Supplemental Methods, Figure Legends, and References

Supplementary Materials and Methods

Conditioned media

Serum-free media was collected two days after transient transfection of MKN28 cells with TFF1-PTT5 plasmid or PTT5 empty vector. The presence of TFF1 in conditioned media was tested by western blot analysis.

Immunofluorescence assay

Primary gastric epithelial cells and MKN28 gastric cancer cells infected with control or TFF1 adenoviruses, or treated with control or TFF1 conditioned media; were plated in 8-well chambers. Cells were washed with PBS and fixed with fresh 4% paraformaldehyde solution for 15 minutes at room temperature. Cells were then washed twice with PBS, followed by incubation in 10% normal goat serum blocking solution (Zymed Laboratories) for 20 minutes at room temperature in a humidified chamber. Cells were then incubated in the specific primary antibodies against β-catenin (Cell Signaling) diluted in PBS (1:400) for 2 hours at room temperature in a humidified chamber. Cells were washed 3 times in PBS and incubated in fluorescein isothiocyanate (FITC)–tagged secondary antibody (1:1,000; Jackson Immunoresearch) for 45 minutes at room temperature in a humidified chamber. The cells were then washed in PBS, mounted with Vectashield/DAPI (Vector Laboratories), and visualized using an Olympus BX51 fluorescence microscope (Olympus Co.). At least 200 cells were counted from each experiment. Total cell number was measured with automatic particle counting in ImageJ

software http://www.uhnresearch.ca/facilities/wcif/imagej/), after setting an automatic threshold range. The image was transformed into a binary image, and the total number of cells in each field was counted using watershed separation. The percentage of β-catenin–positive cells was calculated as the number of cells showing nuclear green staining divided by the total cell number showing DAPI nuclear blue staining × 100.

Quantitative real-time RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen), and single-stranded cDNA was subsequently synthesized using the Advantage RT-for-PCR Kit (Clontech Laboratories Inc). Genes specific for mouse and human primers were designed using the online software Primer 3 (http://frodo.wi.mit.edu/primer3/). The forward and reverse primers were designed to span 2 different exons for each gene (mouse: *Tff1*, *c-Myc, Ccnd1, and Axin-2*; human: *TFF1*, *c-MYC, CCND1, and AXIN-2*). All primers were purchased from Integrated DNA Technologies (Supplemental Table 1). The qRT-PCR was performed using an iCycler (Bio-Rad), with the threshold cycle number determined by use of iCycler software version 3.0. Reactions were performed in triplicate, and the threshold cycle numbers were averaged. The results of the genes were normalized to housekeeping genes, HPRT for human and 18S for mouse, as described previously [1]. Expression ratios were calculated according to the formula 2(Rt–Et)/2(Rn–En)[1], where Rt is the threshold cycle number for the reference gene observed in the test samples, Et is the threshold cycle number for the experimental gene observed in the test samples, Rn is the threshold cycle number for the reference gene observed in the reference

samples, and En is the threshold cycle for the experimental gene observed in the reference samples. Rn and En values were calculated as an average of all reference samples.

Luciferase reporter assay

To monitor the transcriptional activity of the β-catenin/TCF, we used the luciferase reporter constructs, pTopFlash, with six TCF binding sites, and its mutant pFopFlash (Upstate Biotechnology, Waltham, MA). The mutant β-catenin was used as a positive control, and the pFopFlash luciferase reporter to confirm the specificity of β-catenin/TCF. Fugen6 was used for transfection as directed by the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). The luciferase assay was performed following infection, transfection, or treatment with conditioned media. Three hours after adenovirus infection or treatment with conditioned media, the transfection with pTopFlash or pFopFlash was performed. We also transfected the mammalian expression plasmid, pTT5 [2] , in frame with TFF1, or PTT5 empty vector in combination with pTopFlash or pFopFlash. As control for transfection efficiency, we transfected all cells with pSV-β-galactosidase plasmid. The transfected cells were incubated for 48 hours before assaying for luciferase activity and β-galactosidase activity as described previously [3]. The firefly luciferase activity was normalized to β-galactosidase activity and expressed as relative luciferase activity with ± standard error of the mean (SEM).

