

Sox17 and Sox4 Differentially Regulate β -Catenin/T-Cell Factor Activity and Proliferation of Colon Carcinoma Cells[∇]

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Received 21 November 2006/Returned for modification 22 December 2006/Accepted 6 September 2007

The canonical Wnt pathway is necessary for gut epithelial cell proliferation, and aberrant activation of this pathway causes intestinal neoplasia. We report a novel mechanism by which the Sox family of transcription factors regulate the canonical Wnt signaling pathway. We found that some Sox proteins antagonize while others enhance β -catenin/T-cell factor (TCF) activity. *Sox17*, which is expressed in the normal gut epithelium but exhibits reduced expression in intestinal neoplasia, is antagonistic to Wnt signaling. When overexpressed in SW480 colon carcinoma cells, *Sox17* represses β -catenin/TCF activity in a dose-dependent manner and inhibits proliferation. *Sox17* and *Sox4* are expressed in mutually exclusive domains in normal and neoplastic gut tissues, and gain- and loss-of-function studies demonstrate that *Sox4* enhances β -catenin/TCF activity and the proliferation of SW480 cells. In addition to binding β -catenin, both Sox17 and Sox4 physically interact with TCF/lymphoid enhancer factor (LEF) family members via their respective high-mobility-group box domains. Results from gain- and loss-of-function experiments suggest that the interaction of Sox proteins with β -catenin and TCF/LEF proteins regulates the stability of β -catenin and TCF/LEF. In particular, Sox17 promotes the degradation of both β -catenin and TCF proteins via a noncanonical, glycogen synthase kinase 3 β -independent mechanism that can be blocked by proteasome inhibitors. In contrast, Sox4 may function to stabilize β -catenin protein. These findings indicate that Sox proteins can act as both antagonists and agonists of β -catenin/TCF activity, and this mechanism may regulate Wnt signaling responses in many developmental and disease contexts.

The canonical Wnt signaling pathway is involved in many biological processes, ranging from embryonic development to stem cell maintenance in adult tissues, while the dysregulation of Wnt signaling is implicated in human tumorigenesis. The key effector of the canonical Wnt pathway is β -catenin, which forms complexes with T-cell factor (TCF)/lymphoid enhancer factor (LEF) high-mobility-group (HMG) box transcription factors to stimulate the transcription of Wnt-responsive genes (7). While numerous studies have shown that β -catenin is regulated at many levels, less is known about the regulation of TCF/LEF transcription factors.

In the absence of a Wnt signal, levels of cytosolic β -catenin are kept low via the interaction of β -catenin with a protein complex including glycogen synthase kinase 3 β (GSK3 β), adenomatous polyposis coli (APC), and Axin. The phosphorylation of β -catenin by the kinase GSK3 β allows β -catenin to be ubiquitinated and targeted for degradation by the proteasome (1). The binding of a canonical Wnt ligand to the frizzled-lipoprotein receptor-related protein 5/6 receptor complex results in the repression of GSK3 β and the stabilization of β -catenin. Stabilized β -catenin accumulates in the nucleus, where it acts as a cofactor with the HMG box family of TCF/LEF transcription factors to regulate the expression of Wnt

target genes, such as *cyclin D1* and *Cdx-1* (17, 22). Although the formation of a TCF- β -catenin complex is required for the activation of all Wnt target genes (36), Wnt signaling is involved in a wide array of biological processes, including cell proliferation, cellular transformation (14), and embryonic development (24), demonstrating that the output of this pathway is highly influenced by the cellular context.

Given that aberrant activation of the canonical Wnt pathway can lead to unrestricted cell division and tumor formation (12, 26, 28, 31, 40), it is not surprising that this pathway is antagonized by several different mechanisms. For example, several extracellular antagonists that inhibit ligand-receptor interactions have been described previously, including Dickkopf (Dkk), Cerberus, and the secreted frizzled-related proteins (10, 21, 34, 35). In many instances, Wnt signaling is kept in check by a negative-feedback loop in which β -catenin/TCF activity induces the transcription of its own negative regulators, *Axin* and *Dkk1* (4, 20, 39). Finally, in the absence of activated β -catenin, TCF/LEF transcription factors keep Wnt target genes off via their interaction with members of the Grouch family of transcriptional repressors (4, 20, 39).

Structurally related to TCF/LEFs, several members of the Sox family of HMG box transcription factors, including Sox17, Sox3, Sox7, and Sox9, have also been implicated in repressing β -catenin activity by a mechanism that is not well understood (2, 48, 54, 55). In addition to acting as an antagonist, Sox17 cooperates with β -catenin to activate the transcription of its endoderm target genes in *Xenopus laevis* (44). These findings suggest that, dependent on the context, Sox proteins can utilize β -catenin as a cofactor or can antagonize β -catenin/TCF func-

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[∇] Published ahead of print on 17 September 2007.

tion. While the mechanism by which Sox proteins antagonize Wnt signaling is unknown, one possibility is that they compete with TCFs for binding to β -catenin (55).

Here, we report that Sox proteins expressed in normal and neoplastic gut epithelia can modulate canonical Wnt signaling and the proliferation of gastrointestinal tumor cells. While several Sox factors, including Sox17, Sox2, and Sox9, are antagonists of canonical Wnt signaling, others, such as Sox4 and Sox5, promote Wnt signaling activity. Gain- and loss-of-function analyses demonstrate that the Wnt antagonist Sox17 represses colon carcinoma cell proliferation while the agonist Sox4 promotes proliferation. In contrast to a proposed model in which Sox17 protein antagonizes Wnt signaling by competing with TCFs for β -catenin binding, we found that Sox17 interacts with both TCF/LEF and β -catenin and that Sox17 and TCF/LEF proteins interact via their respective HMG domains. Binding experiments suggest that Sox17, TCF, and β -catenin cooperatively interact to form a complex. In contrast, Sox4 can bind to either TCF/LEF or β -catenin alone but does not appear to cooperatively bind both proteins. Structure-function analyses indicate that Sox17 must bind directly to both β -catenin and TCF in order to antagonize Wnt signaling and that Sox17 DNA binding activity is not required. Lastly, functional studies show that Sox17 promotes the degradation of TCF/LEF and β -catenin proteins via a GSK3 β -independent mechanism that can be blocked by proteasome inhibitors. In contrast, Sox4 may function to stabilize β -catenin protein. Together, these findings suggest that Sox transcription factors act in a novel pathway to modulate the stability of β -catenin/TCF proteins and regulate the proliferation of colon carcinoma cells. These results have important implications for how Sox proteins regulate the transcriptional output of Wnt signaling in many developmental and pathological processes.

MATERIALS AND METHODS

DNA constructs. The following constructs were previously used and described: pGEX-3X carrying genes encoding a glutathione S-transferase (GST)- β -catenin fusion protein; pcDNA6 carrying genes encoding human wild-type β -catenin and β -catenin with the S37A mutation [β -catenin(S37A)], human *TCF4*, mouse *Lef1*, mouse *Sox10*, *Xenopus Sox17*, and *Sox3*; and pcDNA1 carrying *Xenopus Tcf3* (55). TCF-luciferase reporter plasmids (TOP-flash and FOP-flash) (17) were generously provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). We subcloned the *TCF4* cDNA into pGEX-3X to generate the TCF4-GST fusion protein. Full-length mouse *Sox17* was amplified from embryonic day 7.5 mouse embryonic day 12.5 mouse cDNA with *Pfx* polymerase (Invitrogen), cloned into pcIG (11), and sequenced. To generate V5-tagged Sox17, the cDNA was subcloned into pcDNA6, and to produce the GST fusion protein, *Sox17* was subcloned into pGEX-3X. Mouse *Sox2*, *Sox4*, *Sox6*, *Sox7*, *Sox9*, and *Sox18* were amplified from whole embryonic day 12.5 mouse cDNA with *Pfx* polymerase (Invitrogen), cloned into pcDNA6, and sequenced. *Xenopus Sox5* was generated by PCR, and we obtained chick *Sox11* from Hugh Woodland (University of Warwick, United Kingdom). Deletions in mouse *Sox17* were generated using PCR, and point mutations in *Sox17* were generated using the Gene Tailor site-directed mutagenesis kit from Invitrogen (Carlsbad, CA). To generate the Sox17 reporter plasmid pH4X8-Luci, annealed oligonucleotides containing four additional copies of a *Sox17* DNA binding site (15) were inserted into the XhoI site of the pH4X4-Luci reporter plasmid.

Cell culture, treatments, cell transfection with plasmids and siRNAs, and luciferase assays. COS, 293T, and SW480 cells were obtained from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (HyClone). Cells were plated into 12-well plates 24 h prior to transfection and were transfected with the TOP-flash plasmid (100 to 150 ng), a β -galactosidase or renilla plasmid (50 ng) as a transfection control, the gene encoding β -catenin(S37A) (100 ng), and the Sox plasmids listed above (100 to 800 ng) by using Fugene 6 (Roche) or

Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The total amount of DNA was kept constant by adding empty-vector DNA. Cell lysates were prepared 24 to 48 h posttransfection, and luciferase activity was measured according to the luciferase assay system (Promega). Transfection efficiency was normalized using either β -galactosidase activity or renilla luciferase activity from the control plasmid. Experiments were done at least three times, and data from a representative example are shown. For small interfering RNA (siRNA) experiments, double-stranded *Sox4* and *Sox17* siRNAs and siRNA negative controls (Stealth RNA interference negative control med GC, catalog no. 12935-300, and BLOCK-iT Alexa Fluor red fluorescent control siRNA, catalog no. 147500-00) were obtained from Invitrogen. SW480 cells were transfected using Lipofectamine 2000 (Invitrogen) in serum and antibiotic-free OptiMEM medium with a 1:1:1 mixture of *Sox4* or *Sox17* siRNAs at doses of 20, 50, and 100 nM. For all experiments, the siRNA control was used at the equivalent concentrations. The cells were harvested at 48 and 72 h after transfection. The transfection efficiency as measured using the BLOCK-iT Alexa Fluor red fluorescent control siRNA was estimated at 80 to 90% after 12 h (data not shown). A combination of three double-stranded *Sox17* siRNA sequences was used (only the sense strand sequences are shown): no. 10620312/116668 B07, GCACGGAAUUUGAACAGUAUCUGCA; no. 10620312/116668 B09, UGCACUUCGUGUGCAGCCUGAGAU; and no. 10620312/116668 B11, CGCGGUAUAAUACUGCAACUAUCCU. A combination of three double-stranded *Sox4* siRNA sequences was used (only the sense strand sequences are shown): no. 10620312/116668 C01, GCAAACGCGUGAAGCUGCUCUCAAAGA; no. 10620312/116668 C03, UCAAAGACAGCGACAAGAUCUUUU; and no. 10620312/116668 C05, GCGACAAGAUCUUUCAUUCGAGA. For protein stability and degradation experiments, leupeptin, lithium chloride, and chloroquine were purchased from Sigma. Epoxomicin was purchased from Calbiochem, dissolved in dimethyl sulfoxide (1,000 \times stock), and diluted to 1 \times in culture medium. The final concentrations were as follows: epoxomicin, 0.1 μ M; leupeptin, 100 μ M; chloroquine, 25 μ M; and LiCl, 40 mM.

