage of gastric tumorigenesis independent of age increase in the *Tff1*-KO gastric cancer mouse model.

TFF1 regulates β -catenin localization, transcriptional activity, and downstream targets

To explore the role of TFF1 in regulating β -catenin, we evaluated β -catenin cellular localization following modulation of TFF1 expression in primary gastric epithelial cells isolated from *Tff1*-WT and *Tff1*-KO mice, and human gastric cancer epithelial cells (MKN28). The immunofluorescence data showed that β -catenin was predominantly localized in the nucleus in *Tff1*-KO mouse gastric epithelial cells more than in wild-type cells ($P < 0.001$) (Figure 2A). In agreement with the *ex vivo* mouse data, the MKN28 cell model results revealed strong nuclear β -catenin staining that was significantly abrogated following reconstitution of TFF1 ($P < 0.001$) (Figure 2B). The results also indicated a substantial increase in β -catenin cell membrane staining in TFF1-expressing cells as compared to control cells (Figure 2B). Western blot analysis data of nuclear and cytosolic protein fractions from MKN28 cells infected with control or TFF1 adenoviruses indicated less nuclear β -catenin expression in TFF1-expressing cells than control cells, consistent with the immunofluorescence data (Supplemental Figure S3). We investigated whether the secreted TFF1 protein could regulate β -catenin expression in MKN28 cells. The immunofluorescence data showed that nuclear β -catenin expression was significantly reduced in cells treated with TFF1 conditioned media as compared to control cells ($P < 0.001$, Figure 3C). To characterize the suppressive effect of TFF1 on β -catenin transcriptional activity, the TCF transcription activity was measured using pTopFlash and its mutant pFopFlash luciferase reporters. The infection of MKN28 cells with the TFF1 adenovirus led to a 50% decrease ($P < 0.01$) in the pTopFlash reporter activity as compared to the control adenovirus (Figure 3A). We confirmed the suppressive effect of TFF1 on β -catenin activity by luciferase reporter assays following treatment of MKN28 cells with TFF1 conditioned media (Figure 3B) or transfection with PTT5 or PTT5-TFF1 plasmids (Figure 3C). Collectively, these data from *ex vivo* and *in vitro* studies indicated that TFF1 decreases the β -catenin nuclear localization and its transcriptional activity in gastric epithelial cells.

To investigate whether the TFF1-induced suppression of β -catenin transcriptional activity leads to downregulation of downstream targets such as *c-Myc* and cyclin D1, we evaluated mRNA and protein expression of these targets in *in vivo* and *in vitro* models. The results revealed a significant increase in mRNA levels of *Ccnd1* and *c-Myc* ($P < 0.01$, Figure 4A), and *Axin-2* ($P < 0.05$, Supplemental Figure S4A) in *Tff1*-KO mice as compared to wild-type.

In accordance with mRNA results, Western blot analysis demonstrated substantially increased c-Myc and cyclin D1 protein expression in the *Tff1*-KO mice as compared to wild-type (Figure 4B). The β -catenin protein level was higher in the *Tff1*-KO mice than wild-type (Figure 4B).

In line with the *in vivo* data, the MKN28 cell model qRT-PCR data showed a significant decrease in mRNA expression of *c-MYC* and *CCND1* ($P < 0.001$, Figure 4C), and *AXIN-2* ($P < 0.001$, Supplemental Figure S4B) in TFF1-expressing cells as compared to control cells. Western blot analysis results confirmed the decrease in protein expression of β -catenin, c-MYC, and cyclin D1 in TFF1-expressing cells as compared to control cells (Figure 4D). To determine if secreted TFF1 is functional, we treated MKN28 cells with conditioned media and evaluated protein expression by Western blot analysis. The results showed that secreted TFF1 induced a decrease in β -catenin, cyclin D1, and c-MYC protein levels (Supplemental Figure S5). To ascertain that *c-Myc* is a β -catenin target gene, we performed c-Myc luciferase reporter assay in MKN28 cell infected with control or TFF1 adenoviruses. The data indicated that TFF1 expression significantly decreased c-Myc transcriptional activity ($P < 0.01$). The c-Myc luciferase reporter with a mutation in the TCF binding site was used as a negative control (Supplemental Figure S6). Taken together, the data demonstrated that TFF1 is a negative regulator of β -catenin activity and downstream targets *in vivo* and *in vitro*.