Western blotting

Cell lysates were prepared in RIPA buffer containing Halt Protease and Phosphatase Inhibitors Cocktail (Pierce Biotechnology Inc.), and were centrifuged at 4,390 g for 10 minutes

at 4°C. Protein concentration was measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories). Equal amounts of proteins (10–15 μg) from each sample were subjected to SDS/PAGE and transferred onto nitrocellulose membranes. Target proteins were detected by using specific antibodies against, p-β-catenin (Ser33/37/Thr41), p-β-catenin (Ser552), β-catenin, CyclinD1, Axin-2, p-AKT (Ser473), AKT, p-GSK3β (Ser9), GSK3β, p-PP2A (Tyr307), c-Myc, and β-actin (Cell Signaling Technology).

For assay of nuclear localization of β-catenin, nuclear and cytoplasmic protein fractions were isolated from MKN28 cells infected with Control or TFF1 adenoviruses, using NE-PER Nuclear and Cytoplasmic Extraction Reagents (*Pierce*) following the manufacturer's instructions. Equal amounts of nuclear and cytoplasmic proteins (10 μg) were loaded onto 10% SDS-PAGE, separated by electrophoresis, and transferred to nitrocellulose membrane. The membranes were incubated with β-catenin antibody (Cell Signaling Technology, Inc.) and TFF1 (Origene). The cytoplasmic and nuclear protein fractions were normalized to β-actin and Histon3 (Cell Signaling Technology, Inc.), respectively.

Proliferation assay

In mouse tissue, proliferating cells were detected using immunohistochemical staining with a mouse monoclonal antibody directed against the Ki-67 antigen (Sigma-Aldrich). In human gastric cancer cell line MKN28, proliferation was measured using the ClickiT® EdU (5-ethynyl-2'-deoxyuridine) Assay Kit (Invitrogen) according to the manufacturer's protocol.

Phosphatase assay immunoprecipitation

Cells were washed twice in Tris buffer (pH 7.5) and total cellular proteins were extracted in lysis buffer containing 20 mmol/L imidazole-HCl, 2 mmol/L EDTA, 2 mmol/L EGTA, pH 7.0 with 10 μg/mL each of aprotinin leupeptin, pepstatin, 1 mmol/L benzamidine, and 1 mmol/L PMSF with no phosphatase inhibitors. The total serine/threonine activity (called also PP2A activity) was measured using a non-radioactive Immunoprecipitation malachite green phosphatase assay kit (Millipore, Billerica, MA). All procedures were performed according to the manufacturer's protocol, and changes in absorbance were recorded at 650 nm in a FluolarStar luminescence microplate reader (BMG Labtech).

Knock down of PP2A catalytic subunit by RNA interference (RNAi)

PP2Ac siRNA (Santa Cruz Biotechnology) was transfected into TFF1-expressing MKN28 cells or control MKN28 cells by using RNAifectin transfection reagent (abmgood, Canada) according to the manufacturer's instructions. A control siRNA (non-homologous to any known gene sequence) was employed as a negative control. The levels of PP2Ac expression were determined by Western blot using a PP2Ac antibody.

Human tissue microarrays and immunohistochemistry

Tissue microarrays containing cores from paraffin-embedded stomach tissue samples (17 normal mucosae, 33 intestinal metaplasia, 19 dysplasia, and 84 adenocarcinoma) were available for immunohistochemical analysis. All tissue samples were collected, coded, and de-

identified using protocols approved by the Vanderbilt University Institutional Review Board. Tissues were stained with H&E, and representative regions were selected for inclusion in a tissue array. Tissue cores with a diameter of 0.6 mm were retrieved from selected regions of the donor blocks and punched to the recipient block using a manual tissue array instrument (Beecher Instruments). Samples were punched in triplicate, and control samples from normal mucosa specimens were punched in each sample row. Sections (5-μm) were transferred to polylysine-coated slides (SuperFrostPlus; Menzel-Glaser) and incubated at 37°C for 2 hours. The resulting tumor tissue array was used for immunohistochemical analysis. The adenocarcinomas collected ranged from well-differentiated (WD) to poorly differentiated (PD), stages I to IV, with a mix of intestinal and diffuse-type tumors. An avidin-biotin immunoperoxidase assay was performed after pretreatment for 20 minutes in a microwave oven with citrate buffer. Rabbit β-catenin antibody (1:200 dilution; Cell Signaling) and mouse Estrogen Inducible Protein pS2 (Tff1) antibody (1:200 dilution; Abcam) were applied at room temperature. The CES was calculated using the formula CES = 4 (intensity – 1) + frequency as described previously [4, 5].