For SW480 cell proliferation assays, we transfected cells by using a Lipofectamine-based Nucleofector technology kit (Amaxa, Gaithersburg, MD). For colony assays, SW480 cells were transfected with pmx-GFP, pBABE-PURO, and either *Sox17*-pcDNA6 or *Sox4*-pcDNA6. Cells were plated at three different dilutions and allowed to recover overnight. Cells were then cultured in three different concentrations of puromycin (0.7, 1.5, and 3 μ g/ml), and selection was continued for 5 days, at which time puromycin-resistant, green fluorescent protein (GFP)-positive colonies were counted. Only colonies containing ≥ 10 GFP-positive cells were counted. To monitor the total cell number, cells were trypsinized and GFP-positive cells were counted using a hemocytometer. Three wells of cells were counted for each time point and condition tested.

Immunohistochemistry and microscopy. Paraffin sections (7 μ m thick) were collected onto glass slides (Superfrost Plus; Fisher), and the slides were heated overnight at 60 $^{\circ}$ C, dewaxed, and equilibrated in phosphate-buffered saline (PBS). We performed antigen retrieval by heating slides in a steam chamber in 10 mM citrate buffer, pH 6, for 20 min. Anti-Sox4 (Sigma; 1:500) and anti-Sox17 (1:500) (33) immunohistochemistry analysis was performed using tissue sections from wild-type CD1 mice or *APC^{mini/+}* mice, which carry a mutation in the *APC* gene that predisposes them to intestinal adenomas. After deparaffinization, rehydration, and antigen retrieval, tissues were blocked for 1 h using 5% normal goat serum in PBS-0.5% Triton X. Sections were incubated overnight at 4 $^{\circ}$ C with antibodies diluted in blocking reagent. On the second day, slides were washed and incubated with goat anti-rabbit biotinylated secondary antibody (Jackson ImmunoResearch) diluted in blocking reagent (1:500) for 1 h at room temperature. Slides were washed and incubated in ABC reagent (Vector Labs) for 30 min at room temperature. Instead of detecting the antigen with the traditional 3,3'-diaminobenzidine chromogen included in the ABC kit, fluorescence detection of antigens was carried out using the tyramide substrate amplification kit as described by the manufacturer (Molecular Probes). Tyramide-488 was used to produce fluorescent green immunostaining. Sections were counterstained with the red nuclear dye PoPro3 (Molecular Probes). A Leica DM IRB inverted microscope was used to capture digital images of GFP-expressing cells of immunofluorescently labeled sections.

SW480 cells were fixed for 10 min in 4% paraformaldehyde and washed extensively in 1 \times PBS. They were then blocked using 5% normal goat serum in PBS-0.5% Triton X. Cells were incubated with primary antibodies overnight at 4 $^{\circ}$ C. On the second day, cells were washed and incubated with the appropriate secondary antibodies directly conjugated to fluorophores.

Western blot analysis. Cells were transfected with *Sox17* (50 to 400 ng), β -catenin gene (100 ng), *TCF4* (100 ng), and/or *Lef1* (100 ng) constructs. Cell lysates were prepared 24 to 48 h posttransfection as described previously (55) and analyzed by standard Western immunoblotting procedures with the follow-

ing antibodies: anti-V5-horseradish peroxidase (anti-V5-HRP; 1:4,000 [Invitrogen]), rabbit anti- β -catenin (1:1,000; Santa Cruz Biotechnology), mouse anti-activated β -catenin (1:1,000; Upstate Biotechnology), mouse antihemagglutinin (anti-HA) antibody (1:1,000; Roche), mouse anti-TCF4 (1:500; Upstate Biotechnology), rabbit anti-Sox4 (1:500; Sigma), mouse antitubulin (1:1,000; Sigma), guinea pig anti-Sox17 (1:1,000), affinity-purified rabbit anti-mouse/human Sox17 (1:5,000), goat anti-rabbit HRP (1:10,000; Jackson ImmunoResearch), goat anti-mouse HRP (1:10,000; Jackson ImmunoResearch), and rabbit anti-XTCF3 (1:3,000) (54).

In vitro protein binding assays. GST-mouse Sox17, GST-TCF4, and GST- β -catenin were expressed in bacteria and purified using glutathione-coupled agarose resin. His-tagged *Xenopus* Sox17 β and His-tagged *Xenopus* Sox17 β consisting of amino acids 1 to 150 (see Fig. 4C) were expressed in bacteria and purified on Ni-agarose resin. β -catenin, TCF4, and LEF1 genes (in pcDNA6) and the *Xenopus* TCF3 gene (in pcDNA1) were transcribed with T7 polymerase, and the proteins were produced in TNT translation reactions (Promega). In vitro-translated proteins were incubated with GST or His fusion proteins coupled to agarose resin as described previously (55), and bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-V5-HRP or anti-HA-HRP antibody or by 35 S labeling autoradiography.

Preparation of nuclear extracts and coimmunoprecipitations. Approximately 10^9 SW480 cells were homogenized in 3.5 ml of cell lysis buffer (250 mM sucrose, 30 mM KCl, 6 mM MgCl₂, 20 mM HEPES [pH 7.9], 0.5 mM EDTA, 0.2 mM NaF, 2 mM Na₃VO₄, and 0.1% Triton X-100 with protease inhibitors). Nuclei were liberated from the cells by 15 gentle strokes of a tight-fitting pestle in a Dounce homogenizer and centrifuged at $500 \times g$ for 10 min at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in nuclear lysis buffer (50 mM Tris [pH 8], 150 mM NaCl, 0.1% NP-40, and 0.5% Triton X-100 with protease inhibitors) and sonicated to lyse the nuclei and thoroughly shear the genomic DNA. The resulting extract was centrifuged at $12,000 \times g$ for 15 min at 4°C, and the supernatant was used as the nuclear fraction. In all experiments, we verified the enrichment of the nuclear fraction with nuclear proteins by using an antihistone antibody (data not shown).

For coimmunoprecipitation, equal amounts of nuclear extract were precleared with 1 μ g of rabbit preimmune serum and protein A-agarose for 1 h at 4°C. The extracts were then incubated for 4 h at 4°C with protein A-agarose and either 1 μ g of a rabbit anti-Sox17 peptide antibody (44) or 1 μ g of anti-HA antibody as a negative control. To demonstrate specific binding with anti-Sox17 antibody, 2 μ g of a competing Sox17 peptide was included in the incubations. Immunoprecipitates were washed four times in nuclear lysis buffer, resolved by SDS-PAGE, and subjected to anti- β -catenin immunoblotting.

***Xenopus* embryo culture and manipulations.** Two-cell-stage *Xenopus* embryos were microinjected with Sox17 α 1, Sox17 α 2, and Sox17 β antisense morpholino oligonucleotides (MO; 20 ng of each) as previously described (6), 20 ng of a β -catenin MO (13), or a synthetic mRNA encoding *Xenopus* Sox17 β (10 pg). At stage 11 (according to a normal table of *X. laevis* development [29]), embryos were harvested and pooled in groups of three for each condition.

RT-PCR analysis. Total RNA was extracted from embryonic tissue or cells by using the TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Roche). cDNA was synthesized with oligo(dT) primers. Real-time reverse transcription-PCR (RT-PCR) and semiquantitative analysis were performed using the Opticon machine (MJ Research-Bio-Rad). The primers used in this study were as follows: human *Sox17* forward, 5'-GAC GAC CAG AGC CAG ACC-3', and human *Sox17* reverse, 5'-CGC CTC GCC CTT CAC C-3' (5); human *Sox4* forward, 5'-TTC AGC AAC CAG CAT TC-3', and human *Sox4* reverse, 5'-TCC CTC TCT CTC GCT TC-3' (37); human *cyclin D1* forward, 5'-AAT GAC CCC GCA CGA TT-3', and human *cyclin D1* reverse, 5'-GCA CAA GAC GCA ACG AAG G-3' (49); human *c-myc* forward, 5'-GGT GAA GTA CAT GAA CTC AGG GC-3', and human *c-myc* reverse, 5'-GGC TTT GAA TCT GCT GGA TTG-3' (43); human β -tubulin gene forward, 5'-GAT ACC TCA CCG TGG CTG CT-3', and human β -tubulin gene reverse, 5'-AGA GGA AAG GGG CAG TTG AGT-3'; *Xnr3* forward, 5'-AAA TCC ATG TGA GCA CCG TTC-3', and *Xnr3* reverse, 5'-GCA TTC TCT GTC TCA TTC TGT G-3'; *Hnf1 β* forward, 5'-GCA TAT GGC ACA GCA GCC ATT-3', and *Hnf1 β* reverse, 5'-CAC CAT GCT TGC AAA GGA CAC-3'; *Edd* forward, 5'-AGA ACG GAG ACT CAG ATC AAT-3', and *Edd* reverse, 5'-TTA CTC ATT GCT CCC ACC ATC-3'; *Plakoglobin* forward, 5'-GCT CGC TGT ACA ACC AGC ATT C-3', and *Plakoglobin* reverse, 5'-GTA GTT CCT CAT GAT CTG AAC C-3'; and ornithine decarboxylase (ODC) gene forward, 5'-GCC ATT GTG AAG ACT CTC TCC AAT C-3', and ODC gene reverse, 5'-TTC GGG TGA TTC CTT GCC AC-3'. SYBR green dye (QIAGEN) was included in the PCR mix. For each experiment and primer pair, a dilution series of cDNA was included in order to

generate a standard curve from which the amount of product in each sample was estimated at the log-linear amplification phase. These values were normalized to the level of expression of the ODC gene (53) or the β -tubulin gene, and the data are presented as a ratio of the expression of each gene to ODC or β -tubulin gene expression. In each experiment, samples with water alone or controls without reverse transcriptase were included and failed to give specific products. Each experiment was repeated at least three times, and results from a representative example are shown.

RESULTS

Sox factors can either repress or enhance β -catenin/TCF activity and are expressed in normal and neoplastic gut tissues. We chose the intestinal epithelium as a model to study Sox17-Wnt interactions because of the well-established role of the Wnt pathway in this tissue and because it has recently been shown that the mRNA levels corresponding to several Sox transcription factors change during cancer progression in the gut (8, 32, 38). Given this link between Sox factors and Wnt signaling in gut tumorigenesis, we analyzed 11 different Sox factors for their ability to affect the canonical Wnt signaling pathway (Fig. 1A) and further analyzed the expression of two of them in normal and neoplastic gut tissues (Fig. 1B and C). In transfection experiments, we coexpressed individual Sox factors with activated, stabilized β -catenin [β -catenin(S37A)] (Fig. 1) and measured Wnt signaling activity by using the TOP-flash reporter plasmid. The activities of Sox factors fell into three categories. Sox2, Sox3, Sox9, Sox10, and Sox17 were strong antagonists of the canonical Wnt signaling pathway. Surprisingly, some Sox factors, including Sox4, Sox5, and Sox11, enhanced β -catenin activity. Other Sox factors, such as Sox6, had no impact on Wnt signaling activity. All of the expressed Sox proteins were detectable by Western blot analysis of transfected cell lysates, and none significantly altered TOP-flash activity in cells not cotransfected with β -catenin (data not shown).