TFF1 regulates gastric epithelial cell proliferation *in vivo* and *in vitro*

The promotion of cellular proliferation is one of the important functions of β -catenin signaling in cancer [27]. To investigate the effect of TFF1-dependent regulation of β -catenin on cell growth and proliferation, we subjected gastric tissues from mice, 4-6 weeks of age and up, to immunohistochemical staining of Ki-67, and MKN28 cells to EdU (5-ethynyl-2'-deoxyuridine) staining. In the wild-type gastric tissues, Ki-67 staining was observed in the basal proliferative region of the glands or crypts (Figure 5A-B). On the other hand, the *Tff1*-KO gastric tissues exhibited Ki-67 staining that extended to the surface of the mucosa (Figure 5A-B). The quantification data demonstrated that in *Tff1*-KO mice 70-80% of gastric epithelial cells exhibited Ki-67 staining per gland; however, in wild type mice 32% or less of cells were positive for Ki-67 per gland (Figure 5A-B). In line with these data, the EdU results showed that incorporation of EdU was significantly lower in MKN28 cells infected with TFF1 adenovirus or treated with TFF1 conditioned media than control cells ($P < 0.01$, Figure 5C-D). These results demonstrated the role of TFF1 in regulating gastric epithelial cell proliferation *in vivo* and *in vitro*.

TFF1 inhibits β -catenin signaling through activation of GSK3 β

Our next step was to investigate if TFF1 inhibits β -catenin through regulation of GSK3 β . Therefore, we transiently expressed TFF1 in MKN28 cells in the presence or absence of LiCl, a specific GSK3 β inhibitor that leads to the phosphorylation of GSK3 β at Ser9 with inhibition of its kinase and subsequent activation of β -catenin. As expected, the luciferase reporter assay data showed that the β -catenin activity in control cells treated with LiCl was significantly higher than non-treated cells ($P < 0.05$, Figure 6A). On the other hand, TFF1-expressing cells exhibited significantly less β -catenin activity than control cells ($P < 0.05$, Figure 6A). However, treatment with LiCl significantly abrogated TFF1 effect, rescuing β -

catenin activity to control level (Figure 6A). The Western blot results indicated that TFF1 expression significantly decreased p-p-GSK3 β (Ser9), p- β -catenin (Ser552), β -catenin, and increased p- β -catenin (Ser33/37/Thr41) protein levels as compared to control. However, in TFF1-expressing cells, LiCl counteracted the effects of TFF1 (Figure 6B). Taken together, these results indicated that TFF1 can inhibit β -catenin signaling through activation of GSK3 β .