c-Myc Luciferase reporter assay

To evaluate the transcriptional activity of c-Myc, we used pBV-Luc reporter plasmid harboring the human c-Myc promoter or derivative plasmids containing mutations in the TCF binding sites on the c-Myc promoter (kind gift of Dr. Alexander Zaika). As control for transfection efficiency, we transfected all cells with pSV-β-galactosidase plasmid. The transfected cells were incubated for 48 hours before assaying for luciferase activity and β-

galactosidase activity as described previously [3]. The firefly luciferase activity was normalized to β-galactosidase activity and expressed as relative luciferase activity with ± standard error of the mean (SEM).

References

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4 Mukherjee K, Peng D, Brifkani Z, Belkhiri A, Pera M, Koyama T*, et al.* Dopamine and cAMP regulated phosphoprotein MW 32 kDa is overexpressed in early stages of gastric tumorigenesis. Surgery 2010;**148**:354-63.

5 Soutto M, Belkhiri A, Piazuelo MB, Schneider BG, Peng D, Jiang A*, et al.* Loss of TFF1 is associated with activation of NF-kappaB-mediated inflammation and gastric neoplasia in mice and humans. J Clin Invest 2011;**121**:1753-67.

Supplemental Figure Legends

Figure S1. *Tff1* mRNA expression in gastric tissues in 4-6 weeks old wild-type, *Tff1*-heterozygotes, and *Tff1*-Knockout mice

Quantitative RT-PCR data demonstrated mRNA levels in the antropyloric gastric mucosa of *Tff1* in 4-6 weeks old wild-type (n=10), *Tff1*-heterozygote (n=10), and *Tff1*-knockout (n=10) mice.

Figure S2. No overall correlation between *c-Myc* and *Ccnd1* mRNA expression and age in *Tff1*-knockout mice

(A-B) Pearson's correlation test of mRNA expression of β-catenin target genes, *c-Myc* **(A)** and *Ccnd1* **(B)** between different age groups. 4-8 weeks old mice exhibit hyperplastic gastric tissue, and 4 months and older mice develop dysplastic or malignant gastric lesions.

Figure S3. TFF1 expression regulates nuclear localization of β-catenin

Western blot analysis of total β-catenin and TFF1 in the nuclear and cytosolic protein fractions of infected MKN28 cells with control or TFF1 adenoviruses is shown. Gel loading was normalized to Histone 3 and β-actin.

Figure S4. Secreted TFF1 attenuates β-catenin and its targets protein expression *in vitro*

MKN28 cells were treated with control or TFF1 conditioned media, and subjected to immunoblot analysis. The data showed a marked decrease of β-catenin, c-Myc, and CyclinD1 protein levels in cells

treated with TFF1 conditioned media relative to control cells. The levels of TFF1 expression in the conditioned media are shown.

Figure S5. TFF1 negatively regulates *AXIN-2* mRNA expression *in vivo* and *in vitro*

(A-B) QRT-PCR data demonstrated mRNA up-regulation of β-catenin target gene *Axin-2* in *Tff1*-KO mice as compared to wild-type **(A)**, and down-regulation of *AXIN-2* in MKN28 cells infected with TFF1 adenovirus as compared to control cells **(B)**.

Figure S6. TFF1 expression negatively regulates transcriptional activation of c-Myc

PBV-Luc-Myc-Wild type and its mutant luciferase reporter assays showing c-Myc transcriptional activity in MKN28 cells infected with control or TFF1 adenoviruses.