It has not previously been demonstrated that different Sox proteins act as antagonists or agonists of the canonical Wnt pathway. Given the role of this pathway in intestinal neoplasia, we reinvestigated whether the expression of Sox family members with different activities correlated with tumor progression by using published microarray data from *APC^{min/+}* intestinal tumors (8, 32, 38). The microarray data indicate that Sox4 and Sox17 are two of the most differentially expressed Sox factors in *APC^{min/+}* tumor tissue versus normal gut samples (Fig. 1B). *Sox4* mRNA levels were significantly elevated in all eight tumor samples, and *Sox17* mRNA levels were reduced in seven of eight tumor samples relative to those in the normal gut samples. *Sox4* clustered with other Wnt pathway genes that showed elevated levels of expression in *APC^{min/+}* tumor tissue, including *cyclin D1* (*CCND1*), *Lef1*, and *TCF4*, consistent with the activated Wnt activity in these tumors. In contrast, *Sox17* expression levels were down regulated in most tumor samples, and *Sox17* clustered with other known Wnt antagonists, such as *Dkk1*. Immunohistochemical analysis of Sox4 and Sox17 in normal and *APC^{min/+}* intestinal tissues revealed that both factors are expressed in the gut epithelium. In normal samples of duodenum, ileum, and jejunum tissue, Sox4 protein appeared to be more restricted to the crypts whereas Sox17 protein was present in both the crypts and villi (Fig. 1C, left panels). Moreover, in *APC^{min/+}* tumors, Sox17 and Sox4 proteins appeared

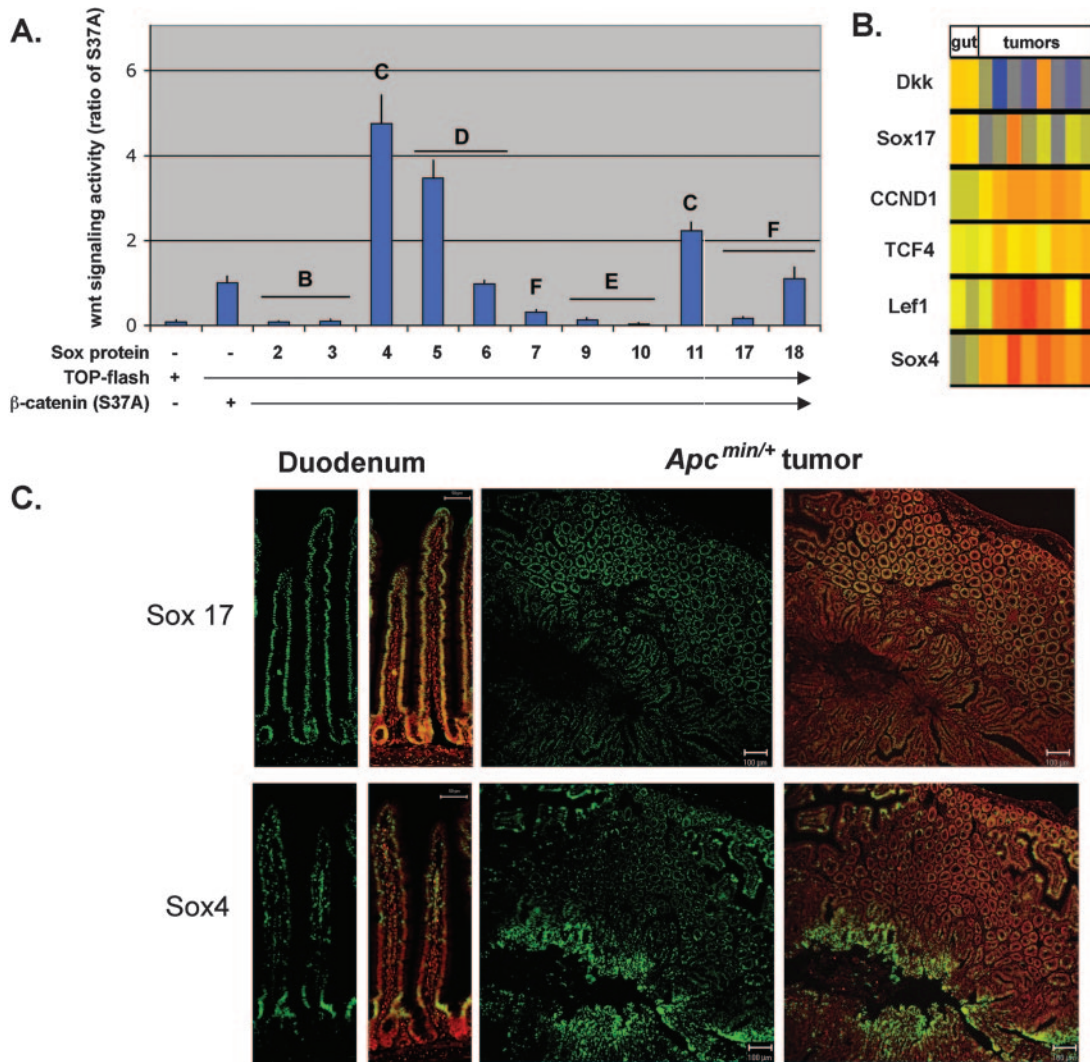


FIG. 1. Different Sox factors are antagonists or agonists of the canonical Wnt pathway and are expressed in the normal and neoplastic gut. (A) Effect of different Sox family members on Wnt signaling activity. 293T cells (shown) and COS cells (not shown) were transfected with *Sox2*, *Sox3*, *Sox4*, *Sox5*, *Sox6*, *Sox7*, *Sox9*, *Sox10*, *Sox11*, *Sox17*, and *Sox18* together with a TCF-luciferase reporter plasmid (TOP-flash) and an expression plasmid encoding a stabilized form of β -catenin (carrying the S37A mutation). Transfection with β -catenin(S37A) alone resulted in significant activation of the reporter plasmid. *Sox2*, *Sox3*, *Sox7*, *Sox9*, *Sox10*, and *Sox17* repressed the β -catenin-mediated activation of TOP-flash, whereas *Sox4*, *Sox5*, and *Sox11* enhanced β -catenin/Wnt activity. Data are shown as ratios of the activity in cotransfected cells to the activity in cells transfected with β -catenin(S37A) alone. Transfection with Sox factors without β -catenin(S37A) did not result in significant TOP-flash activity (data not shown). Letters above the bars denote the Sox subfamily. Error bars indicate standard errors of the means for replicate samples from two separate experiments. -, absent; +, present. (B) Analysis of published microarray data (8, 32, 38). The expression levels of the Wnt antagonists *Dkk* and *Sox17* were reduced in seven of eight tumor samples relative to those in the normal gut samples. The levels of expression of several pro-Wnt signaling genes, *Sox4*, *TCF4*, and *LEF1*, and the Wnt target gene *cyclin D1* (*CCND1*) were elevated in tumor samples relative to those in normal gut samples. The color scale for normalized transcript levels is as follows: red, high; yellow, moderate; and blue, low. (C) Sox4 and Sox17 proteins are expressed in the normal gut epithelium and in *APC^{min/+}* tumors. In the small intestine, Sox17 and Sox4 proteins (green) are expressed in the nuclei of the epithelial cells (a duodenum sample is shown). The Sox4 expression domain is located predominantly in the crypt, whereas Sox17 protein is found throughout the crypt and villus. The right panels show adjacent sections from *APC^{min/+}* tumors in which Sox17 and Sox4 proteins appear to be in mutually exclusive domains. Nuclei are stained with the red nuclear dye PoPro3. Scale bars in duodenum panels, 50 μ m.

to be found in mutually exclusive domains when we stained adjacent tissue sections.

Sox17 and Sox4 have opposite effects on Wnt activity and the proliferation of colon carcinoma cells. The fact that Sox17 represses Wnt signaling and is down regulated in Wnt-dependent tumors while Sox4 enhances Wnt signaling and is expressed at high levels in *APC^{min/+}* tumors suggests that these transcription factors may play opposite functional roles in gut

tumorigenesis. As a rapid way to investigate this idea, we examined the impact of *Sox17* or *Sox4* overexpression and loss of function on β -catenin/TCF activity and on the proliferation of SW480 cells. SW480 cells are transformed colon carcinoma cells that contain a mutant *APC* gene that results in high endogenous β -catenin/TCF activity as measured by a TOP-flash reporter assay (28, 30). Moreover, immunohistochemical analysis revealed endogenous Sox17 and Sox4 protein in