TFF1 suppresses the AKT- β -catenin pathway through PP2A activation

In addition to kinases, protein phosphatases such as PP2A are implicated in β -catenin regulation [28]. The results showed that the PP2A phosphatase activity was significantly higher in TFF1-expressing MKN28 cells than control cells ($P < 0.05$). Of note, the TFF1-induced increase of PP2A activity was completely abrogated upon treatment with okadaic acid (OA), a selective inhibitor of PP2A ($P < 0.05$, Figure 6C). In a control experiment, the treatment of cells with OA decreased PP2A activity by 69% relative to control cells ($P < 0.05$, Figure 6C). To investigate the involvement of PP2A in TFF1- β -catenin signaling, we treated control or TFF1-expressing MKN28 cells with or without OA followed by Western blot analysis. The data showed that, in control cells, there was an increase of p-AKT (Ser473), p-GSK3 β (Ser9), p- β -catenin (Ser552), and β -catenin protein levels after treatment with OA (Figure 6D). As expected, in TFF1-expressing cells, there was a decrease in the expression levels of all these proteins. However, inhibition of PP2A activity with OA counteracted the TFF1-induced decrease of p-GSK3 β (Ser9), p-AKT (Ser473), p- β -catenin (Ser552), and β -catenin protein levels in MKN28 cells (Figure 6D). It has been reported that the phosphorylation of PP2A catalytic subunit at Tyr307 residue indicates inhibition of PP2A [29]. Our Western blot data revealed that transient expression of TFF1 in MKN28 cells decreased p-PP2AC (Tyr307) protein level as compared to control (Figure 6D). Treatment with OA reversed the effect of TFF1 on PP2AC phosphorylation in these cells (Figure 6D). To confirm the role of PP2A in TFF1- β -catenin signaling, we transfected control or TFF1-expressing MKN28 cells with control siRNA or PP2Ac siRNA. Western blot analysis data showed an increase in the protein levels of p-GSK3 β (Ser9), p- β -catenin (Ser552), and β -catenin in MKN28/TFF1 cells transfected with PP2Ac siRNA as compared to MKN28/TFF1 cells transfected with control siRNA (Figure 6E). This confirms the results of PP2A inhibition with OA (Figure 6D). Taken together, the results indicate that activation of PP2A by TFF1 is an important step that mediates TFF1-induced suppression of β -catenin signaling in gastric cancer cells.

TFF1 Loss and β -catenin activation during the multistep human gastric carcinogenesis cascade

Using immunohistochemistry on tissue microarrays, we evaluated the protein expression of TFF1 and β -catenin in human gastric lesions and normal mucosa. The data showed strong immunostaining of TFF1 in normal gastric glands, while β -catenin staining was mostly restricted to the cell membrane (Figure 7A). Conversely, in dysplasia and gastric adenocarcinomas, TFF1 expression was substantially attenuated whereas β -catenin was mostly expressed in the nucleus (Figure 7B-C). Our quantitative results showed a significant decrease in TFF1 expression in intestinal metaplasia (IM), dysplasia (Dys), and adenocarcinoma as compared to normal glands (NG) ($P < 0.001$, Figure 7D). In contrast, we

demonstrated a significant increase in nuclear β -catenin expression along the multistep gastric carcinogenesis cascade from IM, Dys, to adenocarcinoma as compared to NG ($P < 0.001$) (Figure 7E). Of note, we detected a significant inverse correlation between nuclear β -catenin and TFF1 expression ($r = -0.5$; $P < 0.001$) (Figure 7F).

Discussion

Gastric cancer ranks as the fourth most common cancer and the second most frequent cause of cancer deaths worldwide [30]. Understanding of the molecular basis of gastric tumorigenesis is a crucial step for the development of novel anticancer therapeutic approaches. In this study, we investigated the role of the TFF1 protein in regulating β -catenin signaling in gastric tumorigenesis.

Deficient *TFF1* gene expression has been shown to promote gastric tumorigenesis in human and mouse [14, 31]. The development of gastric cancer involves complex and interacting signaling pathways that promote gastric tumorigenesis [2]. In this report, we provide, for the first time, experimental evidence of TFF1 regulation of β -catenin-mediated gastric tumorigenesis in *Tff1*-KO mouse model, and characterize the underlying molecular mechanism in human *in vitro* cell models. The activation of the β -catenin pathway has been reported as an early initiating event in gastric tumorigenesis [32]. In this regard, our findings confirm that β -catenin activation occurs during early stages of tumorigenesis in the *Tff1*-KO mice. The accumulation of nuclear β -catenin started in hyperplastic gastric tissues in 4-6 week old mice. This was maintained during the progression from low-grade dysplasia to high-grade dysplasia and adenocarcinoma, suggesting the importance of β -catenin in all stages of gastric cancer. Immunohistochemical analysis of human gastric tissues demonstrated similar findings supporting the notion that TFF1 loss and nuclear β -catenin expression are early molecular events associated with the onset of the multistep gastric tumorigenesis cascade in human.