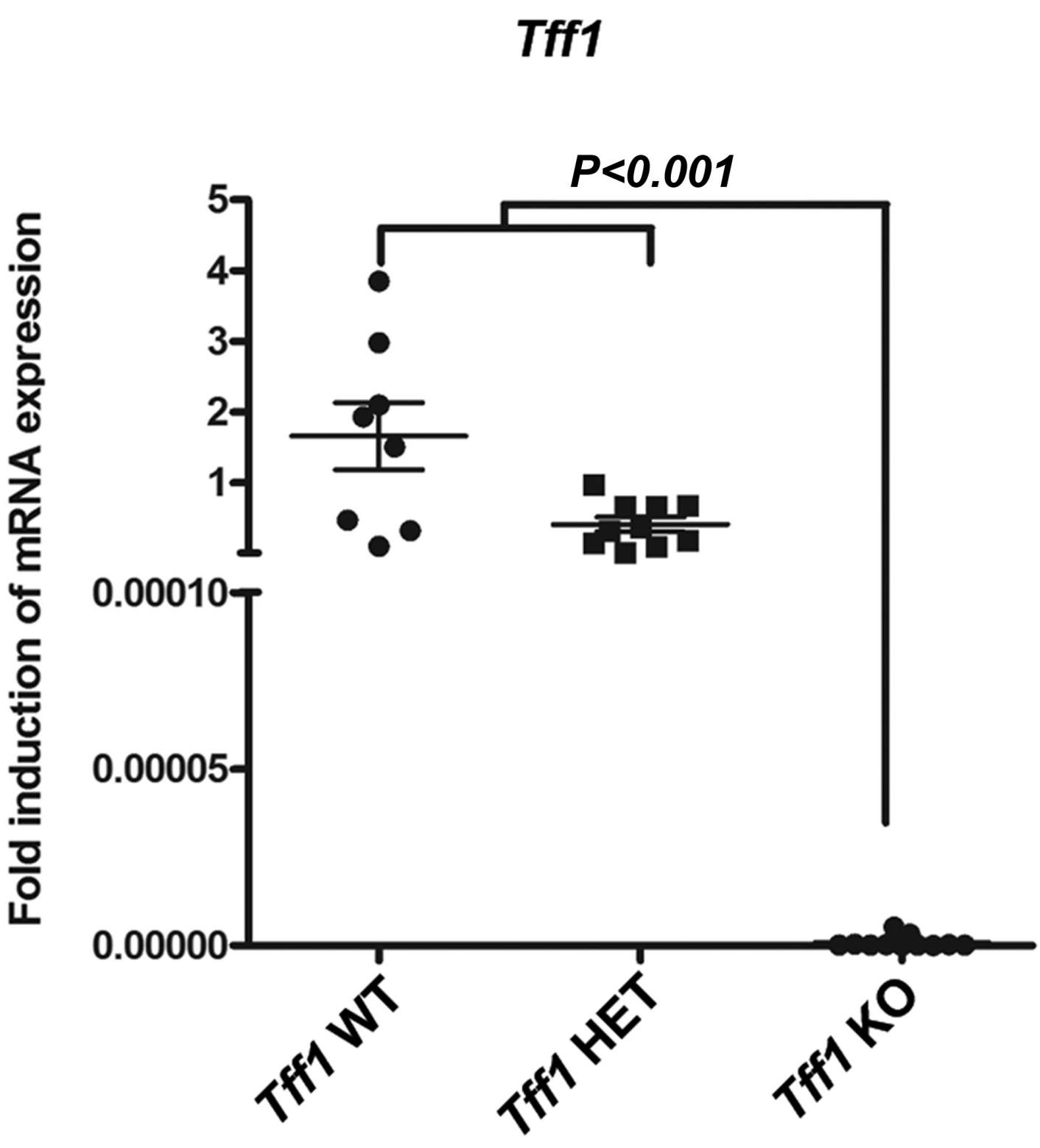

Figure S1. Soutto et al.