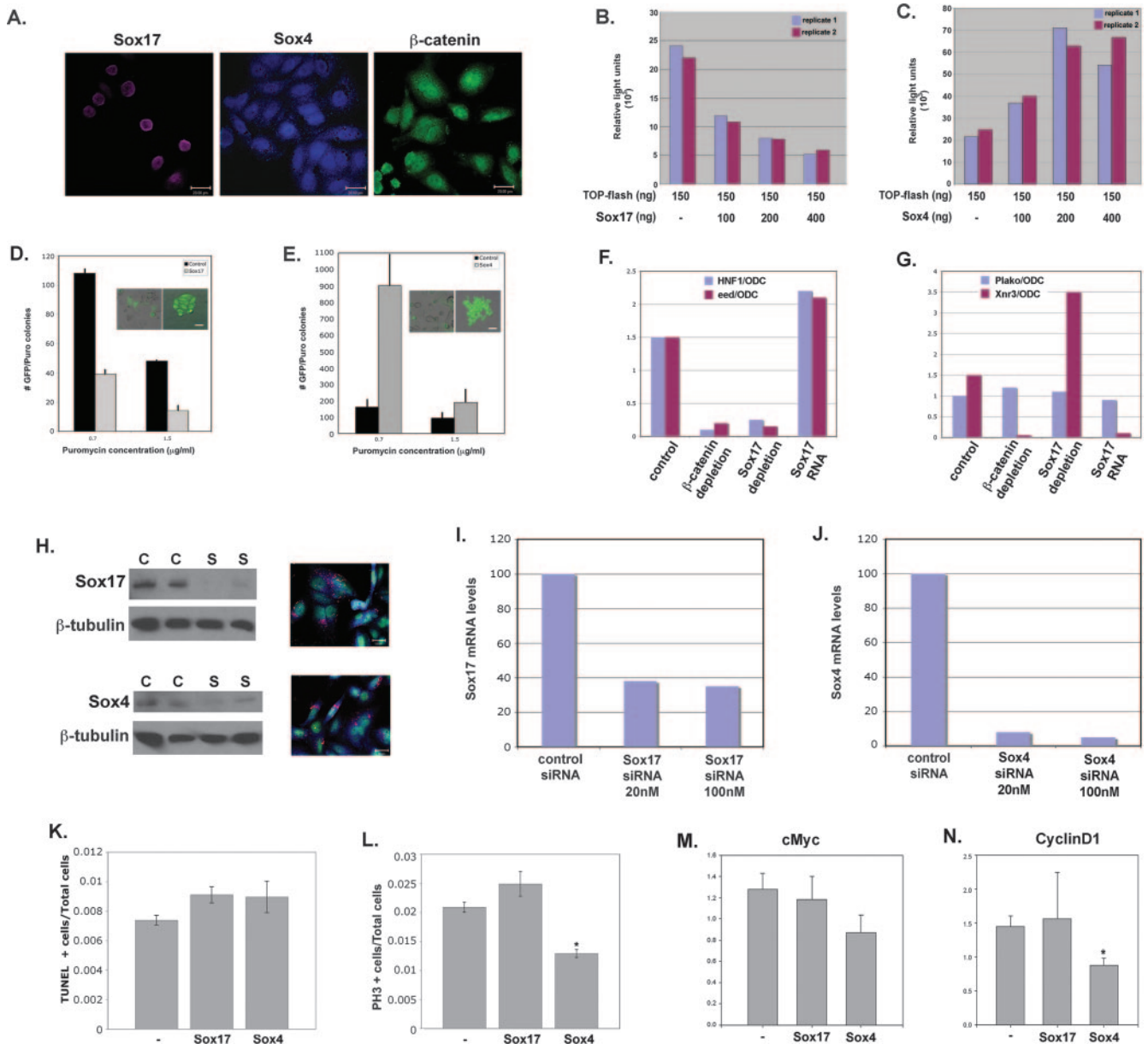


FIG. 2. Sox17 and Sox4 have opposite effects on Wnt activity and proliferation of SW480 colon carcinoma cells. (A) Endogenous Sox17, Sox4, and β -catenin proteins are present in SW480 cells. (B) Sox17 represses endogenous Wnt signaling activity in colon carcinoma cells. Transfection with the TOP-flash reporter alone (lane 1) demonstrates that SW480 cells have high levels of endogenous Wnt/ β -catenin/TCF activity. Cells cotransfected with increasing concentrations of a *Sox17* expression plasmid (100 to 400 ng) showed dose-dependent reduction in endogenous Wnt activity (lanes 2 to 4). TOP-flash activity is expressed in relative light units that were normalized to the transfection efficiency by using a renilla plasmid. The results shown are representative of those from at least three independent experiments. -, absent. (C) *Sox4* enhances endogenous Wnt signaling activity in colon carcinoma cells. SW480 cells that were cotransfected with increasing levels of a *Sox4* expression plasmid exhibited a dose-dependent increase in TOP-flash activity. Results for replicate samples are shown, and experiments were done three times. -, absent. (D) *Sox17* represses the proliferation of SW480 cells. The formation of puromycin-resistant SW480 cell colonies was used to measure the effect of *Sox17* on cell proliferation. SW480 cells were cotransfected with the pBABE-puromycin resistance plasmid, the pmax-GFP plasmid, and either an empty vector or a *Sox17* expression vector and grown in puromycin-containing medium for 5 days. Insets show GFP-positive cells before (left) and after (right) puromycin selection. Only colonies that had ≥ 10 GFP-positive cells were counted. Error bars indicate standard errors of the means of results for three replicate samples. Puro, puromycin. (E) *Sox4* promotes the proliferation of SW480 cells. SW480 cells that expressed *Sox4* formed more colonies than vector-alone controls. Insets show GFP-positive cells before (left) and after (right) puromycin selection. (F and G) The effects of the loss and gain of function of *Sox17* on Wnt and *Sox17* target gene expression in *Xenopus* embryos. MO (20 ng each) previously used to deplete *Xenopus* embryos of *Sox17* (6) or β -catenin (13) were injected into two-cell embryos, and stage 11 embryos were harvested and pooled in groups of three for each condition. Real-time RT-PCR analysis was used to analyze the relative levels of expression of the Wnt target genes *Xnr3* and *Siamois* (data not shown), the *Sox17* target gene *Hnf1 β* , or the ubiquitously expressed gene *Plakoglobin* (*Plako*). All gene expression levels are shown as a ratio of the expression of the indicated gene to that of the ubiquitously expressed ODC gene (44). These results demonstrate that *Sox17* is required to restrict the activity of Wnt/ β -catenin/TCF in *Xenopus* embryos. (H) siRNA-mediated knockdown of *Sox17* and *Sox4* proteins in SW480 cells. SW480 cells were transfected with 50 nM scrambled control siRNA (C) or the Sox siRNA (S). The left panels show Western blot

SW480 cells (Fig. 2A). Sox17 protein was restricted to the nucleus, whereas Sox4 was present in both the nucleus and cytoplasm. For gain-of-function analysis, we transfected SW480 cells with increasing levels of *Sox17* or *Sox4* plasmids. *Sox17* caused a dose-dependent decrease in endogenous β -catenin/TCF activity, whereas *Sox4* caused a dose-dependent increase in β -catenin/TCF activity (Fig. 2B and C). We investigated whether the Sox-mediated regulation of Wnt/ β -catenin/TCF activity correlated with the proliferation of SW480 cells by using a colony formation assay (51). Consistent with its ability to repress Wnt activity, *Sox17* expression caused a significant reduction in the number of SW480 cell colonies (Fig. 2D) but had no impact on apoptosis (data not shown). In addition, we observed a significant decrease in the absolute number of transfected SW480 cells that expressed *Sox17* over the course of 4 days (data not shown). In contrast, *Sox4* had the opposite effect, causing an increase both in colony numbers and in the average number of cells per colony (Fig. 2E). Interestingly, the cells transfected with *Sox17* tended to be flatter and more adherent than cells transfected with *Sox4*, which were often rounder, smaller, and less adherent (Fig. 2D and E insets). These phenotypes are consistent with the possibility that *Sox17* inhibits cellular transformation whereas *Sox4* promotes it.

To investigate the functions of endogenous *Sox17* and *Sox4*, we performed siRNA knockdowns of each factor and quantitatively measured the effects on cell proliferation, apoptosis, and endogenous Wnt target genes (Fig. 2H to N). We transfected SW480 cells with an equimolar mixture of three siRNAs for either *Sox17* or *Sox4* and found that the transfection efficiency was >80%, as determined by the presence of a fluorescently labeled control siRNA (Fig. 2H, right panels). We achieved >60% reduction in *Sox17* mRNA levels (Fig. 2I) and >80% reduction in protein levels (Fig. 2H). *Sox4* mRNA levels were reduced by >90%, whereas Sox4 protein levels were reduced by ~70%. These data suggest that Sox4 protein may be more stable than Sox17. *Sox4* knockdown resulted in a significant reduction in SW480 cell proliferation and an accompanying reduction in the levels of Wnt target genes *c-myc* and *cyclin D1* (Fig. 2K, M, and N), whereas apoptosis was not significantly affected (Fig. 2L). The efficacy and specificity of *Sox4* siRNA knockdown were confirmed by the analysis of two *Sox4* target genes, *Puma* and *Tle-1* (22a), both of which had significantly reduced levels of expression in cells transfected with *Sox4* siRNA (data not shown). *Sox17* knockdown caused a modest increase in proliferation and *cyclin D1* mRNA levels. The fact that *Sox17* does not have more of an effect in colon carcinoma cells is likely due to the presence of other Sox

factors, such as *Sox9*, that are functionally redundant in relation to *Sox17*. For example, *Sox9* is also a Wnt antagonist (Fig. 1) that is expressed in the gut and represses gut cell proliferation (2, 27).

Sox17 antagonizes endogenous TCF/ β -catenin activity in embryos. Sox17 was previously shown to antagonize canonical Wnt signaling through an unidentified mechanism when overexpressed in *Xenopus* embryos (44, 55). To investigate whether endogenous Sox17 may perform a similar function in vivo, we depleted *Xenopus* embryos of either Sox17 or β -catenin by the injection of antisense MO and examined the expression of direct TCF/ β -catenin target genes (Fig. 2F and G) (6, 13). As predicted, the depletion of *Xenopus* embryos of Sox17 resulted in the up regulation of the Wnt target gene *Xnr3*, whereas the overexpression of Sox17 repressed *Xnr3* expression (Fig. 2G) (55). These data further indicate that one normal function of Sox17 is to restrict the activity of β -catenin/TCF. As a control, we demonstrated that the knockdown of β -catenin resulted in the loss of expression of both β -catenin/TCF and β -catenin/Sox17 target genes (*Xnr3* and *Hnf1 β* , respectively) (Fig. 2F and G) (13, 44). Taken together, these findings suggest that Sox proteins normally function to either positively or negatively modulate Wnt/ β -catenin/TCF activity in a variety of cellular contexts.

Sox proteins interact with β -catenin and TCF/LEF proteins. Previous studies demonstrated that *Xenopus* Sox17 binds to β -catenin in vitro (55), suggesting that Sox17-mediated repression in colon carcinoma cells may involve direct interaction with β -catenin. To test this possibility, we performed a coimmunoprecipitation assay using a polyclonal antibody that recognizes mouse and human Sox17 and found that β -catenin can be coimmunoprecipitated with Sox17 from SW480 nuclear extracts (Fig. 3A). This interaction was blocked with a Sox17 peptide used to generate the antibody but not with a control peptide generated from a nonconserved region of *Xenopus* Sox17 β . This finding confirms that the interaction between Sox17 and β -catenin is conserved in *Xenopus* and humans and suggests that this interaction may be involved in the Sox17-mediated repression of Wnt signaling.

One possible mechanism by which Sox17 may antagonize β -catenin/TCF activity is by competing with TCF factors for binding to β -catenin. To investigate this idea, we determined whether TCF4- β -catenin interactions and Sox17- β -catenin interactions were mutually exclusive in pull-down experiments using SW480 lysates with Sox17-GST beads. If Sox17 competes with TCF4 for binding to β -catenin, we would predict that Sox17-GST should pull down only β -catenin without TCF4. Surprisingly, we found that Sox17-GST beads pulled down

analyses of Sox17 and Sox4 proteins, and the right panels are confocal images of transfected SW480 cells showing the typical perinuclear localization of the fluorescently labeled control siRNA (red). Nuclei are stained with SYTOX green. (I and J) siRNA knockdown of endogenous *Sox17* and *Sox4* mRNA levels. Transfection with *Sox17* siRNAs resulted in a 70% reduction of endogenous mRNA, whereas *Sox4* siRNAs effected >90% reduction relative to mRNA levels in cells transfected with a control siRNA. (K) Quantitative assessment of apoptosis in SW480 cells as measured by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining 48 h after transfection with *Sox4* and *Sox17* siRNAs. –, control. (L) Quantitative assessment of proliferation of SW480 cells as measured by phosphohistone-H3 (PH3) staining 48 h after transfection with siRNAs. * indicates significance corresponding to a *P* value of 0.001 versus control samples as calculated using a *t* test analysis. –, control. (M and N) Quantitative RT-PCR analysis of the Wnt target genes *c-myc* (I) and *cyclin D1* (J) in SW480 cells 48 h after transfection with *Sox4* and *Sox17* siRNAs. Quantitative PCR analyses of triplicate biological samples were performed. * indicates significance corresponding to a *P* value of 0.02 versus control samples as calculated using a *t* test analysis. –, control. Scale bars in panels A, D, E, and H, 20 μ m.