The activity of β -catenin signaling depends on the accumulation and translocation of β -catenin to the nucleus, one of the hallmarks for the initiation of tumorigenesis in a variety of human cancers [18, 33, 34]. Our *ex vivo* data demonstrated significant nuclear localization of β -catenin, indicative of its activity, in pyloro-antral epithelial cells from *Tff1*-KO mice. In contrast, the reconstitution of TFF1 expression or TFF1 conditioned media reduced nuclear accumulation of β -catenin and enhanced its cell membrane localization in the *in vitro* cell model. The pTOP-flash luciferase reporter data further validated the role of TFF1 as a negative regulator of β -catenin/TCF transcription activity.

In the nucleus, the β -catenin/TCF transcription complex regulates the expression of several genes that are involved in human carcinogenesis such as *CCND1* and *c-MYC* [18, 35]. Our results demonstrated that loss of *Tff1* gene expression induces an increase of mRNA and protein expression of β -catenin target genes, *c-Myc* and *Ccnd1*, as early as 4-6 weeks of age in the *Tff1*-KO mice. This confirms that activation of β -catenin signaling occurs at early stages of gastric tumorigenesis. Conversely, the reconstitution of TFF1 expression *in vitro* significantly decreased mRNA and protein levels of *c-Myc* and cyclin D1. The β -catenin target genes, *c-Myc* and *Ccnd1*, are known oncogenes that drive cell growth and

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Abbreviations used in this paper

TFF1	trefoil factor 1
GSK3	glycogen synthase kinase 3
PP2A	protein phosphatase 2A
TCF/LEF	T cell factor/lymphoid enhancement factor
OA	okadaic acid
LiCl	lithium chloride

What is already known about this subject?

- *TFF1* is a gastric tumor suppressor gene.
- TFF1 is expressed in the gastric epithelia and secreted by the mucus-secreting pit cells of the corpus and antropyloric regions of the stomach
- Loss of TFF1 leads to the development of gastric cancer.

What are the new findings?

- This study demonstrates that loss of *Tff1* induces β -catenin activation in hyperplastic gastric mucosa and along stages of gastric tumorigenesis in mice
- TFF1 regulates β -catenin localization, transcriptional activity, and downstream targets c-Myc and CyclinD1
- TFF1 regulates gastric epithelial cell proliferation
- TFF1 negatively regulates the AKT- β -catenin pathway through activation of PP2A
- Loss of TFF1 increases nuclear β -catenin expression during human gastric carcinogenesis cascade

How might it impact on clinical practice in the foreseeable future?

- This study provides the role of TFF1 in regulating cell proliferation and tumor development through β -catenin signaling using in vivo and in vitro models of gastric tumorigenesis.
- TFF1 plays a pivotal role in maintaining the integrity of the gastric mucosa, and the fact that TFF1 could suppress proliferation of cancer cells, may offer a rationale for new therapeutic targets and treatment modalities of gastric cancer.