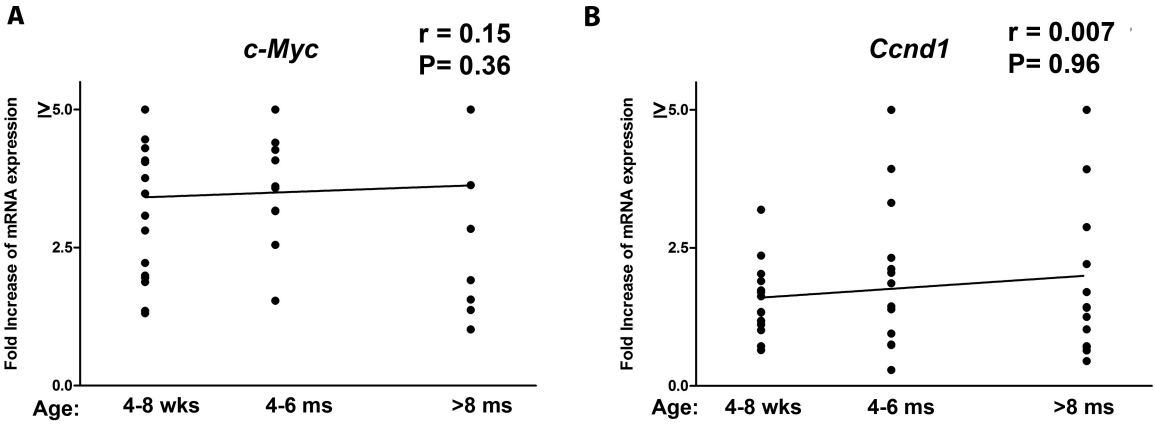

Figure S2. Soutto et al.