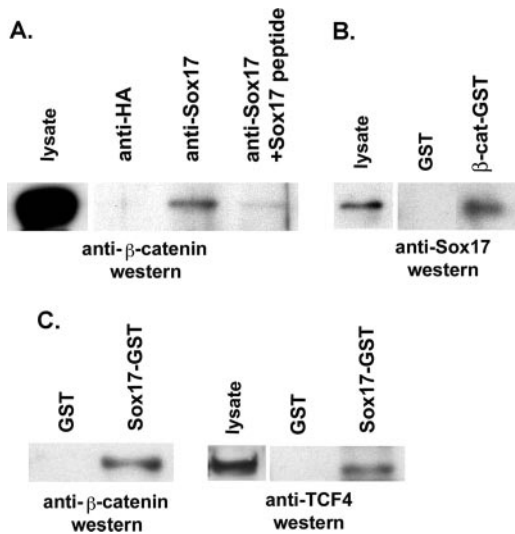


FIG. 3. Endogenous Sox17, β -catenin, and TCF4 proteins interact in SW480 colon carcinoma cell lysates. (A) Sox17 antibodies were used to coimmunoprecipitate interacting proteins from nuclear extracts of SW480 cells. Lysates were immunoprecipitated with a control antibody (lane 1; anti-HA) or with a Sox17 antibody alone (lane 2) or in the presence of a Sox17 peptide or a control peptide (lanes 3 and 4). The Sox17 peptide used to generate the antibody inhibited the immunoprecipitation. (B) β -catenin (β -cat)-GST-linked beads or glutathione beads alone (GST) were incubated with cell lysates from SW480 cells, and precipitates were analyzed by Western blotting with an anti-Sox17 antibody. The Sox17 antibody was raised in rabbits and recognizes both mouse and human Sox17. (C) Sox17-GST beads or GST beads alone (GST) were incubated with cell lysates from SW480 cells, and precipitates were analyzed by Western blotting with anti β -catenin or anti-TCF4 antibodies.

both endogenous β -catenin and TCF4 (Fig. 3C). These findings are the first reported evidence that Sox17 may interact with both TCF and β -catenin proteins and further suggest that Sox17 does not antagonize Wnt signaling by competing with TCF factors for binding to β -catenin.

Sox17 but not Sox4 forms a stabilized protein complex containing both TCF and β -catenin. To further investigate whether Sox and TCF proteins interact, we performed a series of in vitro binding assays with recombinant Sox17, Sox4, β -catenin, and TCF/LEF proteins (Fig. 4 and 5). Using Sox17-GST beads, we determined that Sox17 binds directly to TCF4, TCF3, and LEF1 (Fig. 4A), which indicates that TCF/LEF transcription factors physically interact with Sox proteins.

Given that Sox17 interacts with either TCF or β -catenin protein, we investigated whether these three proteins can simultaneously interact to form a complex. As expected, β -catenin-GST beads pulled down either Sox17 or TCF4 protein (Fig. 4B). When Sox17 and TCF4 proteins were added together, both proteins bound to β -catenin-GST beads but not to control GST beads. Moreover, the interaction between Sox17 and β -catenin appeared to be stabilized in the presence of TCF4 (compare the ratios of input protein to bound protein with and without TCF4). These data suggest that rather than Sox17's and TCF's competing for β -catenin binding, all three proteins interact to form a complex. Consistent with this suggestion, adding increasing amounts of purified Sox17 protein into the binding assay mixture stabilized the interaction of β -catenin and TCF3 in a dose-dependent manner (Fig. 4C). In contrast, a truncated Sox17 protein lacking the C-terminal, β -catenin interaction domain did not stabilize the complex.

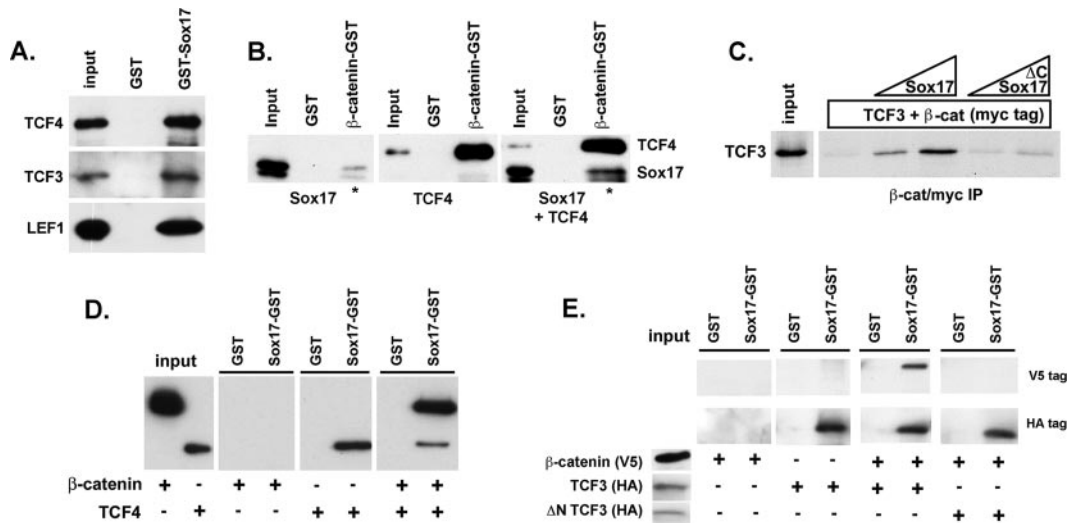


FIG. 4. Sox17 forms a stable, trimeric complex with TCF/LEF and β -catenin proteins. (A) Sox17 interacts with TCF3, TCF4, and LEF proteins. Sox17-GST beads pulled down in vitro-translated, V5 epitope-tagged TCF4, TCF3, and LEF1, and GST beads did not. (B) The interaction between Sox17 and β -catenin is enhanced in the presence of TCF4. Sox17 or TCF4 independently bound to β -catenin-GST beads but not to GST beads alone (panels 1 and 2). The binding of Sox17 to β -catenin-GST beads was enhanced in the presence of TCF4 (compare the ratios of input to bound Sox17 in the presence and absence of TCF4 [lanes marked by *]). β -catenin-interacting proteins were analyzed by Western blotting with an anti-V5 antibody. (C) The interaction between β -catenin (β -cat) and TCF3 is stabilized by Sox17 and requires a Sox17- β -catenin interaction. In vitro-translated, S^{35} -labeled TCF3 protein (input) was incubated with β -catenin (myc tagged) alone or with increasing concentrations of purified His-tagged *Xenopus* Sox17 β or His-tagged *Xenopus* Sox17 consisting of amino acids 1 to 150, which removed the β -catenin interaction domain (Δ C Sox17). β -catenin and its associated proteins were precipitated with an anti-myc antibody, and TCF3 protein was analyzed by SDS-PAGE and autoradiography. IP, immunoprecipitate. (D and E) Under high-stringency conditions, Sox17-GST beads would not efficiently pull down β -catenin. However, in the presence of TCF4 (D) or TCF3 (E), Sox17 was able to pull down β -catenin. This interaction depends on the β -catenin interaction domain of TCF3, which is deleted in the N terminus-lacking TCF3 protein (Δ N TCF3). +, present; -, absent.

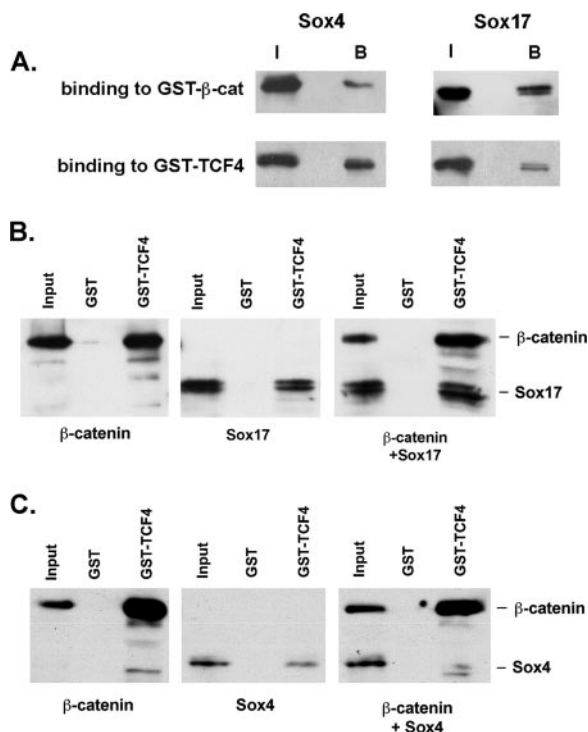


FIG. 5. Sox4 and Sox17 have similar affinities for TCF4 and β -catenin, but Sox4 does not form a stable complex. (A) Sox4 has affinities for TCF4 and β -catenin (β -cat) similar to those of Sox17. The ratios of the input (I) and bound (B) protein fractions are similar for Sox17 and Sox4. (B) Sox17 formed a complex with β -catenin and TCF4. Also, more Sox17 protein was pulled down by TCF4-GST beads in the presence of β -catenin. (C) GST-TCF4 beads were able to pull down either β -catenin or Sox4 but did not efficiently pull down Sox4 in the presence of β -catenin. Moreover, Sox4 binding to TCF4 was somewhat inhibited in the presence of β -catenin, suggesting that Sox4 and β -catenin compete for binding.

To further test the hypothesis that the interaction of all three proteins serves to stabilize a complex, we repeated the *in vitro* binding experiments with higher salt concentrations at which β -catenin would not efficiently bind to GST-Sox17 but at which TCF could (Fig. 4D and E). Under these conditions, we found that the ability of Sox17-GST to pull down β -catenin protein was restored in the presence of TCF4 or TCF3 (Fig. 4D and E). Moreover, we demonstrated that the β -catenin interaction domain of TCF3 was necessary for the ability of Sox17 to pull down β -catenin (Fig. 4E).

In summary, rather than Sox17's and TCF's competing for β -catenin binding, as had been previously proposed, we found that all three proteins interact in a complex. The interaction between TCF/LEF and β -catenin proteins is stabilized in the presence of Sox17, and this stabilization depends on the ability of Sox17 to interact with β -catenin. Similarly, Sox17- β -catenin interactions are stabilized in the presence of TCF/LEF and depend on the ability of Sox17 to interact with TCF/LEF.

The fact that *Sox4* has an impact on β -catenin/TCF activity opposite that of *Sox17* prompted us to investigate whether this difference is manifested at the level of β -catenin/TCF protein-protein interaction. We found that Sox4, like Sox17, could bind directly to TCF4 or β -catenin (Fig. 5A). Interestingly, in three

separate experiments, Sox4 was unable to enhance the binding between TCF4 and β -catenin, as Sox17 could (Fig. 5B and C). In fact, our data suggested that Sox4, TCF/LEF, and β -catenin may interfere with binding to one another (Fig. 5C and data not shown). One implication of these data is that the differences in Sox17 and Sox4 binding to β -catenin and TCF may account for the opposite effects of these proteins on Wnt signaling activity.