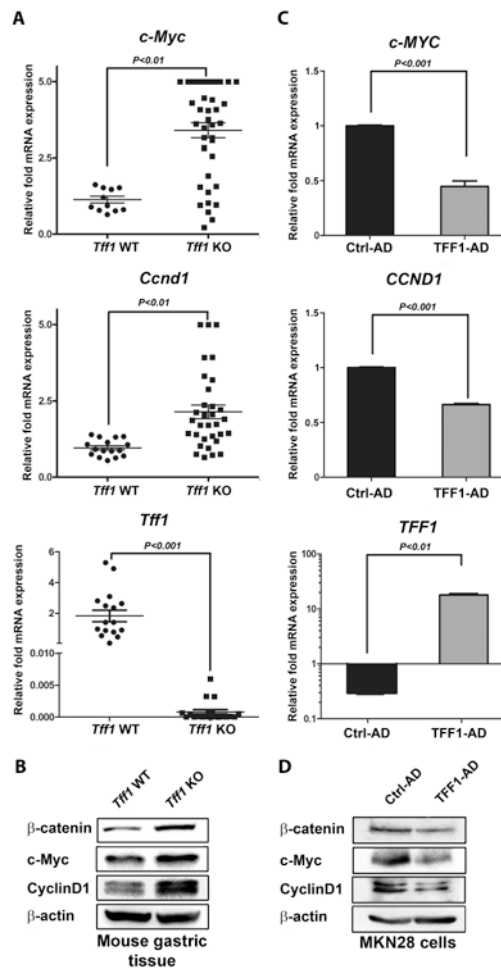


Figure 4. TFF1 negatively regulates mRNA and protein expression of β -catenin target genes *in vivo* and *in vitro*

A) qRT-PCR data demonstrate mRNA upregulation of β -catenin target genes (*c-Myc* and *Ccnd1*) in *Tff1*-KO mice as compared to wild-type. The *Tff1* gene expression was verified in wild-type and *Tff1*-KO mice. Horizontal bars indicate the mean values. $P < 0.05$ was considered statistically significant. **B)** Western blot analysis indicated an increase in protein levels of β -catenin, c-Myc, and cyclin D1 in *Tff1*-KO mice relative to wild-type. **C)** qRT-PCR data indicate mRNA down-regulation of β -catenin target genes (*c-MYC* and *CCND1*) in MKN28 cells infected with TFF1 adenovirus as compared to control. Reconstitution of TFF1 expression in MKN28 cells was confirmed. The bar graphs represent the mean \pm SEM of 3 independent experiments. **D)** Western blot analysis showed that transient expression of TFF1 in MKN28 cells decreased protein levels of β -catenin, c-MYC, and Cyclin D1 as compared to control. Protein loading was normalized for equal levels of β -actin. The Western blot results represent 1 of 3 independent experiments.