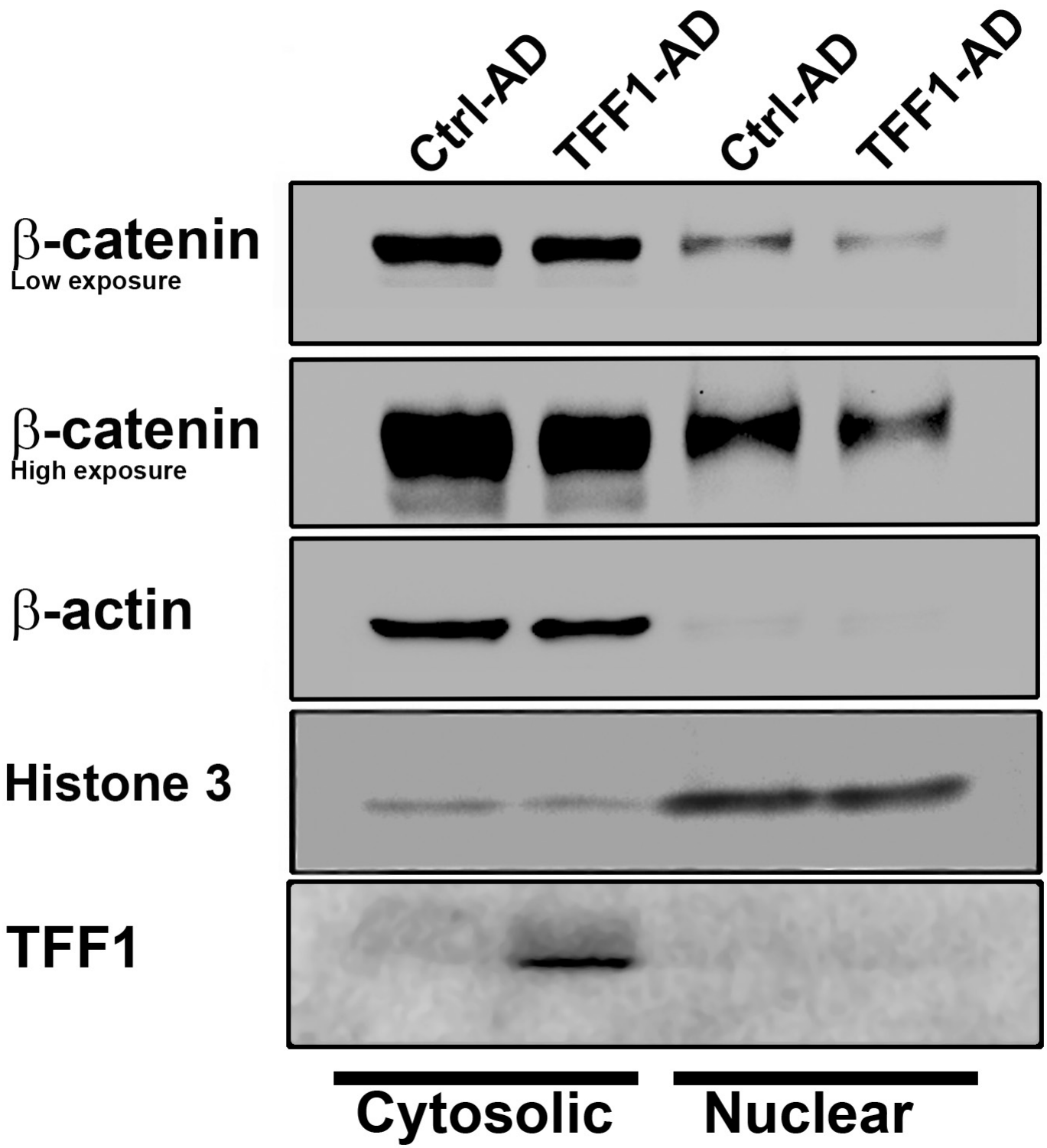

Figure S3. Soutto et al.