Sox17 has distinct TCF and β -catenin interaction domains.

To further investigate the nature of the Sox17- β -catenin-TCF protein complex, we performed reciprocal structure-function binding assays to identify the domains that mediate this interaction. We generated a series of deletions and several point mutations in Sox17 and assayed the ability of mutant proteins to bind to TCF4-GST or β -catenin-GST beads (Fig. 6A). An N-terminal deletion of Sox17 (leaving amino acids 129 to 419 intact) that removed 80% of the HMG domain greatly reduced the ability of the protein to bind to TCF4, whereas the deletion of the C-terminal β -catenin interaction domain (55) had little effect (Fig. 6A). A point mutation (G to R at amino acid 103) was generated in the HMG domain of Sox17, which based on the crystal structure of the HMG domain of LEF1 (23) was predicted to disrupt the α -helical structure of the HMG domain. This mutation abolished the ability of Sox17 to interact with TCF4 but did not reduce β -catenin binding (Fig. 6A). We generated a second point mutation in the HMG domain (M to A at amino acid 76), which was not predicted to affect HMG domain structure (50) but rather impair DNA binding. As predicted, the Sox17 M-to-A mutant form retained the ability to interact with TCF4 and β -catenin proteins but was incapable of activating the transcription of a Sox17 reporter gene (pH4X8-Luci) (Fig. 6A and D). These data suggest that the HMG domain of Sox17 mediates the interaction with TCF4 and is distinct from the β -catenin interaction domain.

In the reciprocal experiment, we generated a series of deletions in XTcf3 and assayed the ability of the mutant forms to bind to His-tagged Sox17 *in vitro* (Fig. 6B). Since all TCF family members tested interact with Sox17, we focused our structure-function analysis on one representative family member, TCF3. The deletion of the N-terminal β -catenin binding motif of TCF3 had no effect on the ability of TCF3 to interact with Sox17. However, the deletion of the HMG domain abolished TCF3 interaction with Sox17 (Fig. 6B). These data indicate that the interaction between Sox17 and TCF factors is mediated via their respective HMG domains. This idea is consistent with the recent finding that the HMG domains of Sox8 and Sox10 interact with DNA binding domains of several other transcription factors (3, 52).

Sox17 requires both its TCF and β -catenin interaction domains, but not transcriptional activity, for Wnt-antagonizing activity. We used the mutant forms of Sox17 to investigate whether the interaction between Sox17, TCF/LEF, and β -catenin was necessary for Wnt-antagonizing activity. We found that Sox17 mutants that did not interact with either TCF/LEF or β -catenin failed to antagonize Wnt signaling in TOP-flash assays (Fig. 6C). One possibility was that Sox17 regulates Wnt signaling indirectly by activating the transcription of a gene encoding a Wnt antagonist. However, the Sox17 mutant protein with the M-to-A mutation (at amino acid 76), which was predicted to have impaired DNA binding ability and cannot

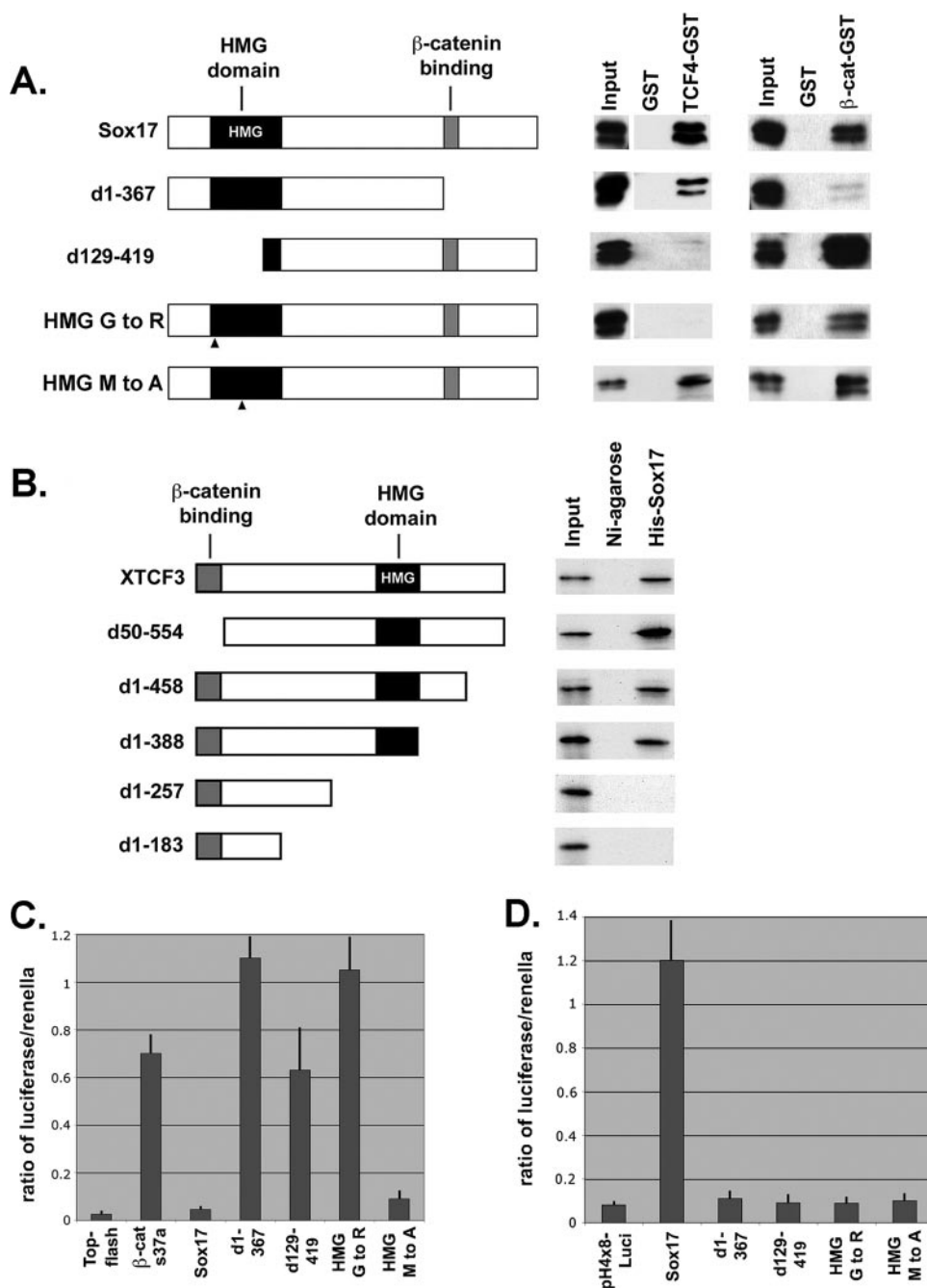


FIG. 6. Structure-function analyses of Sox17-TCF- β -catenin interactions. (A) Sox17 interacts with TCF4 protein via its HMG domain. The left panel shows a schematic diagram of Sox17 deletions and point mutations. HMG mutant 1 carries a stringent mutation (G to R at amino acid 103), and HMG mutant 2 carries a more conservative mutation (M to A at amino acid 76). d1-367, now contains amino acids 1 to 367; d129-419, now contains amino acids 129 to 419. The right panel shows the interaction of in vitro-translated Sox17 mutant proteins (V5 tagged) with GST alone, TCF4-GST, or β -catenin (β -cat)-GST. (B) Identification of protein domains of TCF3 that mediate its interaction with Sox17. The left panel shows a schematic diagram of deletions of *Xenopus* TCF3. The right panel shows the interaction of the in vitro-translated 35 S-labeled TCF3 mutant proteins with His-tagged *Xenopus* Sox17 β or Ni-agarose alone. (C) Sox17 requires interactions with both β -catenin and TCF/LEF to antagonize Wnt activity. COS cells were transfected with β -catenin(S37A) and TOP-flash alone or together with the Sox17 mutant forms, and TOP-flash activity was measured after 48 h. Error bars indicate standard errors of the means of results for replicate samples from at least three separate experiments. (D) Sox17 does not require transcriptional activity to antagonize Wnt activity. 293T cells were cotransfected with Sox17 and derived mutant forms and a Sox17 reporter plasmid (pH4X8-Luci). The transcriptional activity of Sox17 requires a functional DNA binding domain (HMG domain) and a functional transactivation domain (at the C terminus). While the Sox17 HMG mutant form carrying an M-to-A mutation is transcriptionally inactive, it still is able to interact with both TCF4 and β -catenin and antagonize Wnt activity. Error bars indicate standard errors of the means of results for replicate samples from two separate experiments.