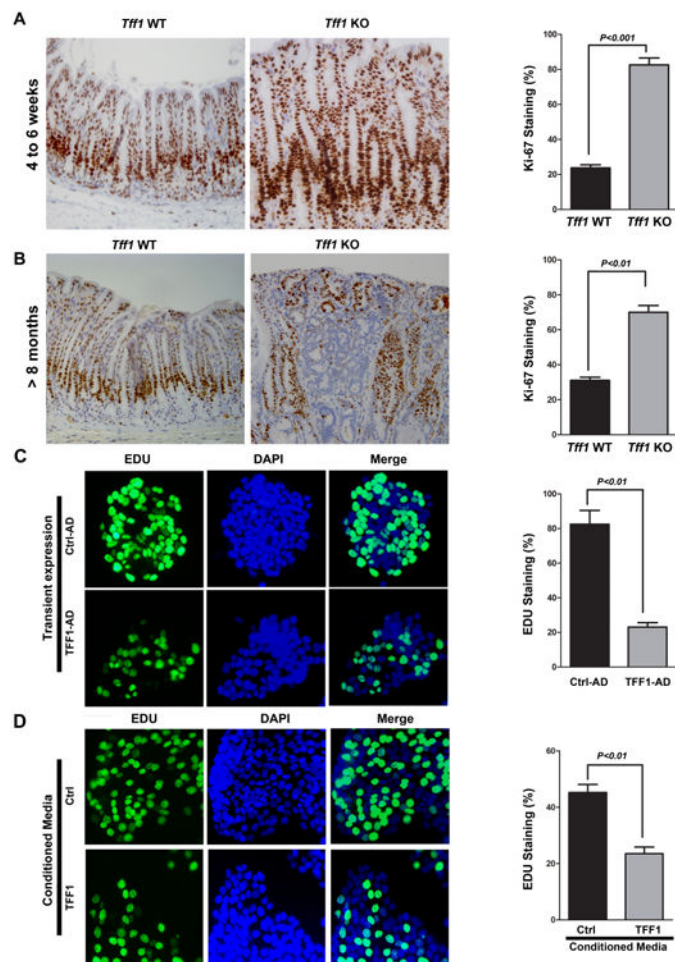


Figure 5. TFF1 regulates gastric cell proliferation *in vivo* and *in vitro*

A-B) Representative images of Ki-67 immunostaining (brown nuclei) in gastric glands of wild-type and *Tff1*-KO mice, 4-6 weeks of age (A) and 8 months and up (B). Original magnification ($\times 200$) is shown. The quantitative data of Ki-67 immunostaining in at least 200 counted cells were presented as percentage \pm SEM in the right panels. **C-D)** Representative images of EdU immunostaining in MKN28 cells infected with control or TFF1 adenoviruses (C), or incubated with TFF1 or control conditioned media (D). Cells were subjected to ClickiT[®] EdU Assay to evaluate cell proliferation. EdU staining is depicted by green fluorescence. DAPI (blue) was used as nuclear counterstain. The quantitative data of at least 200 counted cells indicating a significant decrease of cell proliferation in TFF1-expressing cells or cells treated with TFF1 conditioned media as compared to control cells were presented in the right panels of (C and D).