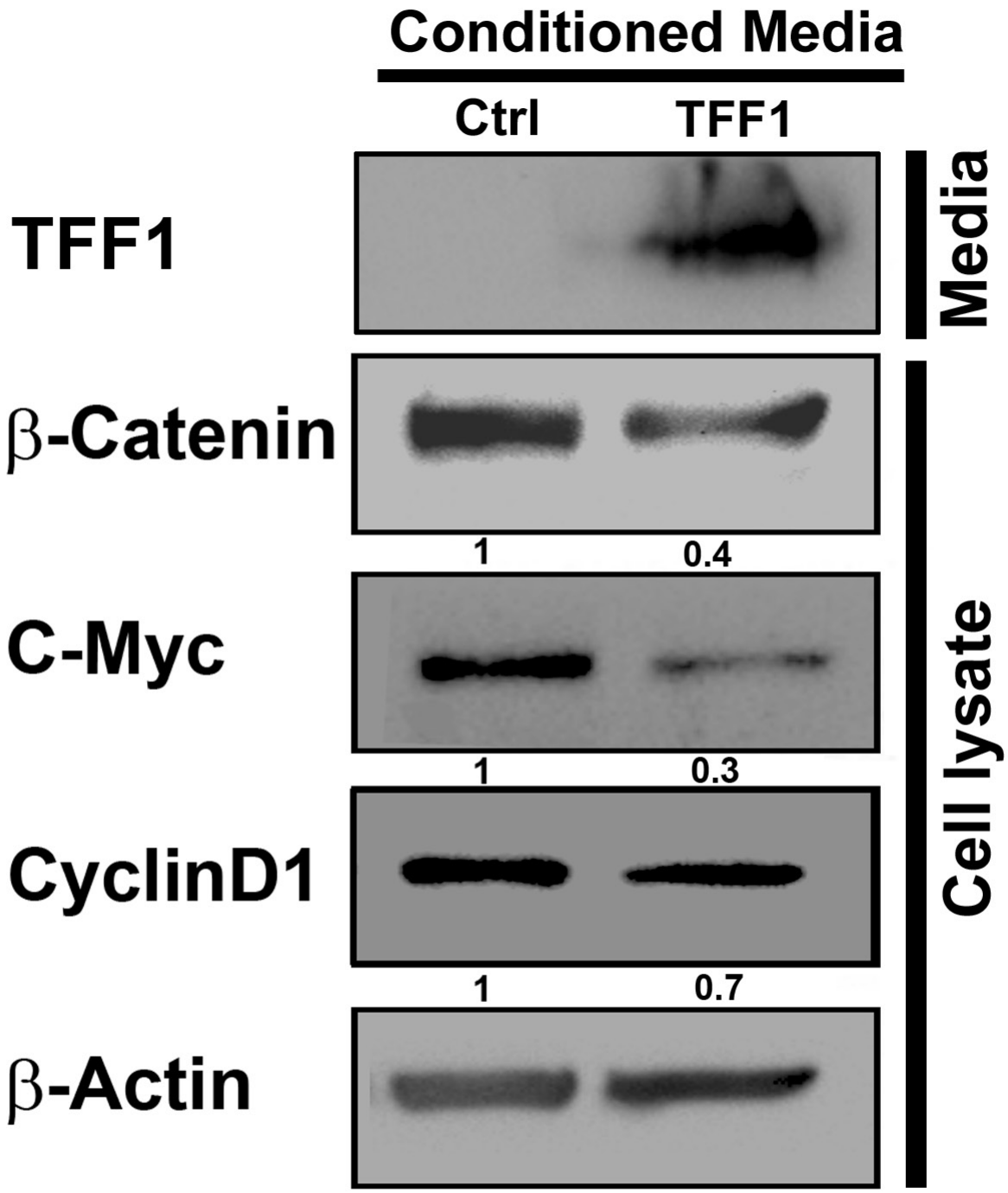

Figure S4. Soutto et al.

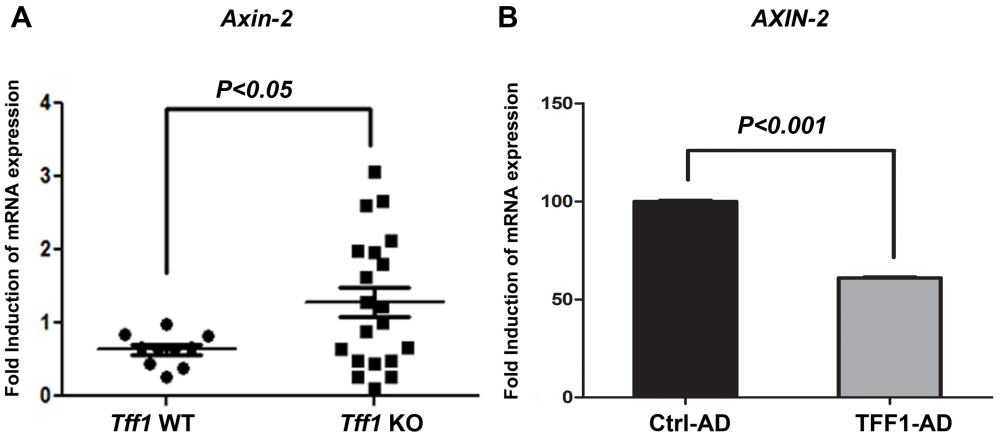

Figure S5. Soutto et al.