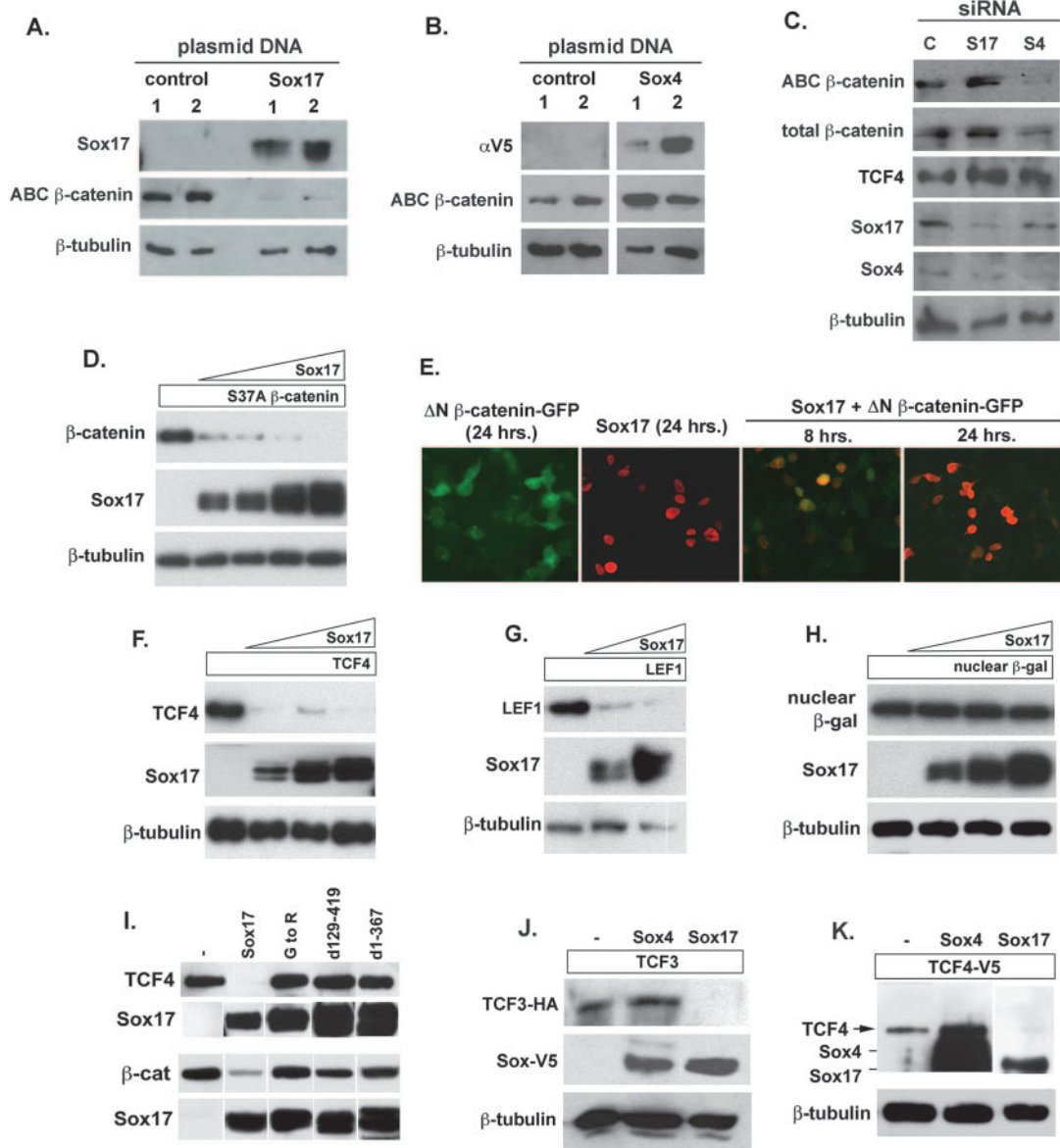


FIG. 7. Sox proteins affect β -catenin and TCF/LEF protein levels. (A and B) Effects of Sox17 and Sox4 overexpression on endogenous β -catenin protein levels in SW480 cells. The overexpression of Sox17 in SW480 cells causes significant reduction in the levels of endogenous unphosphorylated β -catenin protein, which was detected using an antibody specific for unphosphorylated β -catenin (ABC β -catenin). The overexpression of Sox4 caused an increase in the levels of endogenous unphosphorylated β -catenin protein. α V5, anti-V5 antibody. (C) Impact of siRNA knockdown of Sox17 (S17) and Sox4 (S4) on β -catenin and TCF4 levels in SW480 cells. Reduced levels of endogenous Sox4 protein coincide with a significant decrease in unphosphorylated β -catenin protein. Reduced levels of endogenous Sox17 protein coincide with a slight increase in β -catenin and TCF4 protein levels. C, control. (D) Sox17 promotes the degradation of stabilized β -catenin protein in a dose-dependent manner. COS cells (or 293T cells [data not shown]) were transfected with a plasmid encoding stabilized β -catenin (carrying the S37A mutation and tagged with V5 epitope; 100 ng) alone or with increasing amounts of a Sox17 expression plasmid (50, 100, 200, and 400 ng). After 24 to 48 h, cell lysates were harvested and analyzed by Western blotting using anti-V5 (β -catenin), anti-Sox17, and anti- β -tubulin antibodies. (E) Time course of Sox17-mediated reduction in stabilized β -catenin protein. Cells were transfected with a Sox17 plasmid (100 ng) and a plasmid encoding stabilized, GFP-tagged β -catenin (Δ N β -catenin-GFP; 100 ng) alone or together. The expression levels of Sox17 and β -catenin were analyzed 8 and 24 h posttransfection by the analysis of GFP for β -catenin or by immunohistochemistry analysis with a Sox17 antibody. (F and G) Sox17 promotes the degradation of TCF4 (F) and LEF1 (G) proteins in a dose-dependent manner. (H) A Sox17 plasmid (50, 100, and 200 ng) had no effect on the levels of expression of nuclear β -galactosidase protein (β -gal), demonstrating that Sox17 did not generally affect the transcription or translation of plasmids in cotransfected cells. β -tubulin protein was used to indicate that each lane contained roughly equivalent amounts of total protein. (I) The interaction of Sox17 with either TCF4 or β -catenin (β -cat) is required for the degradation of these proteins. COS cells were cotransfected with plasmids encoding Sox17 mutant forms and TCF4 or β -catenin, and degradation was analyzed by Western blotting of cell extracts after 48 h. The relative levels of expression of the V5 epitope-tagged Sox17 mutant forms are shown in the bottom panel. Mutant forms of Sox17 that retained the ability to interact with TCF but failed to interact with β -catenin were unable to promote TCF4 degradation and vice versa, suggesting that interaction with both is required for degradation. -, control. (J and K) Effects of Sox4 versus Sox17 on TCF3 and TCF4 protein levels. Sox4, Sox17, and TCF4 proteins were detected by Western blotting with an anti-V5 antibody and closely comigrate, as indicated in panel K. TCF3 protein was detected using a HA epitope tag, and the Sox proteins were detected using a V5 epitope tag. β -Tubulin protein was used to indicate that each lane contained roughly equivalent amounts of total protein. -, control. For all panels, experiments were repeated at least three times and results from a representative experiment are shown.

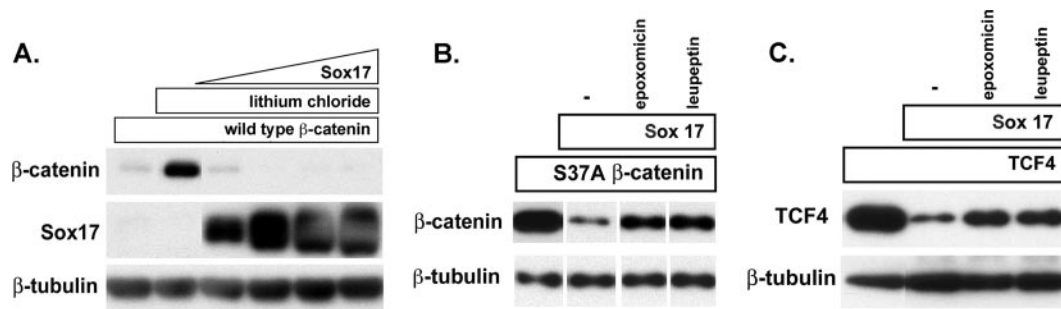


FIG. 8. Sox17-mediated degradation of β -catenin and TCF4 is independent of GSK3 β activity and mediated by the proteasome. (A) LiCl does not affect Sox17-mediated degradation of β -catenin. COS cells were transfected with an expression plasmid encoding wild-type β -catenin (100 ng; myc tagged) in the absence (lane 1) or presence (lanes 2 to 6) of 40 mM LiCl. Cells were cotransfected with increasing amounts of Sox17 plasmid (50, 100, 200, and 400 ng) and the wild-type β -catenin gene in the presence of lithium chloride (lanes 3 to 6). LiCl effectively stabilized wild-type β -catenin protein in the absence of Sox17 but could not inhibit Sox17-mediated degradation of wild-type β -catenin protein. (B and C) The proteasome inhibitor epoxomicin inhibits Sox17-induced degradation of stabilized β -catenin (B) and TCF4 (C). Cells were transfected with the β -catenin(S37A) mutant form (100 ng) or TCF4 (100 ng) alone or together with Sox17 (100 ng). Culturing cotransfected cells with epoxomicin (0.1 μ M) or leupeptin (25 μ M) inhibited Sox17-mediated degradation of β -catenin and TCF4 proteins. The lysosomal acidification inhibitor chloroquine (25 μ M) had no effect (data not shown). –, control.

stimulate the transcription of a Sox17 reporter gene (pH4X8-Luci), was still capable of repressing β -catenin-stimulated TOP-flash activity (Fig. 6C). Importantly, this Sox17 M-to-A mutant protein retained the ability to bind to both TCF/LEF and β -catenin (Fig. 6A). This finding suggests that Sox17 does not antagonize Wnt signaling via transcription but rather does so by direct interaction with TCF/LEF and β -catenin.

Sox factors regulate the degradation of β -catenin and TCF/LEF proteins. While performing transfection experiments with *Sox17* and β -catenin plasmids, we observed reduced β -catenin protein levels in cells cotransfected with *Sox17*. We therefore analyzed the effect of Sox17 overexpression on levels of endogenous β -catenin protein in SW480 cells. We found that the unphosphorylated signaling pool of β -catenin protein was significantly reduced in the presence of elevated levels of Sox17 protein (Fig. 7A) (the activated- β -catenin antibody recognizes unphosphorylated β -catenin). In contrast, β -catenin protein levels were elevated when Sox4 was overexpressed (Fig. 7B). In RNA interference experiments, we found the opposite; a reduction of Sox4 protein coincided with a significant reduction of endogenous unphosphorylated β -catenin protein, and Sox17 siRNAs caused a modest increase in β -catenin protein (Fig. 7C). Similarly, endogenous TCF4 protein levels were slightly elevated by Sox17 siRNAs; however Sox4 siRNAs had no impact on TCF4 levels. These data suggest that Sox factors modulate β -catenin/TCF activity through regulating protein levels.

Endogenous protein levels can be affected by transcription, translation, or protein stability. Our data suggest that Sox proteins may affect β -catenin/TCF protein levels posttranslationally via direct protein-protein interaction. To avoid the effects of Sox proteins on β -catenin and TCF transcription and translation, we studied how Sox factors affect β -catenin and TCF proteins that are expressed from plasmids in cotransfected cells, in which the transcription and translation of these factors should be relatively constant. We first performed a time course experiment to analyze the effects of Sox17 on the signaling pool of β -catenin protein by immunohistochemistry (Fig. 7E). We found that 8 h after cotransfection, both Sox17 and non-phosphorylatable β -catenin protein (stabilized β -catenin-GFP

fusion protein with an N-terminal deletion in β -catenin) were detected in the same cells. In contrast, 24 h posttransfection, Sox17 protein levels were the same but β -catenin-GFP protein was not detectable in most cells. We quantitatively investigated the relationship between Sox17 and β -catenin protein levels by cotransfecting cells with increasing amounts of a *Sox17* plasmid and a constant amount of a β -catenin(S37A)-expressing plasmid (the S37A mutation renders β -catenin nonphosphorylatable and thus stable). Increasing amounts of Sox17 protein directly correlated with a decrease in β -catenin(S37A) protein levels in COS cells (Fig. 7D) and 293T cells (data not shown). Sox17 did not generally inhibit the transcription or translation of plasmids in cotransfected cells since Sox17 had no effect on protein levels driven from a plasmid encoding nuclear β -galactosidase (Fig. 7H). In addition, the mRNA levels corresponding to the β -catenin plasmid in transfected cells were unaffected by *Sox17* (data not shown).