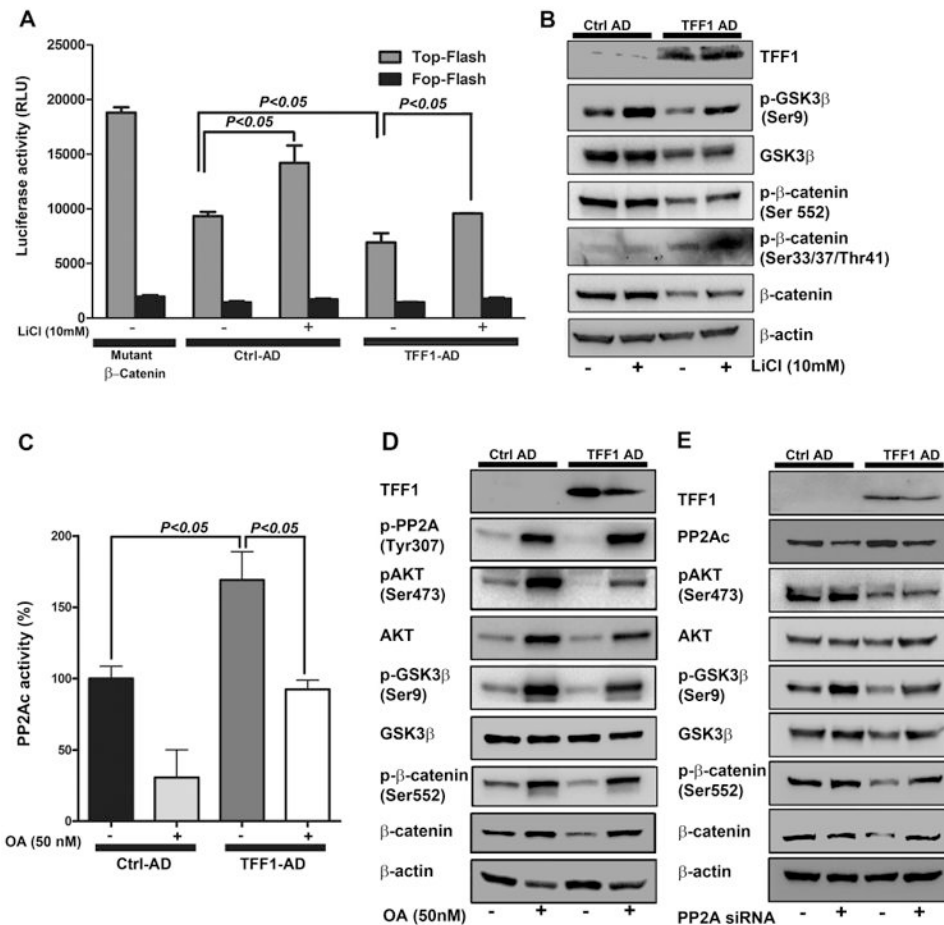


Figure 6. TFF1 suppresses AKT-β-catenin signaling through activation of GSK3β and PP2A
A-B) Reconstitution of TFF1 attenuates β-catenin transcriptional activity and signaling through activation of GSK3β. **A)** p-TOP-flash and its mutant p-FOP-flash luciferase reporter assays showing β-catenin/TCF transcriptional activity in MKN28 cells infected with Control or TFF1 adenoviruses and treated overnight with vehicle or LiCl (10 mM). Data are presented as mean ± SEM of at least three experiments, with each condition performed in triplicate. $P < 0.05$ was considered significant. **B)** Western Blot analysis of p-β-catenin (Ser552) and (Ser33, 37, Thr41), β-catenin, p-GSK3β (Ser9), GSK3β, TFF1 and β-actin proteins. **C, D&E)** Reconstitution of TFF1 suppresses β-catenin signaling through activation of PP2A. **C)** MKN28 cells were infected with control or TFF1 adenoviruses, treated with vehicle or okadaic acid (OA, 50 nM) for 16 h, and subjected to PP2A phosphatase activity assay. Data are presented as mean ± SEM of at least three experiments. **D&E)** MKN28 cells were infected with control or TFF1 adenoviruses treated with vehicle or OA (D) or transfected with control or PP2Ac siRNA (E); all samples were subjected to Western blot analysis. The Western blot results represent 1 of 3 independent experiments.