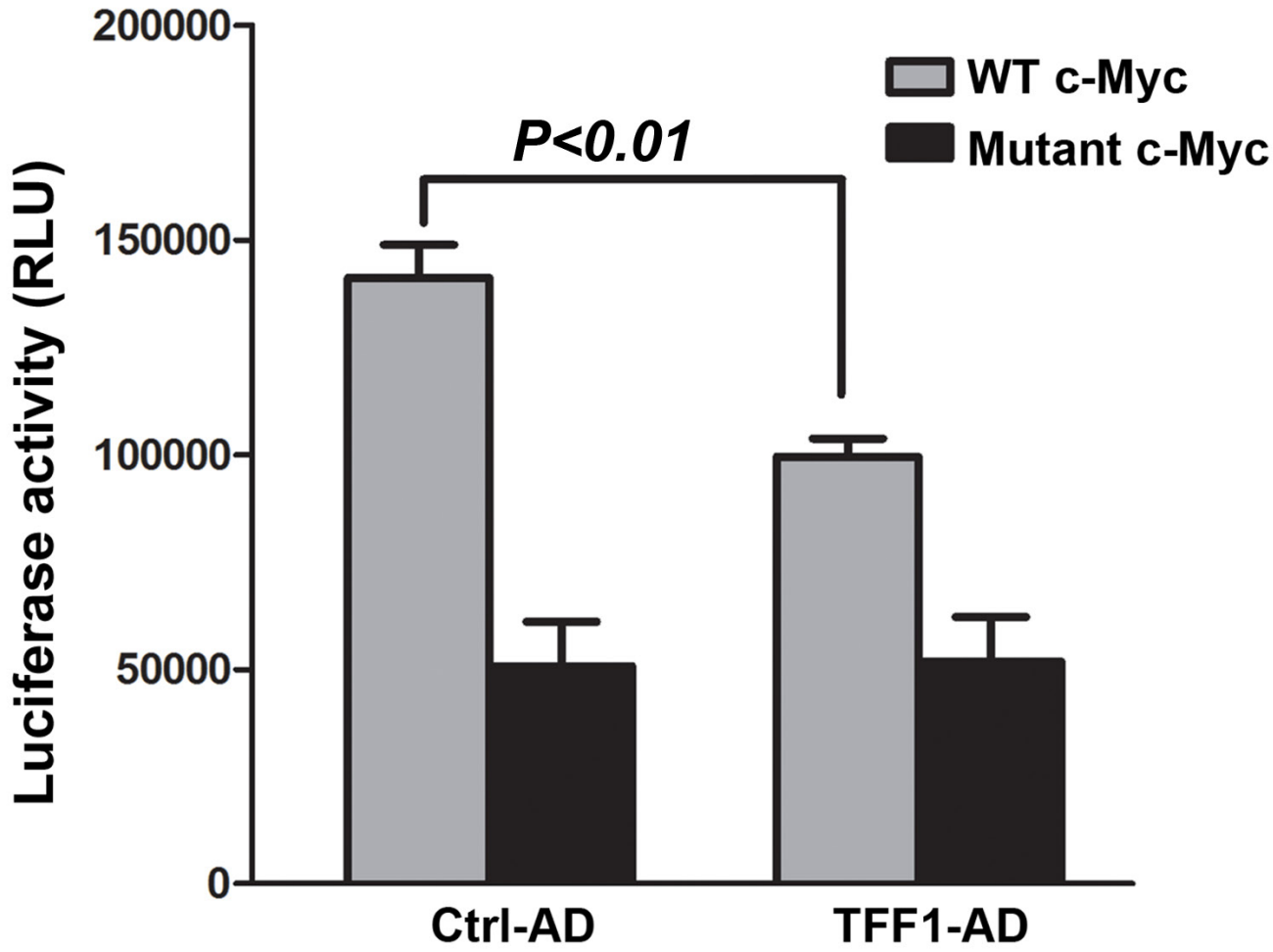

Figure S6. Soutto et al.

Supplemental Table 1.

Mouse primer sequences used in quantitative real time PCR

Gene	Ref. Seq. No.	Forward primer	Reverse primer	Product Size(bp)
Ccnd1	NM_007631	TCCTCTCCAAAATGCCAGAG	GCAGGAGAGGAAGTTGTTGG	188
c-Myc	NM_001177354	TCCTGTACCTCGTCCGATTC	TCTTCAGAGTCGCTGCTGGT	140
Tff1	NM_009362.2	CCCGGGAGAGGATAAATTGT	GCCAGTTCTCTCAGGATGGA	173
Axin-2	NM_015732	CAAGCCTGGCTCCAGAAGAT	TCTCCACAGAAAAAGTAGGTGACA	100

Human primer sequences used in quantitative real time PCR

Gene	Ref. Seq. No.	Forward primer	Reverse primer	Product Size(bp)
CCND1	NM_053056	ACAGATCATCCGCAAACACG	AGGTTCAGGCCTTGCACTG	126
c-MYC	NM_002467	GGTAGTGGAAAACCAGCAG	CAGCAGCTCGAATTTCTTC	199
TFF1	NM_003225.2	GGTCCTGGTGTCCATGCT	ACAGCAGCCCTTATTTGCAC	136
AXIN-2	NM_004655	AGTCAGCAGAGGGACAGGAA	ACACCTGCCAGTTTCTTTGG	114