While it has been previously reported that *Sox9* affects β -catenin protein levels (2), we have demonstrated that Sox17 also interacts with TCF/LEF proteins. Therefore, it is possible that Sox17 may regulate the levels of TCF/LEF proteins. To investigate this possibility, COS cells were cotransfected with TCF4 or LEF1 expression plasmids and increasing levels of a Sox17 plasmid, and extracts were harvested 48 h later (Fig. 7F and G). Interestingly, an increase in Sox17 correlated with a dramatic reduction in TCF4 and LEF1 protein levels in a dose-dependent manner (Fig. 7F and G). To determine if Sox17 needed to interact directly with TCF4 and β -catenin protein to modulate the stability of these proteins, we used the mutant forms of Sox17 depicted in Fig. 6A. Sox17 mutant proteins that did not interact with TCF4 also failed to promote its degradation, and mutant proteins that did not interact with β -catenin did not promote its degradation (Fig. 7I). Interestingly, a Sox17 mutant protein (containing only amino acids 1 to 367) that failed to interact with β -catenin but still interacted with TCF4 was unable to promote TCF4 degradation. The converse was true in that interaction with TCF4 was required for β -catenin degradation. Together, these findings suggest that Sox17 must directly interact with both β -catenin and TCFS

to trigger the degradation of the complex and to antagonize Wnt signaling.

We next investigated whether Sox4 overexpression affected TCF4 or TCF3 protein levels. The Sox and TCF4 proteins were both V5 tagged, which allowed for the direct comparison of protein levels by Western blotting with an anti-V5 antibody. As expected, Sox17 overexpression coincided with a reduction in TCF4 and TCF3. In three separate experiments, Sox4 overexpression either caused no change or effected a slight increase in TCF4 protein levels and had little effect on TCF3 (Fig. 7J and K). Also, Sox4 overexpression had no effect on levels of coexpressed β -catenin(S37A) protein (data not shown). These findings are the first to indicate that Sox factors regulate Wnt signaling by modulating the stability of TCF/LEF family members.

Sox17 stimulates β -catenin and TCF4 protein degradation by the proteasome in a GSK3 β -independent manner. Our data suggest that the *Sox17*-mediated degradation of β -catenin is GSK3 β independent because mutant forms of β -catenin that cannot be phosphorylated by GSK3 β and thus are constitutively stable were still targeted for degradation by *Sox17* (Fig. 7D and E). To confirm this finding, we inhibited GSK3 β activity by including lithium chloride in the culture medium and monitored levels of wild-type β -catenin (myc tagged) alone or in the presence of increasing concentrations of a *Sox17* plasmid. As expected, wild-type β -catenin protein did not accumulate in COS cells (Fig. 8A, lane 1); however, including LiCl (40 mM) in the culture medium resulted in the accumulation of wild-type β -catenin protein (Fig. 8A, lane 2). The coexpression of *Sox17* in LiCl-treated cultures caused a dose-dependent loss of wild-type β -catenin protein. This finding confirms that *Sox17* promotes GSK3 β -independent degradation of wild-type β -catenin protein.

The targeted degradation of many cellular proteins, including β -catenin, is mediated by the proteasome (1). We used the proteasome inhibitor epoxomicin to determine if the *Sox17*-mediated reduction in β -catenin and TCF4 protein levels was due to degradation by the proteasome. We cotransfected cells with *Sox17* and a gene for stabilized β -catenin(S37A) or TCF4 and cultured cells in the presence or absence of epoxomicin (Fig. 8B and C). *Sox17*-mediated degradation of both TCF4 and β -catenin was substantially inhibited by epoxomicin, which selectively inhibits the chymotrypsin-like activity of the proteasome (25). Interestingly, the degradation of β -catenin and TCF4 was also repressed by the protease inhibitor leupeptin, which inhibits the lysosomal protease cathepsin B (42) as well as other serine and cysteine proteases. The specific lysosomal acidification inhibitor chloroquine had no effect on the degradation of either β -catenin or TCF4 (data not shown), suggesting that these proteins are not degraded by the lysosome. These data suggest that the proteasome and possibly a leupeptin-sensitive protease are involved in *Sox17*-mediated degradation of β -catenin and TCF4.

DISCUSSION

The expression of several Sox proteins has been tightly correlated with colon cancer progression. Our data and previously published findings indicate that *Sox4* expression levels are elevated in intestinal tumors whereas the expression of *Sox17* is

reduced in adenomas relative to that in normal gut epithelia (Fig. 1) (8, 32, 38). We have identified some Sox proteins as repressors and others as enhancers of Wnt/ β -catenin signaling activity and the proliferation of colon carcinoma cells, suggesting that Sox proteins may act as tumor suppressors or tumor promoters. While Sox factors are best known for their transcriptional function, we have identified a novel posttranslational mechanism by which Sox proteins regulate Wnt signaling and cell proliferation.

Nontranscriptional functions of Sox factors. We have demonstrated that Sox17 and Sox4 can directly interact with β -catenin and TCF/LEF proteins. In the case of Sox17, it forms a complex with both β -catenin and TCF. This finding is significant because one of the prevailing models is that Sox17 may antagonize Wnt activity by competing with TCFs for binding to β -catenin, in which case Sox17 and TCF binding would be mutually exclusive. Our data show that Sox17 antagonizes the Wnt pathway by promoting the degradation of TCF/LEF and β -catenin proteins via a GSK3 β -independent mechanism. Structure-function analyses indicate that Sox17 must be able to interact directly with both TCF and β -catenin in order to promote their turnover. In contrast, while Sox4 can bind to TCF and β -catenin individually, the three proteins do not appear to form a stable complex. In addition, *Sox4* overexpression does not promote β -catenin/TCF degradation like the overexpression of *Sox17* does. Rather, reduced levels of Sox4 were associated with a reduction of β -catenin protein, and the overexpression of Sox4 either had no impact on TCF4 levels or in some instances caused an increase in TCF4 protein levels. To our knowledge, this is the first demonstration that Sox proteins modulate canonical Wnt signaling by influencing levels of TCF/LEF factors.

In addition to a reduction in β -catenin protein levels, the knockdown of *Sox4* coincided with a decrease in the activation of β -catenin and TCF target genes, such as *cyclin D1*, and reduced the proliferation of colon carcinoma cells, whereas reduced *Sox17* levels had the opposite effect in these cells and in *Xenopus* embryos, resulting in the hyperactivation of Wnt target genes. Together, these data suggest that Sox proteins normally function to modulate β -catenin/TCF activity in vivo. One possible mechanism by which Sox4 may stabilize β -catenin was suggested by the finding of Lee et al. (19), who demonstrated that TCF3 can stabilize β -catenin protein by preventing its association with Axin and APC.

Transcriptional functions of Sox, β -catenin, and TCF proteins. Although we have demonstrated that the ability of Sox17 to antagonize β -catenin and TCF is separable from its role as a transcription factor, it is possible that the transcriptional function of Sox proteins may also have an impact on Wnt signaling in some contexts. For example, Sox and TCF proteins may compete for common DNA binding sites or shared cofactors. In *Xenopus*, there is evidence that Sox3 and TCF bind to adjacent DNA sites to coordinately regulate the expression of the Wnt and β -catenin target gene *Xnr5* (54). In addition, it has recently been shown that the HMG domain of Sox factors can interact with other transcription factors, including homeodomain proteins, zinc finger proteins, and basic helix-loop-helix and leucine zipper proteins (3, 52), each of which may affect the ability of Sox factors to bind DNA or interact with cofactors such as β -catenin and TCF proteins. Thus, in addition to

Sox proteins' regulating β -catenin and TCF protein stability, Sox-TCF complexes may regulate target gene transcription.

In the case of Sox17, there is evidence that it has two distinct functions, to repress or limit β -catenin and TCF activity and to use β -catenin as a cofactor to activate Sox17 target gene expression in *Xenopus* embryos. The depletion of *Xenopus* embryos of endogenous Sox17 protein results in the hyperactivation of Wnt target genes, and the depletion of β -catenin results in reduced Sox17 target gene expression. Although transcriptional targets of Sox17 in the intestinal epithelium are unknown, we postulate that a similar mechanism may act there. Together, the present and previously published findings show that Sox17 can either utilize β -catenin as a transcriptional cofactor or promote the degradation of β -catenin and TCFs. Thus, Sox17 may radically shift the transcriptional output of the Wnt signaling pathway from TCF to Sox target genes. Ultimately, the function of Sox17 as a Wnt antagonist or a transcriptional activator may depend on the relative levels of Sox17, β -catenin, and TCF/LEF. In a case in which the signaling pool of β -catenin is in excess, we predict that high Sox17 levels could both repress Wnt signaling by promoting the degradation of β -catenin-TCF complexes and activate the expression of Sox17 target genes.

Sox proteins and cancer. Oncogenic, activating mutations in β -catenin stabilize the protein by preventing its phosphorylation by GSK3 β and subsequent degradation and are associated with the formation of colorectal carcinoma (26, 28, 46). Our data show that *Sox17* promotes the degradation of oncogenic forms of β -catenin, suggesting its possible role as a tumor suppressor. Consistent with such a function, *Sox17* expression levels are down regulated in *APC^{min/+}* tumors and in colon cancer as adenomas progress to carcinomas (32). In addition, *Sox17* mRNA was almost undetectable in 66 human primary tumors derived from various tissues (16). Finally, our functional analysis of *Sox17* clearly indicates that this factor represses colon carcinoma cell proliferation and Wnt signaling, possibly via targeting β -catenin and TCF for degradation. These findings suggest that down regulating Wnt antagonists like *Sox17* may be an obligatory step in tumor formation and progression.

In contrast to those of *Sox17*, elevated *Sox4* levels are correlated with cancer in many contexts; *Sox4* was identified as one of the most highly tumor-associated genes in an insertional mutagenesis screen for oncogenes (47). Elevated *Sox4* levels are associated with small-cell lung carcinomas and medulloblastomas (9, 18, 45) and *APC^{min/+}* adenomas (Fig. 1) (8, 32, 38). Our data provide a novel mechanism by which *Sox4* may act as an oncogene, namely, by stimulating Wnt signaling, possibly by regulating β -catenin or TCF protein stability and/or activity. Together, our findings suggest a novel mechanism by which some Sox proteins may act as protooncogenes while others may act as tumor suppressors by regulating β -catenin and TCF protein activity.

Mammals have over 20 Sox proteins that are found in many cell types (41). The fact that most Sox proteins can have an impact on canonical Wnt responses in TOP-flash assays suggests that Sox-mediated regulation of β -catenin and TCF proteins is a broadly utilized mechanism that may regulate Wnt signaling in a variety of physiological and pathological contexts, ranging from embryonic development to cancer. More-

over, this novel GSK3 β -independent means to degrade TCF/LEF and β -catenin proteins represents a new molecular target for drug therapies that target the canonical Wnt pathway.

ACKNOWLEDGMENTS

We thank Gail Deutsch, Janet Heasman, and Xinhua Lin for comments on the manuscript and members of the Wells and Zorn labs for advice. We thank Richard Harland, Mike Klymkowsky, and Hugh Woodland for reagents. We thank Michael Weiss for advice on HMG box mutations. We also thank Michael Kleimeyer and Bruce Aronow for help with the clustering analysis depicted in Fig. 1.

This research was supported by a career development award and a postdoctoral fellowship from the Juvenile Diabetes Research Foundation (J.M.W. and S.-C.J.L.), an NIH training grant in developmental and perinatal endocrinology, no. T32 HD07463 (J.R.S.), and the NIH grants HD42572 (A.M.Z.) and GM072915 (J.M.W.).

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