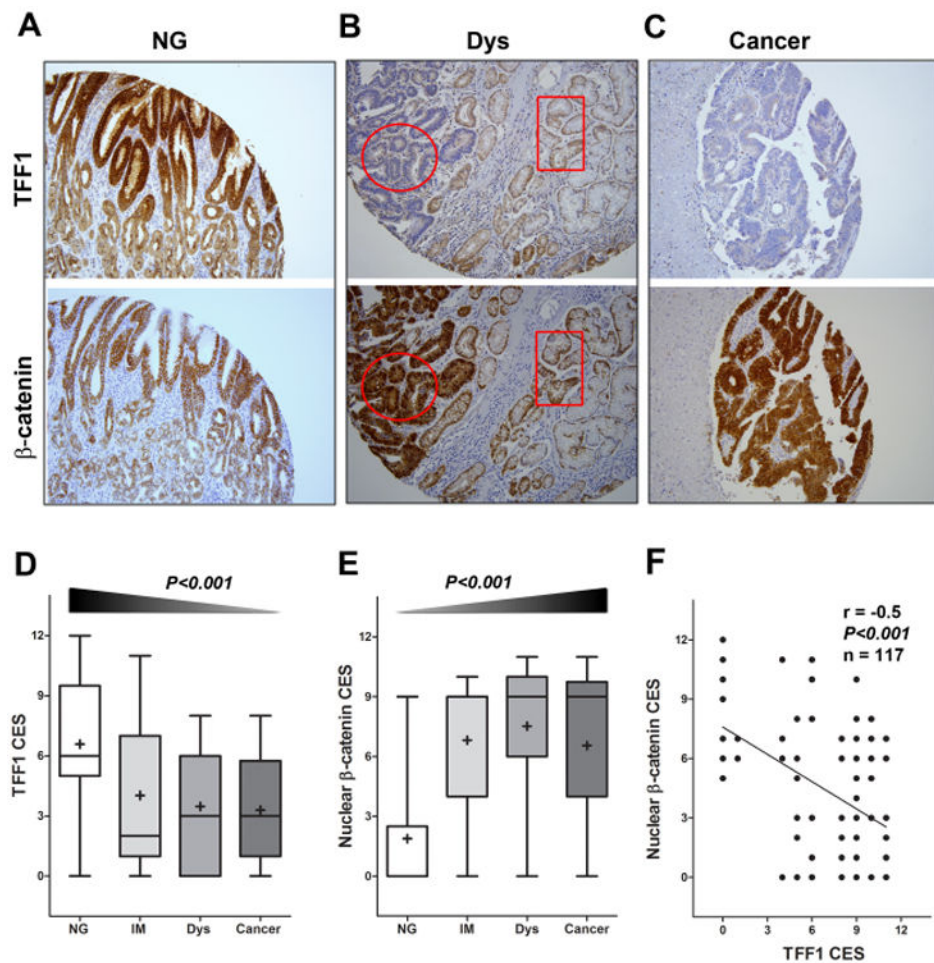


Figure 7. Immunohistochemistry for β-catenin and TFF1 in human tissue samples

A-C) Immunohistochemical staining for TFF1 (upper panels) and β-catenin (lower panels) was performed on serial tissue sections from human gastric mucosa with (A) normal gland (NG), (B) dysplasia (Dys), and (C) cancer. A progressive decrease of TFF1 expression was observed from normal mucosa to adenocarcinoma, along with a progressive increase of nuclear localization of β-catenin expression. The circular and rectangular areas demonstrate an inverse relationship in the immunostaining of TFF1 and β-catenin in serial sections from the same tissue. Original magnification ($\times 200$) is shown. **D-E)** The graphs summarize the immunohistochemical staining of TFF1 and nuclear β-catenin results on gastric tissue microarrays, respectively. **F)** The results from Pearson's correlation test between TFF1 and β-catenin nuclear staining are shown.

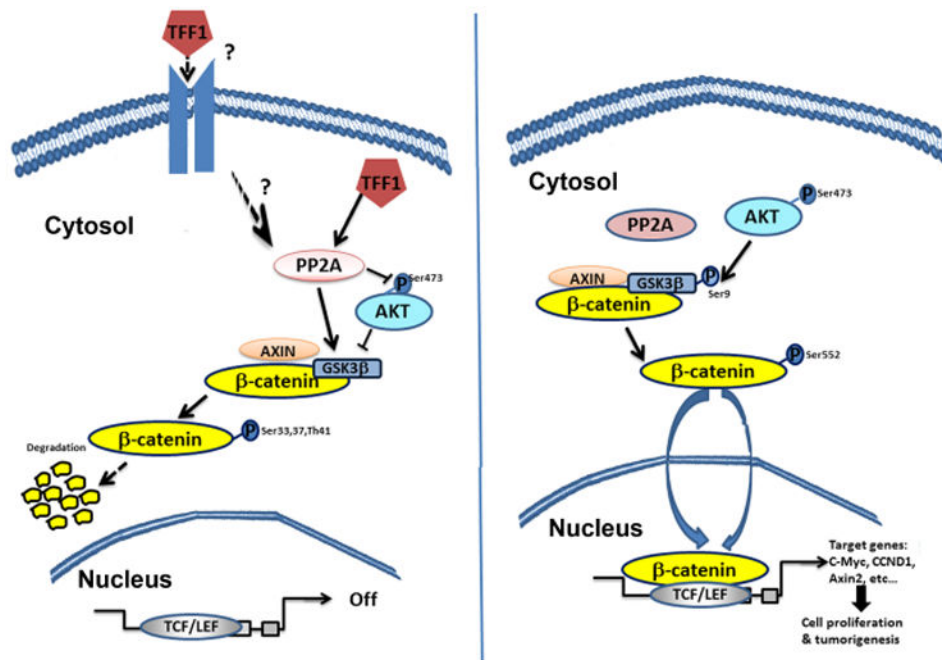


Figure 8. Schematic representation of the role of TFF1 in suppressing AKT- β -catenin pathway through activation of PP2A in gastric epithelial cells

In normal gastric epithelial cells, TFF1 activates PP2A leading to dephosphorylation of AKT, activation of GSK3 β , and degradation of the β -catenin protein. With the silencing of the *TFF1* gene in gastric cancer, β -catenin becomes active and translocates into the nucleus where it binds to TCF/LEF transcription factors and transactivate β -catenin target genes involved in proliferation and tumorigenesis.